Histology of the inflammatory response of carp (*Cyprinus carpio* L.) to various stimuli

by

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DECLARATION

I, hereby, declare that this thesis is the results of my research carried out at the Institute of Aquaculture, University of Stirling. It has been prepared entirely by myself and has not been submitted for any other degree. Any results or comments of other workers used in this thesis, has been specifically acknowledged by reference.

Candidate: Issa Sharifpour Date & Signature.....20.7.97

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Assistant supervisor: Prof. R.H Richards Date & Signature.....

DEDICATED TO:

My wife, Farzaneh

Who proved to be very patient and helpful during my study. She tenderly looked after every thing and every body at home, putting my needs first and letting me devotedly pursue and complete this investigation. As a woman, wife and mother, She deserves the best rewards from ALLAH (God). Words are unable to express how deeply I am indebted to her. May ALLAH give her what she deserves.

IN THE NAME OF GOD

THE COMPASSIONATE , THE MERCIFUL

My God! *To You is due all praise, always and forever, eternally, increasing, not diminishing, as You like and please.*

My God! *If You hold me for my offences and crimes, I will hold on to You for Your forgiveness, and if You hold me for my sins, I will hold on to You for Your pardon. If You cast me into the Fire, I will announce to its inmates that I love You. So do not disappoint me of Your Mercy, and do not block me from Your kindness.*

My God! *Place me among Your friends in the position of one who hopes for an increase in Your love, who never neglect giving thanks to You, and who do not depreciate Your commands.*

My God! *Provide me with a heart, the passion of which may bring it near to You; with a tongue, the truth of which may be offered up to You; and with a vision whose character may bring it nigh to You.*

Amen!

(From The Munajat of Sha'ban)

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ABSTRACT

The present investigation was carried out to study the inflammatory response of carp (*Cyprinus carpio* L.) to various stimuli. The stimuli were; surgical wound, talcum powder, Freund's complete adjuvant (FCA), a bacterium *Aeromonas hydrophila*, and spores of fungus *Aphanomyces invaderis*. Following exposure to thes stimuli, fish were sacrificed over a period of time, and sequential tissue samples were processed for histopathological examinations.

The surgical wounds at 27°C, re-epithelialized at 4 hours. Macrophage infiltration began at 2 hours, and myophagia at 6 hours. Fibroplasia and muscle regeneration were initiated at 2 days. After 16 days epidermis was normal and dermis was completely linked. The wounded area restored its main components by 16 to 24 days after wounding.

Injection of talcum powder at 26.5°C, and FCA at 24°C, produced chronic granulomatous inflammatory reaction. The following events started in both experiments at the same time; myophagia at 6 hours, macrophages had changed into epithelioid cells, active fibroplasia and muscle regeneration at 3 days. Regenerated muscles filled the defects by 14 to 28 days post-injection (p.i.). Langhans and foreign body giant cells, were observed after 3 days in talc and 4 days in FCA experiment. New capillaries formed at 3 days in talc and 2 days in FCA study. At the end of the experiments (42 days) the encapsulation of the irritants was very advanced, and the granulomata were entirely surrounded by normal muscle tissue.

Inoculation of the *Aeromonas hydrophila* at 27.5°C, provoked a lethal acute inflammatory response within 48 hours. The surviving fish showed well developed capacity for dealing with bacteria. An ulcerative wound developed by 48 hours in the

surviving fish. Polymorphonuclear cells (PMNs) were observed at 1 hour p.i. and remained up to 7 days. PMNs were one of the dominant inflammatory cells and participated in myophagia and micro-abscess formation. The acute inflammation then developed into chronic inflammation characterised by fibroplasia which was active at 5 days. Process of wound healing began and developed by 5-10 days and was completed by scar formation at 28 days.

A chronic inflammatory response occurred after inoculation of spores of the fungus *Aphanomyces invaderis* at 27°C. Limited growth of the fungus in tissue occurred in the early stages p.i. but was then halted by fish's defence mechanisms. Macrophages had changed into epithelioid cells at 3 days p.i. Presence of Langhans, foreign body and intermediate giant cells, muscle regeneration, fibroplasia, and vascularization also started at 3 days. Developing granulomata formed by 10 days, and fully matured granulomata were observed by 18 days.

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AIM OF THE PRESENT STUDY

Today, the human population is growing and demands an increasing supply of protein which has encouraged aquaculture to develop and apply technologies that utilise natural resources more efficiently. Carp pond culture is one of the more effective ways of producing protein which will continue to be exploited (Horvath *et al.* 1992). Carp (*Cyprinus carpio* L.) is cultured in many countries under a wide range of geographic climatic and technological conditions. Carp culture is considerably developed and disease generally could result as a consequence of this development.

The skin and underlying muscle of carp not only protect against a wide range of infections, they are also of physiological important to the fish and economic importance to the farmers. Furthermore, since disease, in general, is one of the major limiting factors on the successful expansion of fish farming, basic information about the host reaction against the different irritants and pathogens could be considered as a major guide and help for fish pathologists for diagnostic purposes, and developing solutions or treatments for the diseases.

There is little or no basic histopathological knowledge on the inflammatory response and capability of wound healing of carp. Therefore, the present study was conducted in order to define the basic inflammatory responses, and wound healing process in healthy carp to a series of standard physical (surgical wound), chemical (talcum powder) and biological (bacteria and fungus) stimuli, involving tissue inflammation and repair.

CHAPTER 1:

THE BIOLOGY OF CARP (*Cyprinus carpio* L.)

1.1 INTRODUCTION

The carp (*Cyprinus carpio* L. 1758) is a freshwater teleost fish belonging to the family Cyprinidae. This fish is found in lakes, rivers, reservoirs, and other inland bodies of water (Panek 1987). It is the most widely cultivated species throughout the world. In 1989, the world production of the common carp (*Cyprinus carpio*) and three Chinese carps; grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*) reached 4 million tonnes (excluding the production of Indian major carps), representing more than 65% of the fresh water finfish production and 50% of the global finfish aquaculture production (Kestemont 1995). Carp production has shown an increase of 30% from 1986 (New 1991), and Chamberlain (1993) reported that the world production of carp was about 5 million tonnes in 1990, which came mostly from developing countries where the various carp species are grown in fertilised ponds without feeding or with low-level supplementary feeding. The popularity and world-wide application of carp production systems can be attributed to the fact that protein production is highly energy-efficient in these systems (Varadi 1995).

Most carps are cultured for food. Other uses of the different carp species are as follows; bighead carp can be a source of oil used in the manufacture of pure medicated organic soap. The head of bighead carp consists of 20% oil with vitamins A, D and B complex. This oil in different grade (refined) can be used for vitamin and livestock feed preparations, manufacture of perfumes, cosmetics, soap and paints. Carp meat can also be used as extender in preparation of hot-dogs and sausages, as substitute for corn starch, and in the preparation of fish protein concentrate for baby food. The internal organs of carp are used as inoculants and liquefied hormonal fertiliser for agriculture

while the spines and meat on the tail are dried and ground as feed appetiser. The head of the common carp is a good source of pituitary gland, the extracts of which are utilised to induce early breeding and fry production in carps and other species of fish. The grass carp is utilised as biological control for aquatic weeds, while the Japanese "Koi" carp is an extremely valuable ornamental fish (Samson & Guzman 1990).

The culture of carp is an ancient activity that started probably one millennium BC, and common carp is now one of the few truly domesticated fish species (Billard & Gall 1995). The purpose of its introduction or transfer includes from aquaculture and sport, improvement of wild stocks or ornament (Biro 1995).

Among the carps, common carp is probably the most wide spread species (Jeney & Jeney 1995). Its annual production was 1,112,726 tonnes in 1990 (FAO 1992). In a review on aquaculture in Asia, New & Csavas (1993) gave a corrected figure (850,530 tonnes) for common carp production in 1990 as well as a forecast for Asia for the year 2000: 1,073,704 tonnes. Today, nearly 100 tonnes of carp fillets are marketed. Other filleting plants have sprung up and related activities have developed (Vallod 1995).

Due to its wide distribution and its easy rearing, carp is generally used as a model for research on physiology (especially on thermal adaption), nutrition and processing, and substantial information has been gained (Fauconneau *et al.* 1995).

1.2 Taxonomy

Cultured carps belong, taxonomically, to the family Cyprinidae. This group includes seven major species: The common carp (*Cyprinus carpio*), the grass carp (*Ctenopharyngodon idella*), the silver carp (*Hypophthalmichthys molitrix*), and the bighead carp (*Aristichthys nobilis*) commonly referred to as "Chinese carps"; and the

catla (*Catla catla*), the rohu (*Labeo rohita*), and the mrigal (*Cirrhinus mrigala*) commonly referred to as "Indian major carps" (Jhingran & Pulling 1985). Other Cyprinids of importance for aquaculture are: the crucian carp (*Carassius carassius*), the gold fish (*Carassius auratus*), the mud carp (*Cirrhinus moritorella*), and the tench (*Tinca tinca*) (Hulata 1995). Also Indian minor carps; *Labeo pangusia*, *Labeo bata*, *Labeo angra*, *Cirrhinus reba* and *Puntius sarana* are of importance (Jhingran 1978).

Cyprinidae is one of the six families of the order Cypriniformes. The Cypriniformes is one of the four orders constituting the series Otophysi, mostly freshwater group (apart from some catfish), which together with the Anotophysi (Gonorhynicformes) forms the superorder Ostariophysi (Howes 1991).

Cyprinids are known to the angler, aquarist and fishery biologist under the common names of carps, barbs, minnow, roaches, rudds, daces, bitterlings, rasboras, danios and gudgeons. There are some 1700 valid species in at least 220 genera, making the family the most specious of all freshwater teleost fishes (Howes 1991).

1.3 Original range and distribution

The carp is one of the most widely cultivated warm-water fish which has been introduced into several parts of the world (81 countries) (Welcomme 1988; Holcik 1991). They are found in almost all area of the world but do not naturally occur in the polar regions, South America or Australia (Michaels 1988). Carp is cultured under a wide range of geographic, climatic and technological conditions (Jeney & Jeney 1995).

Carp occur in a wide range of freshwater habitats from clear mountain lakes to degraded rivers. They are found in lakes, large and small rivers, large reservoirs, shallow ponds, still pools, swamps and bogs, large slow-moving rivers, fast-flowing

streams and even some tidal and torrential rivers, creeks, underground water courses, and estuaries (Panek 1987; Howes 1991).

The original range of the common carp probably was limited to the Asia watersheds of the Black, Caspian and Aral seas. It may have been present in portions of western Europe, in the Volga River, and in eastern Asia from the Amur River southward to Burma. However, transfers of carp from Danube River to Greece and Italy during the Roman Empire and the widespread culture of carp throughout Europe during the Middle ages have obscured its original geographic range. There is no evidence to support the common belief that it was introduced to Europe from an original range in China (Panek 1987). Michaels (1988) stated that it is generally accepted that *Cyprinus carpio* originated in the region of the Caspian Sea; from there it is believed to have been brought west by Roman soldiers, to the Black Sea and River Danube. It also spread eastwards to China probably introduced by soldiers of the Chinese imperial army.

1.4 Ecological characteristics

Carp are thermophilic, but will tolerate extreme, long-lasting cold as well as rapid fluctuations of temperature. The metabolism of carp and consequently its demand for food slow down gradually along with the decrease in temperatures, and practically stop at a water temperature of 4°C. The capacity for rapid growth which is characteristic of the species, manifests best at a water temperature above 20°C. A variety of studies have determined that growth in this species is retarded with decreasing temperature until, at ≤ 10°C, aphagia ensues. Under such aphagic thermal conditions, carp will lose weight, even though provided with feed adequate enough to

support growth. It has been suggested a positive correlation exists between growth hormone (GH) secretion and temperature. Since carps have an exceptional environment tolerance, they can survive long exposure to a temperature range of < 1°C to 40°C, and also rapid temperature changes. Carp show high tolerance to variations in the ion concentrations of the water. They can live in brackish water and have been grown routinely at 5ppt salinity, and at up to 12ppt experimentally, and also in waters of pH from 5-9. They are also less sensitive to fluctuations in oxygen level and tolerate low dissolved oxygen and withstand prolonged anoxia under ice. They can be efficiently cultured even at an oxygen concentration of 3-4 mg/l (fish kills may occur at oxygen concentrations of 0.3-0.5 mg/l). They are extremely tolerant of turbidity caused by suspended clay, silt, or other particulate matter. Carp can grow fast with occasional specimens reaching body weights of 20 kg (Kim *et al.* 1975; Wood & Ghannudi 1985; Billard & Marcel 1986; Panek 1987; Howes 1991; Horvath *et al.* 1992; Teskeredzic *et al.* 1995).

1.5 Anatomy and physiology

Sarig (1966) described common carp as follows; "dorsal fin long, 3-4 spines and 17-22 soft rays. 35-39 scales in the lateral line. In wild form, the body is elongated, moderately deep, slightly compressed. Squamation complete. Mouth directed forwards, protrusible, surrounded by fleshy lips. Two pairs of fleshy projections barbels on the each side of upper lip. Lateral line complete. Domesticated form is stocky with a high back, squamation very much reduced (mirror carp), or almost completely absent (leather carp). Upper side olive-green to yellow-green; flanks greenish clay-yellow or brassy-yellow; underside yellowish; fins opaque grey-green or

brownish, sometimes reddish. Female dumpy at spawning time; male develops weak snout tubercles at spawning time".

Almost all species of carp have scales but none have teeth in the jaws, instead they have curved bones in the throat which are used for grinding some of their food (Michaels 1988).

The common carp has many features in common with other minnows: a weberain apparatus (it is made of a series of small bones and ligaments connecting the swim bladder with the inner ear which improves the sense of hearing), cycloid scales, pharyngeal teeth in the throat, but no teeth on the jaw. The pharyngeal teeth are well adapted for crushing and grinding food. All minnows lack true fin spines, but the carp has the first ray of the dorsal and anal fins modified into a hard, serrated bony structure, which can inflict a deep, painful wound on the careless fish handler. The common carp often exceed 60cm in length and 4.5Kg in weight. It is a strong and vigorous fish whose body height is about one-fourth its length. The body is laterally compressed. Head is small and triangular with a blunt snout and a thick nose plate covered by very sensitive skin known as "carp tongue". The small and horizontal mouth, located below the snout is protrusible. The posterior barbels are usually longer than the anterior. The colour and scale pattern of the carp are highly variable, depending on the amount of genetic selection that has occurred. Domesticated carp, selected for ornamental purposes, often show a wide range of white, gold and black colours (the koi of Hawaii and China). They maybe completely scaled (common carp), have only a few isolated large scales (mirror carp), or be almost scaleless (leather carp) (Panek 1987).

Much of the ecological survival of the common carp has been attributed to its well-developed senses of hearing, smell, and taste. The carp's sense of hearing is

certainly improved over that of many other fishes by the evolutionary development of the Weberian apparatus which is made up of a series of small bones and ligaments connecting the swim bladder with the inner ear. The amplified vibrations via swim bladder then carried to the brain, allowing the fish to hear and respond to sounds. The carp has also a well-developed sense of smell. Water enters an olfactory bulb through paired openings, the nares, carrying odorous materials to receptor cells that are embedded in the epithelium of the nasal cavity. The acute sense of smell is used by carp for the avoidance of predators. The sense of taste in carp is similar to that of higher animals. Specialised taste buds in the skin of the snout, mouth, lips, and throat are connected to the brain by special nerves. Tests have shown that carp can discriminate between salty, bitter, and sweet substances, as well as many extracts of fish skin and other fish tissues. Carp can feed more efficiently in the dark by smell and taste than many sight-feeders, they enjoy a competitive advantage in turbid waters (Panek 1987).

1.6 Feeding and growth

Cyprinids feed at all levels of the tropic chain: phytoplankton, macro and micro-zooplankton, benthos, macrophytes and even fish (Billard & Marcel 1986).

Carps are known as notorious species in aquaculture, due to the combination of several factors such as; feeding habits at a low level of the food chain, high survival and growth performances under culture conditions, and tolerance to high variations in water quality and disease (Kestemont 1995).

Carps feed almost all hours of day and night, if the temperature is suitable (Sarig 1966). The nutritional requirements of carp differ little from those established for

carnivorous fish, despite the particularities of their digestive tract: absence of stomach and long intestine. Thus when these figures are expressed in absolute terms, i.e. as quantity of intake per day, the protein needs are similar to those of other fish (Wilson 1985).

Carp feed at different levels in the water from the bottom to the surface. Most often, they root around in silty bottoms in shallow waters and suck up quantities of silt, then spit it out and select insects or other items of food from the water. They also utilise plankton suspended in midwater and some times eat insects or plankton caught in the surface film. Most active feeding occurs at sunrise and sunset when waters are calm and there is less disturbance by potential predators. Young carp feed primarily on animal foods such as small crustacea, snails, and midge larvae. Zooplankton are also an important source of nutrition. The actual plant material represented a small portion of the diet. Adult carp are truly omnivorous, consuming varying amounts of plants and animal foods. Plant material in the diet varies among populations and includes both rooted plants and algae. Organic debris can also be an important part of the carp's diet. Dominant animal foods of adult carp include midge larvae, crustaceans, small snails, and freshwater clams. Fish and fish eggs have seldom been noted in carp stomachs. Carp in lakes and ponds differ from those in streams by showing preference for the abundant planktonic crustaceans typical of lake communities (Panek 1987). In addition to benethic and zooplanktonic organisms, they also feed on seeds of plants and water weeds, detritus, etc. (Horvath *et al.* 1992).

The young fry possesses an underdeveloped digestion system which is best served by obtaining nutrients from living organisms. Thus it is essential to ensure the presence of very large numbers of food organisms of the appropriate size to provide

adequate nutrition. Later as the digestive system develops, the fry can consume mixed feed, including appropriate sizes of fine artificial feeds known as flours. This feed should be supplementary to the live feed and should not form a complete diet. Feeding should commence on the day of stocking. Although the fry will not feed on the flour immediately, it will not be wasted as the planktonic organisms will consume the finest grains. Hence, initially, only the plankton is being fed. Simultaneously, however, the fish are becoming accustomed to the taste of the feed and become used to a mixed diet. As the fish grow, the artificial feed becomes increasingly important until the stage is reached when it is virtually the sole diet. Because of this the quality and content of the feed is very important. It is particularly important at the early stages to provide proteins originating from animal sources and Soya (Horvath *et al.* 1992).

In fish ponds, fish are kept in much higher densities than in the natural environment. As a result the natural food supply of the pond is not capable of providing adequate nutrition for the weight of fish. Additional (supplementary) feeds therefore have to be added to the pond by the farmer to compensate for this overpopulation. In the case of carp farming, many varieties of cereal grains are suitable as a source of supplementary feed. The appetite of fish varies in response to many factors and consequently the daily ration of feed changes, according to temperatures, throughout the growing season. Appetite will obviously depend on temperature but other factors are also important (Horvath *et al.* 1992).

Carp have been shown to survive up to 47 years in captivity. A world record carp of about 37 Kg was reported from South Africa. Growth rate of carp depend upon a variety of biological and environmental factors. In addition to genetically defined limits, growth can be influenced by temperature, length of the growing season, water

chemistry, and the availability of food. Growth tends to slow when the fish reach maturity and more energy is diverted into reproduction (Panek 1987).

The wild carp grow slowly while domesticated forms utilise artificially fed cereals and natural food well, giving rapid growth. It is extremely advantageous that as a farmed animal 50-60% of the feed requirements of carp can be satisfied with cereals, the other 40-50% being made up from small animals living in the ponds (lower crustaceans, larvae of insects, molluscs, etc.) (Horvath *et al.* 1992).

Fish grow fast during their life stages of active feeding. This active period is during spring, summer and early autumn months (in Europe) when the water temperature remains steadily above 12-14°C. There is no growth during the non-feeding period, in fact some loss body weight may occur. During this period, fish withdraw to the bottom of water layers where, by maintaining their metabolism at its minimum level, they survive the cold winter season. The growth rate naturally is not determined solely by temperature and/or age of fish. It is significantly influenced by a number of other factors, e.g. stocking density, quality and quantity of food, oxygen concentrations, etc. (Horvath *et al.* 1992).

1.7 Reproduction

Cyprinids have various modes of reproduction. They mate in pairs or are polyandric, and they lay eggs on many different substrates, even on other animals, or in running water (Billard & Marcel 1986).

The common carp is heterosexual. Differentiation in the size of the testicles often occurs, the right male gonad being usually smaller than the left one, as is also found in the female's ovaries (Sarig 1966).

Carp mature and spawn at different ages, according to sex and growth rate. Fast-growing males may mature in 1 year, but females in populations of slowly growing fish may take up to 5 years before maturing. Most spawning occurs in late spring or early summer when the water temperature rises. Spawning occurs both day and night over several weeks as the water temperature rises. Carp do not build nests nor protect their eggs or young. The lack of parental care is compensated for by the production of large numbers of eggs. Larger fish generally produce more eggs. Eggs from females and milt from males are released into the water naturally. The eggs sink and adhere to rooted vegetation, algae, or firm substrates, and generally occur in clusters of several hundred. Eggs develop to the eyed stage, when pigmented eyes are visible through the egg capsule. After hatching, the larvae are heavily pigmented on the head and back and prominently so along the belly. During the first few days of life, these small larvae remain attached to vegetation until they have completely absorbed their yolk sacs. Small carp remain in areas protected from wave action and predation. Young carp are seldom observed. They prefer to hide rather than find safety in flight. They will often bury themselves in mud or sand as a means of avoiding predatory birds. Such behaviour, in addition to high fecundity, enable carp to rapidly establish populations in suitable habitats (Panek 1987). The reproduction capacity of carp is extremely high, and during one season 0.5-1 million fry maybe produced from one female (Horvath *et al.* 1992).

The common carp has been cultivated for several thousand years and is now widely distributed. For a long time of the carp culture, reproduction was not controlled and spawning occurred spontaneously in rearing ponds. Fry were also released in rivers and lakes. Controlled reproduction really started in the 1950s, when research on

reproduction and gamete biology began. In several production systems male and female carp are introduced into spawning ponds or spawning tanks and reproduce naturally. The change in environment, mostly a rise in temperature, and also the presence of spawning substrates, induce ovulation followed by oviposition and fertilisation. Eggs are attached to various spawning substrates. Incubation and hatching may occur in the spawning pond or tank, or substrates with attached eggs maybe transferred to various incubators (Billard *et al.* 1995).

Throughout the world a wide variety of methods are used to produce carp fry. Many are based on simple techniques unchanged over hundreds of years, whereas in contrast, others involve sophisticated technology used to produce vast numbers of fry throughout the year. The methods chosen to produce seed will depend upon the resources of the farm or country and also upon the economics of the enterprise (Horvath *et al.* 1992).

When the majority of carps are cultured in tanks, they do not generally receive sufficient environmental stimuli such as water flow to breed naturally. This makes it necessary to use additional hormones to induce ovulation. Traditionally, freshly extracted pituitary glands are taken from donor fish and, after extraction, the hormones are immediately injected into the mature fish. However, several types of hormone preparations are commercially available such as; fresh pituitary glands, dried pituitary extract, whole freeze-dried glands, purified human chorionic gonadotrophin (HCG), and crude HCG (Price 1989).

Induced spawning is used in carp species which can mature but cannot spawn in captivity (major Indian and Chinese carp) as well as for those which can spawn when environmental required conditions are provided (common carp). However, in common

carp, occurrence and success of spontaneous spawning are not consistent and the use of induced spawning techniques allow fish farmers to optimise broodstock management by increasing the quantity of fry produced. Hormonally induced spawning is commonly used for carp and related species and knowledge of endocrinology of reproduction is required to improve the use of these techniques. The success of these techniques can be improved by also manipulating environmental conditions (temperature, photoperiod, water quality, social factors) (Weil *et al.* 1986).

1.8 Culture systems

Kestemont (1995) described that the notoriety of carp species in aquaculture has led to development of numerous and different culture and production systems in both temperate and tropical regions. The different carp production systems are as follows:

1) Extensive common carp monoculture production in earthen ponds: the degree of culture intensity in this system varies. There are low stocking densities with no supplemental feeding or fertilisation in highly extensive production systems.

2) Intensive monoculture: in this system, feeding is entirely with artificial feed and water with strong aeration or running water (raceways). The common carp is the best species reared in intensive monoculture, the other carp species being usually cultivated in polyculture.

3) Integrated carp monoculture system: traditional carp monoculture has been associated with agriculture (rice, cereals), or farming (duck) in several regions. In the agro-aquaculture integrated systems, some distinctions maybe made according to whether integration is direct, indirect, parallel, or sequential.

4) Carp polyculture and integrated fish farming: this system is the culture of several fish species in the same pond that feed on different natural resources, to utilise the fish production potential of a pond. It is an important management technique.

5) Openwater carp polyculture in cages and enclosures: this system includes cage and pen culture. It became popular during the 1980s in China. Market-size fish culture in cages is carried out with Chinese carp, common carp or tilapia. The traditional polyculture concept is practised in pen culture system where large-sized fingerling (>13cm) and 2-years-old yearlings are grown to market-size.

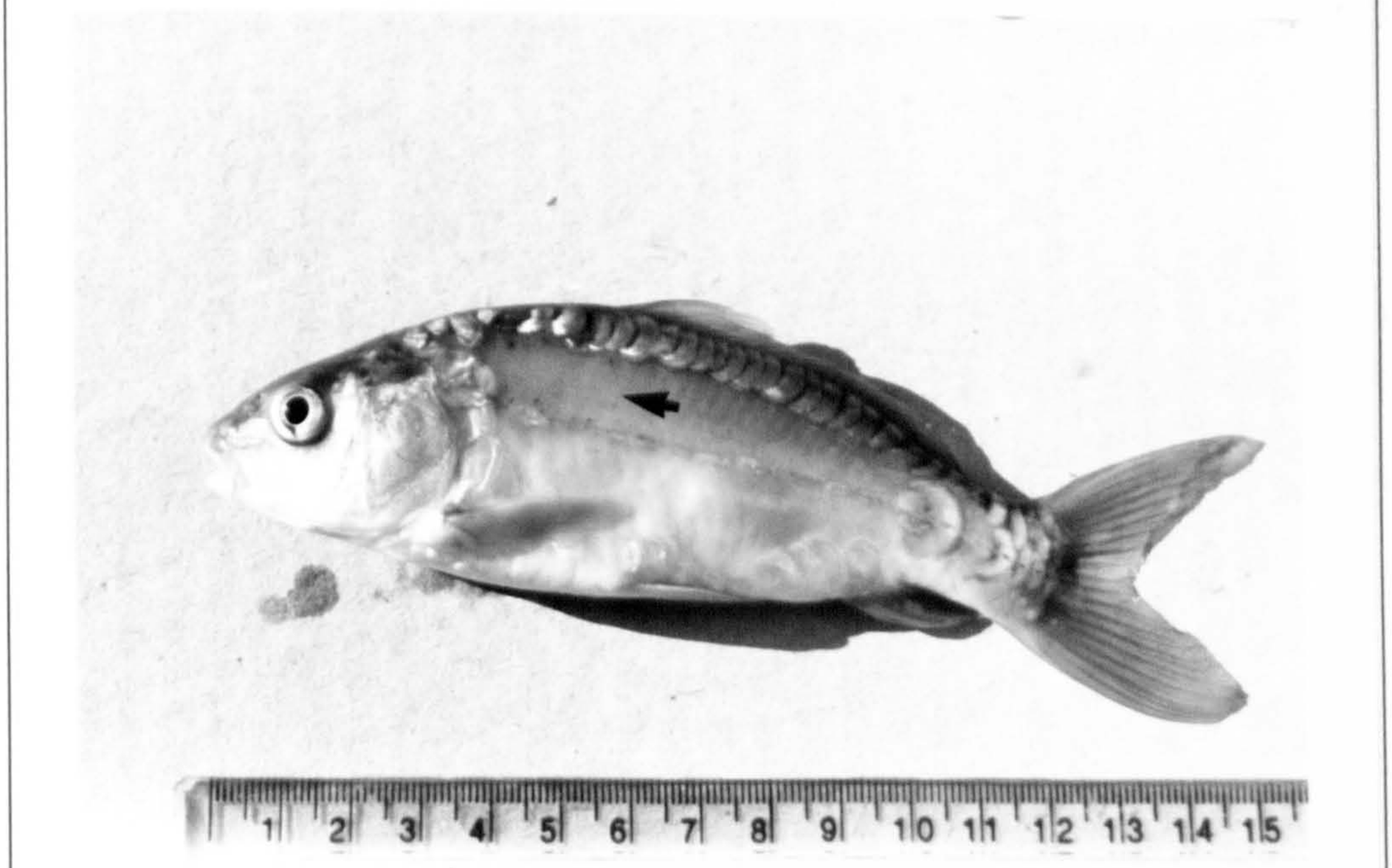
Briefly, the techniques of carp culture are highly diversified, ranging from the extensive production systems in pond or openwater with no fertilisation or supplemental feeding, to highly intensive systems in concrete tanks or cages. Like production of other animals, carp production invariably has environmental effects, but compared with other fish species (e.g. salmonids), carp culture has limited negative impacts on the environment, and may play a positive role in well-managed integrated systems, by recycling efficiently the wastes from other livestock animals. The carp is well adapted to intensive cultivation in ponds and it seems that intensive pond culture has limited environmental impacts compared with other intensive production systems (Kestemont 1995).

1.9 Mirror carp (*Cyprinus carpio* Var. *specularis* L.)

The main morphological characteristic of the mirror carp is the scattered, linear or nude scale-pattern. The mirror carp is also characterised by its protruding eyes, relatively short body and high back (Sumantadinata 1995) (Fig. 1.1). The mirror carp

was used as experimental fish in 4 experiments, and the common carp in 1 experiment of the present study.

Fig. 1.1 The normal mirror carp. Arrow head shows the site of incision or injection made on the experimental fish during the present study.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CHAPTER 2:

FISH SKIN AND MUSCLE

2.1 INTRODUCTION

The carp (*Cyprinus carpio* L.) is widely cultured fish species and is of a great importance for the aquaculture in many countries. In most countries of Europe and Asia, and in some area of Central America, the common carp is the most important cultured fish (Horvath *et al.* 1992).

The skin is an important part of the body of fish and its functions have been described by many workers (Chapter 4). Briefly, it is the first line of defence mechanism and protective capacity against pathogens, a complex limiting membrane, a mechanical protection for body and preservation and regulation of the homeostasis of the internal environment. It entraps micro-organisms and parasites by mucus, has immunological function in fish immunology, and responds quickly to stressors. The early recognition of possible danger by sensory receptors, providing a wide range of mechanisms against potentially invasive agents by lysozymes, proteolytic enzymes, complement component and antibodies, are other functions of the skin. Sensory receptors including specialised taste and touch receptors are present on the skin. Therefore the teleosts skin is a vital tissue involved in the protection and maintenance of homeostasis, and also locomotion (mucus acts as a lubricant, reduces the friction between the fish and its environment) of the fish (Pickering & Macey 1977). The skin as a cover for body of fish, and a protecting barrier for the fish, is also considered as a main criterion for fish health.

Musculoskeletal system (flesh) has also an important part in body locomotion and furthermore as human food. Muscle accounts for 40-60% of the total body mass of fish, more than in any other group of vertebrates (Wieser 1991). Different muscle tissues comprising different fibre types are found in fish. These tissues together with

adipose tissues compose the edible part of fish and are responsible for most of protein retention (Fauconneau *et al.* 1995).

Since the present investigation was carried out using skin and muscle of carp as substrate for making surgical wounds and injection of different stimuli, therefore it is necessary to know the structure and functions of the normal skin and muscle of carp.

2.2 SKIN AND MUSCLE

2.2.1 Skin

As in all vertebrates, the external surface of the teleost fish is continuous with that of the urogenital, digestive and respiratory systems (Bullock & Roberts 1975). The skin is the primary barrier against the environment and the first line of defence mechanism against diseases. Its condition is also important to allow normal physiological function.

Aspects of the structure and physiology of the skin of a wide range of teleost fishes have been studied by many workers (Brown & Wellings 1970; Whitear 1970; Fishelson 1972; Hawkes 1974; Bullock & Roberts 1975; Roberts 1989). Although the finer detail of the structure of the skin varies among the different groups of teleost fish, it has a number of features in common. Teleost fish skin is composed of layers of cuticle, epidermis, basement membrane, dermis and hypodermis.

Although there have been numerous studies on the fish skin, literature on the skin of carp is limited (Singh & Mittal 1990). The skin of carp is composed of the layers similar to those of other teleost fish. Also subcutaneous muscles are under the hypodermis (Fig. 2.1). The detail of the normal histology of the carp skin has been studied by Liewes (1977). He carried out the study by sampling skin from a variety of sites on the body, head and fins and defined the carp integument. Also Singh & Mittal (1990) carried out a comparative study of the epidermis of the common carp and three Indian major carp. Their investigation was on the skin covering the head region of the carp which is devoid of scales.

The details of carp skin which have been described by Liewes (1977) and Singh & Mittal (1990) will be used in this chapter to describe the skin of carp.

2.2.1.1 Cuticle

Cuticle is the external coating layer of skin comprised mainly of mucopolysaccharide, approximately 1μm thick and normally secreted largely from the surface epidermal cells rather than from goblet mucous cells. It is a complex of cell protoplasm, sloughed cells and mucus that has been released onto the surface. Under normal conditions mucus is a minor component compared with the contribution from the Malpighian cells. Cuticle has capability of resecretion and repair, and protects fish skin against abrasion and infection by pathogenic organisms, or by parasites (Whitear 1970; Bullock & Roberts 1975; Ellis *et al.* 1989).

Cuticular layer also contains specific immunoglobulins (Fletcher & Grant 1969; Baldo & Fletcher 1973), lysozyme that differs in amount considerably between marine species (Fletcher & White 1973), and free fatty acids (Lewis 1970) which are believed to have protective activities (Roberts 1989). The cuticle has a very delicate attachment to the surface of the layer of Malpighian cells, therefore it is lost during histological preparation, resulting in difficulty in demonstration in histological sections (Whitear 1970; Hawkes 1974).

2.2.1.2 Epidermis

Epidermis is the outermost living tissue. It is an avascular layer of cells, primarily of ectodermal origin, overlying the mesenchymal dermis (Leydig 1850 cited in Bullock & Roberts 1975).

In adults of most species the epidermis is a weakly stratified squamous epithelium that covers the whole body surface and also the fins (Bullock & Roberts 1975). Epidermis of the fish is living and capable of mitotic division at all levels

(Henrickson 1967; Roberts *et al.* 1970). The thickness of the epidermis varies with species and also with the position on body, stage of reproductive cycle and age of the fish. It is usually thicker in those fish which are scaleless or with only a few scales and also over the fins (Bullock & Roberts 1975).

The basal layer of epidermis comprises a layer of cuboidal or columnar cells with oval or spherical nuclei (Henrickson & Matoltsy 1968*a,b*; Mittal & Munshi 1971; Merrilees 1974) which rest on a single basal glycoprotein membrane (Roberts *et al.* 1971*b*). Peleteiro and Richards (1985) showed that there are clear spaces between basal cells and lymphocytes either isolated or in small groups could be observed in these clear spaces attached or close to the basal layer. Peleteiro and Richards (1990) also showed in an electron microscopical observation of skin of rainbow trout that several types of phagocytic cells were present in the epidermis, most notably macrophages, some of which were seen crossing the basal lamina.

The fundamental structural component of the mid-layer of epidermis of the teleost fish is the filament-containing Malpighian cells. It also contains mucous cells, club cells, granular cells and other cells eg. rodlet cells, and special sensory structures.

The epidermis in carp, which varies in thickness depending on the site of the body, could be divided into some layers which are generally not distinct (Fig. 2.2). These layers from the base outwards are;

1) Basal Malpighian cells, attached to the basement membrane.

2) Malpighian cells with dark nucleus.

3) Club cells. Also Malpighian cells are seen in this layer vertically orientated.

4) Horizontally orientated Malpighian cells.

5) Mucous cells which are supported by Malpighian cells with a round nucleus.

Variations in the structure of the epidermis could be observed in different sites of the body. The basal layer Malpighian cells are arranged in a single layer on a thin non-cellular basement membrane. They are cuboidal to cylindrical in shape, apically placed and sometimes have intercellular space between them.

The middle layer epithelial cells are arranged in several layers. They are, in general polygonal with centrally placed rounded nuclei. Where the large and voluminous club cells are present, the Malpighian cells are vertically elongated with elongated nuclei, due to lateral pressure. The Malpighian cells towards the top of the epidermis are more horizontally orientated and generally acquire a polygonal shape with rounded nuclei. They are arranged in several layers in the outer regions of the dermis. In the superficial layer, the Malpighian cells appear very much flattened with flattened nuclei.

2.2.1.2.1 Malpighian cells

These cells are always present in the teleost epidermis. In fish, the filament-containing cells (Malpighian cells) are usually rounded cells and very similar in structure whether they come from the basal or mid-layers of the epidermis but different from the outermost layer that are horizontal and flattened (Roberts 1989). Bullock *et al.* (1978) showed that all Malpighian cells are vital, actively respiring and capable of division. The highest concentration of dividing cells is in the supra-basal layer. Peleteiro & Richards (1990) suggested that filament-containing cells are also capable of phagocytosis.

Malpighian cells in carp are described in 2.2.1.2.

2.2.1.2.2 Mucous cells

Mucous cells are derived from the basal layer of the epidermis (Van Oosten 1957; Harris & Hunt 1973). They are found in the epidermis of all teleosts and vary in the number and size in different species and site. Immature mucous cells are rounded but they flatten laterally and increase in their size and secretory material as they mature and approach the surface (Bullock & Roberts 1975). They are large, clear, ovoid, spherical or flask-shaped in haematoxylin and eosin stain (Pickering 1974).

Presence of high-molecular-weight, gel forming macromolecules in mucus makes fish slippery to touch (Shephard 1994). The predominant gel-forming macromolecules are glycoproteins (mucins) (Asakawa 1970; Harris & Hunt 1973; Fletcher *et al.* 1976). In invertebrates mucopolysaccharides contribute to slimes (Hunt 1970). The glycoproteins in fish mucus appear to be similar to mammalian mucins (Harris & Hunt 1973). They maybe neutral but sialic acid (a carboxylated monosaccharide) is often present. (Enomoto *et al.* 1964; Harris & Hunt 1973; Pickering 1974; Fletcher *et al.* 1976; Pickering & Macey 1977). Other materials which have often been shown in fish mucus are; glycosaminoglycans, lysozyme, immunoglobulins, complement, carbonic anhydras, lectins, crinotoxins, calmodulin and proteolytic enzymes (cited by Shephard 1994). Roberts *et al.* (1973) reported that mucous cells in epidermis of plaice larvae contain glycoproteins without acid groups, and the sulphated acid groups appear at about 30 days after hatching. It seems that the composition and origin of fish mucus varies (Pickering 1977; Pickering & Macey 1977).

Mucous cells in carp could be seen in large numbers mainly in the outer regions of the epidermis but only a few mucous cells are in the lower levels of the epidermis.

The younger mucous cells, with usually a round shape and a round eccentrically-placed nucleus close to the cell wall, are between the Malpighian cells mainly in the middle layer and above the basal layer. The older mucous cells (goblet cells) with a flattened basally-placed nucleus and a more pyriform shape can be seen in the top layer secreting their contents onto the epidermal surface. The secretory contents of the mucous cells contain a mixture of neutral and acidic (non-sulphated and sulphated) mucopolysaccharides, but a few mucous cells contain only neutral mucopolysaccharides. Therefore they have different staining properties. In PAS stain, neutral mucopolysaccharides are red and acidic mucopolysaccharides are purple and dark blue.

2.2.1.2.3 Club cells

Club cells have been described as "club cells", "Leydig cells", "goblet cells" and "giant cells", but the term "club cell" is commonly used in the literature. They take their origin from columnar basal cells (Mittal & Munshi 1970). Club cells are a group of large and usually round cells found in the lower and middle layers of the epidermis of some groups of teleost fish. They appear to secrete a potent alarm substance when the fish is under stress eg. in the presence of a predator, and in this way the other fish may be alerted (Bullock & Roberts 1975; Roberts 1989). Club cells are large and capacious in size with two nuclei (some species) close to each others arranged irregularly in some layers. In most of the fixatives used the cytoplasm of the club cells shows shrinkage and the cell outlines are not clearly distinguishable (Mittal & Munshi 1970).

Club cells in carp are large, voluminous, round to ovoid with a slightly eosinophilic pale staining cytoplasm and a single rounded, or irregular shaped, centrally placed nucleus. They are found in the middle layers and very occasionally at the surface of the epidermis, but are never found close to the basement membrane. The club cells very near to the surface of the epidermis are generally rounded and smaller than those in the deeper layers. The contents of the club cells exhibit variable degrees of shrinkage and vacuolisation with various fixatives, appear homogeneous and stain pink with H & E and PAS.

2.2.1.2.4 Granule cells

Granule cells are found in the epidermis of many teleost species. They are round with single large eosinophilic granules, and are scattered between Malpighian cells (Bullock & Roberts 1975). The only reliable criterion for identifying these cells is the presence of refractile eosinophilic granules within their cytoplasm (Ellis *et al.* 1989). The nucleus in these cells is irregular and pushed to one side of the cell by the large granule (Phromsuthirak 1977). The position of these cells in the epidermis varies in different species. They are basal in some species and in some other are near the epidermal surface. There are some reports indicating their poorly phagocytic activities, but it is still not clear whether or not they have a role in antigen sampling and processing (Ellis *et al.* 1989; Barnett *et al.* 1996).

These cells in carp are round or oval with an either centrally or eccentrically placed nucleus and hyaline, eosinophilic granules in their cytoplasm. The number of granular cells are relatively higher in sites of the body with a good blood supply. The

large refractile eosinophilic cytoplasmic granules of these cells in H & E staining is a good criterion for identification (Liewes 1977).

2.2.1.2.5 Lymphocytes

These cells are described in chapter 3.

2.2.1.2.6 Rodlet cells

Rodlet cells occur in both freshwater and marine teleost tissue (Smith *et al.* 1995*a*) and consisted of a thick capsule enclosing a number of small, dense rodlets. The nucleus of the rodlet cell is displaced to one end (Flood *et al.* 1975; Mattey *et al.* 1979). Rodlets are moderately PAS-positive, but the capsule is non-reactive. These cells are various in morphology among species (Smith *et al.* 1994). Rodlet cells connect to the adjacent cells by the desmosomes and tight junctions. The luminal part of the cells contain many vesicles and tubules, but the basal part of cells have some club-like inclusions. A layer of small, elongated mitochondria occurs between these two layers (Leknes 1986). They liberate proteins which maybe enzymes or antibiotic material (Leino 1982).

Rodlet cells in carp are oval, with the nucleus in a polar position. The cytoplasm contains elongated membrane-bound granules. The granules contain a tubular structure which sometimes contain a very electron-dense inclusion (Cenini 1984).

Since 1892, when Thelohan described the rodlet cell as a parasite, whether they are normal component of tissue or a parasite has been discussed. "A normal tissue component", "a type of granulocyte", "not pathological", "a commensal parasitic

organism", "a normal tissue constituent engaged in holocrine secretion", and "migrating secretory cells", are different descriptions by many workers until 1979 (Smith *et al.* 1995*b*). Smith *et al.* (1995*b*) also suggested that rodlet cells are normal component of teleost tissue, and possibly represent a form of matured granulocytes. Richards *et al.* (1994) reported that previous observation suggested that they may have a role in ion transport and osmoregulation, secretion and lubrication, a pH buffering capability and/or antibiotic effects. TEM observation in carp demonstrated phagocytosis of rodlet cells and liberation of rodlets within the spleen and pronephros, which may indicate that these cells are parasitic and not of fish origin. It has also been suggested that rodlet cells maybe a stress-induced type of fish cell produced in response to adverse environmental conditions (Smith *et al.* 1994). Iger *et al* (1994) reported presence of rodlet cells in the epidermis of stressed trout (*Oncorhynchus mykiss*) with a strong peroxidase activity in the rodlets of these cells. However, their function is yet unclear, but they show clear similarity to the morphology, behaviour and distribution of granulocytes in other vertebrates (Smith *et al.* 1994).

2.2.1.2.7 Sensory organs

Three groups of sensory organs are present throughout the epidermis of teleost fish and their position varies in different species. These groups are; Group A, with free superficial neuromasts and canal organs, contained within the lateral line canal; Group B, taste receptor organs (taste buds); Group C, chemical receptors.

The free neuromasts on the integument in higher concentration are found around the snout, head and the opercula. The upper part of the neuromast is composed of a flexible jelly-like structure named the cupula which is attached to the apical edge. It is

very sensitive to mechanical damage (Bullock & Roberts 1975). The cupular substance is comprised of a microfibrillar network which knitted with the bundle and connect with the stereocilia and kinocilia that probably serve the mechanical transmission of cupular shearing to the sensory cells (Flock 1965). The sensory components of the neuromast consists of a cluster of pear-shaped mechanoreceptor cells, the apical surface of which bear numerous bundles of sensory hairs. This sensory component is encased in fusiform supporting cells that are situated near the periphery of the neuromasts extend around the basal ends of the sensory cells. The latero-distal surface of the neuromast is capped by a thin layer of flattened mantle cells (Bullock & Roberts 1975).

Flock (1971) has demonstrated by scanning electron microscope that both free neuromasts and lateral line canal organs have a similar ultrastructure, but Dijkgraaf (1963 cited in Bullock & Roberts 1975) reported that the main differences between them were that the canal organs become directly affected by external currents whereas the free neuromasts may require stimulation by local displacement of the canal fluid.

In addition to the mouth, pharynx, gill cavity and gill arches (Hara 1971) the taste organs are also present on the integument around the snout and lips and, in some fishes, over the whole body surface and even an appendages such as the fins and barbels (Gobiidae and Gadidae) (Herrick 1904). Taste buds are spherical or flask-shaped and surrounded by the epithelioid cells and are made up of different cell-types, namely, receptor or sensory cells and basal or supporting cells (Cordier 1964; Hirata 1966; Bardach & Atema 1971; Murray 1973; Kapoor *et al.* 1975; Reutter 1978). The receptor cells are fusiform and the apical edge of the cell bears a sensory hair in contact with the surrounding medium. The basal cells lie at the base of the taste bud and are connected

to the supporting cells by protoplasmic threads (Herrick 1904). The nerve plexus is located between the basal cells and the sensory cells (Reutter 1982).

The chemical receptors are on the oral epithelium and upon the body surface (Bullock & Roberts 1975). Ultrastructurally the chemosensory cells are different from the surrounding filament-containing cells. They are single units with a centrally placed nucleus, and a distal process terminating in a microvillus at the apical edge (Whitear 1971).

In carp, the free neuromasts and taste buds are observed in the epidermis. The free neuromasts are only found in the skin of the head. They were not encountered in sections of skin from the lateral line region. Also the taste buds could be found in the skin of all part of the body. They could be seen in very large number in the epithelium of the barbels, skin of the head and on the edges of the fins.

The free neuromasts in carp could be seen on a considerably lower and much wider dermal papilla than the taste buds, because they lie on the bottom of a hollow in the epidermis. The supporting cells with a more round nucleus are basally placed in the free neuromast while the sensory cells with a larger and more elongated nucleus are placed in the middle of the free neuromast. The cupula, consisted of the joined sensory hairs of the sensory cells is difficult to be seen because they easily tear from the skin during processing.

The taste buds in carp are either restricted to the outer regions supported by prominent dermal papillae or traverse the whole thickness of the epidermis, and they show moderate reaction for neutral mucopolysaccharides. The taste buds consist of two cells types. One cell type are spindle shaped sensory cells with a large elongated nuclei which have a sensory hair extending to the epithelial surface. Another cell type are

supporting cells with round nuclei. The Malpighian cells around the taste buds correspond to the space available.

2.2.1.3 Basement membrane

The basement membrane is located under the epidermis (Fig. 2.2) and its thickness varies in different parts of the body (Phromsuthirak 1977). It is more prominent on scaleless fish and has a strongly PAS-positive reaction suggesting a very high proportion of carbohydrate. It is very complex and ultrastructurally made up of the condensed basal plasmalemmata of the innermost epithelial cells, a clear electron-lucent zone and the basement membrane proper, a layer of electron-dense fine fibrillar material which is probably the site of insertion for reticular fibres from the dermis. Its morphology depends on the site and species. It plays a significant role in the strength of the skin and its important physiological role in osmotic homeostasis of the internal milieu in hypotonic and hypertonic environments (Roberts *et al.* 1970; Bullock & Roberts 1975). It was suggested by Fleischmajer & Billingham (1968) and Phromsuthirak (1976) that the basement membrane maybe formed by the combined action of both the epithelium and the connective tissue from the dermis.

2.2.1.4 Dermis

The dermis is the middle layer of skin which is derivative of the mesoderm (Gaines & Rogers 1975) composed of two distinct layers, stratum spongiosum and stratum compactum. Stratum spongiosum is the upper layer adjacent to the basement membrane and stratum compactum is the lower layer of dermis close to the hypodermis (Roberts 1989). These two layers are clearly seen in most teleosts, although in the

scaleless fish dermis differentiation is not so pronounced (Mittal & Munshi 1971; Grizzle & Rogers 1976). The dermis has a number of important functions. It provides the structural strength of the skin by collagen fibres and scales and supplies the outer limits of the tissues with vascular and neural components. It also contains the pigment cells (Bullock & Roberts 1975).

2.2.1.4.1 Stratum spongiosum

The stratum spongiosum is a loose network of collagen fibres. It contains a variety of vascular and neural components and also scales and fibrous tissue. The cellular components of this layer include the pigment cells, mononuclear cells, mast cells and the cells of the scale synthesis (Bullock & Roberts 1975).

In carp, the thickness of stratum spongiosum varies considerably with the area of the body. In the collagen network of stratum spongiosum in carp, in addition to pigment cells, blood capillaries and nerve bundles, also fat cells could be observed where the stratum spongiosum is reasonably thick (Fig. 2.2). Blood capillaries are at all levels in this layer of dermis. Pigment cells are under the layer of straight collagen fibres which are below and parallel with the basement membrane. These cells are generally arranged linearly in the skin of the body, but they are more widely disposed through the stratum spongiosum in the skin of the head and mouth.

At the sites where free neuromasts and taste buds in the epidermis could be observed, a core of dermal tissue extends up into the epidermis, reaching the base of the sensory organ.

2.2.1.4.1.1 Scales

The scales are flexible calcified plates lying within shallow scale pockets and can be observed in the outer loose connective tissue of the epidermis (Bullock & Roberts 1975). They are arranged in an overlapping pattern in diagonal rows. This pattern forms during the early stages of life and is retained during the life of the fish. The scales have capability for replacement if they are lost, even if the scale pocket is severely damaged (Reif 1978). Two main types of scale are designated; cycloid and ctenoid, which are differentiated by their surface pattern. There is no scale in naked species and some other species are covered by large bony shields or heavy dermal spines (Bullock & Roberts 1975).

Scales are composed of an inner fibrillar layer and outer calcified layer, both derived from mesoderm and are similar to bone in their calcification (Hyman 1962; Neave 1936*a,b*; Brown & Wellings 1969; Van Oosten 1957). The deeper layers are only collagen fibres embedded in the matrix material. Collagen of the scale differs from that of the dermis in its staining properties (Brown & Wellings 1969).

Ultrastructurally, scale is composed of three basic components; collagen fibres, a matrix of albuminoid material diffused between collagen fibres, and an inorganic deposit mainly calcium phosphate and calcium carbonate giving the scales their hardness and rigidity (Brown & Wellings 1969). Scale growth continues throughout life by addition of new material at the edges causing new rings similar to the annual rings formed in the trunk of the growing tree (Neave 1936*b*; Van Oosten 1957). In many species, notably the salmonids, they maybe used to determine the individual's age (Bullock & Roberts 1975).

Scales in carp are cycloid and placed within the stratum spongiosum nearly parallel to the stratum compactum, and are never seen embedded in the compact collagen layer. They are embedded rather loosely and superficially and the border of a scale is covered by the next scale. The epithelium covering the scales is not different from that on the unscaled skin. Between this epithelium and the scale, a thin layer of spongiosum can be seen.

2.2.1.4.1.2 Pigment containing cells

The colour changes in fish to match the environment, or due to sexual activity or in response to disease, are produced by specific pigment cells of the stratum spongiosum known as chromatophores. Chromatophores can change the colour of fish by their absorptive and reflective properties (Bullock & Roberts 1975; Roberts 1989). They usually are classified according to the nature of their pigments as melanophores, lipophores, iridophores and leucophores (Fujii 1969; Hawkes 1974). Bagnara & Hadley (1973) have described the comparative physiology of the chromatophores of teleosts and other poikilotherms in some detail. Also Roberts (1975*a*) reviewed the morphology and ultrastructure of the melanin-containing cells and their relation to disease in details.

2.2.1.4.1.2.1 Melanophores

Melanophores, dark pigment-containing cells, are derived from melanocytes and contain large numbers of melanosomes or mature melanin granules. They are asteroid cells in the stratum spongiosum of the dermis which participate with other chromatophores in the rapid colour changes by intracellular displacement of

melanosomes (Roberts 1975*a*). Melanocytes are mainly in the deeper layers of the stratum spongiosum and in the hypodermis. They are more dendritic than asteroid (Roberts 1975*a*). Melanocytes are immature melanophores actively producing melanin but capable of becoming melanophores and moving up into the functional melanophore layer (Bullock & Roberts 1975).

2.2.1.4.1.2.2 Lipophores

Lipophores are chromatophores containing pigments soluble in organic solvent, but insoluble in water. They are subdivided into erythrophores which contain red pigments, and xanthophores containing yellow pigments. These pigments are mainly carotenoids (Roberts 1989). The absence of carotenoids from the diet eventually results in loss of the lipophore colour pigment (Bullock & Roberts 1975).

2.2.1.4.1.2.3 Iridophores and leucophores

Iridophores contain purines, usually guanine, in the form of reflective plates responsible for the white and silver colours. The purine is contained in the form of platelets of reflecting material in the cytoplasm of these cells (Bullock & Roberts 1975). The orientation of the arrays of platelets may vary markedly with the site on the body (Harris & Hunt 1974, cited by Bullock & Roberts 1975). Leucophores are very similar to iridophores, but their guanine is arranged much more irregularly (Fujii 1969).

2.2.1.4.2 Stratum compactum

The stratum compactum is a dense matrix of a series of layers of collagen fibres at right angles to each other, over the hypodermis. Columns of collagen bearing the

vessels and nerves run through the stratum compactum vertically at regular intervals to serve the stratum spongiosum. Although fibroblasts are normally the only cellular component distributed between the collagen fibres, melanocytes and mast cells are found where the matrix is penetrated by nerves and vessels (Bullock & Roberts 1975). There is an inverse relationship between the thickness of the stratum compactum and the development of the scales (Mittal & Munshi 1971).

The stratum compactum in carp is composed of a wave-like collagen fibres. The thickness of this layer varies on different areas of the body. The lateral side of the body has a rather thick and distinct stratum compactum, while it is considerably thinner in the fins and in the skin covering the skull.

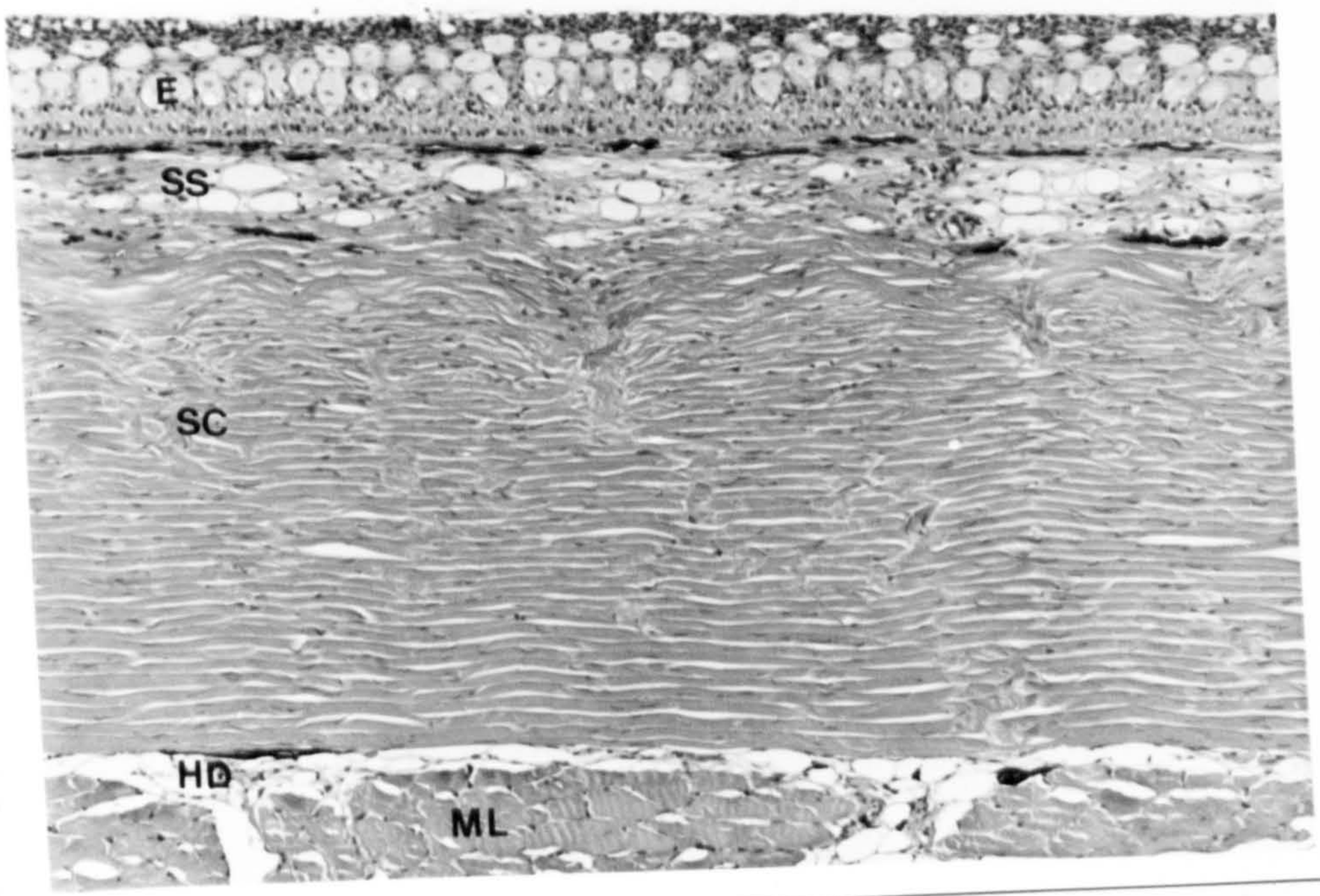
2.2.2.1.5 Hypodermis

This layer is derived from the mesoderm (Gaines & Rogers 1975) situated under the stratum compactum. It is composed of loose connective tissues, richly infiltrated with fat cells and also the main branches of dermal blood vessels and nerves (Mittal & Munshi 1974). The flexibility of its structure allows considerable movement of the integument between the stratum compactum and the superficial myotomes. In addition of vessels and nerves, the presence of a large numbers of fat cells and chromatophores is the most obvious feature of the hypodermis (Bullock & Roberts 1975).

The hypodermis in carp is thick in the skin around the lateral line, but it is absent in the skin covering the fins and the bony part of the head. Although there are some loose connective tissue fibres, blood vessels and nerves in this layer, the main component of the hypodermis is fat cells.

Fig. 2.1 This picture shows normal skin of mirror carp. The skin of mirror carp composed of cuticle, epidermis (E), dermis [stratum spongiosum (SS), stratum compactum (SC)] and hypodermis (HD). Also subcutaneous muscles (ML) are under the hypodermis. (H & E, X 110).

Fig. 2.2 This picture is an enlargement of Fig. 2.1 which shows epidermis (E) and stratum spongiosum (SS) of dermis. The epidermis composed of malpighian cells (MC), club cells (CC) and mucous cells (Mu). Basement membrane (arrows) is located between epidermis and stratum spongiosum. Loose collagen network (arrow head), pigment cells (P), capillaries (C) and fat cells (FC) are observed in stratum spongiosum. (H & E, X 440).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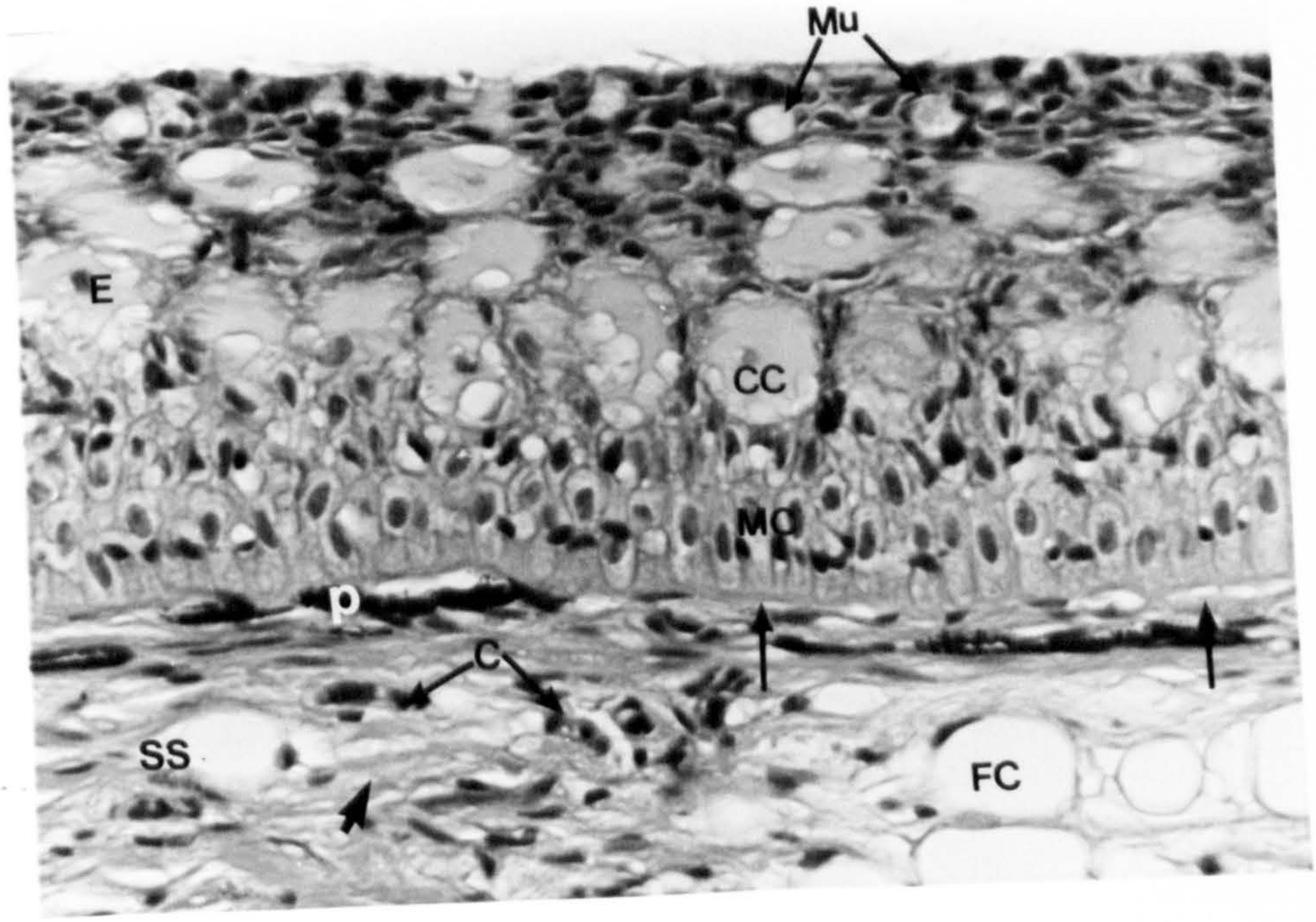
Mu
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2.2.2 Muscle

Fish like all other vertebrates have three types of muscle. These are smooth (gut muscle), cardiac (heart muscle) and skeletal muscle. The skeletal muscles are effectively attached to the firm skeleton and are primarily used in movement of skeletal components and in locomotion (Lagler *et al.* 1962).

Most fish swim by sequential contraction of the muscle blocks or myomeres of the trunk. The most obvious feature of the muscle in these fish is the folding and interlocking of the myomeres. Some fish swim by waving motions of certain fins. The appropriate muscles in these fish are highly developed, but the main myomeres maybe considerably reduced. In most teleosts, the skeletal muscles have two main subdivisions; the red muscle fibres and the white muscle fibres. The pink fibres are also found in some teleosts (Roberts 1989). In fish, the red and white muscle fibres are more clearly distinct from one another than in mammalian muscle, where the two types of fibres are normally mixed (Bilinski 1969). They are located in distinct anatomical regions, and can be characterised according to their histochemical and immunohistochemical properties (Vegetti *et al.* 1993). Kilarski (1990) in a histochemical study of the myotomal muscles in the roach (*Rutilus rutilus* L.) also showed three main muscle regions: red, intermediate and white, which were distinguished on the basis of glycogen content, succinate dehydrogenase (SDH), and myofibrillar ATPase (mATPase) activity. He also found a tonic-like fibre and the presence of a transitional zone with two fibre types, and the mosaic organisation of the white fibre region. He described tonic-like fibres as arranged in the form of a thin sheet of single fibres that covers both lateral sides of the red muscle triangular wedge, and partly separates the two phasic muscle zones from each other.

The myotomes which comprise the bundles of white muscle fibres are bound together by connective tissue (perimysium). The individual muscle fibre is surrounded by a delicate endomysium. In the normal myofibre the nuclei of each fibre lies immediately below the sarcolemma (Roberts 1989). The muscle perimysium has an important role as a retaining barrier among the muscle bundles. It limits the spread of necrosis and noxious agents in a muscle (Finn & Nielson 1971*a*; Anderson & Roberts 1975).

Skeletal muscle in carp is composed of different muscles: red superficial muscle, a pink muscle and a deep white muscle. These tissues contain very different fibre types (Fauconneau *et al.* 1995). The characteristics of the three skeletal muscle types in Cyprinids are mainly the consequence of specificities in fibre type (Johnston *et al.* 1977; Sanger 1992). In higher teleost, including carp, red and white fibres are innervated multiply, and probably even polyneurally (Bone 1978). In contrast to white fibres, red fibres do not generate action potentials but are activated by junction potentials (Hagiwara & Takahashi 1967). Red fibres generally have been considered to be slow tonic fibres (Hess 1970; Bone 1978).

2.2.2.1 White muscle fibres

The white muscle fibres constitute the major fraction of the myotomal muscle mass (Kilarski 1990). The white muscle is a compact mass muscle consisting mainly of fibres capable of fast contraction with poor blood supply and absence of myoglobin in the cells. They are anaerobic and used during strenuous swimming, particularly in bursts of activity or fast speed swimming, and for the rapid manoeuvres involved in hunting and flight. They constitute up to 90% of the total swimming musculature.

Vascularization in white muscles is not as developed as in the red muscle fibres, and the nerve endings in white muscle are usually terminal where the white fibres arise from the myosepta (Roberts 1989; Wieser 1991).

White muscle in carp is characterised by a higher ATPase activity than red muscle. White muscle is recruited only at high sustained swimming speeds. It also permits a burst of swimming activities. The presence of fibres of different size in white muscle gives it a classical mosaic appearance and a specific distribution pattern (Fauconneau *et al.* 1995).

2.2.2.2 Red muscle fibres

The red muscle of fish is usually situated under the skin, along the lateral line although some fish have developed deep-seated red muscle (Bilinski 1969). Red muscle fibres are aerobic and slow-contracting, similar to those of mammalian muscle. They are active during low speed swimming and are abundantly supplied with blood. The innervation of the red muscle fibres occur with the nerve endings in the middle of the muscle fibres (Roberts 1989). The red muscle in all the species so far examined has a simple segmental structure, in contrast to the complex overlapping arrangement of white muscle (Kilarski 1990). Red muscle fibres are usually little more than 10% of the muscle mass, and come into action for slower sustained swimming (Wieser 1991).

Red muscle in carp is richly vascularized and thus contains a high amount of myoglobin. It also contains a very large amount of lipid (Johnston *et al.* 1977; Sanger 1992). Red muscle is always active (Fauconneau *et al.* 1995).

2.2.2.3 Pink muscle fibres

In the Salmonidae and, to an extent, in Cyprinidae the pink fibres are found sandwiched between the red and white muscle fibres. The pink fibres have an innervation intermediate between red and white muscle fibres (Roberts 1989). Also Bilinski (1974) has reported that in Salmonids, the greater part of the myomere is made up of a mosaic of white and pink fibres rather than white fibres only. Kilarski (1990) described pink muscle fibres as intermediate fibres which separate the superficial red and deep white zones of the lateral muscle. They are usually small in relation to the total amount of lateral muscle, and show some histochemical similarities to both red and white layers.

The pink fibres in carp are intermediate between red and white fibres for energy metabolism, contractile characteristics and speed of contraction (Akster 1983; Rowlerson *et al.* 1985). At moderate swimming, speed contractions are observed in pink muscle (Fauconneau *et al.* 1995). The pink muscle in carp which is recruited at intermediate swimming speeds (Johnston *et al.* 1977), maybe of functional significance for post larval stages, which are characterised by a different mode of swimming compared to the larval fish (Batty 1984).

CHAPTER 3:

INFLAMMATORY RESPONSE IN FISH

3.1 Inflammation and inflammatory response

The term " inflammation" has been used since the earliest days of medicine. It is the sequence of events which begins following sublethal injury to a living tissue and ends with complete healing (Zweifach *et al.* 1965; Ogilive 1967; Cappell & Anderson 1971). According to Winter (1964), the inflammatory response is the basic protection mechanism to tissue damage of whatever cause. These responses have much in common, no matter what the original stimulus.

Inflammation occurs whenever pharmacodynamic amines, especially those found in mast cells, are released by any agent. Inflammatory changes are a universal non-specific response to physical traumata such as heat, mechanical trauma and ionising radiation, chemical injuries (organic and inorganic), death of cells from circulatory insufficiency, the immune reactions such as the cell mediated immunity tissue rejection reaction, and damage due to infective agents such as bacteria, fungi, etc. The time scale over which the lesion develops, depends on the nature of the insult and its persistence. If the insult is of short duration, then the tissue may return to normal rapidly, but if it is prolonged there are usually proliferative changes which gradually dominate over the exudative and cellular changes of the acute reaction (Ogilive 1967; Cappell & Anderson 1971; Roberts 1989).

The most important signs of inflammation in the higher animals which have been recognised since Roman times are; heat, redness, swelling, pain and loss of function. Each of these clinical signs can be related to one or other of the pathophysiological features of the inflammatory process (Roberts 1989). It is doubtful if heat can be considered a cardinal sign in the cold-blooded animals, swelling is limited, and it is difficult to demonstrate a pain response.

The main requirements for the development of an inflammatory response are that the basic structural integrity of the tissue concerned is maintained despite the injury or insult, and that a functional blood supply is maintained. Although the histopathological appearance of the inflammatory lesion depends on the tissue, the time scale, the cause of injury and the environmental temperature in cold-blooded animals, there is a general pattern to the inflammatory response at all evolutionary levels (Roberts 1989).

The changes occurring in inflammation are grouped into two classes, those typical of the acute inflammatory response, and the changes characterising chronic inflammation. They are not mutually exclusive and may occur together within the same tissue (Morehead 1965; Cappell & Anderson 1971).

3.1.1 Acute inflammation

Acute inflammation occurs in response to most stimuli. It is characterised by a sudden and short duration inflammatory process (Smith & Piper 1972). The acute inflammatory response consists of a triphasic event; a vascular, an exudative and a cellular component with a time limit of each of these phenomena which is directly correlated with environmental temperature in poikilotherms (Roberts & Bullock 1976). Changes in the amount and quality of blood reaching the affected area without any proliferation, is a circulatory phenomenon involved in acute inflammatory response (Smith & Piper 1972), which is responsible for the cardinal clinical signs of inflammation in mammals (Ward 1971). The redness occurs following the dilation of blood vessels due to stimulation of vasodilator nerves and release of vasoactive amines (Spector & Willoughby 1965). The heat (in mammals) is due to the increased blood

flow to the area (Ward 1971). The swelling is due to the increase of permeability of the walls of the capillaries and venules to larger plasma molecular components (Cappell & Anderson 1971), escape of serous fluids (Wright 1958) and migration of phagocytic leukocytes from blood vessels (diapedesis) into interstitial spaces in tissue (Addison 1843; Clark & Clark 1935; Marchesi & Florey 1960; Marchesi 1961). The degree of cellular migration depends, to a considerable extent, on the nature of stimulus (Roberts 1989). The pain in mammals is due to the pressure on nerve endings and/or the action of mediators such as bradikinin on these structures (Ward 1971). The serum proteins which pass into the tissue, play their roles in the inflammatory response by their antibody and complementary components. Prothrombin from thrombocytes activates fibrinogen to polymerise into fibrin. Fibrin provides a stroma of fibrous strands which limits the lesion (Roberts 1989).

In an acute inflammatory response different cells leave the blood for the tissue namely; neutrophils (polymorphonuclear leukocytes), monocytes or mononuclear macrophages and lymphocytes (Roberts 1989). During the early phase of the inflammatory process, the predominant cell in all exudates is the neutrophil. They are replaced by mononuclear cells as the inflammation progresses. If the inflammatory process is prolonged, lymphocytes may become quite numerous (Cappell & Anderson 1971). A significant difference in inflammatory response in fish from the mammalian response is the relatively minor role of the neutrophils (polymorphonuclear leukocytes). These are present in most inflammatory responses for a short time. The exact period depends on the type of insult and the temperature (Roberts & Bullock 1976).

The results of acute inflammation maybe complete resolution and healing, development of exudative or necrotic lesions, or progression to chronic inflammation, or death (Cappell & Anderson 1971; Smith & Piper 1972; Roberts 1989).

3.1.2 Chronic inflammation

If an acute inflammatory lesion does not resolve quickly, then chronic inflammation develops. Chronic inflammation is characterised by proliferation of local and neighbouring supporting tissues to produce a stroma of collagen. This proliferation also results from the successful resolution of an extensive acute inflammation insult in which case it develops into scar tissue, but if the acute inflammatory insult continues, the inflammatory and reparative responses take place together and chronic inflammation occurs (Cappell & Anderson 1971; Roberts 1989). A chronic inflammatory lesion needs not to be produced subsequent to an acute inflammatory process, and features of the chronic response may present from the beginning (Willis 1950; Morehead 1965; Cappell & Anderson 1971).

In general, chronic inflammatory responses are distinguished from the acute forms by the lesser prominence of vascular and granulocytic components and by the greater participation of macrophages and fibroblasts (Wright 1958).

Chronic inflammatory lesions characteristically contain macrophages, lymphocytes, fibroblasts, plasma cells and may in addition possess giant cells, especially in the presence of foreign bodies (Willis 1950; Wright 1958). The features of the chronic inflammatory response of fish are similar to those of mammals (Roberts & Bullock 1976).

3.1.3 Granulomatous inflammation

Granulomatous inflammation was recognised as a distinct entity in the early 19th century (Long 1965). It is a type of chronic inflammatory response to certain agents and commonly covers all forms of long-lasting inflammation. Originally the term "granuloma" descriptively defined tiny granular white bodies which could be seen in the tissue, as a result of systemic spread of the tuberculous bacilli. The term was later redefined according to certain microscopic criteria, to include all lesions showing features of chronic inflammation (Morehead 1965; Spector 1969; Ward 1971). A proliferative lesion which progresses to fibrosis is found in many fish diseases. These lesions which are white to yellow and may have a cheesy or hard consistency or even be calcified, are forms of granuloma. Granulomatous inflammation is characterised by the infiltration of the lesion by phagocytic cells (Roberts 1989).

A granuloma consists of a compact (organised) collection of epithelioid cells which surround the irritant, and is not necessarily accompanied by accessory features such as necrosis. If necrosis is not present, the lesion can be referred to as a pure granuloma and if it is present then the lesion is referred to as a complex granuloma. Organisation and activation of the mononuclear phagocytes in a granulomatous inflammation, separates granulomas from simple chronic inflammation (Adams 1976). Granulomatous inflammation is highly effective in surrounding irritants, and also immobilising and destroying some of the pathogens (Forbus 1943).

The granulomatous lesion develops as a central zone of necrotic cell material containing the initiating agent, with a surrounding layer of macrophages and other inflammatory cells. As the lesion matures the macrophages form into layers around the irritant and resemble an epithelium. These cells are large with pale staining nuclei, ill-

defined cytoplasm and communicating intercellular process. The superficial resemblance to the cells of epithelial tissue resulted in them being referred to as epithelioid cells (Morehead 1965; Carr 1973; Roberts 1989).

It is the macrophages which possess the remarkable capacity to form giant cells. These are found surrounding particular foreign bodies. They form a cellular defence against bodies which are too large to be ingested by single phagocytic cells and also against very resistant forms of micro-organisms such as *Mycobacterium* spp. (Willis 1950; Wright 1958). In fish, as in mammals, certain types of particularly resistant foreign bodies are said to produce a very distinctive type of chronic inflammatory response characterised by alternation of macrophages to form epithelioid cells around the stimulus. Subsequent fusion of these epithelioid cells form characteristic multinucleated giant cells (Roberts & Bullock 1976).

Also there is proliferation of fibroblasts, which actively produce collagen and appear very large compared with the small densely staining fibroblasts of normal fibrous tissue.

As the lesion develops, lymphocytes usually appear within the fibrous tissue, taking part in a cell-mediated immune response. A number of adjacent granulomata maybe interlinked by fibrous tissue and produce a very large lesion which eventually develops into an encapsulated, hard, avascular nodule (Roberts 1989).

3.2 Types of cells in inflammatory lesions

Several types of cells are involved in an inflammatory response in teleost fish. Their identification is essential to the histological study of the inflammatory reaction. The cells that are usually found in an area of inflammation (acute to chronic) are;

thrombocytes, neutrophils or polymorphonuclear leukocytes (PMNs), lymphocytes, plasma cells, macrophages, epithelioid cells, giant cells, fibroblasts, melanin-containing cells and eosinophilic granular cells (EGCs).

3.2.1 Thrombocytes

There are great variations in cell size and shape and staining characteristics of nucleus and cytoplasm of thrombocytes among the various fish species (Saunders 1968) and even seasonal variation in numbers (Gardner & Yevich 1969). They can be divided into four main morphological types; spiked, spindle, oval and lone nucleus forms. The function of thrombocytes is the clotting of the circulating fluids, and has been described in plaice by Wardle (1971). These cells secrete thromboplastin, the enzyme which polymerises fibrinogen (Roberts 1989). Phagocytosis by thrombocytes was reported by Yokoyama (1960) and Fange (1968). Ferguson (1976) found carbon particles present in the thrombocytes of plaice after intravenous injection. Ellis (1977) suggested that these cells are unlikely to be capable of phagocytosis proper, and their uptake of particulate matter maybe due to mechanical entrapment in the cytoplasmic labyrinthine vesicles which communicate with the environment via stomata in the cell membrane.

Thrombocytes are easily recognisable in carp (*C. carpio*) by their fusiform shape in longitudinal sections, while they are similar in appearance to the lymphocytes in transverse sections. It also possible to identify them sometimes by the presence of small vesicles in series and/or microtubules below the plasma membrane (Cenini 1984).

3.2.2 Neutrophils or polymorphonuclear leukocytes (PMNs)

In mammals they are found in blood and bone marrow and comprise about 65% of blood leukocytes. The major granulopoietic organ in teleost fish is the kidney though the spleen may play a major role. The numbers of circulating neutrophils in fishes vary to a considerable extent, and constitutes a smaller proportion than in mammals (Ellis 1977). Although they often have fewer lobes to the multi-lobed nucleus, they have very similar morphology and histochemical staining properties to mammalian neutrophils (Ellis 1976; Ferguson 1976; Cannon *et al.* 1980). Typically the mature neutrophil is a large rounded cell containing basophilic cytoplasm, which possesses granules that do not stain markedly with either basic or acid dyes at neutral pH (Ellis 1977). In mammals they are major phagocytic cells and appear rapidly at an inflammatory site (Ellis 1977), while there are different opinions regarding their function in fish. Neutrophils of plaice appeared not to be phagocytic towards carbon particles (Ellis 1976), and rainbow trout neutrophils did not take part in phagocytic activity in an inflammatory site caused by bacteria (Klontz 1972). Finn & Nielson (1971*a,b*) reported phagocytic neutrophils in rainbow trout during experimentally induced bacterial inflammation. Gold fish neutrophils were regarded as being highly phagocytic for thorotrast by Weinreb & Weinreb (1969) and for bacteria by Watson *et al.* (1963). Phromsuthirak (1977) showed in three-spine stickleback that neutrophils were phagocytic to carbon introduced into the blood although they were not obviously phagocytic within wounds. Neutrophils may frequently be seen in the earliest stages of inflammatory lesions and are usually the first cells to leave the vessels for the site of injury (Roberts 1989). They are generally only present for a short time and are of limited significance (Klontz & Anderson 1968).

The carp neutrophils show an eccentric, kidney-shaped nucleus. The cytoplasm contains a few mitochondria and varying proportions of large, electron-dense (dark) and small, less dense (light) rod-like granules surrounded by a membrane. All types of granules are uniformly distributed within the cytoplasm (Cenini 1984; Suzuki 1986).

3.2.3 Lymphocytes

Lymphocytes are found in the circulation, the lymphoid organs, and other tissues, particularly during inflammatory events (Ellis 1989). Also integumental membranes of fish should be regarded as immune-reactive tissues since under certain pathological or physiological circumstances, the number of lymphocytes present may increase significantly (Peleteiro & Richards 1985). They are highly differentiated cells which respond to immunological stimuli (Ellis 1977). White (1963) called them immuno-competent cells. The morphology of lymphocytes is similar throughout the vertebrates, though there maybe variation in the size range from one species to another. They have been divided into the categories of large and small lymphocytes (Ellis 1977). The nucleus of lymphocytes occupies virtually the whole of the cell leaving only a narrow rim of basophilic cytoplasm in which there are a few mitochondria and isolated ribosomes (Ferguson 1976). It has been suggested that lymphocytes in fish can recognise and migrate to appropriate tissue environments (Ellis & De Sousa 1974). They have been shown to carry *Ig* (*IgM*) in their surface membranes and can be transformed into antibody-producing cells (plasma cells) or their precursors.

Lymphocytes in carp are the smallest leukocytes, characterised by a large, sometimes indented, nucleus surrounded by a thin rim of cytoplasm with occasional

vesicles. The nuclear chromatin sometimes shows a cross-hatched appearance, with separation between dark and light phases (Cenini 1984).

3.2.4 Plasma cells

These cells, derived from *B* lymphocytes in birds and mammals, are responsible for the production of humoral antibodies. Typically they possess an eccentric nucleus with prominent nucleolus and pyroninophilic cytoplasm packed with rough-surfaced endoplasmic reticulum (Ellis 1977). Evidence for the existence of plasma cells in fish comes from the immuno-fluorescent technique (Ellis 1976). Also Timur, M. (1975) recorded the presence of plasma cells in plaice. He described plasma cells as more or less oval in outline with a dense characteristically eccentric nucleus and condensed nuclear chromatin around the periphery of the nucleus. Cytoplasm of these cells in the Unna-Pappenheim staining method is dark pink to purple. The nuclei of some plasma cells shows a cart wheel chromatin configuration (Russell 1974).

Plasma cells in carp are characterised by an abundant rough endoplasmic reticulum (RER) in cytoplasm, and a well developed Golgi apparatus. The large and mainly euchromatic nucleus frequently show a slightly eccentric distinct nucleolus (Cenini 1984).

3.2.5 Macrophages

Macrophages are typically found in connective and other tissues and are not normally found as a component population of circulating leukocytes (Ellis 1977). They are mononucleated tissue cells, derived from circulating monocytes, which adhere to glass and plastic, are characteristically highly phagocytic or pinocytic and have an

undulating membrane (Laskin & Lechevalier 1972). The size of macrophages is very variable depending on species and state of digestion of any ingested material (Ellis 1976; Russell 1974). Their surface is irregular and in section they have several finger-like pseudopodia. The large nucleus is fairly irregular in outline with slightly marginated nuclear chromatin (Timur, M. 1975). Staining of the cytoplasm varies in colour and density depending on the physiological state of the cell. Large macrophages maybe irregular in shape with foamy cytoplasm when replete with phagocytosed material (Russell 1974). Macrophages act as protectors of the body and also as scavengers. They have large phagosomes and digest all sorts of digestible foreign materials, necrotic debris and even engulf red blood cells and where possible convert them into a soluble form so that they can be utilised by the body, eliminated as waste products, or at least be used to prime the immune system to stimulate an immune response (Ellis 1976 & 1977 & 1981; Phromsuthirak 1977). During an inflammatory response, macrophages become activated, that is they grow in size, undergo metabolic changes and develop increased phagocytic and killing properties. Their stimulation and responsiveness is non-specific but they affect and are affected by products and cells of the specific immune system (Ellis 1980 & 1981). They appear to be the dominant infiltrating cell in most cellular inflammatory responses in teleosts and are capable of developing into epithelioid cells and multinucleated giant cells (Roberts 1989).

The carp macrophages normally show pseudopodia, and contain a reniform, largely euchromatic nucleus, generally in a rather eccentric position with often prominent nucleolus. The cytoplasm is rich in mitochondria and contains well developed rough endoplasmic reticulum (RER). There are also many clear vesicles of

variable size, and occasional small, round, oval or elongated granules in the cytoplasm (Cenini 1984; Suzuki 1986).

3.2.6 Epithelioid cells

Epithelioid cells are a characteristic feature of many types of granulomatous inflammation. They appear as polygonal cells with an elongated, large, regular and pale nucleus, and cloudy eosinophilic cytoplasm with a variety of vesicles and large vacuoles. The outline of epithelioid cells tends to intermingle with that of its neighbours (Timur, M. 1975). It seems likely that the majority of epithelioid cells in lesions such as tuberculosis are derived form macrophages that have not ingested bacteria since macrophages can be prevented from developing into epithelioid cells if they phagocytose. It appears, therefore, that macrophages turn into epithelioid cells when they become immobilised at the site of inflammation without being called upon to undertake phagocytosis (Papadimitriou & Spector 1971).

3.2.7 Giant cells

Giant cells have an electron-dense cytoplasm and appear to be formed by macrophages (Dumont & Sheldon 1965). Giant cells, produced by fusion of macrophages, occur readily in all fish species, although there are temperature-dependent time scales governing their appearance and regression (Timur, M. 1977). They are generally irregular in outline and vaguely circular, oval or spherical. Their multiple nuclei are either distributed at the periphery, in a horse shoe configuration, which are known as Langhans types, or distributed throughout the cytoplasm which are known as foreign body types (Timur, G. 1975). Milne (1972) also explained the Touton type of

giant cell in humans which is seen in the lipid granulomata. The cell is circular and contains a perfect ring of nuclei situated midway between the centre of the cell and periphery. Another type of giant cell is the asteroid giant cell seen in Pleuronectid fish with a large structure usually found in groups and associated with granulomata (Roberts & Bullock 1976). Timur, G. (1975) introduced a further type of giant cell in plaice named "intermediate giant cell" with its nuclei at the periphery of the cytoplasm and also scattered in the middle of the cytoplasm. It was produced in response to a foreign body, talcum powder (magnesium silicate). The numbers of nuclei in giant cells vary from two to over 100 and they usually develop in association with material which is not readily digested (Roberts 1989). Giant cells are capable of phagocytosis, but appeared to have a lower phagocytic performance than surrounding macrophages (Secombes 1985).

3.2.8 Fibroblasts

Fibroblasts are an immature form of fibrocytes. Different sources have been reported as the source of fibroblasts such as; the mesenchymal perivascular connective tissue, those fibroblasts present in the endomysium of the striped muscle bundles (Mittal & Munshi 1974), metaplasia of mononuclear cells of the blood stream, the local mesenchymal cells (Mittal *et al.* 1978), the fibrocytes in the perimysium or from haematogenous cells derived from the vessels in the perimysium (Finn & Nielson 1971*a,b*). The view of local origin of fibroblasts is supported by majority of investigators (Edwards & Dunphy 1958; Dunphy 1963; Grillo 1963; Hadfield 1963; McMinn 1967). They considered the loose areolar perivascular tissue as the origin of fibroblasts. Fibroblasts appear in chronic inflammation and are highly active in the

production of collagen fibres. Active fibroblasts are swollen, elongated and their nuclear chromatin is denser at the periphery (Timur, M. 1975), whereas normal fibroblasts are spindle shaped, with finely speckled nuclei of a wide variation of size and density (Russell 1974; Timur, M. 1975).

3.2.9 Melanin-containing cells

Melanin is found in many parts of the body of fish. Melanin-containing cells are occasionally found in the epidermis but in high numbers in the dermis. The pigment melanin-containing cells of fish are the melanocytes and melanophores. Melanocytes actively produce melanin, and are capable of becoming melanophores and moving up into the functional melanophore layer. Melanophores are more regular, asteroid structures whereas melanocytes are more dendritic than asteroid. Melanocytes are found mainly in the deeper layers of the stratum spongiosum and in the hypodermis. They are responsible for synthesis of melanin in the melanosomes. They are also found in small groups at various levels of the circulatory system, especially in association with the larger veins and lymphatic. Loss of control of melanophores with consequent change of skin colour would appear to be a common clinical indication of disease within a fish population (Roberts 1975*a*).

Melanin-containing cells have been reported in fish in connection with certain pathological states. They have been observed in injured tissue of gold fish (Mawdesley-Thomas & Young 1967) and in the encystment response to metacercaria in the plaice (McQueen *et al.* 1973). Melanin-containing cells may play a role in bactericidal mechanisms (Ellis 1981), and also take part in a reaction against irradiation (photoprotection) by dispersion within dendritic processes (in low irradiation) or

aggregation in a dense patches (in high irradiation) in the skin (Bullock 1988). They also play a defensive role by their capacity for H_2O_2 generation (Edelstein 1971), and camouflage for fish.

The cells of the melanin-macrophage centres are distributed throughout the interstitial haematopoietic tissue of the kidney, the white pulp of the spleen and the pre-portal tissue of the liver. They are usually nodular with a delicate capsule and are frequently seen in close association with foci of lymphocytes. Melanin -macrophage centre cells are ultrastructurally very complex. They have indented nuclei and large numbers of membrane-bound vacuoles containing a variety of materials, and pigment granules in groups. The amount of melanin contained within the melanin-macrophage centre cells has little relation to the degree of pigmentation of the skin. They are considered a component of the reticulo-endothelial system participating in the defensive system of the fish. Melanin-macrophages can phagocytose breakdown products of haemoglobin (Roberts 1975*a*). Thorpe & Roberts (1972) reported that during severe septicaemia in brown trout, melanin-containing macrophages displaced from the kidney and spleen into the circulation. They may or may not contain granules of black pigment but the yellow-brown pigment, unstained in H & E sections, is always present. The yellow-brown pigments are PAS and ZN positive indicating that chemically they are of the melanin series (lipofuscin or ceroid) (Roberts 1975*a*).

3.2.10 Eosinophilic granular cells (EGCs)

Eosinophilic granular cells occur in abundance particularly in association with surface epithelial covering of the gill, skin, intestinal tract, and haemopoietic tissues of many fishes but they are rarely seen in fish blood (Roberts *et al.* 1972; Blackstock &

Pickering 1980; Ellis *et al.* 1989; Powell *et al.* 1990). It is generally assumed that EGCs are derived from haemopoietic tissue (Powell *et al.* 1990). They are characteristically packed with large refractile granules and the only criterion for identifying the EGCs of fishes has been the presence of these fairly large eosinophilic cytoplasmic granules. They are considered to play a role in defence mechanisms in mammals by phagocytosing antibody/antigen complexes. They may therefore have an important role in maintaining homeostasis during infection and are particularly numerous when antigens are continually being released as in parasitic diseases (Ellis *et al.* 1989). It has also been reported that they are histaminogenic (Ellis 1981 & 1985), and maybe similar to mammalian mast cells (Barber & westerman 1978). EGCs in fish have been implicated in both specific immunological and general defence processes (Powell *et al.* 1990). They also have been shown to degranulate after exposure to UV light (Roberts & Bullock 1981) and pathogenic bacteria (Ellis 1985; Powell *et al.* 1993). They also accumulate heavy metals (Zia & McDonald 1994) and have been reported to be part of the trout immune response to parasitic infections (Sharp *et al.* 1989). Barnett *et al.* (1996) described the presence, characteristics, and behaviour of motile granulocytes within and on the epidermis of *Oreochromis mossambicus* gill filaments and identified them as EGCs. They also confirmed the migratory ability of EGCs and stated that recent experiments suggest that these cells are poorly phagocytic.

EGCs in carp are round cells with a rather small, eccentric, oval nucleus with a considerable number of fine cytoplasmic vesicles. The main characteristic of these cells is the abundant, round to elongated cytoplasmic granules which are membrane-bound, uniformly distributed in the cytoplasm (Cenini 1984).

CHAPTER 4:

WOUND HEALING IN CARP (*Cyprinus carpio* L.)

4.1 INTRODUCTION

Fish have a skin which is very well adapted to their physiological requirements in terms of a limiting barrier (Roberts & Bullock 1976). The skin, as a complex limiting membrane serves many purposes in fish. It is the only barrier to the osmotic pressure differences between the internal and external environment. This barrier serves to preserve and regulate the homeostasis of the internal environment of the fish by osmoregulatory capacity of the epidermis, especially in the larvae, by respiratory and excretory mechanism (Bullock & Roberts 1975). Epidermal integrity is vital to fish in maintaining osmotic balance and excluding micro-organisms (Ellis 1989). Also the dermis plays a very important role in mechanical protection of the body (Bullock & Roberts 1975). Thermal adjustment to the environment, and osmoregulation, especially in the embryo, are made in part through the skin. It may also serve as a respiratory organ and as a receptor of stimuli (Van Oosten 1957; Jakowska 1963; Rosen & Cornford 1971; Cameron & Endean 1973). The protective capacity against pathogens is another principle role of skin. In addition to the scales and fibrous tissue of the dermis which prevent or limit penetration or spreading parasites, a wide range of other mechanisms limit, inhibit or destroy potentially invasive agents. The skin of fish is also metabolically very active tissue, that responds quickly to stressors (Whitear 1986; Iger *et al.* 1992). The skin contains the sensory receptors which allow early recognition of possible danger. It also contains specialised taste and touch receptors in specific sites of the body (Bullock & Roberts 1975).

Skin has a complex role in modulating the relationship of fish to their environment. It is a basic tenet of fish pathology that the maintenance of the integrity of the surface of the skin and gill is one of the main criteria for health. The aquatic

environment, and the cuticle of skin, contains large numbers of potentially pathogenic organisms (Bullock 1988).

The complete integrity of the skin of fish is its first line of defence against disease. Thus it is important to know the reaction of the fish to any insult which breaches this integrity. The present study was carried out in order to characterise the pattern of inflammatory response and wound healing process in the inflicted skin and underlying muscles of carp (*Cyprinus carpio* L.) without complication of secondary infection.

4.2 LITERATURE REVIEW

4.2.1 Wound healing in fish

Any tissue insult, microbial or parasitic lesion will lead to ulceration of the epidermis. The original cause of the lesion may then be masked by invasion of opportunist pathogens. One of the significant effects of such ulceration is breaching the membrane enclosing the internal environment with resultant osmotic effects (Roberts 1989). If the lesion is extensive or is increased in size either by the effects of pathogens or osmotic damage, the fish may die rapidly due to extensive loss of body fluids and resulting osmotic effects (Hickey 1979; McVicar & White 1979). The importance of rapid dermal healing for restoration of osmotic control after injury has been discussed by Hickey (1979). The capability for wound healing and the quantitation of repair in fish is related to the several variables (Mawdesley-Thomas & Bucke 1973), including degree of damage and the temperature (Bisset 1946; Finn & Nielson 1971*b*; Anderson & Roberts 1975), dietary status and stress factors (Halver 1972; Jauncy *et al.* 1985). Both larval and mature fish show rapid epidermal repair in skin wounds to prevent osmotic damage and invasion of pathogens (Mittal & Munshi 1974; Anderson & Roberts 1975; Phromsuthirak 1977; Bullock *et al.* 1978*a,b*; Hickey 1982; Rai & Mittal 1983; Jauncy *et al.* 1985; Chinabut 1989).

The inflammatory reaction after wounding has been studied by Finn & Nielson (1971*a,b*), Mawdesley-Thomas & Bucke (1973), Roberts *et al.* (1973*a*), Mittal & Munshi (1974), Anderson & Roberts (1975), Roberts & Bullock (1976), Phromsuthirak (1977), Mittal *et al.* (1978), Hickey (1982), Bereiter-Hahn (1986), Roubal & Bullock (1988), Chinabut(1989), Bullock & Roberts (1992).

Wound repair consists of one rapid and one slower event. Re-epithelialization of the wound and elimination of the injured tissue is the first step which is rapid, and re-organisation of the dermal connective tissue is the second stage which occurs much slower than the first step. The rapid closure is an important homeostatic mechanism for an organism surrounded by an aquatic environment which is not iso-osmotic (Bereiter-Hahn 1986). The mechanisms leading to wound repair in fish are quicker than in higher animals. These mechanisms are very important to their survival in terms of integrity of organisms and maintaining a satisfactory osmotic balance whether in fresh or salt water (Bullock & Roberts 1992).

The epithelial covering of the wound is less a temperature-dependent mechanism than other processes in teleosts (Bullock *et al.* 1978*a*; Bullock & Roberts 1980). Once a single layer of epidermis is formed over the defect by cellular migration towards the wound gap, and the osmotic and infection barrier restored, then slower, and more temperature-dependent, re-epithelialization can be achieved by normal mitotic proliferation. This is accompanied by fibrous scar formation and reconstitution of the dermal wound (Roberts 1989).

Skin wound healing in experimentally damaged stickleback at 20±2°C, was studied at the ultrastructural level by Phromsuthirak (1977). He showed that epidermis from both sides of the wound moved in through the incision and spread out towards the wound gap. The wound was closed when cells at the surface of the epidermis met across the gap, forming a plug of epidermal tissue which was then invaded by dermal tissue from either side. He mentioned that the migrating epithelial cells were phagocytic. After wounding, more leukocyte type cells migrated into the epidermis from the blood. It was confirmed that neutrophils which reached a peak in 24 hours

after wounding and decline during the second day, were phagocytic although not to the same extent as the macrophages. The migration of epithelial cells took place within 1 hour after wounding and complete closure of the incision took 3 days, but was unsupported by either basement membrane or dermal substance. He concluded that in the stickleback, the damaged epidermis was healed and normal by day 8 after wounding.

Different epidermal wound-closing and healing times have reported by different authors (mentioned above).

The presence of lymphocyte cells in the epidermis of the teleost fish was reported by Percy (1970), Mittal & Munshi (1971, 1974), Bullock & Roberts (1975), Peleteiro & Richards (1985) and lymphocytes have also been detected in the skin mucus by Mittal & Whitear (1979) and Ourth (1980). Mittal and Munshi (1974) found in the fresh-water siluroid fish (*Rita rita*), that lymphatic spaces on the 4th day after wounding become gorged with lymphocytes which may play an important role in the defence mechanism. This result has also been observed in *Heteropneustes fossilis* by Mittal *et al.* (1978). Mittal & Munshi (1979) also reported the secretion of large amounts of mucus in catfish immediately after wounding. They suggested that the increase in the secretory activity of the mucous cells 30 minutes after wounding may serve a protective function preventing pathogens from invading the body through the damaged surface. It may also establishes a barrier to protect from osmotic effects across the wound, thus playing an important role in re-establishing the osmoregulation between the internal and external environments of the body.

Replacement of melanin, scales, deep muscle and fascia in trout appeared to occur concurrently with repair of the dermis (Ashley *et al.* 1975).

4.2.1.1 Migration of the epidermis and re-epithelialization

The migration of the epidermal cells at the edges of the skin defect, soon after wounding, has been reported by most of the scientists who have studied on the healing of fish skin.

Mittal & Munshi (1974) reported an increasing in volume of the epidermal cells at the edge of the surgical wound of siluroid fish (*Rita rita*). These cells were seen migrating towards the wound gap. They suggested that detachment of basal layer of Malpighian cells from the underlying basement membrane makes the cells move freely towards the wound gap, resulting in quicker epithelialization of the wound. This suggestion was supported by Mittal and his co-worker (1978) in their study on healing of wounds in the skin of *Heteropneustes fossilis*. Phromsuthirak (1977), using the electron microscopy, also showed migration of epidermis into defect area of three-spined stickleback (*Gasterosteus aculeatus*) within 1 hour after wounding.

Bullock *et al.* (1978*b*) showed that 30 minutes after inducing a small surgical wound in plaice at 10°C, initial changes in the morphology of the epidermis were observed comprising of flattening of the Malpighian cells at the edge of the wound which was followed by rapid migration of a layer of the Malpighian cells over the exudate surface from the edges. It was a complete closure of the wound gap by 9 hours after wounding. The coverage of the defect was rapid even at low temperatures but was purely due to migration from the periphery, following detachment of the desmosal junctions, and did not involved mitotic division of cells.

Hickey (1982) found the same mechanism of epidermis migration from the wound periphery, spreading over the defect surface of the larvae skin. This mass

migration of cells from the wound periphery was without a rapid phase of cell division before or during closure of the wound.

According to Bereiter-Hahn (1986) the re-epithelialization process in teleosts comprises five stages as follows; 1) filling the wound cavity with tissue debris and mucus; 2) detachment of the epidermis from the basal lamina quickly after injury; 3) migration of epithelial cells over the wound cavity with loosening of intercellular contacts; 4) reconstituting the epithelial continuity and forming the primary wound closure by contacting moving cell sheets each other over the defect area; 5) growth, differentiation and remodelling of the cell mass which occurs several days after the wounding.

Bullock & Roberts (1980) showed that the presence of pathogenic bacteria on the surface of the lesion in the skin of rainbow trout, completely inhibited any migration of peripheral epidermal cells resulting in the expansion of bacterial ulceration, as peripheral epidermis sloughed. It has also been showed that the mechanism of cell migration is not inhibited by anti-inflammatory treatments (Roubal & Bullock 1988) or by deficiency of vitamin C (Jauncey *et al.* 1985).

Roberts (1989) also reported that there is a loss of the intercellular desmosomal attachments of the Malpighian cells immediately after wounding. Then a tongue of Malpighian cells migrates as a thin layer over the surface of the dermal limit to the lesion. He explained that when this migration, which forms at least a single layer of epidermis over the defect as rapidly as possible, occurs, the adjacent normal epidermis is correspondingly reduced in thickness.

Bullock & Roberts (1992) exposed surgically damaged skin of Atlantic salmon (*Salmo salar* L.) to low levels of ultraviolet-B radiation, which is the wavelengths of

solar radiation associated with sunburning and the induction of pathological changes on the skin. They found that the basic mechanism of superficial wound healing, characterised by epidermal migration, was unaffected but the migrating cells were more vulnerable to the necrotising effects of the irradiation than the surrounding donor epithelium.

Turnbull *et al.* (1996) in their study on dorsal fin rot in farmed Atlantic salmon (*Salmo salar* L.), parr reported three main types of superficial damage: areas lacking epithelium, areas of thin epithelium and clefts through the epithelium which was defined, using SEM study, as bite wounds almost surgical in nature which are the main type of injury in dorsal fin rot of Atlantic salmon. They described that following injury, the damaged cells maybe lost, undergo degeneration and sloughing, or may recover. The migrating epithelial cells rapidly smoothed the edges of the wound, resulting in the protrusion of the necrotic tissue from the wound. The extending of the superficial epithelial cells at the edge of the wound into the wound, occurred concurrently with the processes of repair and sloughing of the damaged tissue.

4.2.1.2 Fibrosis in fish

Hadfield (1951) suggested that granulation tissue is the only convenient term in common use for the immature and highly fertile mesenchyme which invades and subsequently replaces dead, dying, degenerated, ill-nourished, time-expired and useless tissue in any situation in the body and under a large number of pathological conditions. The essential function of granulation tissue is to replace dead material by living mesenchyme. The newly formed, active, proliferating capillary blood vessels of granulation tissue are primarily responsible for its capacity to invade. The general

physiological process of mesenchymatous replacement would thus appear to have two phases, the first being one of capillary vascularisation which creates an environment in which cells can multiply, and the second phase is characterised by a process of capillary devascularisation which is just as spectacular as the vascularisation of the first phase.

Mittal & Munshi (1974) in their study on the regeneration and repair of superficial wounds in the skin of *Rita rita* showed that fibroblasts maybe come from the mesenchymal perivascular connective tissue. Also they suggested the possibility of the origin of new fibroblasts from those present in the endomysium of the muscle bundles. The loosening of the endomysium which is a result of the lytic processes involved in wound healing, facilitates the migration and proliferation of new fibroblasts.

Mittal and his co-workers (1978) in their study on the healing of wounds in the skin of *Heteropneustes fossilis* described the origin of the fibroblasts in the granulation tissue. According to some of scientists, fibroblasts are derived by metaplasia of mononuclear cells of the blood stream. It was later suggested that fibroblasts are derived from the local mesenchymal cells. The view of local origin of fibroblasts was supported by a majority of investigators (Edwards & Dunphy 1958; Dunphy 1963; Grillo 1963; Hadfield 1963; McMinn 1967). Mittal *et al* (1978) also demonstrated that the spaces left following the disintegration of injured muscle bundles gradually gets occupied by fibroblasts and capillaries resulting in the formation of granulation tissue. They also showed that 6 days after wounding the fibroblasts in the granulation tissue become more compact and secrete the collagen fibres as part of the process of maturation. The maturation of the fibres first appears at the wound margins and then gradually towards the centre and the deeper layers resulting in the gradual contraction of the wound.

Finn & Nielson (1971*a*) suggested that perimysium appears to be a source of fibrocytes and fibroblasts. They also reported (1971*b*) that the initial appearance of fibroblasts and presumably the beginning of fibroplasia are usually delayed at the lower temperatures.

Anderson & Roberts (1975) suggested that fibro-granulation tissue and replacement fibrosis are more extensive in wounds produced by a prolonged inflammatory stimulus. They showed that the simple non-continuous trauma of a surgical incision is a weak stimulus to fibroplasia comparing with a prolonged stimulus. They also reported that fibrosis develops more rapidly at higher temperatures than at lower temperatures.

It has been demonstrated that vitamin C or ascorbic acid is a vital co-factor in the hydroxylation of proline to hydroxyproline and thus in synthesis of collagen (Halver 1972; Sato *et al.* 1982*a,b*).

4.2.1.3 Muscle regeneration in fish

Lesions, whether traumatic, due to bacterial or parasitic effects, will cause central migration of nuclei and flocculation of sarcoplasm, but spread of actual infection within myotomes is generally limited to a single myotome, by the intermyotomal fascia. One of the first indications of pathological change in muscle is the central migration of the nuclei of the muscle fibres. If the muscle damage is not intense and extensive, the healing takes place by regeneration of fibres or budding from stimulated satellite cells. Also with any type of teleost inflammation, the rate and quality of the host response to muscle damage is temperature-dependent (Roberts 1989).

The satellite cells which are described by Mauro (1961), Przybylski & Bumberg (1966) Shafiq *et al.* (1967), Church (1969) and King (1975) in various vertebrates, located in between the muscle fibre and its basement membrane. It is generally assumed that these cells maybe activated, proliferate and later differentiate into muscle cells.

Mawdesley-Thomas (1975) reported that the regenerative processes in fish muscle are similar to higher vertebrates and include sarcolemmal sprouting and nuclear reduplication. They explained that in areas of regenerating muscle, a sarcolemmal tube is seen containing many nuclei closely aligned within the tube. These tubes usually are narrowed towards each end and easily mistaken for young or immature fibroblasts since both cell types are frequently seen in the areas of muscle damage.

Mittal *et al.* (1978) also reported muscle regeneration in *Heteropneustes fossilis*. They explained that at first, new muscle bundles appear at the margins of the wound gap near the intact muscle bundles and then gradually appear towards the centre. These muscle bundles then enlarge but they are always smaller than normal muscle bundles.

Chinabut (1989) showed myofibrillar regeneration in wounded snakehead (*Channa striatus*) in the form of sarcoplasmic budding among the area of fibrosis.

Similar to fibrosis, wound healing in muscle is vitamin C-dependent. Deficiency of vitamin C may result in failure of muscle regeneration and wound healing (Halver 1972).

4.2.2 The effect of temperature on wound healing

Fish are poikilotherms or ectotherms, lower vertebrates whose body temperature is a reflection of the ambient temperature of their environment (Roberts 1975*b*).

Temperature effects not only the protective mechanisms of the host but also the metabolism of a potential pathogen (Finn & Nielson 1971*a*). Alternations in temperature may enhance or cause delay to the rate of multiplication of micro-organisms, increase or decrease the amount of dissolved oxygen in the water, the host's metabolic rate or the rate at which their body's defensive mechanisms act and antibody formation takes place. The effects of temperature are also important to all aspects of the physiology of the teleosts and control the rate of feeding, the ability to escape from predators, oxygen requirements, and many other factors (Roberts 1975*b*). The interaction between temperature and disease has two major components.

Roberts and his co-workers (1971, 1973*a*) have investigated the effects of temperature on the rate of healing of natural and surgically inflicted ulcers in Atlantic salmon (*Salmo salar*) and plaice. They showed considerable differences between the rates of healing and density of the connective tissue in such wounds. At 4°C healing was very slow with little cellular inflammatory response below the epidermis. Although epithelial healing was slow, it was still relatively rapid compared with that of the underlying connective tissue. At 14°C there was rapid epithelialization and fibrosis. Since the multiplication of most pathogens increases greatly over the range 4°C to 14°C, relatively slow healing rates at low temperatures are not so important because most bacteria cannot multiply sufficiently rapidly to take advantage of the breach, whereas at higher temperatures it is of prime importance for the host to seal the lesion as quickly as possible and prevent access by the potentially rapidly-growing micro-organisms.

Finn & Nielson (1971*a*) have studied the effect of temperature on the inflammatory response of rainbow trout (*Oncorhynchus mykiss*) tissues to

Staphylococci, Freund's complete adjuvant and burning. They found that the effect of low temperature was to delay the appearance of macrophages within degenerated muscle fibres, the clearance of bacteria and necrotic tissue from the lesions, and the beginning of fibroplasia. These quantitative changes in fish kept at 5°C, were delayed by as much as 50 percent compared with the responses in fish kept at 15°C. Roberts *et al.* (1973*a*) confirmed these findings in their studies on the development of the tagging lesion in salmon parr (*Salmo salar*) held at different temperatures. Similarly, Ferguson (1976) found that the activity of the fixed macrophages of the reticuloendothelial system was inhibited at lower temperatures.

Timur, M. (1975) in his study on the carrageenin granuloma in the plaice found a delay in fibroplastic activity and also a less intensive inflammatory cell response with reduction in temperature. He showed that there was a much longer period of myophagic activity before the removal of necrotic sarcoplasmic debris. At higher temperature, the myophagia was completed before significant epithelioid cell development took place, whereas both were seen side by side from the 42nd to the 56th day at low temperature. He has also mentioned that the temperature reduction retarded epithelioid and giant cell development.

Anderson & Roberts (1975) compared the effects of temperature on wound healing in a tropical and a temperate teleost. They showed that the wounds were covered in less than 2 hours at warm temperatures and within 24 hours at cold temperatures. They suggested that the wound healing in both tropical and temperate temperature range, is proportional to ambient temperature.

Bullock *et al.* (1978*b*) showed that the defect in the experimentally wounded plaice was closed within 12 hours at 5°C, while it took 9 hours at 10°C and the

thickness of the migrated epidermal cover was much thicker at 15°C than 5°C. Hickey (1982) also reported that the rate of epidermal migration over skin wounds in plaice was almost doubled with a 10°C rise in temperature.

4.2.3 Vitamin C requirements in relation to wound healing

Vitamin C or L-ascorbic acid is a white, odourless, crystalline compound, soluble in water, but insoluble in fat solvents. It is very heat labile and prone to atmospheric oxidation (Gresham & Jennings 1962; Harper 1971).

Inability of higher vertebrates such as man, monkey and guinea pigs to synthesise L-ascorbic acid is due to absence of the enzyme L-gulono-γ-lactone oxidase in hepatic and renal tissues (Burns 1957). This enzyme converts L-gulonolactone to L-ascorbic acid (Kitamura 1969). Those animal that lack the ability to synthesise vitamin C must regularly ingest this vitamin for good growth and health (Ashley *et al.* 1975). Most fish are also unable to synthesise vitamin C and therefore have to rely on a dietary exogenous source (Chatterjee 1973; Wilson & Poe 1973). Ascorbic acid is an essential co-factor for the hydroxylation of the amino acids, proline and lysine, essential for normal maturation of collagen and also forming the basal membrane on which collagen, cartilage and bone are formed. It also plays a very major role in tissue synthesis and growth processes and mediates rapid tissue repair in trauma or disease conditions (Halver 1972; Sato *et al.* 1982*a,b*; Roberts 1989). Collagen is essential for the development of callus, for repair of fractured bones, and also for provision, maintenance and increasing of the dense collagenous fibrous tissue (Roberts 1989).

Wound healing in muscle and especially fibrosis is critically dependent on availability of vitamin C, and in the reports on the effects of ascorbic acid deficiency in

fish, failure of wound healing is a principal finding (Halver *et al.* 1969; Halver 1972; Sato *et al.* 1982*a,b*; Jauncey *et al.* 1985).

Halver (1972) reported that ascorbic acid is rapidly absorbed and is distributed throughout the tissues of growing rainbow trout and is rapidly fixed in those areas involved in collagen and cartilage formation. He showed that the ascorbic acid deficient rainbow trout and coho salmon could not synthesise enough collagen for normal wound repair. He concluded that the rate of repair of inflicted wounds is directly dependent upon dietary ascorbic acid intake.

Ashley and co-workers (1975) showed that wounded salmon and trout which were fed vitamin C had many delicate collagen fibres three weeks after wounding, but those given no ascorbic acid had little or no collagen in the wound. Also wound repair was generally poor in trout fed 5 mg or 10 mg vitamin C, while salmon with the same levels repaired wounds, to some extent, better. But both species repaired wounds almost as well as control fish when fed 40 mg or more of ascorbic acid per 100 grams of diet. Timur M. (1975) stated that fish with carrageenin granulomas on an excess of vitamin C did not affect the general feature of the granuloma, but the degree of fibroplasia increased and started 5 days earlier than in the fish fed on ordinary diet. Lim & Lovell (1978) explained that catfish (*Ictalurus Punctatus*) fed a diet devoid of vitamin C had replaced muscle with dense immature collagen fibres, whereas fish fed ascorbic acid in excess of 30 mg per kg diet produced mature collagen.

Sato *et al.* (1982*a*) studied effects of dietary vitamin C levels on collagen formation in rainbow trout. They reported that the ratio of hydroxyproline to proline content of collagen fraction of the skin and bone were significantly lower in the fish group receiving low ascorbic acid diets. They also showed that the minimum dietary

ascorbic acid requirement to maintain a normal collagen formation in the tissue of the experimental fish was estimated to be 5 to 10 mg per 100 g diet.

According to Soliman *et al.* (1985), tilapia with a very mild and small insults and with ascorbic acid deficiency showed that collagen deposition in the wounds of those fish fed the diet devoid of ascorbic acid, was inhibited and delayed when compared to those fish which were fed adequate or excess dietary vitamin C. However, the epidermal closure by rapid epithelial cell migration was unaffected by the deficiency and was achieved irrespective of vitamin C level. Jauncey and his co-worker (1985) in their study on tilapia fingerlings (*Oreochromis niloticus*) confirmed these results. They showed that the epithelial elements of the healing process developed irrespective of the vitamin C level but although fibroblast activity was marked in all fish, collagenization was very much slower in the deficient group and the lesion did not mature.

Chavez de Martinez & Richards (1991) described the histopathological changes of vitamin C deficiency in the Mexican native cichlid (*Cichlasoma urophthalmus*). The fry were fed with diets deficient in vitamin C without any insult. The epidermis of these fish showed an extensive inflammatory response with spongiosis and also degenerative changes in the basal cells. Inflammation, changes in the number and position of the nuclei, vacuolation and necrosis, granular degeneration and fibrous loss, were a range of pathological changes observed in the muscles.

In undamaged fishes, vitamin C has significant roles in preventing deficiency signs such as structural deformities, retarded growth, and haemorrhages (cited by Jauncey *et al.* 1985).

4.3 PILOT STUDY

A pilot study was carried out with a small number of fish to determine optimum techniques for wounding, sampling, fixation, trimming, processing, wax embedding, sectioning and staining the sections for histological examinations.

The mirror carp were anaesthetised and wounded by sterilised scalpel blade vertically in the dorsal myotomal muscle between the lateral line and scale row. The wound was made in the line with the first ray of the dorsal fin, as a marker.

The wounded fish were sacrificed by an overdose of anaesthesia, then a block of lesion bearing area was cut out and fixed in 10% buffered formalin.

The fixed tissues were then trimmed and cut into three pieces, each piece composed of a part of lesion. These pieces of tissue were processed in an automatic processor. After processing, blocks of tissue were embedded into wax and then were sectioned at 5 μm. The sections were stained using standard haematoxylin and eosin (H & E) for microscopic examination.

A number of problems with the methodology were highlighted in this pilot study. The most important of these problems are as follows:

Due to cutting the tissue into three pieces, and handing the tissues, the wounded area, especially epithelium was disrupted. Also re-epithelialization was not seen within 24 hours after wounding because of tearing the delicate epithelium apart by cutting the sampled tissues into three pieces.

Sectioning the blocks of tissues without decalcification was difficult and separated the epidermis, dermis and muscles.

No problems with the rest of methods and also designed aquaria system, etc. were encountered.

The second stage of this pilot study was conducted with another small number of fish. They were wounded and sampled by the same procedure used in the first stage, but the tissues were trimmed and cut into two pieces after processing, to be assured to have a wedge of lesion in each piece of tissue. Also this was done after processing rather than before, to avoid damage to the delicate epithelium. The tissues were decalcified by a decalcifying solution, after embedding into wax, to allow easier sectioning.

The result of the microscopic examination of the sections showed that the healing process was very rapid especially at the early stages of the repairing process, therefore it was necessary to arrange a sampling time scale so that it would cover the all steps of the healing process.

The result of the second stage of pilot study was much more satisfactory and reliable. This information allowed the experiment to be designed for the definitive study.

4.4 DEFINITIVE STUDY

4.4.1 Materials and Methods

4.4.1.1 Fish

Mirror carp were obtained from BAFRU (Bangladesh Aquaculture and Fisheries Resources Unit), at Howietoun Fish Farm, affiliated to the Institute of Aquaculture, University of Stirling. Thirty fish were stocked in glass aquaria for at least two weeks prior to experimentation to allow acclimation to the laboratory conditions. The average length of fish was 22 cm (± 4 cm). Fish were not fed during acclimation or the experiment.

4.4.1.2 Aquaria and water

The fish were held in a recirculating glass aquaria system, 122 × 32 × 42 cm. The water system consisted of dechlorinated, aerated fresh water, at an average temperature of 27°C (± 3°C). Aeration, circulation and filtration of water was supplied via a standard Fluval 303 external power aquarium filter system (ASKOLL, Italy) and air pump. Although filtration allowing mechanical, biological and chemical water quality control, further monitoring was carried out using the Dry-Tablet Master Test Kit (Aquarium Pharmaceuticals, Inc.). This allowed measurement of ammonia (NH_3 / NH_{4+}), nitrite (NO_{2-}), nitrate (NO_{3-}) and pH. Depending on the results of the water quality measurement, between 10-50 percent of the water was changed, as necessary.

4.4.1.3 Wounding procedure

Benzocaine (Ethyl-4-aminobenzoate), 100 g/l ethanol (Ross & Ross 1984), was used (0.5 ml/l water) to anaesthetise the fish. A vertical surgical (1.5-2 cm long by 0.5 cm deep) wound was made by sterilised scalpel blade into the left dorsal myotomal muscle of each anaesthetised fish. The wound was always made in line with the first ray of the dorsal fin, below the scale row, as a marker for subsequent sampling (Fig. 1.1). After wounding, fish were returned to the aquaria where they remained until sacrificed.

4.4.1.4 Sampling procedure

At least one wounded fish was sampled at 30 minutes, 1, 2, 4, 6, 8, 10, 12 and 24 hours. Thereafter, fish were sampled at 2, 4, 6, 8, 16, 24, 32, 40, 50 and 60 days after wounding. In addition, extra fish samples were taken, in repeat experiments, where good specimens were not available and also at times where particularly significant features required to be re-examined.

4.4.1.5 Histological procedures

Fish were sacrificed by an overdose of benzocaine. Then a block of 1.5 × 2 cm of the left dorsal myotomal muscle around the lesion area was cut out and fixed in cold 10% buffered formalin for at least 24 hours prior to cassetting. The fixative was changed with fresh fixative at least once during the fixation period. The fixed and cassetted tissues were processed in an automatic tissue processor using standard procedure. After processing, tissues were trimmed and cut into two pieces having a wedge of lesion in each piece of tissue. These trimmed blocks were embedded into wax

(a mixture of purified paraffin wax and plastic polymers formulated for use in routine histological techniques, melting point 56-57°C). Because the tissues were hard, blocks of the wax infiltrated tissue were decalcified with RDC solution (Rapid Decalcifier from CellPath plc, Herts, England). After decalcification, blocks of tissue were cut at 5 µm using a Leica Rotary Microtome (appendix 1.1).

4.4.1.6 Staining procedures

The standard H & E staining method (appendix 2.1.1) was used for staining the sections. Also Periodic Acid Schiff (PAS) and PAS with tartrazine in cellosolve counterstain methods (appendix 2.1.2) were used for specific tissue features. The stained sections were mounted in Pertex mounting medium (CellPath plc, Herts, England) with a cover glass for microscopic examination.

4.4.2 Results

4.4.2.1 Gross pathology

After wounding the skin, the muscle retracted from both sides, causing a "V" shaped gap "  " on the skin. The size of the gap depended on the tension of skin in individual fish and depth of the incision. It was considered as a normal response to wounding. The amount of haemorrhage after incision was variable and ceased within 2-5 minutes. This was, to some extent, dependent on the degree of fish activity. Because the fish had been deliberately chosen not to have scales on most parts of the body (mirror carp), the site of incision was obvious to the naked eye during the period of study. There was no colour change around the wound in the first few minutes after wounding, however, in less than one hour it became lighter than the normal colour of skin. After about 2 hours, the edges of the lesion became black, the area close to the wound was light coloured and beyond the darkened area, normal colour of skin was regained. This black colour of the edge of the lesion was visible to the naked eye until the end of the experiment. After 2-7 days the site of incision appeared as a white streak in the centre of a darker area of skin.

4.4.2.2 Histopathology

30 minutes

The obvious feature in the lesion, 30 minutes after wounding, was a large focus of haemorrhage in the defect, from the depths of the muscle to the surface. The exudate in the lesion was comprised of red blood cells and white cells, principally thrombocytes and lymphocytes. Marked accumulation of thrombocytes and also associated fibrinous exudate was obvious. There was a predilection of fibrinous exudate towards the edges of the lesion, where the accumulation of fibrin and thrombocytes appeared to act as a surface cover preventing further tissue fluid loss into the wound. Strands of degenerating nuclear material, in the necrotic debris between two cut edges of the lesion, were seen. The epidermis, far from the lesion, was thinned and the number of mucous cells and club cells was reduced close to the defect, so the epidermis seemed composed almost entirely of Malpighian cells with obvious cellular oedema. A single celled layer of epidermis was seen on the edge of the defect (Fig. 4.1). This appeared to be migrating into the wound surface and the inward migrating tip of epidermis was observed to extend onto the surface fibrin of the exudate as a substrate for its movement. The cut edge of the stratum compactum of the dermis was very sharply defined. Muscles close to the edges of the lesion were degenerate. Sarcoplasmic changes comprised flocculent degeneration or even complete disruption and loss of sarcolemmal outline and karyolysis of the nuclei. There was some haemorrhage into the degenerating muscles and hyperaemia of the local capillaries. Muscle changes were limited to a single myotome. The intermyotomal fascia provided an obvious limit to such muscle changes.

1 hour

Epithelialization was active and more epidermal cells were seen migrating towards the wound gap (Fig. 4.2). Fibrin was more obvious as a limiting membrane to the lesion. Cellularity of the damaged muscle area and the area adjacent to that, had increased and the fibrin was covering the edges of the ruptured myofibrils, close to the centre of the lesion. Marked haemorrhage into the degenerating muscles was seen.

2 hours

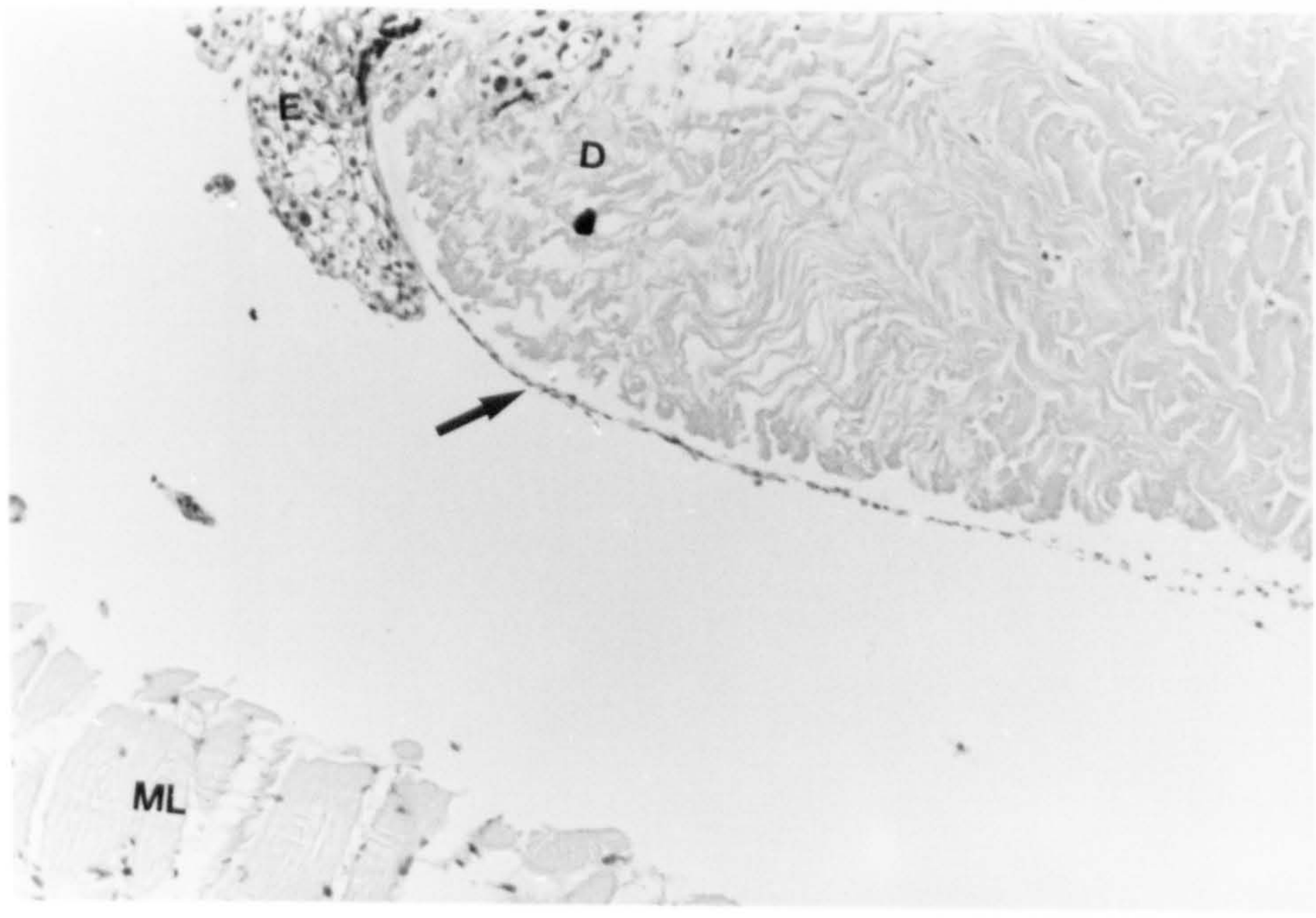
Epidermis was migrating further down the wound (Fig. 4.3) and the normal epidermis close to the centre of the lesion was oedematous. Degeneration of muscles and cellular infiltration were obvious (Fig. 4.4). Macrophages were first detected in the degenerated muscle area close to the centre of the lesion and also in the epidermis. There was a relatively large number of macrophages in the lesion. A number of polymorphonuclear cells (PMNs) were seen in the area of clot of fibrin near the epidermis. Also lymphocytes were seen in the lesion area. Haemorrhage and hyperaemia of the local blood vessels was obvious.

4 hours

At four hours after wounding, epithelialization was completed (Fig. 4.5). New epithelium was thickening in the defect with pink staining Malpighian cells, accompanied by a number of macrophages and eosinophilic cells in the thickened rim of the epidermis adjacent to the newly covered area. There was no basement membrane

Fig. 4.1 A single celled layer of epidermis was seen migrating down (arrow) into the wound gap at 30 minutes after wounding. (PAS + tartrazine, X 220).
E=epidermis, D=dermis, ML=muscle

Fig. 4.2 More epidermal cells were seen migrating (arrow) towards the wound gap after 1 hour. (PAS + tartrazine, X 110).
E=epidermis, D=dermis


E
D
ML

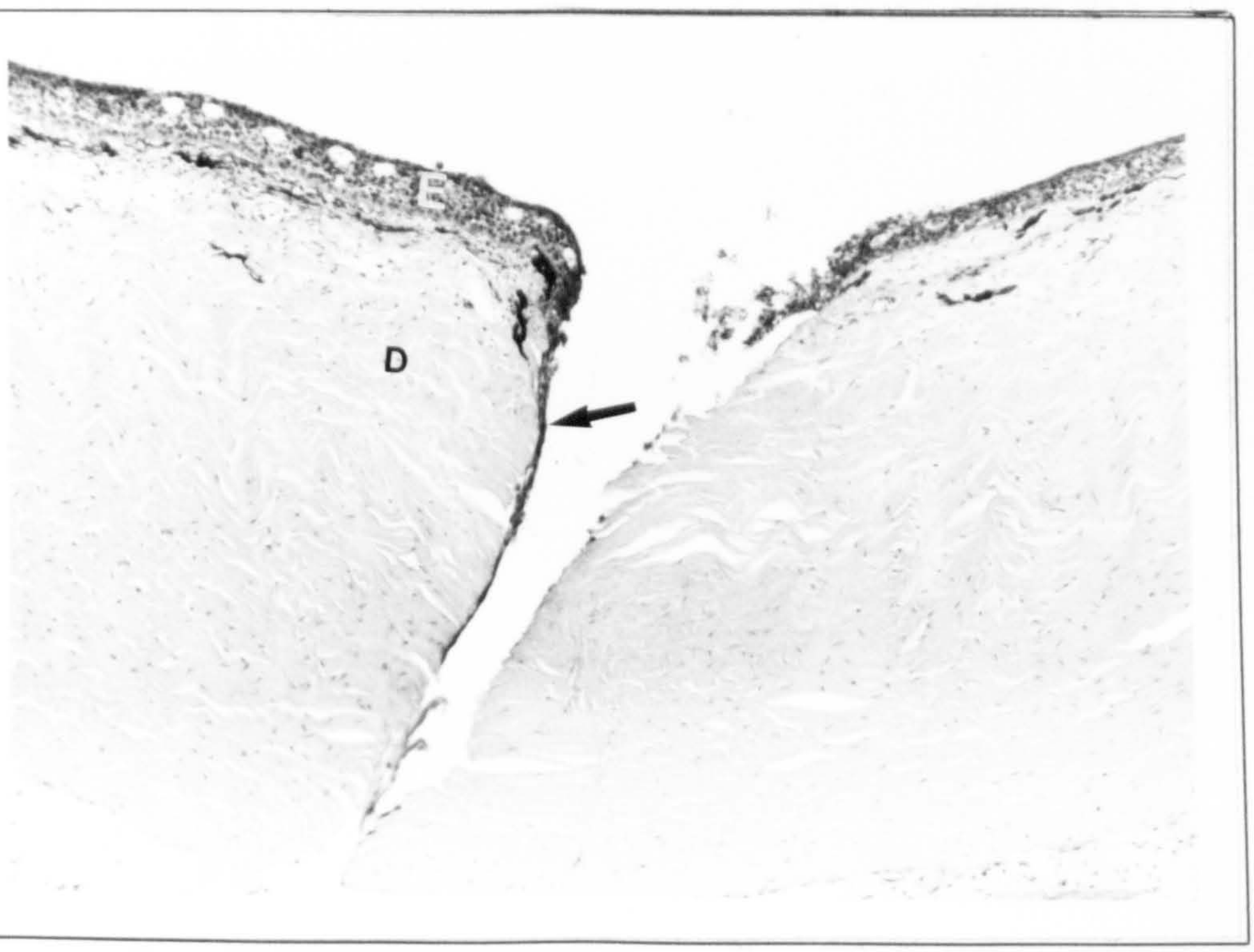
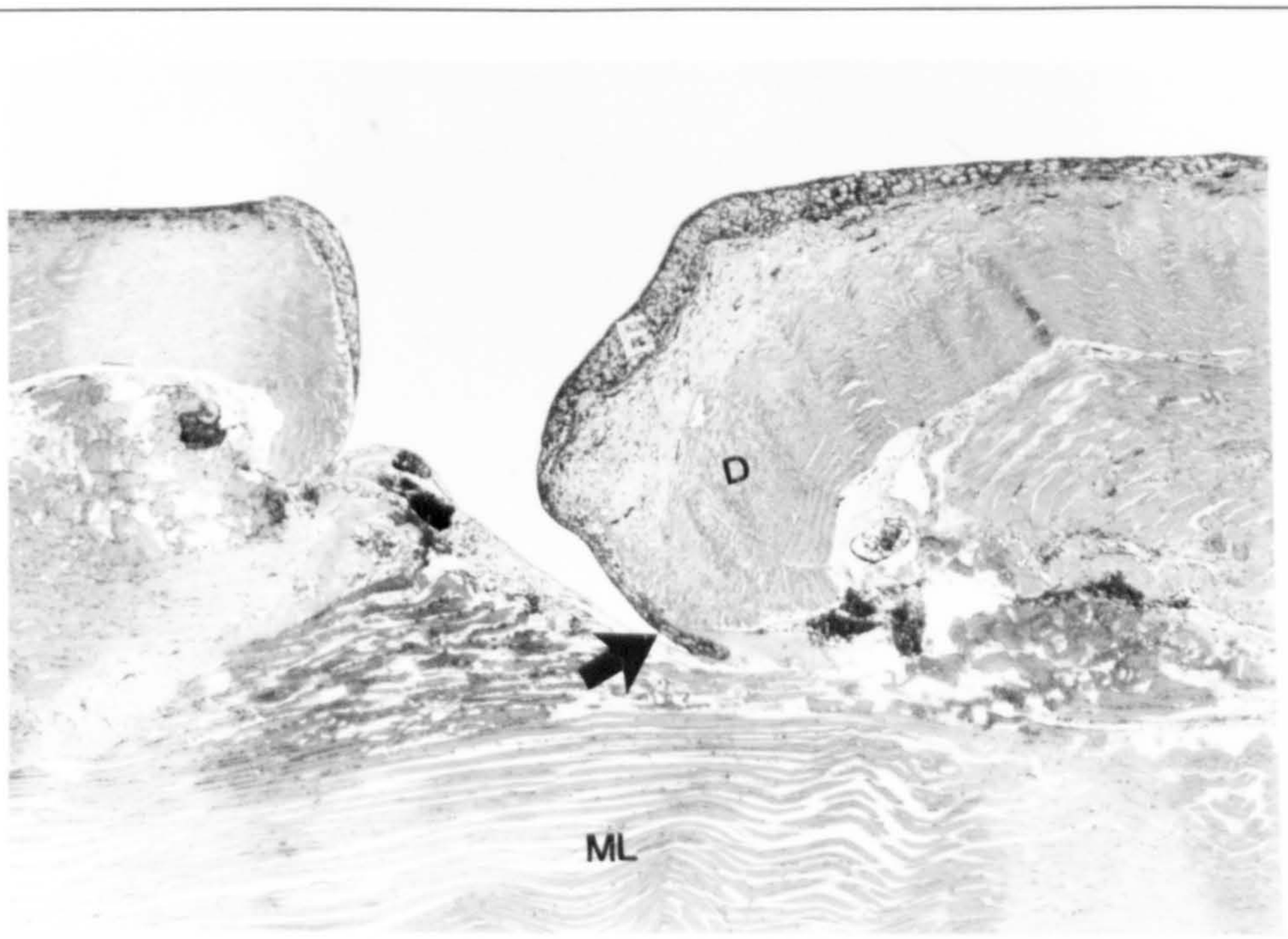

E
D

Fig. 4.3 By 2 hours after wounding, the epithelialization was active and epidermal cells were migrating further down the defect (arrow head). (H & E, X 44).
E=epidermis, D=dermis, ML=muscle

Fig. 4.4 This picture shows muscle degeneration (MD) and presence of inflammatory cells (arrows) in the lesion area at 2 hours post-wounding. (H & E, X 220).


D
ML

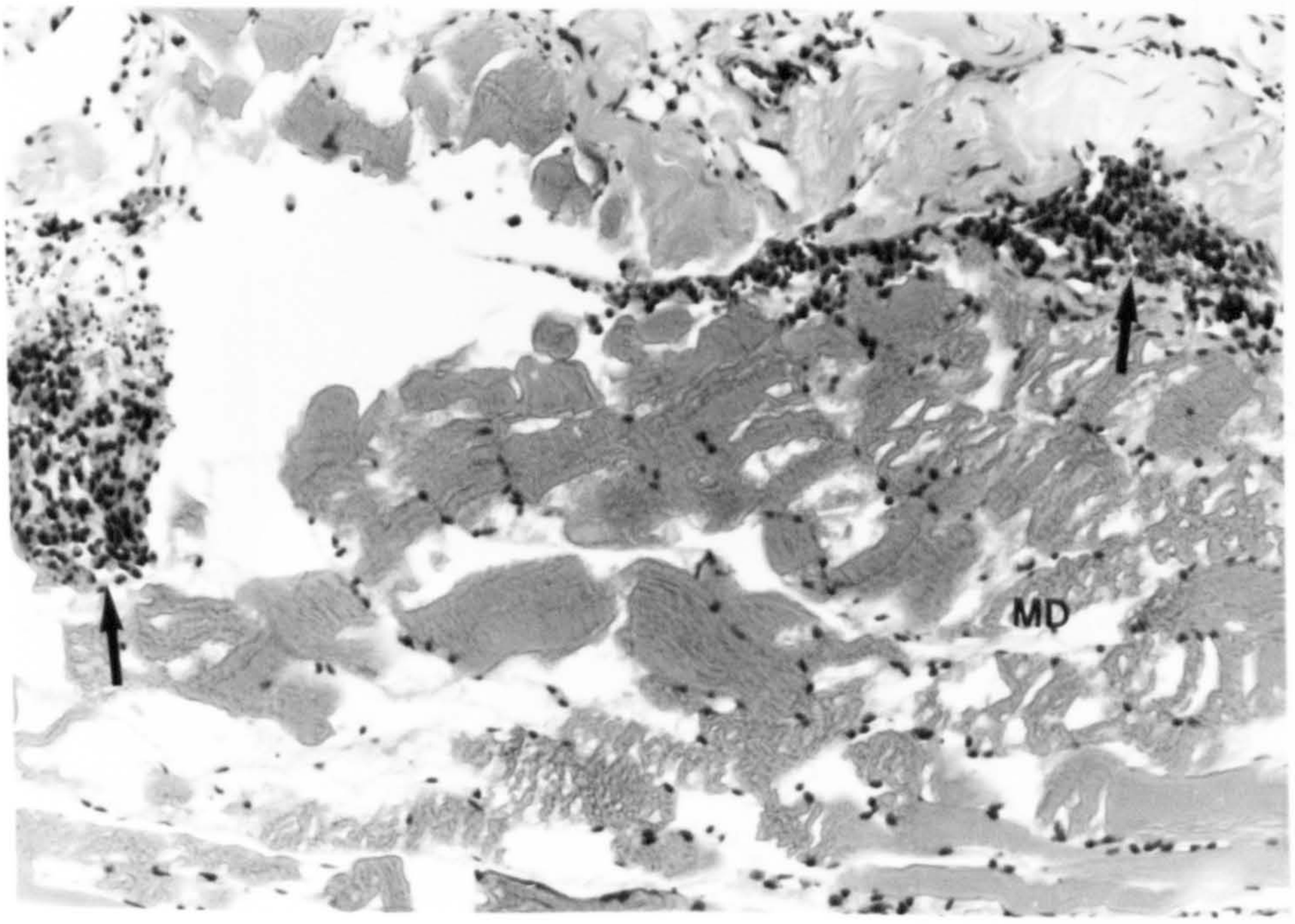
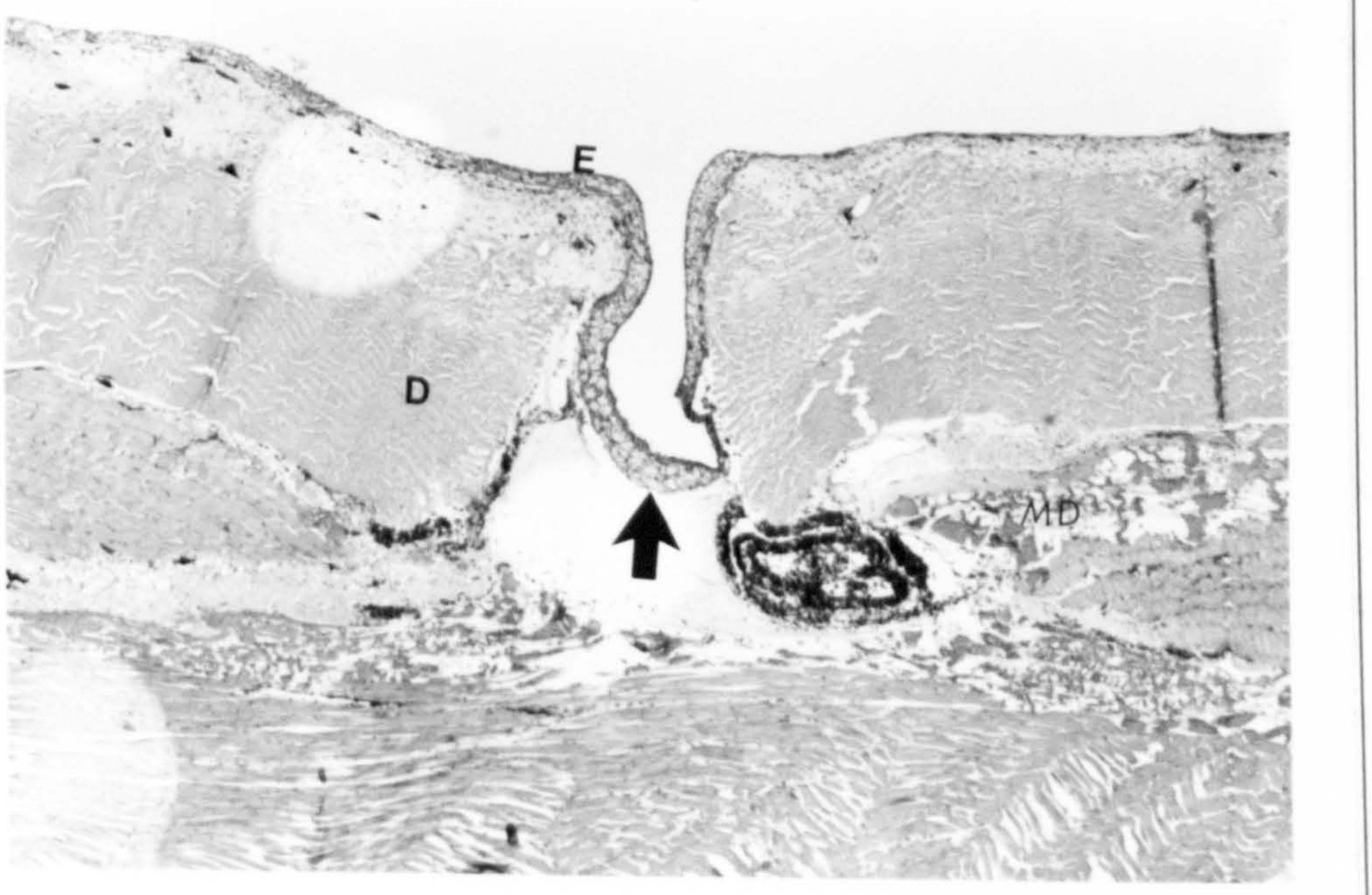
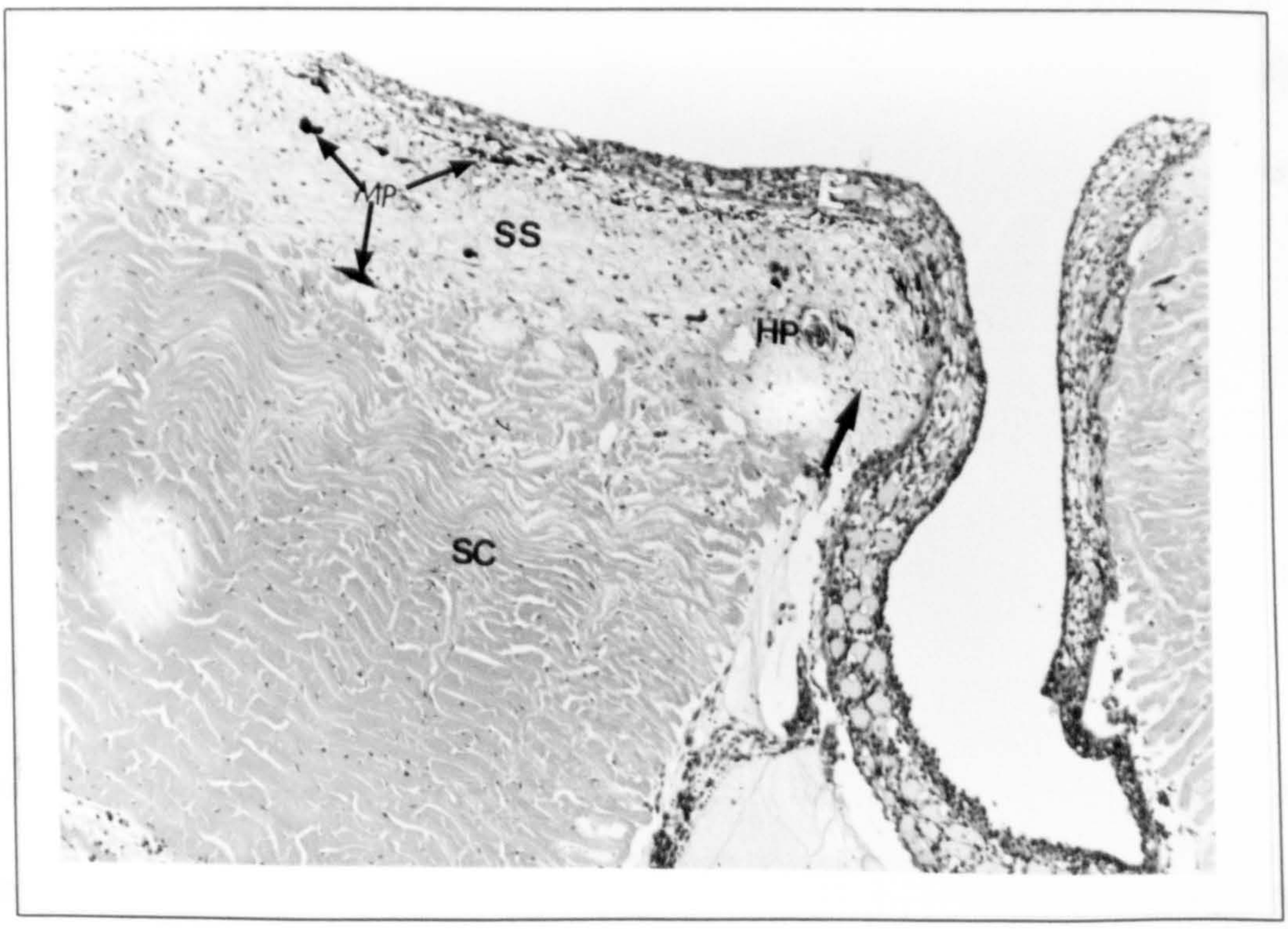

MD

Fig. 4.5 By 4 hours after wounding, epithelialization was completed (arrow head). (H & E, X 44).
E=epidermis, D=dermis, MD=muscle degeneration

Fig. 4.6 This picture is an enlargement of Fig. 4.5 (4 hours p.w.) which shows oedema (arrow) of the stratum spongiosum (SS) with hyperaemia of blood vessels (HP) and some cellularity and melanin pigments (MP). (H & E, X 110).
E=epidermis, SC=stratum compactum


E
D
M.D


MP
SS
HP
SC

under the newly formed epidermis. The stratum spongiosum was oedematous, with hyperaemia and some cellularity and melanin containing cells (Fig. 4.6). The muscles were infiltrated with more macrophages. Lymphocytes and some PMNs were evident in the area. There was a very large area of sarcoplasmic debris, while there was ingrowth of the new capillaries into the area of inflammatory activity.

6 hours

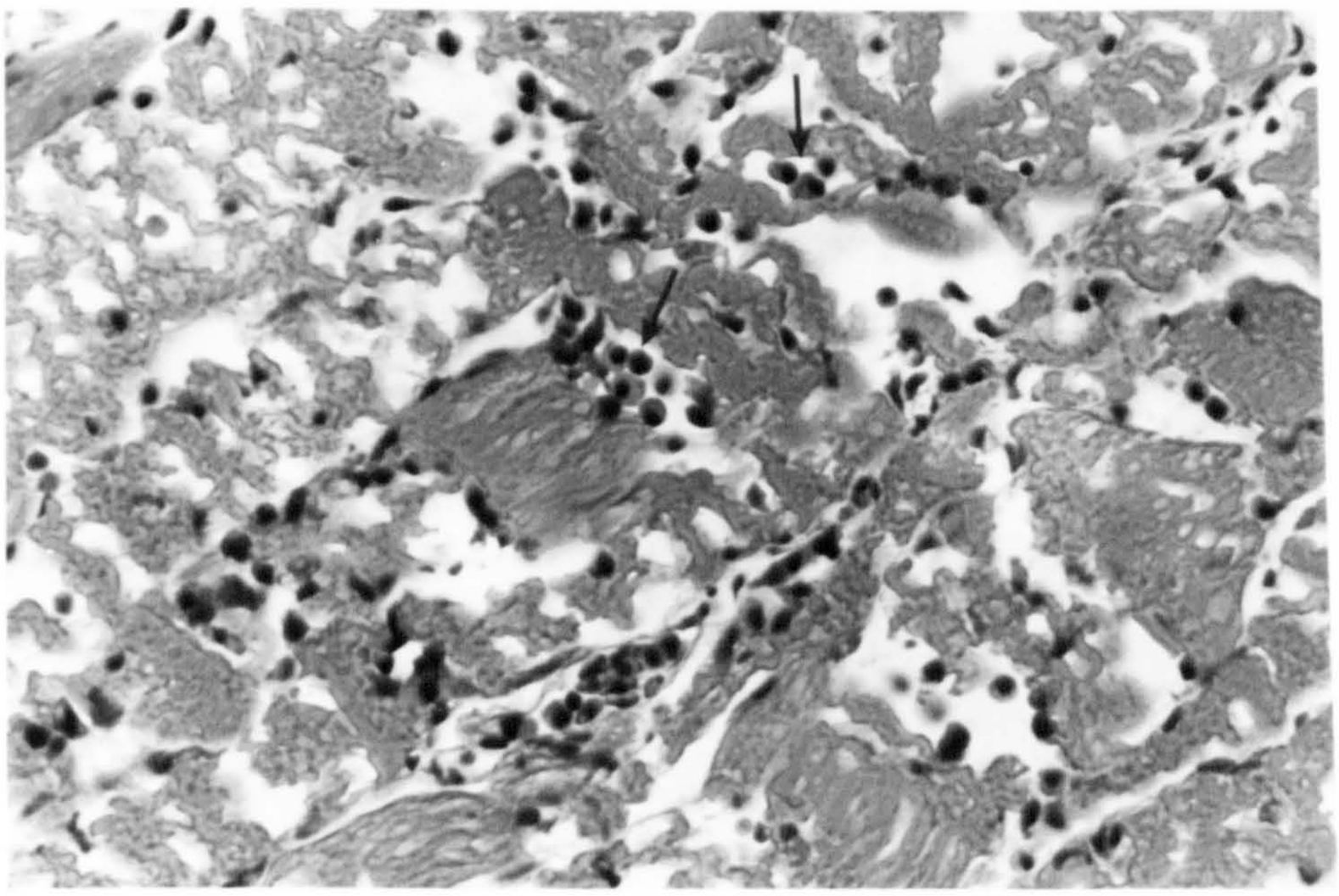
At this stage, the new epidermis was oedematous in the centre of the defect, and regeneration of basement membrane under the new epidermis was initiated. The fibrin thrombocyte layer as a cover for the cut surface of the wound was condensed and became a dark basophilic surface over the edge of the muscles. There was also cellularity and organisation of the fibrin on the underside of the epidermis in what would ultimately become the replacement stratum spongiosum. The initiation of myophagia accompanied by increasing the number of macrophages and vascularization, both in the muscles and stratum spongiosum, and also cellular infiltration from adjacent blood vessels were observed (Fig. 4.7). In addition of lymphocytes some PMNs were seen in the clots of fibrin under the new epidermis.

8 hours

The replacement cover was still thick and oedematous over the lesion. Myophagia was active and also macrophages in the new epidermis were seen. First presence of the new fibroblasts, swollen cells with pale staining nuclei, in the intermyotomal fascia adjoining the affected myotome was the main change in this stage (Fig. 4.8). More PMNs were seen in the lesion area and also some lymphocytes were

Fig. 4.7 The degenerated muscles at 6 hours p.w. showed myophagia (arrows). (H & E, X 440).

Fig. 4.8 First presence of the enlarged fibroblasts with pale staining nuclei (arrows) in the intermyotomal fascia adjoining the affected myotome at 8 hours after injury, showed initiation of fibroblasts activity. Blood vessels (BV) were seen associated with melanin pigments (MP). (H & E, X 440).




MP
BV

evident. The melanin within perivascular tissue of the fascia was marked and larger vessels of the fascial area were very obviously associated with melanocytes. New capillaries were actively growing into the lesion area.

10-12 hours

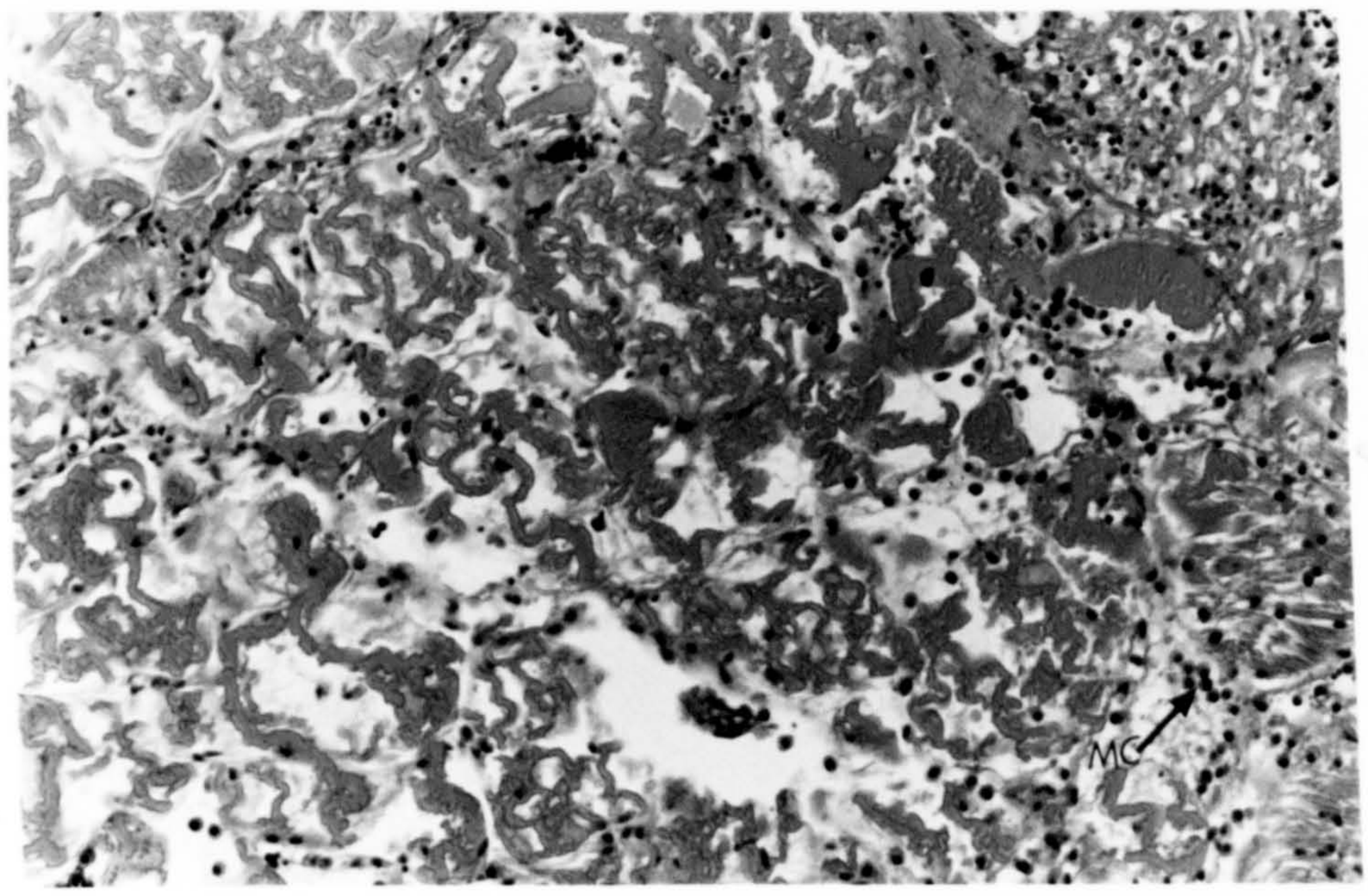
The inflammatory response at these two stages were similar. New epidermis still showed intracellular oedema. Myophagia was very active. PMNs were seen throughout the lesion area and also located along and within the degenerated muscles. Presence of lymphocytes in the lesion area was evident. Fibroblasts activity in the damaged muscle area was seen. New capillaries were actively growing into the lesion area and contained white cells as well as red cells. Large blood vessels were associated with melanocytes and contained a large number of red and white blood cells.

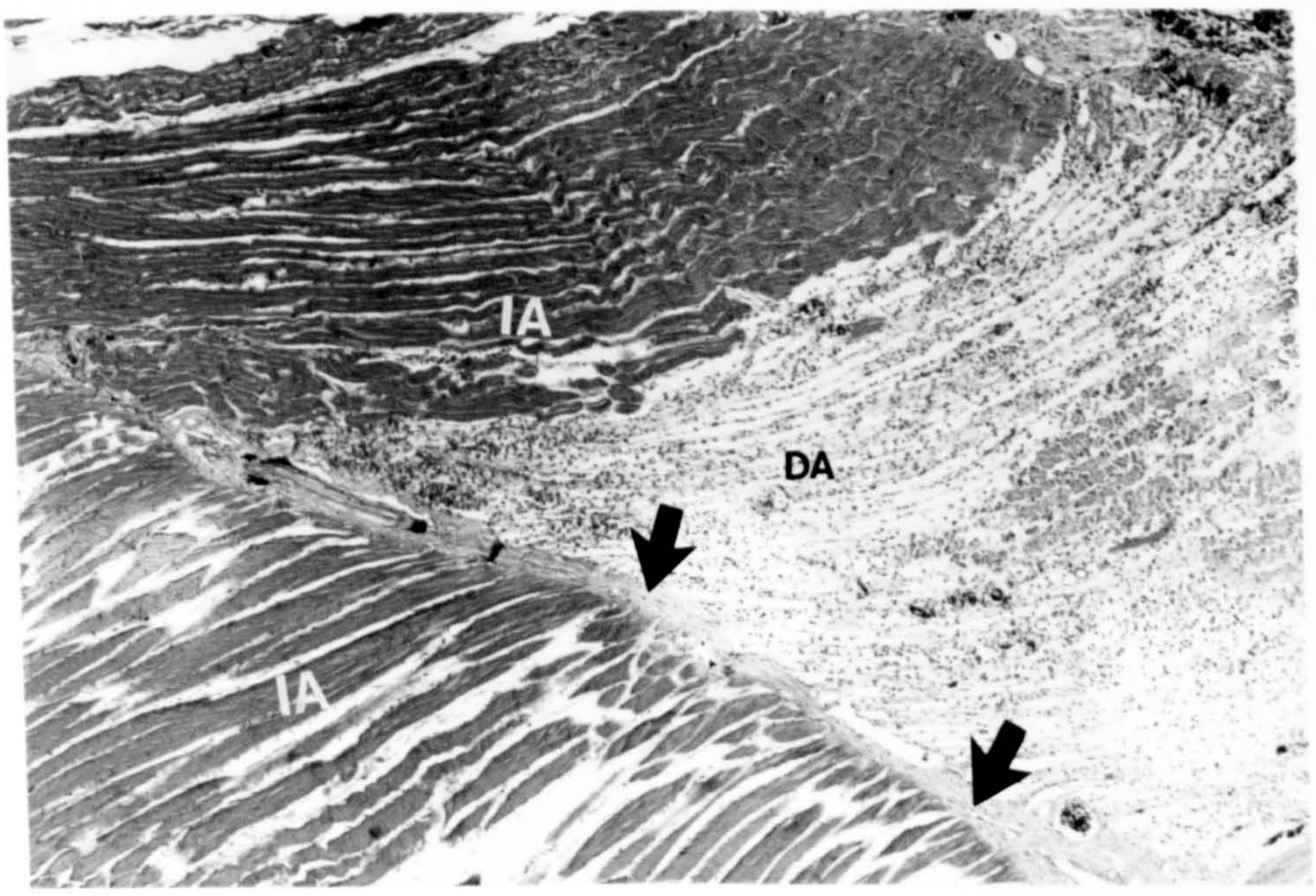
24 hours

Intracellular oedema was still as marked in new epidermis. It was supplied with some mucous cells and club cells, but eosinophilic component was not reduced. Myophagia was active along with increasing numbers of macrophages (Fig. 4.9). Lymphocytes and PMNs were observed in the lesion area. Myofibrils which had been slightly damaged had started redeveloping their peripheral nucleation. Some fibroplasia was seen in the lesion area.

Fig. 4.9 This picture demonstrates degenerate muscles, and more active myophagia along with increasing in the number of macrophages (MC) within the lesion area at 24 hours p.w. (H & E, X 220).

Fig. 4.10 At 2 days p.w. the perimysium appeared as a retaining barrier in limiting the spread of the lesion (arrow heads). (H & E, X 44).
DA=damaged area, IA=intact area


MC


1A
DA
1A

2 days

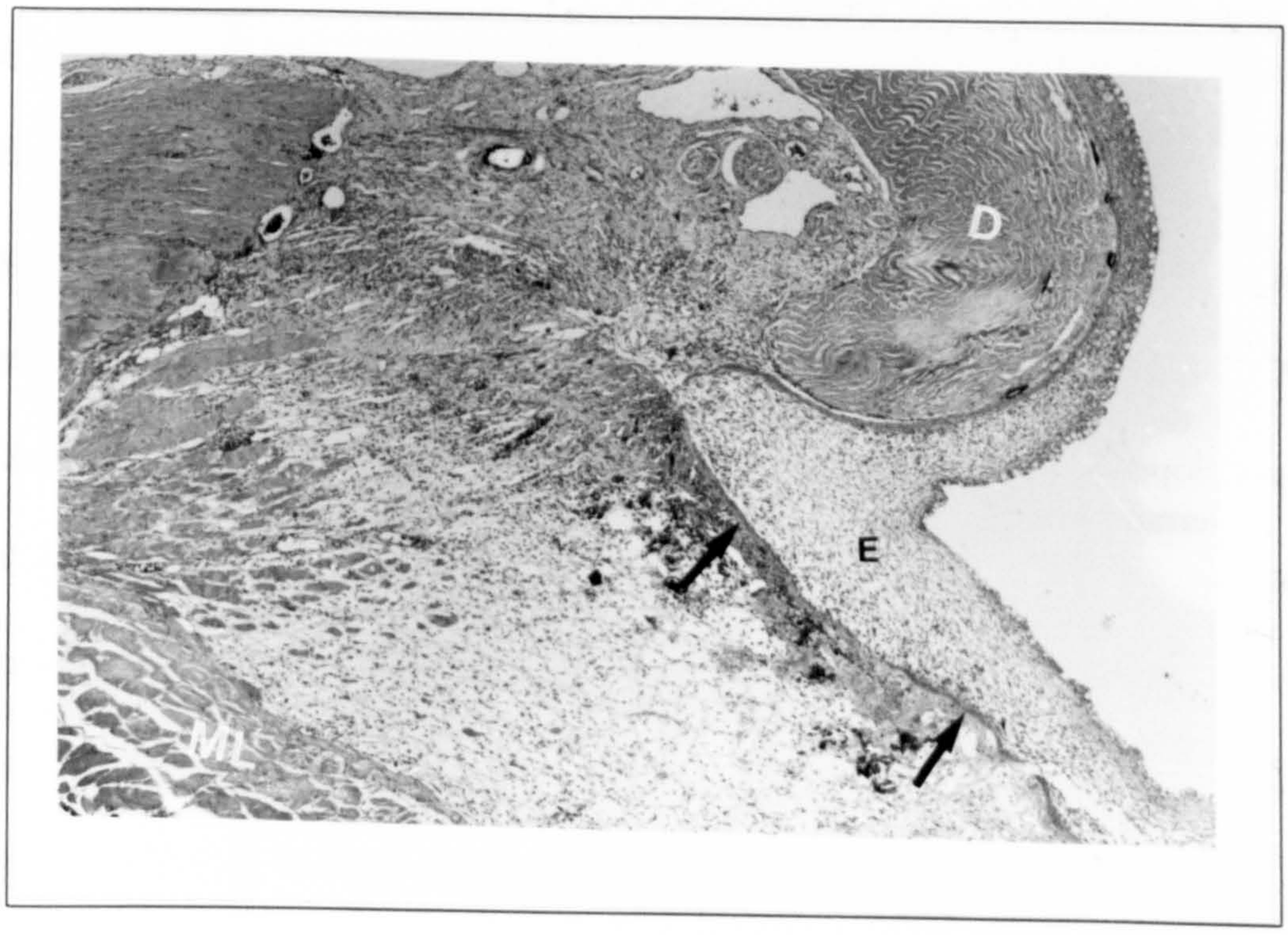
Myophagia, fibroplasia and muscle regeneration were the main features of this stage. Intracellular oedema in new epidermis was still as marked, especially in the centre of the lesion. The epidermis was fully supplied with mucous cells, but club cells were not present at a normal level. The eosinophilic component of the epidermis was reduced and mostly was limited to the centre of the lesion. Limiting the spread of the lesion by perimysium was very obvious (Fig. 4.10). Regeneration of basement membrane under the new epidermis was, to a considerable extent, completed. Main area of activity was still at the base of the lesion. Exudate with fibrin, red blood cells and inflammatory cells were observed on the underside of the centre of the lesion. Presence of lymphocytes and some PMNs in the lesion area was evident. First presence of the new muscle buds in the damaged muscle area was the characteristic feature in this stage.

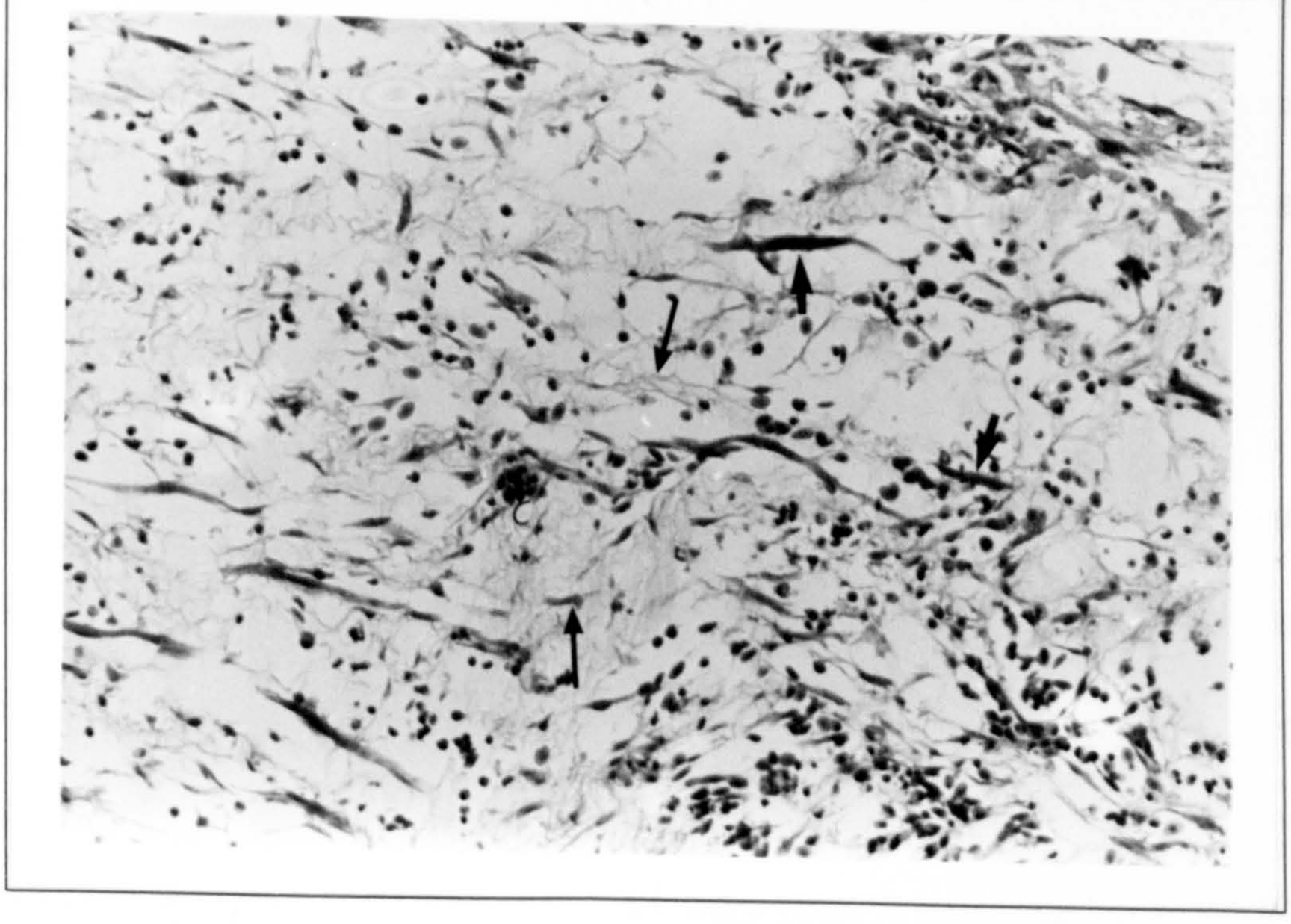
4 days

The picture of epidermis was similar to that of the 2 days after wounding. Basement membrane under the new epidermis was fully developed (Fig. 4.11). Myophagia was completed and there was minimal macrophage activity. Some PMNs in the lesion area were observed. Also presence of lymphocytes in the fibrous area was evident. Fibroplasia and fibrosis were dominant features of the lesion. In the fibrous replacement area of the muscle, considerable number of small muscle fibres and small basophilic muscle buds were also evident. They were more obvious adjacent to an area of normal muscle, rather than in the middle of the damaged area (Fig. 4.12). More active areas of fibrous replacement were vascular.

Fig. 4.11 New epidermis (E) over the area of the defect was thickened and oedematous at 4 days p.w. Basement membrane under the new epidermis (arrows) was fully developed, and myophagia was completed. (H & E, X 44).
D=dermis, ML=muscle

Fig. 4.12 This picture shows regenerated small muscle fibres (arrow heads) along with fibrosis (arrows), and new capillaries (C) in the healing area at 4 days p.w. (H & E, X 220).


D
E
ML



6 days

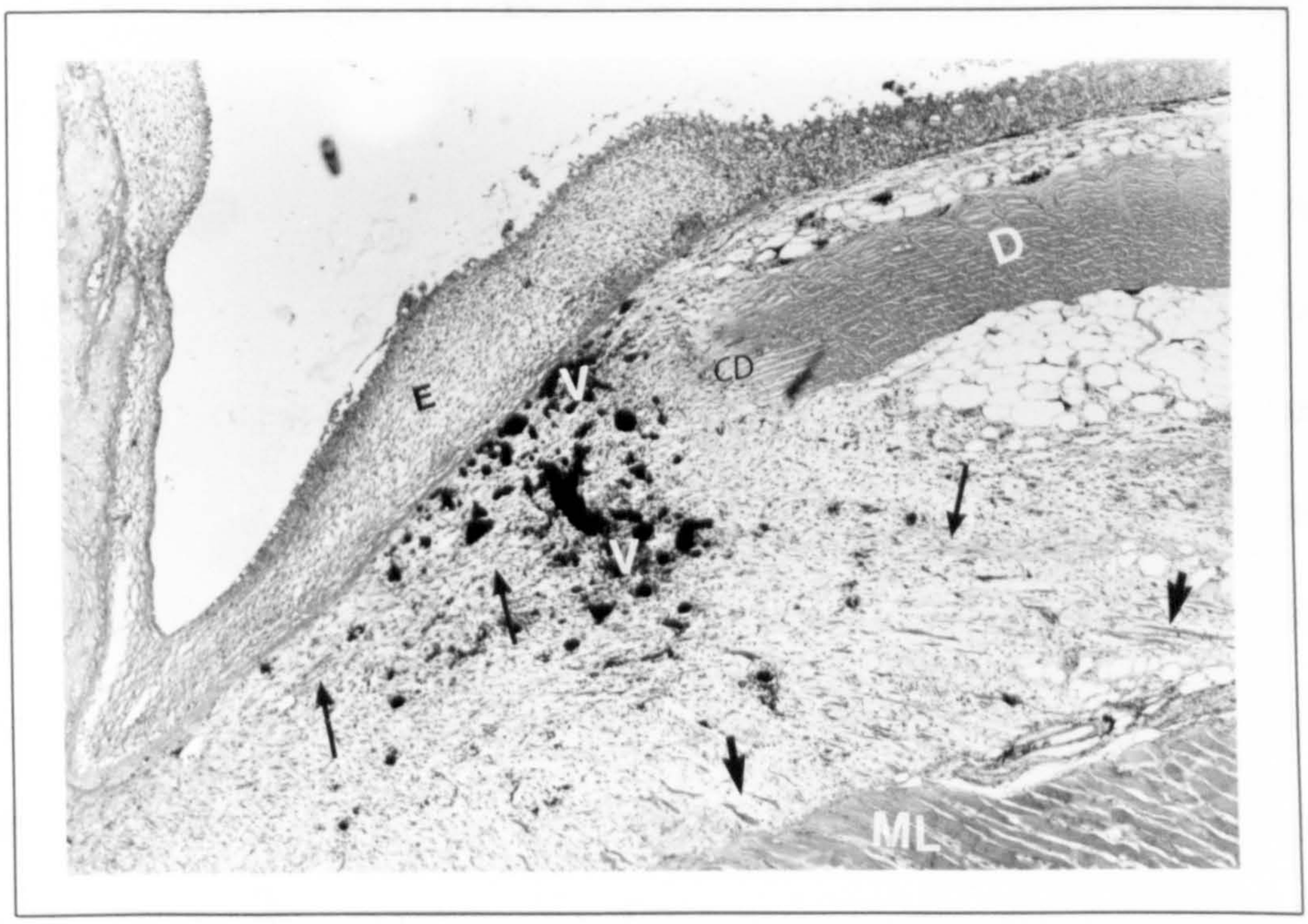
The epidermis was still oedematous and thicker over the main lesion area. The cut edges of dermis slightly started linking by a length of active fibrous tissue. There was fibrosis dominated with active collagen formation, involving the whole of damaged areas of muscle, accompanied by very active vascularization especially between two cut edges of dermis, underneath the new epidermis (Fig. 4.13). Muscle regeneration was active and new fibres were obvious. The number of macrophages was considerably reduced in the lesion area but presence of PMNs in the fibrous area was very obvious. Also active lymphocytes were evident in the lesion area. A distinct thickening of the melanin layer was seen under the initial part of the new epidermis, immediately adjacent to the normal epidermis.

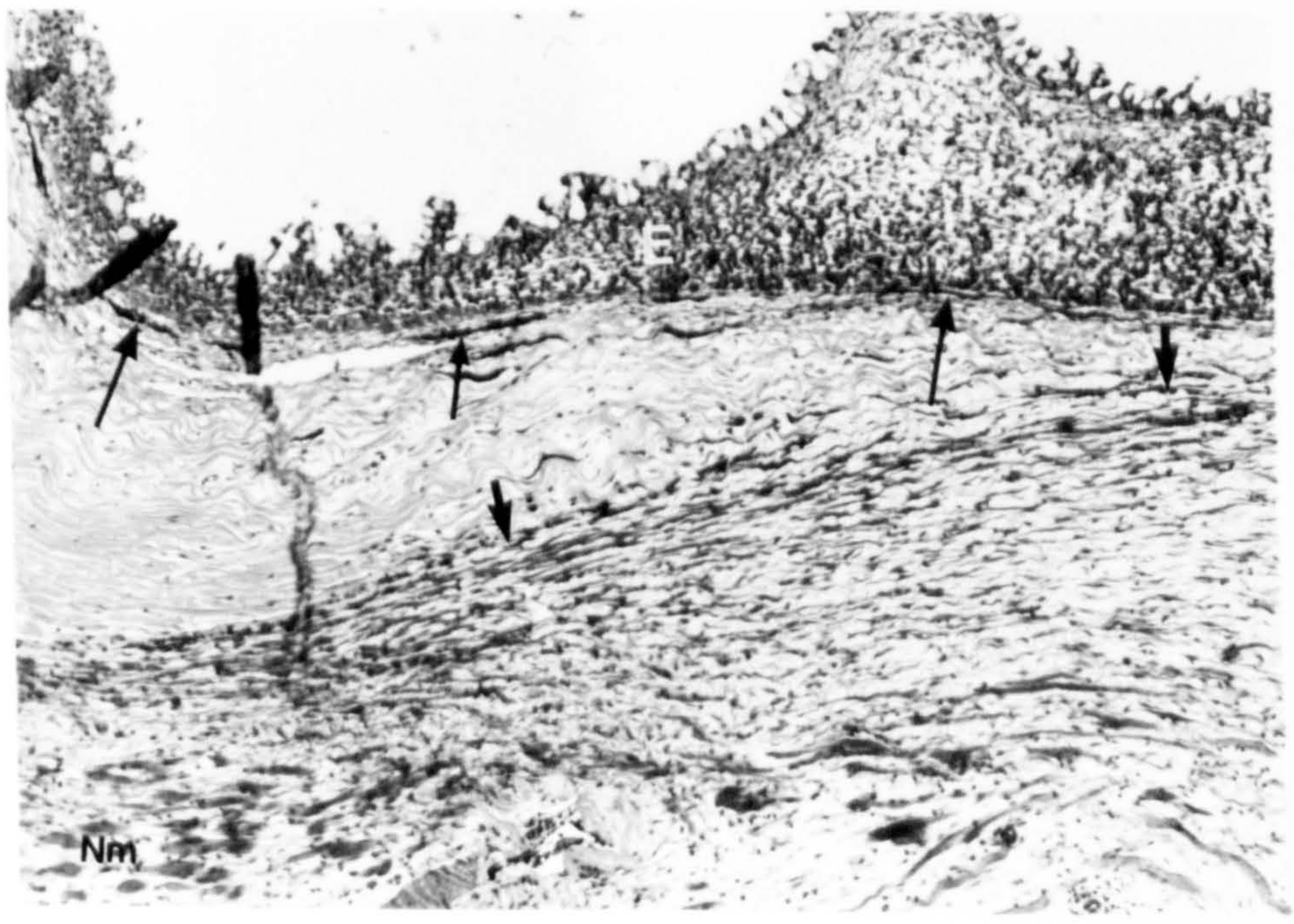
8 days

The epidermis was almost similar to that of the normal fish but still thicker over the centre of the lesion area. The overlying epidermis was mostly normal. There was a very obvious development of a melanin layer immediately below the basement membrane of the new epidermis and some in the underside fibrous tissue (Fig. 4.14). The dermis was more distinctly linked between two cut edges by active fibrous tissue. The growth of myofibrillar elements into the granulation tissue was very obvious, and the stratum spongiosum had a very active fibrous tissue activity serviced by capillary development. Lymphocyte activity and presence of some PMNs in the lesion area were evident.

Fig. 4.13 By 6 days after the incision, the new epidermis (E) was still oedematous and thicker over the lesion area. The cut edges of dermis (CD) slightly started linking. Active muscle regeneration (arrow heads), fibrosis (arrows), and vascularization (V) were obvious at this stage. (H & E, X 44).
D=dermis, ML=muscle

Fig. 4.14 A distinct development of melanin layer (arrows) immediately below the new epidermis (E) was seen at 8 days after the incision. The cut edges of dermis was more linked by active fibrous tissue (arrow heads). Also the growth of new muscles (NM) was very obvious. (H & E, X110).


E
V
CD
D
V
ML


E
Nm

16 days

By this time, the progress of the muscular and dermal regeneration led to integrity of both, across the area of the defect, being almost completed. Epidermis was normal and the red component cells had disappeared from the outer layer, but there were no club cells in a very small part of the epidermis that was exactly over the damaged dermis and muscle areas had been replaced by fibrous tissue. The two cut edges of dermis were completely closed and linked together. Stratum spongiosum was developing normal melanocyte structures. It was still more oedematous and cellular than the normal. Melanin and ceroid containing cells and lymphocytes were present in the fibrous tissue and a small part of the epidermis and also adjacent to blood vessels. Myofibrillar extension across the defect was seen. A layer of melanin was seen under the whole new epidermis and also in the fibrous tissue between two cut edges of dermis.

24 days

Epidermis was almost fully developed, but club cells were still not at normal numbers over the replaced fibrous tissue in the damaged dermis and muscle areas. Dermis was very developed with a strong link, but there was still a small part of fibrous tissue between two cut edges (Fig. 4.15). All the defect area under the dermis was filled by new muscle bundles and fibrous tissue. Melanin pigments were scattered in the damaged dermis and area of the replaced fibrous tissue. Also a number of melanin and ceroid containing cells were seen in the fibrous tissue and adjacent to blood vessels. Lymphocyte activity was evident in the lesion area.

32 days

The picture of this stage was similar to that of the 24th day with more consolidation of the damaged areas (Fig. 4.16).

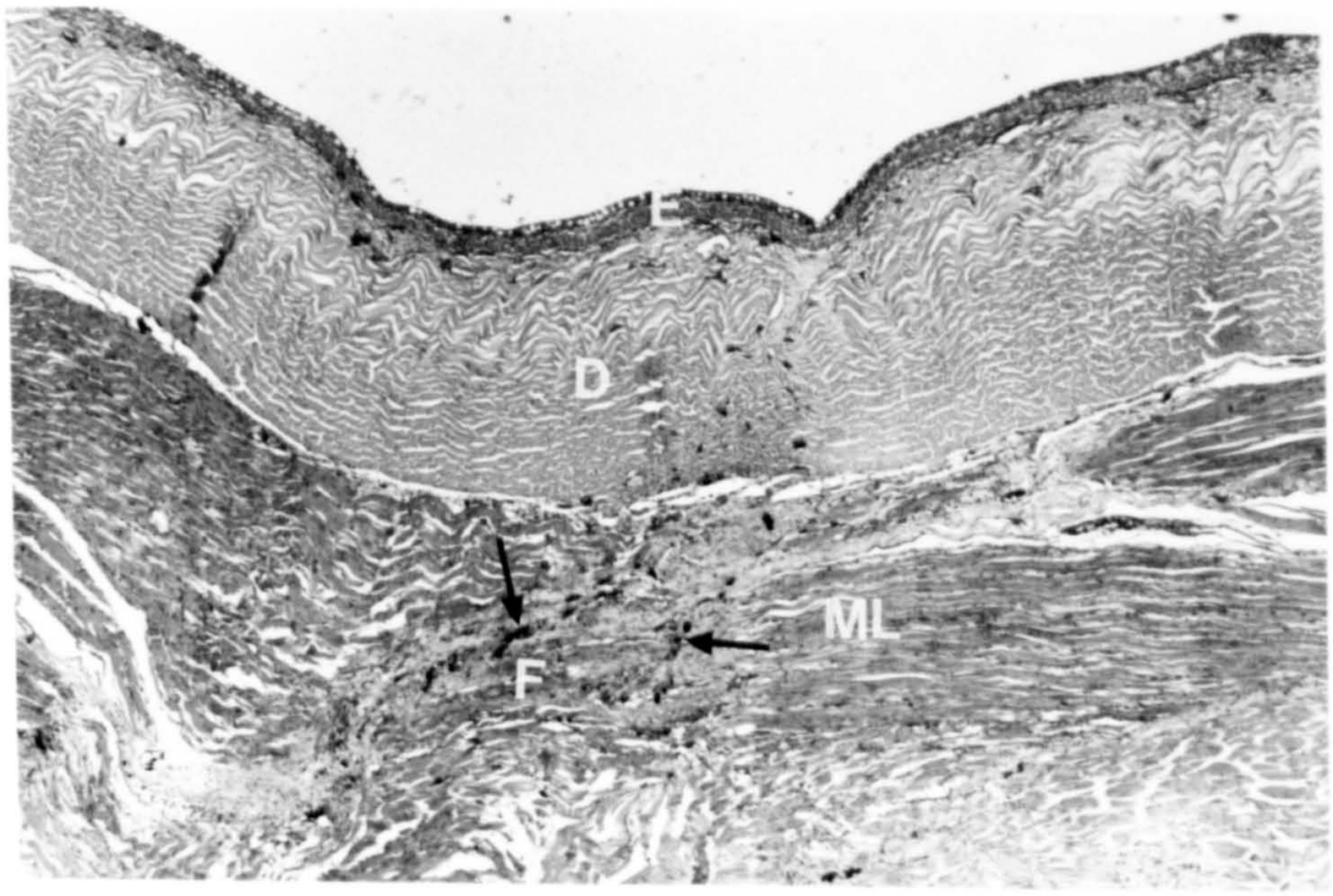
40, 50, 60 days

These three stages of the experiment were carried out to investigate the progress of completion of the club cells in number, and also the degree of dermis linking in the damaged area in the late stages of wound healing. The club cells in epidermis more increased in number in each stage than the previous stage, so that at 60 days after wounding the epidermis was completely normal. Linking of two cut edges of dermis was more strong and consistent in each stage than the previous stage, but even by the last stage of the experiment the dermis in the damaged area was not the same as a normal dermis, in consistency of collagen fibres. There were considerable melanin pigments in the fibrous tissue and new dermis. Also there were a few ceroid and melanin containing cells and macrophages in the area of the lesion. Lymphocytes activity was evident in the lesion area up to the final sampling at 60 days.

Fig. 4.15 By 24 days after wounding, new epidermis (E) was very developed and the cut dermis (CD) was completely joined. Melanin pigments (arrows) were scattered in the newly joined cut dermis and muscle area. (H & E, X 110).

Fig. 4.16 This picture shows the wound area after 32 days. Integrity of epidermis (E) and dermis (D) is similar to a normal skin. Regenerated muscle fibres (ML) filled the defect area. A small area of replaced fibrous tissue (F) with scattered melanin pigments (arrows) is obvious in the picture. (H & E, X 110).


CD


E
D
ML
F

4.5 DISCUSSION

Observations of the gross pathology of the lesion showed that the edges of lesion became black within 2 hours after wounding and areas close to the wound became brighter than the normal colour of skin. These changes in colour after incision of the skin, may be due to the passive dispersal of pigments (Mittal & Munshi 1974), under neural control (Sage 1970; Roberts 1975*a*) or destruction of melanophore control (Laird *et al.* 1975). Amiri (1991) reported that both melanosome aggregation and dispersion are controlled by an adregenic mechanism in a spinal nerve section in the European minnow (*Phoxinus phoxinus* L.). The changes in colour after wounding in this study was similar to that had been reported in skin wounds of snakehead (Chinabut 1989), catfish (Mittal *et al.* 1978 ; Mittal & Munshi 1974) , and salmon (Roberts *et al.* 1971).

One of the most important and interesting features of the wound healing, is the rapid process of epithelialization. Following the infliction of the skin, the epidermal cells started migrating and this resulted in the bridging of the cut epidermis and the covering of the wound gap. The epithelialization of the wounded fish in this study was completed within 4 hours which was slower than that of snakehead at 28°C, studied by Chinabut (1989), but very rapid in comparison with some fish species which have already been studied (table 4.1). Mittal & Munshi (1974) and Hickey (1982) correlated the rate of epithelialization with the thickness of the epidermis and suggested that if the epidermis is thick, the epithelialization of the wound is quicker than that of the thin epidermis. The results of the present study do not support this view. Epithelialization of the wound was completed within 2 hours in snakehead (Chinabut 1989), 4 hours in catfish (Mittal *et al.*1978) and 4 hours in carp (present study), despite having a thinner

epidermis than some other fishes (table 4.2). The findings of this study show that migration of epidermal cells takes place towards the wound gap using the fibrinous exudate as a substrate. At the time of migration, the epidermis, far from the cut edges, became thin with elongation of the Malpighian cells. These findings support the results of the previous studies about wound healing in fish (Mittal & Munshi 1974; Anderson & Roberts 1975; Phromsuthirak 1977; Bullock *et al.* 1978; Mittal *et al.* 1978; Hickey 1982; Chinabut 1989; Bullock & Roberts 1992). Many workers believe that epithelialization in fish takes place much quicker than in mammals and other vertebrates (Mittal & Munshi 1974; Bullock *et al.* 1978; Mittal *et al.* 1978). It can be said that more rapid epithelialization in fish may be because of their aquatic medium and the mucoid nature of the skin that the wound remains moist. This view is supported by, Krawozyk (1971) and Rovee *et al.* (1972). They observed that epithelialization of mammal skin in moist conditions is much quicker than in terrestrial conditions. It is suggested by Winter (1964) that on a moist wound, the epidermis moves through the exudate above the fibrous tissue but on a dry wound, the fibrinous clot that lays in the path, blocks epidermis movement. Therefore in fish that the wound always remains moist, the faster rate of epithelialization would be expected. According to the previous works on wound healing in fish, listed in table (4.2), the rate of epithelialization in carp (present study) is faster than the majority of other species studied. The ambient temperature is one of the important factors that are involved with the rate of epithelial migration in fish (Roberts *et al.* 1971; Anderson & Roberts 1975; Bullock *et al.* 1978). Therefore the rapid rate of epithelialization in carp in 27°C (present study) can be readily explained. Stress appears to have less influence on the rate of wound healing than temperature (Anderson & Roberts 1975).

Table 4.1: The complete epithelialization time requirement in different fish species.

Species of fish	Time required (hr.)	Temp. (°C)	Size of wound (mm)*	References
Misgurnus fossilis	12	NA	NA	Van Oosten 1957
Cobistis taenia	24	NA	NA	Kudokotsev & Silkima 1967
Protopterus annectens *Protopterus aethiopicus*	24	21	NA	Conant 1970
Rita rita	4-6	NA	5 x 2-3	Mittal & Munshi 1974
Salmo salar	3	23	4.5 x 1	Anderson & Roberts 1975
Gasterosteus aculeatus	72	NA	1.3 x ?	Phromsuthirak 1977
Pleuronectes platessa	9 12 12	10 5 15	5 x 1 " "	Bullock *et al.* 1978*b*
Heteropneustes fossilis	4	NA	5 x 2-3	Mittal *et al.* 1978
Pleuronectes platessa (Larvae)	4-12	10-11	0.1-6.6 mm^2	Hickey 1982
Clupea harengus (Larvae)	4-6	10-11	0.4-0.6 mm^2	Hickey 1982
Salmo salar (Larvae)	4-8	10-11	0.6-1.2 mm^2	Hickey 1982
Mystus vittatus	5	NA	4 x 2	Saxena & Kulshrestha 1982
Salmo salar	4	NA	NA	Rai & Mittal 1983
Oreochromus niloticus	5	28	4 x 2	Jauncey *et al.* 1985
Salmo salar	8	4	2-3 x ?	Roubal & Bullock 1988
Channa striatus	2	28	5 x 3	Chinabut 1989
Salmo salar	12	NA	5 x 1	Bullock & Roberts 1991
Cyprinus carpio	4	27	15-20 x5	Present study

NA=not available, ?=not indicated by author (s), *=long × deep

Table 4.2: Thickness of the epidermis and the time required for complete epithelialization of wound in different fishes.

Species of fish	Length (cm)	Average thickness of the epidermis (μm)	Temp. (° C)	Time required for complete epithelialization (hr.)
Rita rita	15.5	490 (Mittal 1968)	NA	4-6 (Mittal & Munshi 1974)
Misgurnus fossilis	18.2-27	338.7 (Jakubowski 1958)	NA	12 (Van Oosten 1957)
Heteropneustes fossilis	18	98 (Mittal & Munshi 1971)	NA	4 (Mittal *et al*. 1978)
Pleuronectes platessa (Larvae)	0.6-0.7	2-6 (Hickey 1982)	10-11	4 (Hickey 1982)
	1-1.2	3-7 " "	10-11	6-10 " "
	2.2-2.6	7-14 " "	10-11	11-12 " "
Clupea harengus (Larvae)	1.9-2.1	3-7 (Hickey 1982)	10-11	4-6 (Hickey 1982)
Salmo salar (Larvae)	2.4-2.7	7-12 (Hickey 1982)	10-11	4-6 (Hickey 1982)
Channa striatus	14-17	32-40 (Chinabut 1989)	28	2 (Chinabut 1989)
Cyprinus carpio	18-26	95 (present study)	27	4 (present study)

NA=not available

The rapid epidermal covering of wounds have a major survival advantage in the aqueous environment. Rapid epidermal coverage is an obvious factor in helping reduce fluid, protein and ion loss by outflow and osmosis from wounds and also in limiting the entry of potential pathogens. The early appearance of mucous cells within the healing epidermis may be of significance in that antibodies have been found in mucus (Fletcher & Grant 1969; Bradshaw *et al.* 1971; Harris 1972; Fletcher & White 1973*b*; Ourth 1980; Hjelmeland *et al.* 1983). The quicker rate of epithelialization in fish may also be correlated with the late formation of granulation tissue (Mittal & Munshi 1974).

Phromcuthirak (1977) reported that by 3 hours after wounding a basement membrane-like substance could be seen in the wounded three spined stickleback (*Gasterosteus aculeatus*) between the migrating epidermis and the underside of the dermis. The basal membrane had attained a thickness of 105 nm, compared with the original thickness of 160 nm in undamaged basement membrane by 40 days after wounding. Hickey (1982) in his study on wound healing in fish larvae reported that the normal skin structure was restored with regeneration of a new basement membrane and dermis within 3 weeks after wounding. In the present study, using special staining, it was observed that basement membrane did not exist under the new epidermis before closuring the wound gap at 4 hours after wounding. After this time it began to regenerate underneath the oedematous new epidermis and was completed within 2-4 days after wounding.

Club cells in this study were the last cell type to appear, develop and complete in number during the healing and re-epithelialization. According to Trevisan & Pederzoli (1984), club cells are the last cells to differentiate from the Malpighian cells during the larval stages. Mittal & Munshi (1969) showed histochemically that the

secretions of club cells are not mucoid in nature. The late appearance of these cells during the larval stages and re-epithelialization after wounding shows that maybe their role in defence and protective mechanisms is less than Malpighian cells or mucous cells.

Mittal & Munshi (1974) reported that the process of fibre formation and repairing the dermis in *Rita rita* started at 7-8 days after wounding and recovered completely after 13-26 days. Mittal *et al.* (1978) also found that this period in *Heteropneustes fossilis* started 5 days after wounding and the damaged dermis repaired completely by 25 days. This period in snakehead reported by Chinabut (1989), was 1-3 days after wounding at 28°C which damaged dermis started to healing and completed by 5 days. The linking between cut edges of the dermis slightly began at 6 days after injury in the present study and completely rejoined by fibrous tissue by 16 days.

It has been demonstrated that L-ascorbic acid (vitamin C) is a necessary co-factor for collagen formation (Halver 1972). In this study, the experimental fish were not fed during the course of the experiment. Nevertheless, the repairing time for dermis in comparison with the above mentioned fish, which were fed during the experiment, is reasonable. It could be also said that if the fish receive a diet with adequate vitamin C, it maybe able to repair the damaged tissue quicker than the time achieved in this study. However, it can take more than a year, depends on the ambient temperature, to restore normal architecture of dermis (R.J. Roberts 1995, personal communication). The non-collagen related changes (i.e. epithelialization) appeared to occur quite normally irrespective of the vitamin C level in fish.

With the gradual repair of dermal tissue which plays an important role in strengthening the skin and protecting the body, the overlying and thickness of the new epidermis reduced during the stages of healing.

Macrophages were first found 2 hours after wounding in the damaged muscle area and active myophagia began to take place with increase in the number of macrophages 6 hours after wounding throughout the muscle degenerated area. A large number of macrophages accumulated within the muscle degenerated area of snakehead by 3 hours at 28°C (Chinabut 1989) and Nile tilapia by 10 hours at 28°C (Jauncey *et al.* 1985), maximum number of macrophages in three spined stickleback by 3 days (Phromsuthirak 1977), first appearance of macrophages in salmon at 23°C, and catfish (*Mystus vittatus*) within 24 hours (Anderson & Roberts 1975; Saxena & Kulshrestha 1982 respectively) and plaice larvae by 5 days after wounding at 10-11°C, which was reported by Hickey (1982). The accumulation of macrophages within the wound area in carp (present study) after wounding, is slower than in snakehead, reported by Chinabut (1989), but faster than that had been reported by other workers. Disappearance of macrophages from the wound area in the present study was within 6-24 days, while Chinabut (1989) has reported 6-12 days and Anderson & Roberts (1975) reported 16-18 days after wounding. Initiation of myophagia was seen at 6 hours and myophagia was completed in 4 days after injury in the present study. Anderson & Roberts (1975) reported commencement of myophagia in salmon, in 24 hours and was completed in 16-18 days after wounding, but Timur, M.(1975) reported myophagia beginning within 3-5 days and was completed in 21-28 days after injecting of carrageenin into the plaice muscle at 10°C. Chinabut (1989) found initiation of

myophagia in snakehead in 1-2 hours and was nearly completed in 10 hours after injury.

Presence of PMNs adjacent or in the lesion reported by previous workers are as follows; 8-12 hours after wounding (Chinabut 1989), 1-3 hours (Phromsuthirak 1977), 48 hours (Anderson & Roberts 1975), 3 days at 4°C and 2 days at 12°C in salmon parr (Roberts *et al.* 1973*a*). Some other workers (Mittal & Munshi 1974; Mittal *et al.* 1978; Hickey 1982; Saxena & Kulshrestha 1982) also reported that PMNs have never been significant in the process of wound healing. Phromsuthirak (1977) showed in his study that the number of PMNs in the wound area increased significantly from 12-24 hours after wounding. The number of PMNs decreasing by day 3 and it was normal by day 8 after wounding. According to Phromsuthirak (1977), the maximum presence number of neutrophils might vary from fish to fish and between areas of the lesion. Hines & Spira (1973) reported that neutrophilia in the mirror carp occurred within 48 hours and lasted for about 8 days after an experimental infection with *Ichthyophthirius multifilis*. The PMNs appeared in the clot of fibrin, near the epidermis, 2 hours after injury in the present experiment. Thereafter they gradually increased in the lesion area so that they were seen along and within the degenerated muscle area at 10 hours. However, their presence around and, to some extent, within the degenerated muscles was considerable and remained for 8 days in the damaged area. According to Hine & Wain (1988) mature neutrophils (toxic neutrophils) and immature neutrophils which prematurely release into the peripheral circulation is a non-specific response to stressors as well as to inflammation and disease. Phromsuthirak (1977) reported that PMNs are phagocytic, although not to the same extent as the macrophages. Roberts *et al.* (1973*a*) also reported that PMN activity around the necrotic sarcoplasm was considerable until the

15th day after wounding in salmon. Watson *et al.* (1963); Weinreb & Weinreb (1969); Finn & Nielson (1971*b*) and Davies & Haynes (1975) reported that neutrophils are phagocytic cells while Klontz (1972) and Ellis (1976) showed that neutrophils do not take part in phagocytic activity towards carbon particle and bacteria. However, in the present study PMNs seemed to be myophagic along with the macrophages.

Mittal & Munshi (1974) and Mittal *et al.* (1978) in catfish and Chinabut (1989) in snakehead, reported the enlargement of lymphatic space, which filled with lymphocytes. Lymphocytes were observed in the lesion area during the whole course of the present experiment. Also they were seen in the epidermis and between the muscles. Their number was increasing gradually during the healing period. The enlargement of the lymphatic spaces and the increase in the number of lymphocytes after the injury could be an indication of an immunological reaction of the defence mechanism (Mittal & Munshi 1974; Ellis 1977; Mittal *et al.* 1978).

First appearance of new fibroblasts adjacent to blood vessels and in the intermyotomal fascia adjoining the affected myotome, was at 8 hours, that is a rapid rate of fibroblasts forming in comparison with some other fish species. The peak of developing of granulation tissue was within 4-6 days after wounding that had been replaced later by the new muscle fibres within 4-16 days. Chinabut (1989) reported appearance of inactive form of fibroblasts at 4-5 hours in the snakehead lesion at 28°C and peak of developing of granulation tissue at 3-5 days and replacement by the new muscle fibres within 4-14 days. The results of the present study show slightly slower rate in fibroblasts and granulation tissue forming, and muscle fibre replacement than in snakehead, but it is much faster than in fresh water catfish (Mittal & Munshi 1974; Mittal *et al.* 1978; Saxena & Kulshrestha 1982), three spined stickleback

(Phromsuthirak 1977), Nile tilapia at 28°C (Jauncey *et al.* 1985) that all were reported within 3 days, and also much faster than in salmon at 23°C (Anderson & Roberts 1975) and plaice larvae at 10-11°C (Hickey 1982) that were reported 55 hours and 11 days respectively.

Sarcoplasmic budding or myofibrillar regeneration in the damaged muscle area in this study, was seen in 2 days after injury. This is slower than 8-12 hours in snakehead at 28°C which was reported by Chinabut (1989), but it is faster than the rate of sarcoplasmic budding recorded within 4-8 days after wounding in *Rita rita* (Mittal & Munshi 1974), salmon at 23°C (Anderson & Roberts 1975), catfish (Mittal *et al.* 1978), and tilapia at 28°C (Jauncey *et al.* 1985). Muscle regeneration in carp wound area in this investigation, was completed in 16 days, but Chinabut (1989) reported that in snakehead at 28°C, was almost completed in 14 days after wounding. It is necessary to mention that the rate of muscle regeneration and temperature are strongly correlated. Anderson & Roberts (1975) reported that sarcoplasmic regeneration in salmon took place within 4-7 days at 23°C but 8-38 days at 5°C.

Melanin pigments within perivascular tissue and larger vessels of the fascial area and also in the intermyotomal fascia adjoining the affected myotome were seen at 8 hours after injury in this study, but it was not seen in the muscle area replaced by fibrous tissue until 8th day. At 8 days, development of a melanin layer below the basement membrane of the new epidermis and under the fibrous tissue was obvious. Thereafter the melanin pigments in the healing area and also in the dermal area greatly increased, as the healing process developed. They mostly appeared in a dendritic shape through the area. Anderson & Roberts (1975) reported observation of melanin pigments in salmon in the wound area within 4-6 days and Mittal *et al.* (1978) also

reported the same period in catfish, but Hickey (1982) observed nodules of dark pigments in skin wounds in plaice larvae after 20-30 days. Chinabut (1989) reported presence of the melanin pigments in snakehead in the epidermal cells within 4-5 hours and in the necrotic muscle fibre area by 6-7 hours and also melanin layer below the basement membrane within 9-12 hours after wounding. Melanophores play a part in the development of most skin lesions because of their presence immediately below the delicate epidermis where they are vulnerable to traumatic damage of many kinds (Roberts 1975*a*). When the skin is wounded by predators, or in the process of tagging, the melanosomes released from the damaged pigment cells are engulfed relatively rapidly, and macrophages laden with such granules can then be seen in considerable numbers, migrating through the epidermis of the healing lesion and opening on the surface (Roberts *et al.* 1973*a*). In addition to melanins role in camouflage for fish, Edelstein (1971) reported that melanin and related pigments play a defensive role in many organisms by their capacity for H_2O_2 generation. Ellis (1981) also suggested that the melanin may play a role in bactericidal mechanisms or as a modulator of bactericidal mechanisms which involve the production of free radicals by phagocytic cells. Bullock (1988) reported that in the irradiation (UV) experiments on turbot skin there was no evidence of an increase in sunburn cell numbers in cells in close proximity to the melanocytes although, on occasion, where two or more melanocytes were situated closely, localised hyperplasia did occur; sunburn cells were not a major feature of the reaction. Hyperplasia of this type was interpreted as being an immediate localised response to radiation as the melanocytes swell and push their melanosomes through the dendritic processes. However, dispersal of the melanosomes within the dendritic processes with a concomitant swelling of the melanocyte nuclei was a reaction

at the lower doses of irradiation, but at higher doses the melanocytes appeared to aggregate and form dense patches. Roberts (1989) described that lack of a keratinized layer in fish skin and the presence of dividing cells in all layers makes fish skin more readily damaged by Ultraviolet-B emissions. This is compounded by the fact that the fish epidermis normally does not contain protective melanin-containing cells. The presence and dispersion of melanin pigments throughout the newly healed area of the lesion, prevents the penetration of Ultraviolet radiation into the underlying tissue, considering that damaged skin, and newly regenerated epidermis, and joined cut dermis are not able and strong enough to prevent the penetration of UV (R.J. Roberts 1997, personal communication).

In this investigation, vascularization started at 6 hours after incision, both in the degenerating muscle area and in the stratum spongiosum. Along with the healing and contraction of the wound, the capillaries gradually reduced and in late stages of healing it became difficult to locate the capillaries in the healed area with a very few exceptions. Chinabut (1989) reported that in snakehead at 28°C, vascularization developed after 2 hours in the muscle area and 3 hours in the dermis. This process was observed in 3 days in litid catfish (Mittal *et al.* 1978) and mystid catfish (Saxena & Kulshrestha 1982), 31 hours in Atlantic salmon at 23°C (Anderson & Roberts 1975) and 20 hours in catfish (Mittal & Munshi 1974).

The relatively rapid appearance of blood capillaries in the wound area in carp, in the present study, has a major role in rapid process of wound healing. It can cause an active migration of defence cells to the wound area to prevent the invasion of bacteria into the underlying tissues and also prevent absorption of toxins. It can also supply the nutrients for the actively proliferating cells in the lesion area.

CHAPTER 5:

CHRONIC INFLAMMATION IN RESPONSE TO TALC (MAGNESIUM SILICATE) INJECTION IN CARP (*Cyprinus carpio* L.)

5.1 INTRODUCTION

Talc (magnesium silicate) is known as French chalk, soapstone or steatite and is a naturally occurring magnesium silicate [3(MgO), 4(SiO_2), H_2O], belonging to crystal system number 4 (Kingzett, 1940). It is also known as a causative agent of granuloma formation and giant cell production in mammals. There are large numbers of reports on granulomatous lesions caused by talc, naturally or experimentally, in mammals while very few investigations has been carried out in fish. Shattuck described the essential features of the silica granuloma when he presented the first case report in 1917. The first explanation of the pathogenesis of granulomata due to silicaceous material was advanced by Kettle (1932), who suggested that the presence of finely divided silica produced a colloidal solution which acted as a tissue poison. Also Gardner (1932 & 1937) showed experimentally that the type of reaction is dependent on the size of the particles introduced. He confirmed that the introduction of silica into tissues can produce all the type of cellular response found in tuberculosis. Talc can cause granuloma in various intestinal and pelvic organs (German 1943). In the skin, localised silica granulomas due to glove talc have been observed in surgical scars (Fienberg 1937; Gardner 1937), and as the result of the impregnation of the skin by silicaceous particles such as glass or sand, following an injury (Crossland 1955; Epstein 1955). Once the talc is deposited in the tissue, it may be dormant even for years. The standard hospital talc (as previously used by surgeons for dusting gloves) shows under the microscope a marked similarity in shape and size to the particles of talc present in the tissues of the human body (Roberts 1947).

Wolke and Trainor (1970) reported a naturally occurring granulomatous enteritis in white sucker fish (*Catostomus commersoni*) associated with diatoms (silicon oxide).

Timur, G. (1975) used talc powder along with some other inflammatory agents to study giant cells in inflammatory lesions of the plaice (*Pleuronectes platessa*). An experimental assessment on granulomatous nodules in rainbow trout (*O. mykiss*) and turbot (*Scophthalmus maximus*), using talc as inert particles and BCG as immunogenic material, has been reported by Balouet & Baudin Laurencin (1986). In all the above mentioned studies on fish, the workers reported granulomas caused due to talc.

The majority of the information about inflammatory response against talc is derived from human or other mammals study. There is little information available regarding inflammatory response against talc in fish. The aim of the present experiment is to define the inflammatory reactions of carp (C*yprinus carpio*) after injection of the talc. Talc has been chosen as an inorganic irritant to use in the present study because of its easy availability, and capability of granuloma production and also to compare the results with the other stimuli used in the other experiments.

5.2 LITERATURE REVIEW

5.2.1 Granulomatous inflammation

Granulomatous inflammation has been described in detail in chapter 3. It is well recognised that granulomatous inflammation maybe caused by a variety of different agents. Many foreign substances when injected or implanted in the body by trauma, could provoke a granulomatous reaction (Jubb & Kennedy 1970).

5.2.1.1 Infective granulomas

This kind of granuloma maybe caused by bacteria such as *Mycobacterium* spp. and *Renibacterium* spp. or certain fungi, notably *Ichthyophonus hoferi* and *Aspergillus* spp., or Parasites such as microsporidians, myxosporidians and helminths (Roberts 1989; Balouet & Baudin Laurencin 1986). The classical infective granuloma of homeothermic and poikilothermic vertebrates is that seen in tuberculosis where histologically there is central necrosis and a peripheral collar of epithelioid cells, giant cells, fibroblasts and lymphocytes (Francis 1958).

A detailed record and explanations about *Mycobacterium* spp. and fungus granulomas is given in chapters 6 and 8 respectively.

Differential diagnoses must consider different agents responsible for the granulomata formation. Positive diagnosis is possible for mycobacteriosis by means of the Ziehl-Neelsen method for acid-fast organisms which reveals the presence of the bacillus in the central zone and within macrophages. This central zone of necrosis is also Periodic Acid-Schiff (PAS)-positive (Amlacher 1961). *Flavobacterium* spp.

produce a similar reaction to *Mycobacterium*, but these bacteria are not acid-fast. The mycelia and spore stages of *Ichthyophonus hoferi*, a fungus of marine fishes which produces granuloma, are PAS-positive. Various migrating helminths usually elicit a granulomatous response which is dominated by eosinophilic granular cells. Protozoans (microsporidians and myxosporidians) maybe identified in a granulomatous lesion by means of the Giemsa stain (Wolke 1975).

5.2.1.2 Foreign body granulomas

Many organic and inorganic substances can be causative agents of foreign body granulomata. Granulomata in fish have been described in response to a variety of agents including inert materials (Timur, M. 1975; Timur, G. 1975).

The well known examples of these agents are; Freund's incomplete adjuvant, carrageenin (marine algae), marking tags, silicaceous diatoms (silicon dioxide), silicaquartz, talcum powder (magnesium silicate), beryllium, aluminium hydroxide, starch, lipids, glass fibre, fibrous silicate (asbestos), nylon, tattoos, barium sulphate, paraffin oil, colloidal substances, polystyrene beads, turpentine, and many others.

5.2.1.2.1 Freund's incomplete adjuvant

Freund's incomplete adjuvant contains mineral oil and emulsifying agent. It is used to aid production of high titres of serum antibody in experimental immunology studies (Freund *et al.* 1948). It produces a specific cellular reaction in the injection area. It also produces a cellular reaction in internal organs such as spleen, kidney and liver when injected intravenously. This specific cellular reaction consists of usual

components of the chronic granuloma (Steiner *et al.* 1960; Spector & Lykke 1966; Spector & Willoughby 1968).

Chronic granulomatous inflammatory lesions were experimentally induced in plaice (*Pleuronectes platessa*) by Timur, G. (1975). She used complete and incomplete Freund's adjuvant as a part of irritants in her study and showed that both produced chronic granulomas composed of macrophages, lymphocytes, plasma cells, epithelioid cells and giant cells. The absence of *Mycobacterium* from Freund's incomplete adjuvant did not affect the production of granulomas, but there were significantly fewer lymphocytes and plasma cells seen at all stages in incomplete adjuvant as compared with complete adjuvant.

5.2.1.2.2 Carrageenin

Carrageenin is an extract of seaweed (*Chondrus crispus*) with a high molecular weigh and is a long chain polymer of sulphated galactose units (Smith & Cook 1953; Smith *et al.* 1954). It has been widely used as an irritant to induce chronic inflammation in man and animals (Robertson & Schwartz 1953; Williams 1957; Ryan & Spector 1969).

The injection of carrageenin produces a local response consisting of massive macrophage infiltration, as a granuloma, followed by deposition and finally by resorption of connective tissue (Jackson 1957; Chvapil & Cmuchalova 1961).

Timur, M. (1975) studied the granuloma in the plaice induced by carrageenin. He also reviewed the literature on the inflammatory response in mammals and fish. Timur, M. *et al.* (1977) reported that in mammals the carrageenin granuloma is relatively constant between species and its component cells were also observed in their

study in the plaice. The results of their investigation were analogous to those described in higher animals but plasma cells and giant cells, often absent in higher animals, were present in the plaice. Timur, M. *et al.* (1985) later showed that all of the stages of carrageenin granulation were considerably delayed at 5°C compared to similar lesion reported at 10°C (Timur, M. *et al.* 1977).

5.2.1.2.3 Marking tags

Marking tags are especially used to study biological and behavioural aspects of animals. In fish, it has been used to study aspects of the anadromous life cycle, and to distinguish fish of different origins (Roberts *et al.* 1973*a*), or measurement of movement (Young *et al.* 1972).

Roberts and his co-workers (1973*a,b,c*) have investigated the histopathology of tagging in the Atlantic salmon (*Salmo salar* L.). They used a plastic monofilament trace, carrying a plastic identification tag inserted through the dorsal myotomes below the dorsal fin. They found that inflammatory phenomena were closely comparable with those of mammals with a slower time scale. The cellular response at the early stage lesion after tagging comprised mainly of macrophages and a few PMNs. A firmly established fibro-granulation tissue was extant later. This fibro-granulation tissue appeared to be among the most significant factors in the isolation of the tissues damaged at tagging from the normal tissues. Eight days after tagging early granuloma formation was evident and then developed to reach maturity at 25 days when they consisted also of multinucleated giant cells of foreign body type. They concluded from the study on the returned tagged fish, one or two years later, that the tagging lesion is quite a severe traumatic insult to the young fish. Roberts *et al.* (1973*c*) also showed that mycotic

infection at or around the time of tagging, infection due to bacteria localised at the tagging wound and generalised infection, developed in tagged fish used in their studies.

Young *et al.* (1972) passed silver wires through dorsal muscle of brown trout (*Salmo trutta*) to keep a small sonic tag on the body of fish. This technique was used to measure the pattern and levels of swimming activity of fish in their natural environment, but they did not study its histopathological effects on the fish tissues.

5.2.1.2.4 Silicaceous diatoms (natural)

A division of the algae known as the chrysophyta contains unicellular plants with cell walls composed of hydrated amorphous silica (silicon dioxide), are commonly referred to as diatoms. They are ubiquitous in aquatic and soil environments (Smith 1950; Lewin 1957; Patric & Reimer 1966). Silicon oxide is known to produce severe granulomatous reaction in the lung and other organs of man and domestic animals (Gardner 1937).

Wolke & Trainor (1971) described a granulomatous enteritis in the white sucker fish (*Catostomus commersoni*) in response to silicaceous diatoms. The granuloma comprised of epithelioid cells, lymphocytes and giant cells. Also ovoid or rectangular striated refractile bodies were found free in the lumen of the gut. They concluded that maybe there was a pre-existing lesion which allowed entrance of the diatoms to the intestinal submucosa, where they were able to elicit the observed reaction as foreign bodies due to their silicon content.

5.2.1.2.5 Silica (experimental)

Silica reaction can result from the contamination of wounds with particles of silica. Silica can be derived from soil or glass which contains silicon dioxide. Such wounds appear to heal but the interval between the causative accident and the development of the silica granuloma varied in man from several months to many years (Epstein 1955; Shelly & Hurley 1960). Roberts (1947) reported silicaceous granulomata in human occurring in appendectomy scars and fallopian tubes. A large number of granulomata containing epithelioid cells, giant cells and lymphocytes were observed histologically. Also numerous small particles of silica close to the giant cells and within them was revealed with the polarising microscope.

Tye *et al.* (1966) also described talc granulomas of the skin of human. They suggested that talcum powder gained entrance through the skin at the sites of draining or incised furuncles. The granulomatous lesion was composed of epithelioid cells and giant cells. Polarised light, microscopic and X-ray studies confirmed talc as the crystal particles. Also, electron microscopic studies showed the electron dense lamella to be morphologically identical to that of talc in many phagocytic cells. These studies confirmed the talc as the causal agent of the foreign body granulomata formation.

A similar lesion consisted of fibrous tissue in which there were many giant cells containing refractile crystals of talc was described by McCallum & Hall (1970). Sommerville & Milne (1950) in a case study of human injury observed a granulomatous lesion consisting of epithelioid cells and giant cells with a peripheral rim of lymphocytes. The giant cells were related to particles of crystalline material which with polarising microscope examination were seen to be refractile.

Timur, G. (1975) studied the development of giant cells and granuloma in plaice (*Pleuronectes platessa*) in response to inoculation of complete and incomplete Freund's adjuvant, *Mycobacterium* spp., talc (magnesium silicate) and beryllium oxide. The chronic inflammatory response of the plaice showed many features of similarity to that of higher vertebrates, but the rate of response is temperature-dependent in fish, so that the higher the temperature, the greater and quicker the response would be achieved. She noted that all agents used in her investigation produced chronic granulomata and described that the cellular inflammatory response consisted of a few neutrophil leukocytes, occasional lymphocytes, macrophages and two types of giant cell (Langhans and foreign body) were evident in the granulomata. The giant cells present in talc and beryllium oxide experiments bore large distinctive talc particles and amounts of beryllium within their cytoplasm, respectively.

Balouet & Baudin Laurencin (1986) in their study on granulomatous nodules in fish investigated the aetiology and histogenesis of granulomatous nodules in rainbow trout (*O. mykiss*) and turbot (*Scophthalmus maximus*) in response to inert (talc particles) and immunogenic (BCG) material. They identified two basic types of granulomatous nodule; solid homogeneous and cystic (necrotic). Solid homogeneous nodules consisted of concentrically arranged epithelioid cells. The centre of the nodule contained similar or larger whirling cells with clear or eosinophilic cytoplasm with some pigment granules. Around the epithelioid cells there was some vascular congestion mixed with a non-specific inflammatory reaction, lymphocytic or histiocytic with multinucleated giant cells. Cystic or necrotic granulomatous nodules were larger than the solid nodules and the centre was necrotic with granular or nuclear debris. A peripheral lamellar cell arrangement was around the necrotic centre. The non-specific inflammatory reaction

persisted, or was replaced by a fibrous capsule. Both talc crystals and bacteria were observed in the nodules.

5.2.1.3 Unknown aetiology granulomas

There are some chronic granulomas with indistinct aetiology. Sarcoid granuloma (sarcoidosis) as one of these granulomas in human has been described by Longcope & Freiman (1952), Wanstrup & Christensen (1966), Hirsch *et al.* (1967), Montgomery (1967), and Spector (1977). They characterised sarcoid as a focal granuloma composed of epithelioid cells and Langhans type giant cells which are large and irregular shape and occasionally containing asteroid inclusion bodies. In addition to epithelioid and giant cells as the most prominent histological elements in the sarcoid granuloma, a narrow zone of lymphocytes and occasional plasma cells at the periphery of the epithelioid cells maybe present.

Lamas *et al.* (1996) reported orange nodules of different sizes on the skin and fins of cultured turbot (*Scophthalmus maximus*). These nodules were located mainly in the dermis and hypodermis and were composed of large numbers of aggregates of pigment-laden macrophages surrounded by a mixture of epithelioid cells and spindle-shaped cells and occasional giant cells. Their histochemical studies suggested that the orange pigments were lipofuscin-like substances. They suggested that these substances or their precursors were included in the food, that affected fish were not able to metabolise them. They finally concluded that the ultimate cause of these nodules has not yet been recognised.

5.3 PILOT EXPERIMENT

A pilot experiment was carried out with a small number of fish to determine the optimum dose rate of talc suspension for injection and sacrifice time, and also the optimum method of sampling, trimming, and sectioning the tissues.

The fish were inoculated with 0.15 ml of 3% talc suspension by 1 ml syringe and 25 gauge needle in the left and right dorsal myotomal muscle. One fish was sacrificed at 8 days after injection and the blocks including the inoculated areas were cut out and fixed in 10% buffered formalin for 24 hours.

Each tissue was trimmed and cut from the middle, into two pieces. Thereafter these tissues were processed using standard procedure for routine histology. After processing, each piece of tissue was embedded in wax. These waxed blocks of tissue were decalcified and 5 μm sections taken. Sections were stained using haematoxylin and eosin (H & E).

The lesion in this sample was small and the histopathological picture of the lesion comprised some cellular infiltration, epithelioid cells and a very few multinucleated giant cells with refractile talc particles.

Regarding the results of this pilot experiment, a number of problems with the methodology used was highlighted. Therefore some changes in the protocol were made to ensure a greater cellular response in the definitive experiment.

An increase in dose rate of irritant to 0.3 ml for each injection was used and also the needle gauge was changed to 23, to inoculate more irritant into the fish body readily, so as to have a greater cellular reaction. It was also decided to sample two fish at each time and reduce the interval to cover all stages of the inflammatory response.

In this pilot experiment, first, each fixed tissue was trimmed and cut into two pieces, and thereafter processed. There was a problem at the embedding time to ensure the correct orientation of the injection site, because the tissues were dried and, to some extent, shrunk. Therefore this method was changed in the main study and tissues were trimmed and cut into two pieces after processing but before embedding into wax.

The muscles of the fish in this pilot experiment were very emaciated and the muscle bundles were small and separated from each other. There was also a disruption in the muscles adjacent to the damaged area. Therefore the traumatic and pathological changes in the muscles were not very clear. The reason of this problem probably was that the fish were not fed during the course of acclimation and pilot experiment, so it was decided to feed fish during the course of the main experiment.

5.4 DEFINITIVE EXPERIMENT

Following on the findings of the pilot experiment, the definitive experiment was carried out.

5.4.1 Materials and methods

5.4.1.1 Fish

Forty carp were obtained from the same fish farm mentioned in chapter 4 and held in glass aquaria to acclimatise, for at least two weeks before the experiment began. The average length of fish was 21 cm (± 7 cm). Fish were fed on a pelleted diet (Fry 02, Trouw Aquaculture, Inverbreakie, Invergordon, Ross-Shire, Scotland) once per day.

5.4.1.2 Aquaria and water

The aquaria and water system were the same as those described in chapter 4. The average temperature of water was 26.5°C (± 3°C). Faecal material were removed from aquaria every day and 10-20% of the water was changed as necessary.

5.4.1.3 Injection procedure

Talcum powder (magnesium silicate) was prepared for injection. Two grams of talcum powder suspended in 100 ml distilled water was centrifuged 3 times for purification. Then a 3% suspension was made using normal saline. This suspension was then sterilised by autoclave. The anaesthetised fish (0.5 ml/l of 10% benzocaine) were inoculated with 0.3 ml of talc suspension into the left side of dorsal myotomal muscle (Fig. 1.1), using a 1 ml syringe and 23 gauge needle. A scale in line with the first ray of

the dorsal fin was removed before injection either for easy injection and as a marker for subsequent sampling. Control fish were injected intramuscularly on the left myotome with the same volume of sterile normal saline. After injection the fish were returned to the aquaria until sacrificed.

5.4.1.4 Sampling and histological procedures

Two injected fish were sacrificed by an overdose of 10% benzocaine at 6 hours, 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after injection. The blocks of tissue encompassing the inoculation area were immediately removed and fixed in cold 10% buffered formalin for at least 24 hours before cassetting. The fixative was changed with fresh fixative at least once during the fixation period. The fixed and cassetted tissues were processed in an automatic tissue processor using standard procedure. After processing, tissues were cut down the middle into two pieces to expose the site of injection for sectioning. These trimmed blocks were then embedded into wax, decalcified by RDC (Rapid Decalcifier from CellPath plc, Herts, England), and 5 μm sections prepared using a Leica Rotary Microtome (appendix 1.1).

5.4.1.5 Staining procedure

The standard H & E staining method (appendix 2.1.1) was used for staining of the sections. Pertex mounting medium (CellPath plc, Herts, England) with a coverglass was used to mount the stained sections for microscopic examination.

5.4.2 Results

5.4.2.1 Gross pathology

There were no clinical signs or gross lesions observed in the fish, after injection.

5.4.2.2 Histopathology

6 hours

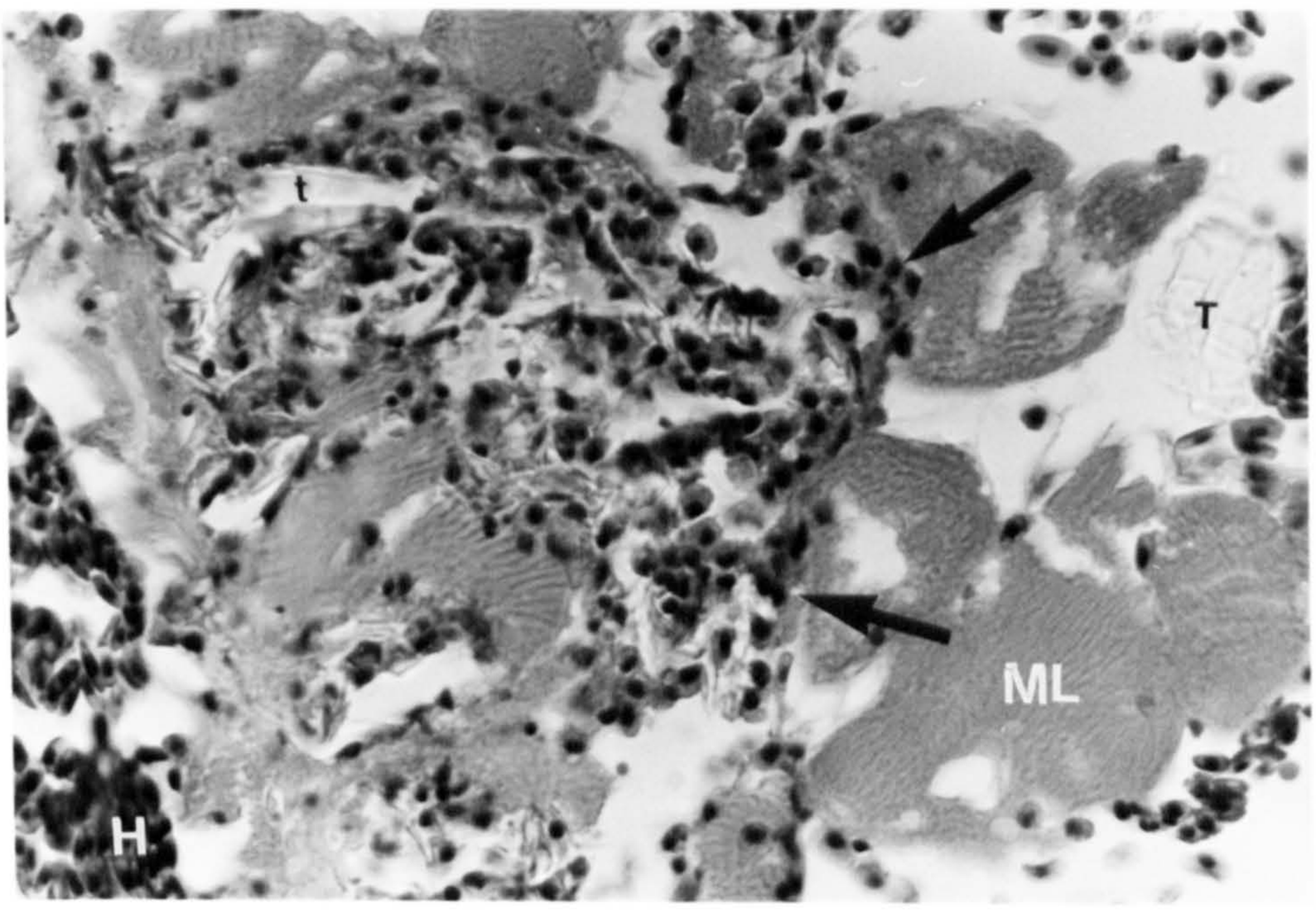
By 6 hours after inoculation of talc suspension, there was an acute inflammatory response, consisting of haemorrhage and exudate with considerable numbers of inflammatory cells. Muscle fibres in the area of injection showed degeneration, and myophagia was active in the damaged muscle area (Fig. 5.1). Numbers of macrophages and melanocytes were found around the blood vessels adjacent to the damaged area. Refractile talc particles were seen lying free and also between muscle bundles in the site of injection.

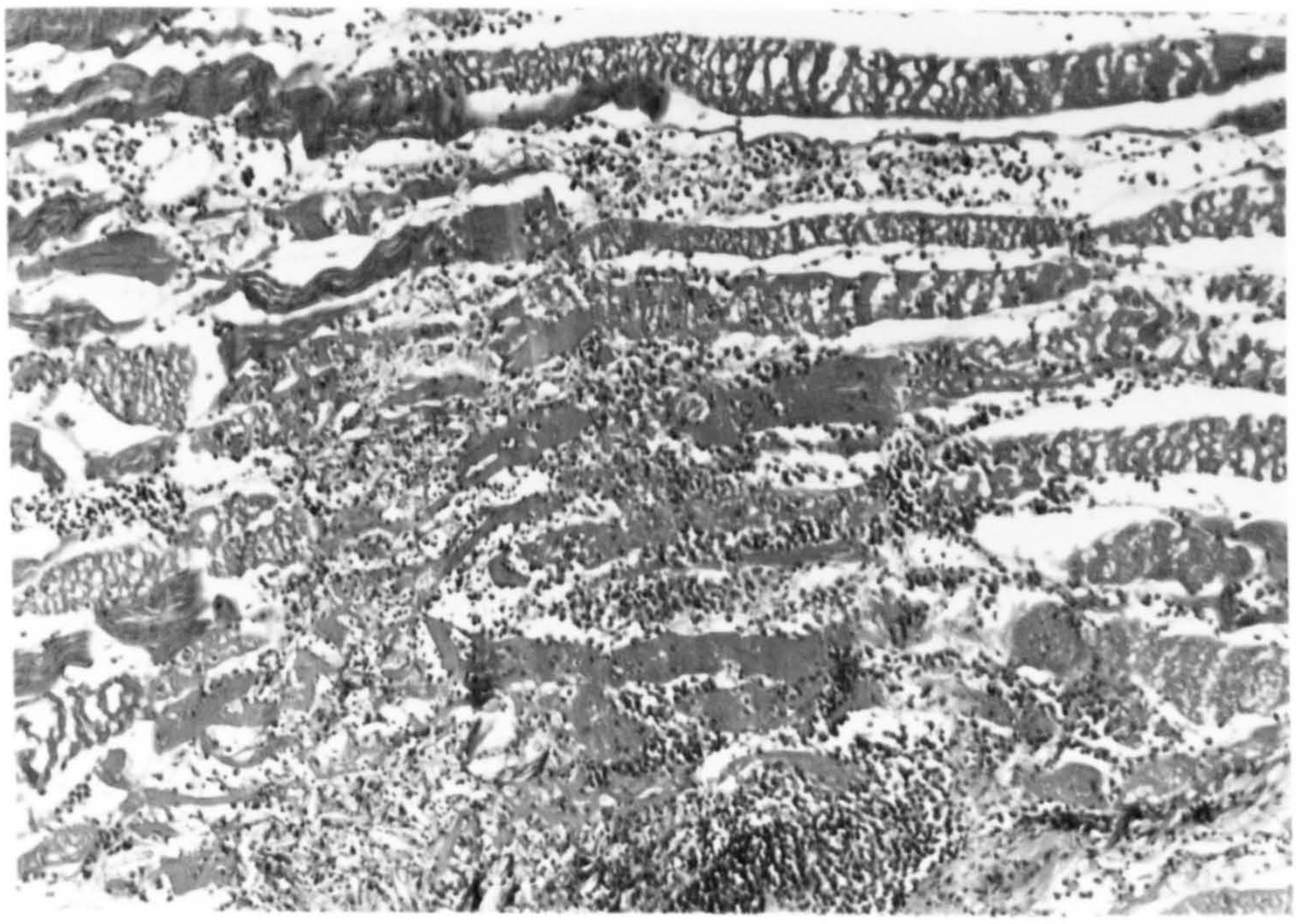
24 hours

The principal feature in this stage was presence of a large numbers of macrophages and active myophagia in the damaged muscle area. Also extensive muscle degeneration, exudate and large numbers of thrombocytes, lymphocytes, macrophages and polymorphonuclear cells (PMNs) were obvious in the lesion area (Fig. 5.2). Talc crystals were seen between and within the muscles, in the area of injection.

Fig. 5.1 By 6 hours after injection of talc, considerable numbers of inflammatory cells had infiltrated the lesion area. Myophagia was active (arrows), and refractile talc particles were seen lying free (T) and also between muscle bundles (t). (H & E, X 440). ML=muscle, H=haemorrhage

Fig. 5.2 An extensive muscle degeneration and large numbers of inflammatory cells were obvious at 24 hours p.i. (H & E, X 110).


t
T
ML
H



2 days

At this stage, more cellular infiltration, degenerating muscles and myophagia were obvious. Some PMNs and haemorrhage were observed in the lesion area and blood vessels were associated with melanin pigments. Exudate and strands of fibrin associated with thrombocytes was extensively present in the lesion area. There was evidence of macrophages which intermingled to make a pattern of epithelioid cells (Fig. 5.3). Talc particles were scattered between and within damaged muscles.

3 days

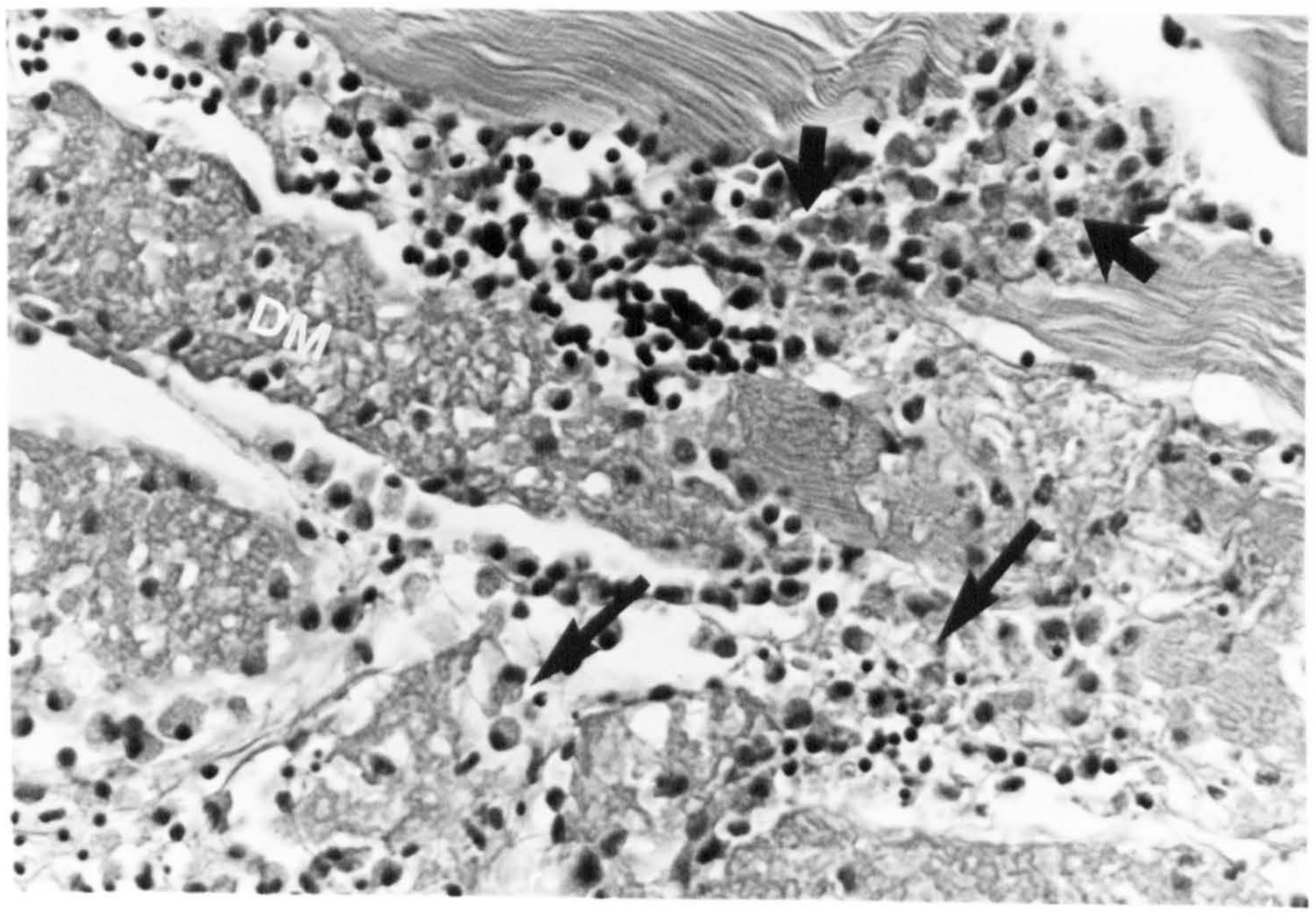
The main feature in this stage was the first presence of epithelioid cells and giant cells (Langhans and foreign body types) in the damaged area surrounding talc fragments (Fig. 5.4). Degenerated muscles, haemorrhage, large numbers of inflammatory cells such as macrophages, PMNs, lymphocytes and active fibroblasts were observed. Also new capillaries, fibroplasia and myophagia were obvious in the lesion area. Basophilic small new muscle buds were seen in the damaged area (Fig. 5.5). Blood vessels were congested and associated with melanin pigments. Talc particles were scattered in the lesion area.

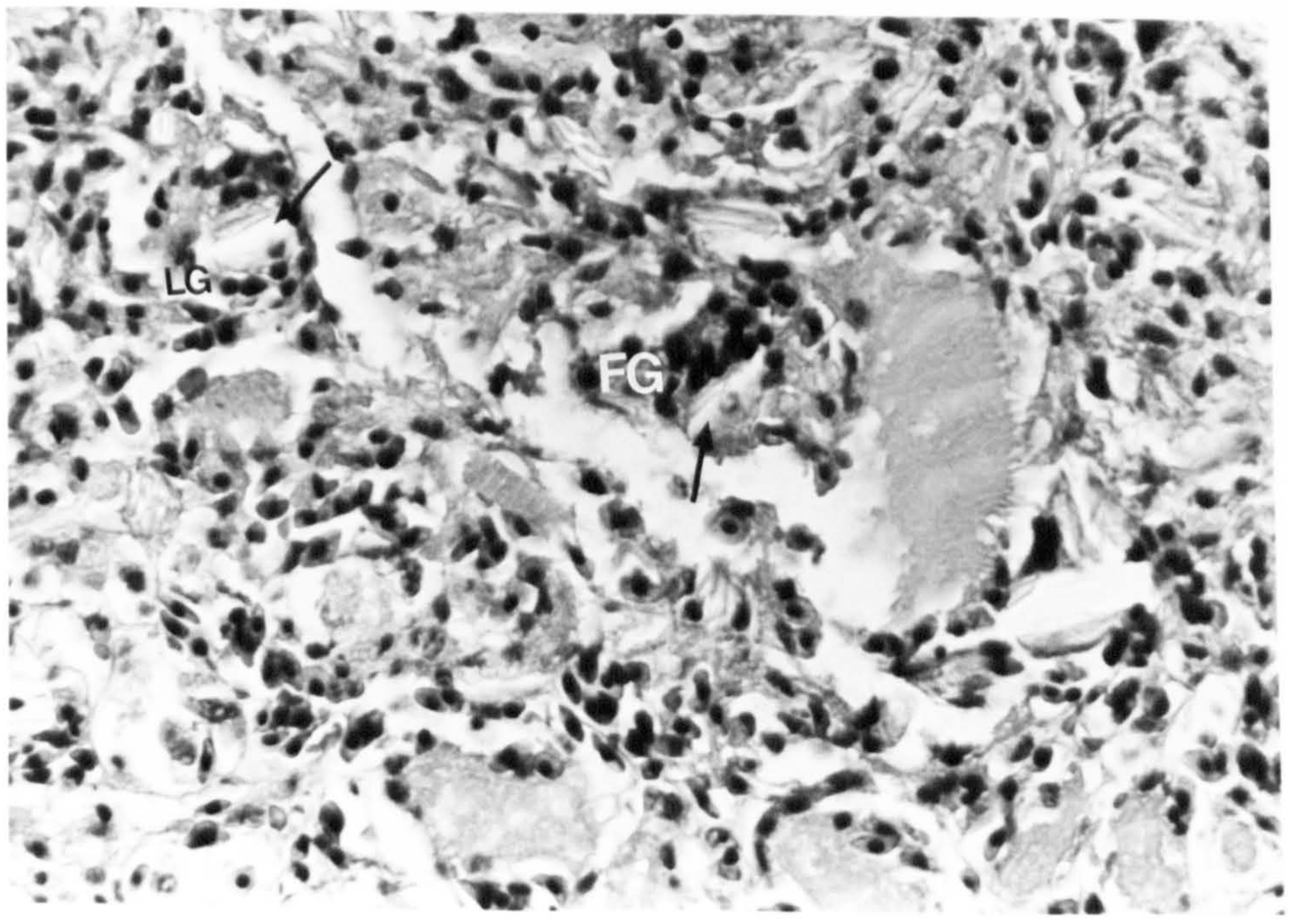
4 days

In this stage, there were haemorrhage and inflammatory cells consists of macrophages, PMNs, lymphocytes and epithelioid cells in the lesion area. Myophagia was completed. Also, active fibroblasts and fibroplasia with vascularization and new muscle fibres were observed. The dominant feature in this stage was increasing the

Fig. 5.3 At 2 days p.i., macrophages started to intermingle to make an epithelioid cell configuration (arrow heads). Myophagia was active (arrows). (H & E, X 440). DM=degenerated muscle

Fig. 5.4 This picture demonstrates the first presence of Langhans type (LG) and foreign body types (FG) of giant cell 3 days p.i. in the lesion area, surrounding talc particles (arrows). (H & E, X 440).


DM


LG
FG

number of epithelioid cells and also presence of the horse shoe (Langhans) and foreign body types of giant cells in the damaged area. Talc crystals, lying free or surrounded by epithelioid cells, were seen in the lesion.

5-6 days

The picture at these stages showed similar features to that at 4th day, with the exception that fibroplasia and collagen formation, vascularization and muscle regeneration were more active than 4th day (Fig. 5.6). Also dense melanin pigments were in some parts of the lesion area and around the blood vessels at 6 days.

7 days

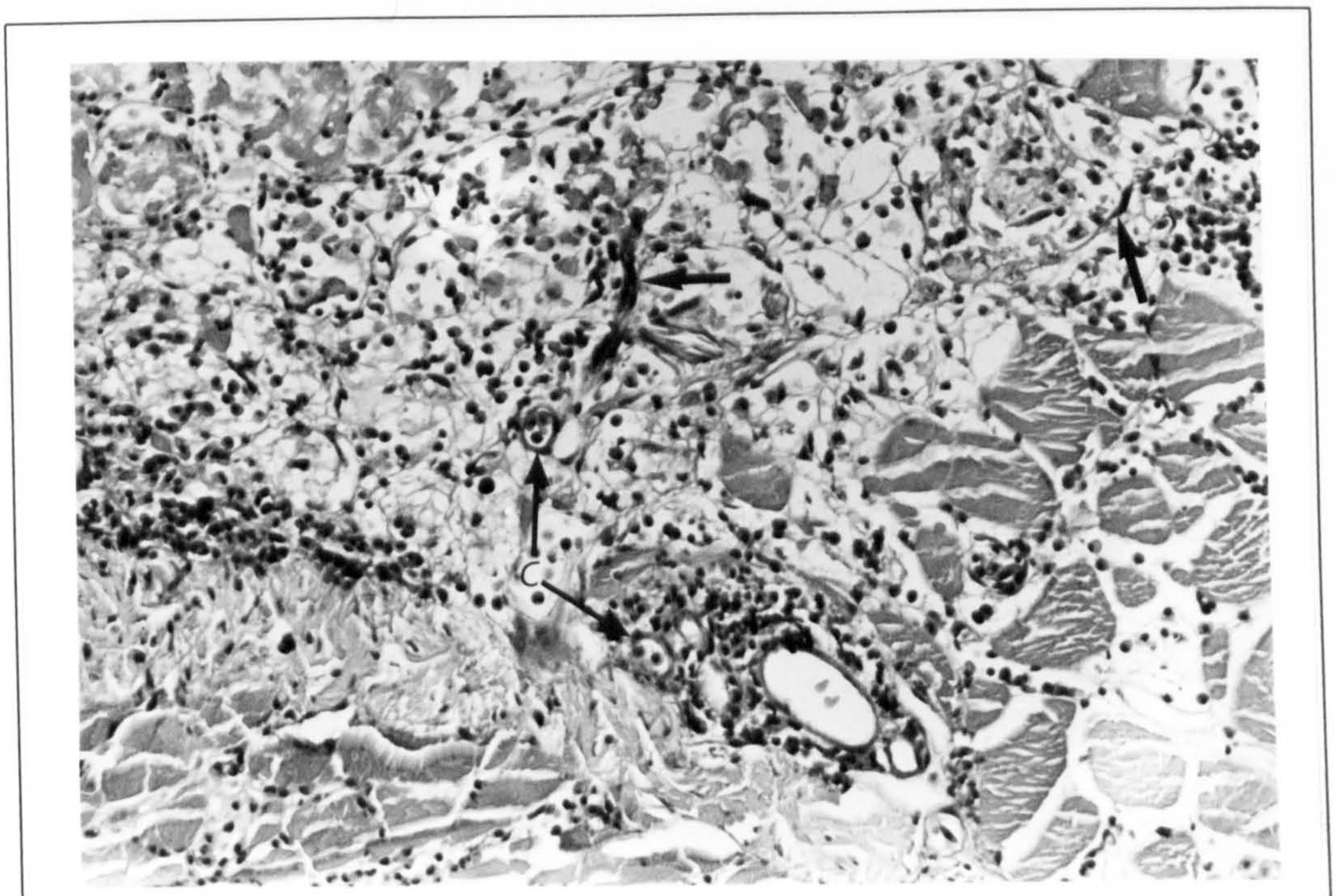
Presence of the inflammatory cells, new muscle fibres, new capillaries, some haemorrhages, fibrosis and collagen formation, giant cells and refractile talc particles in the lesion area were observed (Fig. 5.7). More new muscle bundles were evident in the damaged area. Dense melanin was seen around the blood vessels and in some areas of the lesion.

10 days

By the 10th day the inflammatory area consisted of a number of inflammatory cells. Epithelioid cells, giant cells, fibroblasts and more marked fibrosis were prominent (Fig. 5.8). Dense epithelioid cells sequestrated large amount of talc particles, resembling granuloma (Fig. 5.9). Refractile talc crystals were seen within the epithelioid and giant cells and also between the muscles in the lesion area.

Fig. 5.5 At 3 days p.i., small new muscle buds (arrows), and new capillaries (C) were observed in the damaged area. (H & E, X 220).

Fig. 5.6 This picture shows the grown new muscle buds (arrows) and also active fibroplasia (F) in 5 days p.i. Inflammatory cells and new capillaries (C) are obvious in the lesion area. (H & E, X 440).


C

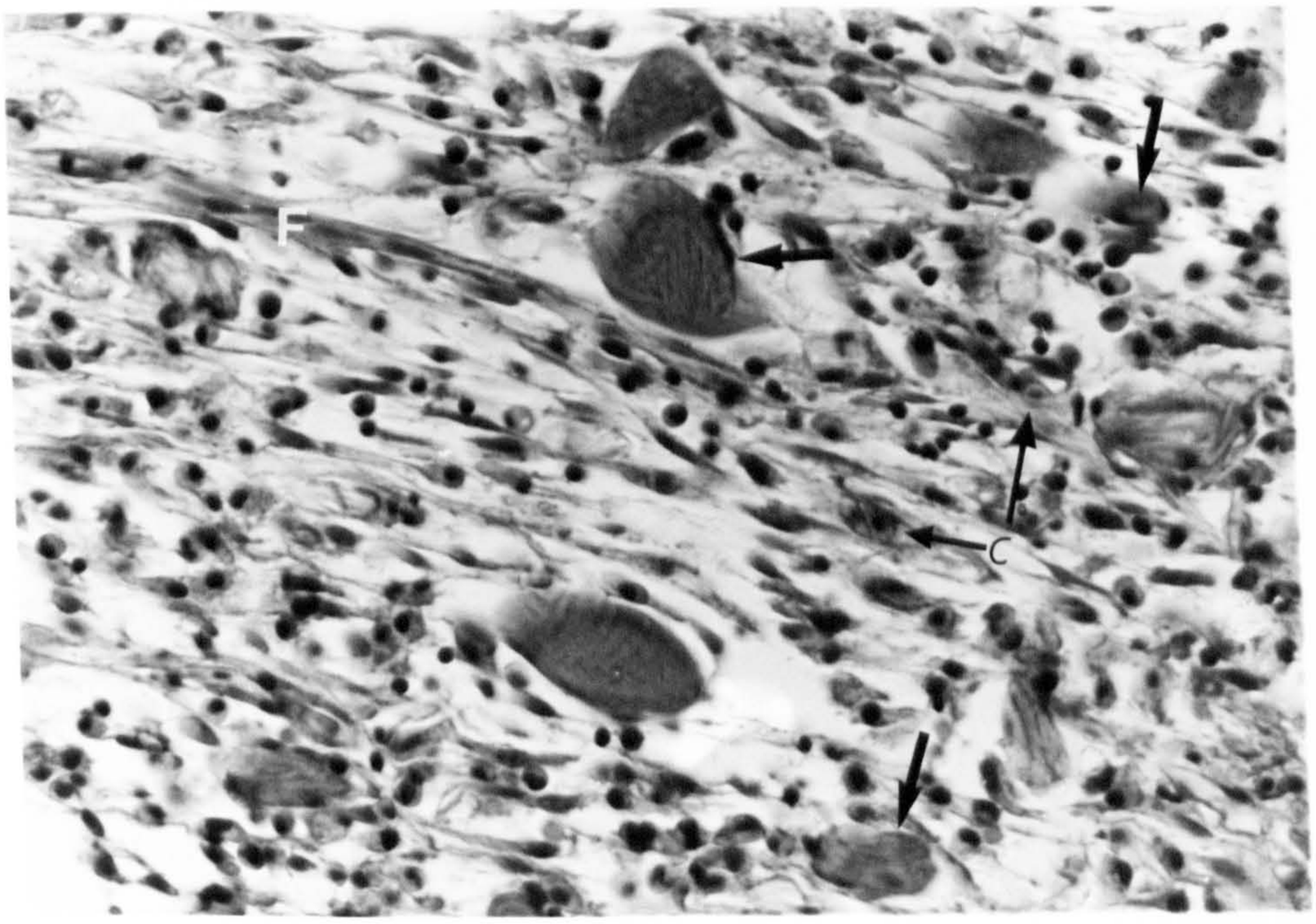
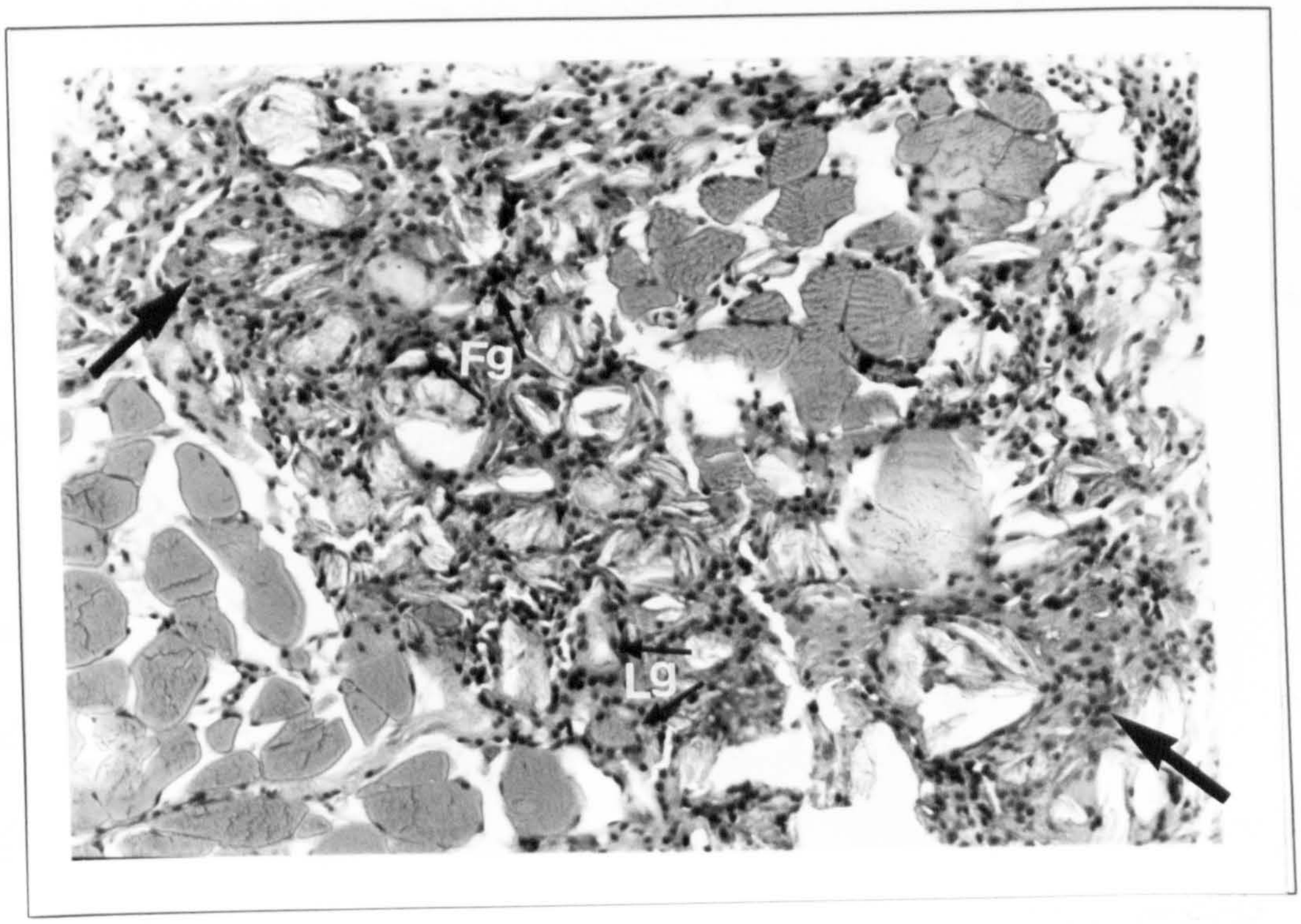
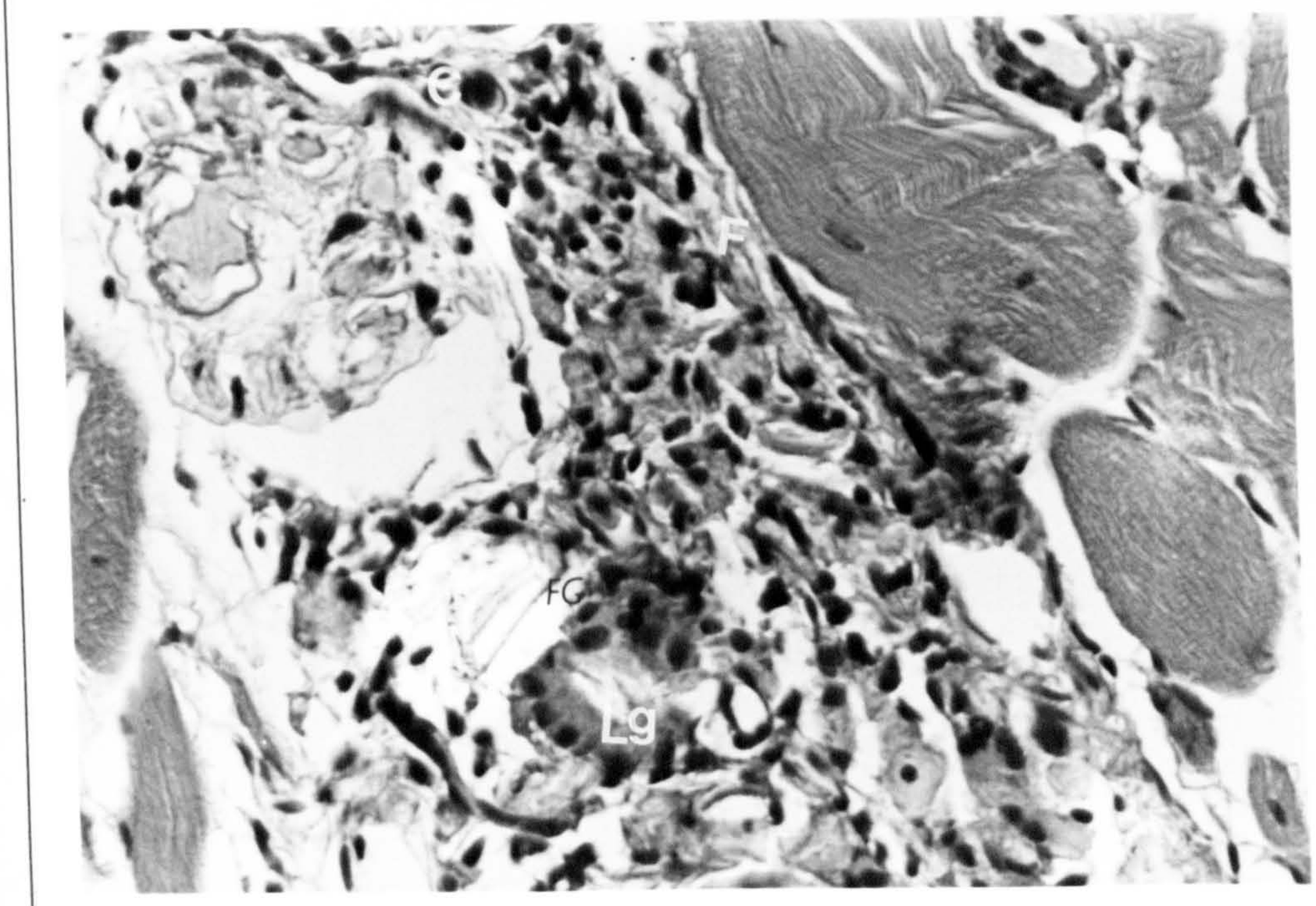

F
C

Fig. 5.7 At 7 days p.i., epithelioid cells (arrows) were seen surrounding talc particles. More Langhans (Lg) and foreign body (Fg) giant cells were observed in the area. (H & E, X 220).

Fig. 5.8 By 10 days p.i. presence of both types of giant cells, Langhans (Lg) and foreign body (FG), fibroplasia (F) and new capillaries (C) were observed. (H & E, X 440).


Fg
Lg


F
FG
Lg

14 days

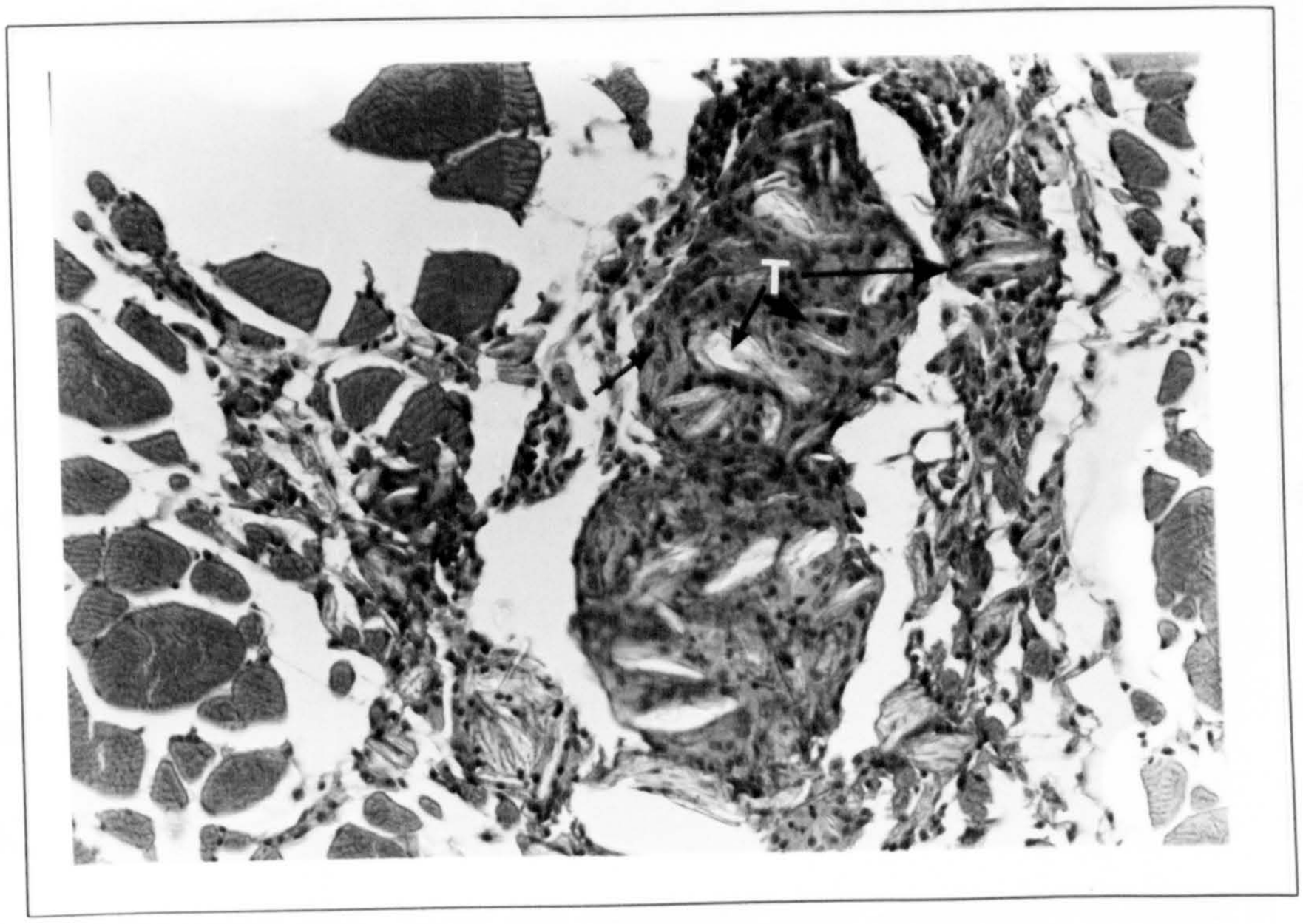
At this stage, the inflamed area contained a number of new capillaries showing diapedesis and some haemorrhages, also macrophages, lymphocytes and fibroblasts were obvious. Fibrous tissue tended to surround the epithelioid cells which were sequestrating talc particles and in some sites a layer of fibrous tissue was seen around them (Fig. 5.10). Also aggregate epithelioid cells bearing talc crystals and containing giant cells in a stroma of epithelioid cells, with some necrotic materials and debris in the centre were obvious in the lesion area. A number of macrophages were seen intermingled with epithelioid cells and foci of lymphocytes were evident around the granulomata. Muscle regeneration was almost completed. Talc particles were seen lying free or within the cytoplasm of epithelioid cells and giant cells.

18 days

A dominant feature at this time was increasing in size, number and level of activity of lymphocytes and fibroblasts in the inflammatory area (Fig. 5.11). New thin walled blood vessels and capillaries in the granuloma showed diapedesis and some haemorrhage. Macrophages, epithelioid cells, giant cells, active fibroblasts and collagen fibres between the cellular elements were seen in the damaged area. Necrotic materials and refractile talc crystals were seen sequestrated in the centre of granulomata. Intermingled macrophages with epithelioid cells that had been observed on the 14th day, had disappeared.

Fig. 5.9 Dense epithelioid cells sequestrated large amount of talc particles, resembling granuloma at 10 days p.i. Talc crystals (T) were also seen within giant cells (arrows). (H & E, X 220).

Fig. 5.10 By 14 days, fibrous tissue (arrow) started to surround the epithelioid cells which were sequestrating talc particles. (H & E, X 220).
G=giant cell, T=talc particle


T

G
I

22 days

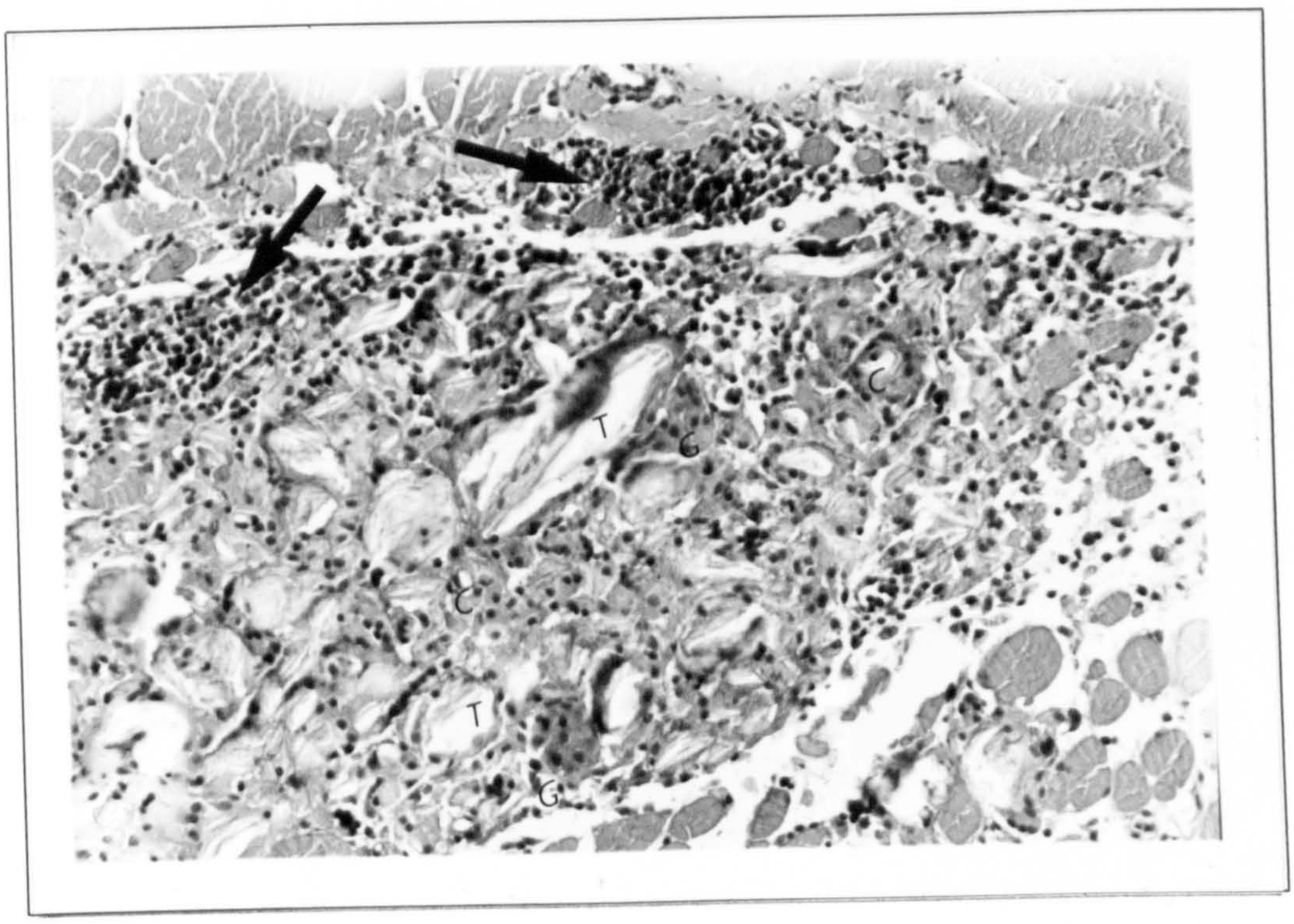
Granulomata containing talc particles and a pink granular deposit with nuclear debris from effete macrophages and epithelioid cells, surrounded by fibrous tissue, and also a high level of lymphocytes and fibroblast activity were prominent features of this stage. Some lymphocytes were seen leading into the lesion area from between the adjacent muscle fibres. Initiation of interlinking between the granulomata was seen (Fig. 5.12). Some melanin pigments were arranged along the same plane as the fibrous tissue and within the granulomata. The number of macrophages was very reduced. Epithelioid cells, a few giant cells, fibroblasts, large and small lymphocytes, collagen fibres, and talc particles in the granulomata or within the epithelioid cells, were observed in the defect area. Some new capillaries in the lesion area were evident.

28 days

The characteristic feature in this stage was various sized granulomata in the lesion area (Fig. 5.13). These granulomata comprised talc particles, largely necrotic macrophages and epithelioid debris and some melanin pigments, surrounded by an epithelioid capsule. A few giant cells were seen among the epithelioid cells. The outer most layer of these granulomata was layers of fibrous tissue (Fig. 5.14). Fibrous tissue interlinking small granulomata was also seen. A significant decrease in the number of lymphocytes and macrophages, in comparison with that of the 22nd day was obvious. New capillaries in the fibrous tissue were congested. Melanin pigments associated with the blood vessels, adjacent to, and within the damaged area were seen. Fibroblasts and collagen fibres were prominent in the damaged area and also around the granulomata.

Fig. 5.11 A dominant feature at 18 days p.i. was increasing in size, number and level of activity of lymphocytes and fibroblasts. Foci of lymphocytes (arrows) and scattered lymphocytes are obvious in the lesion area in this picture. (H & E, X 220).
G=giant cell, C=capillary, T=talc particle

Fig. 5.12 Initiation of fusing and interlinking between the follicular granulomata was observed at 22 days p.i. (H & E, X 440).
G=giant cell, T=talc particle


T
G
C
C
T
G

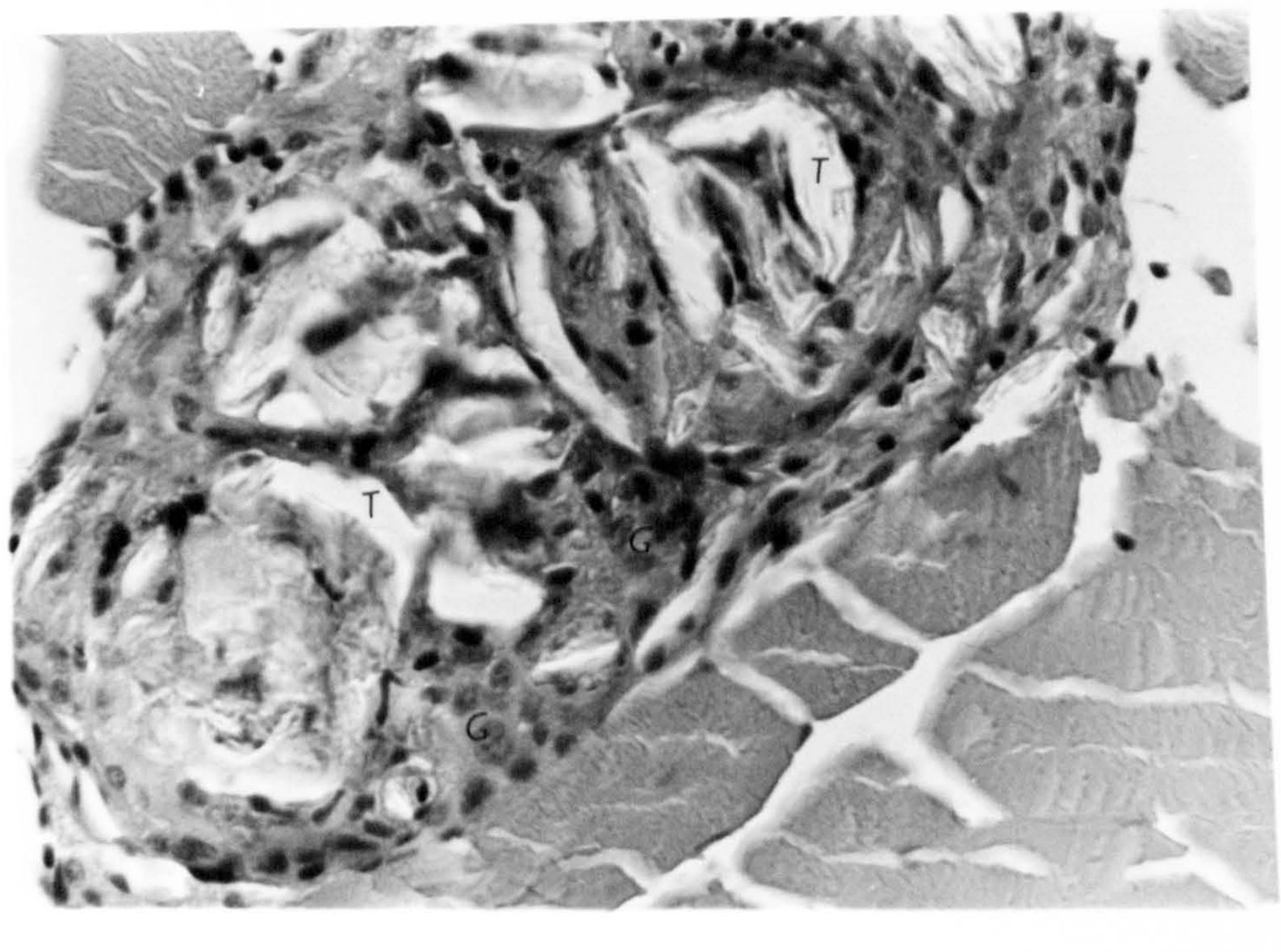
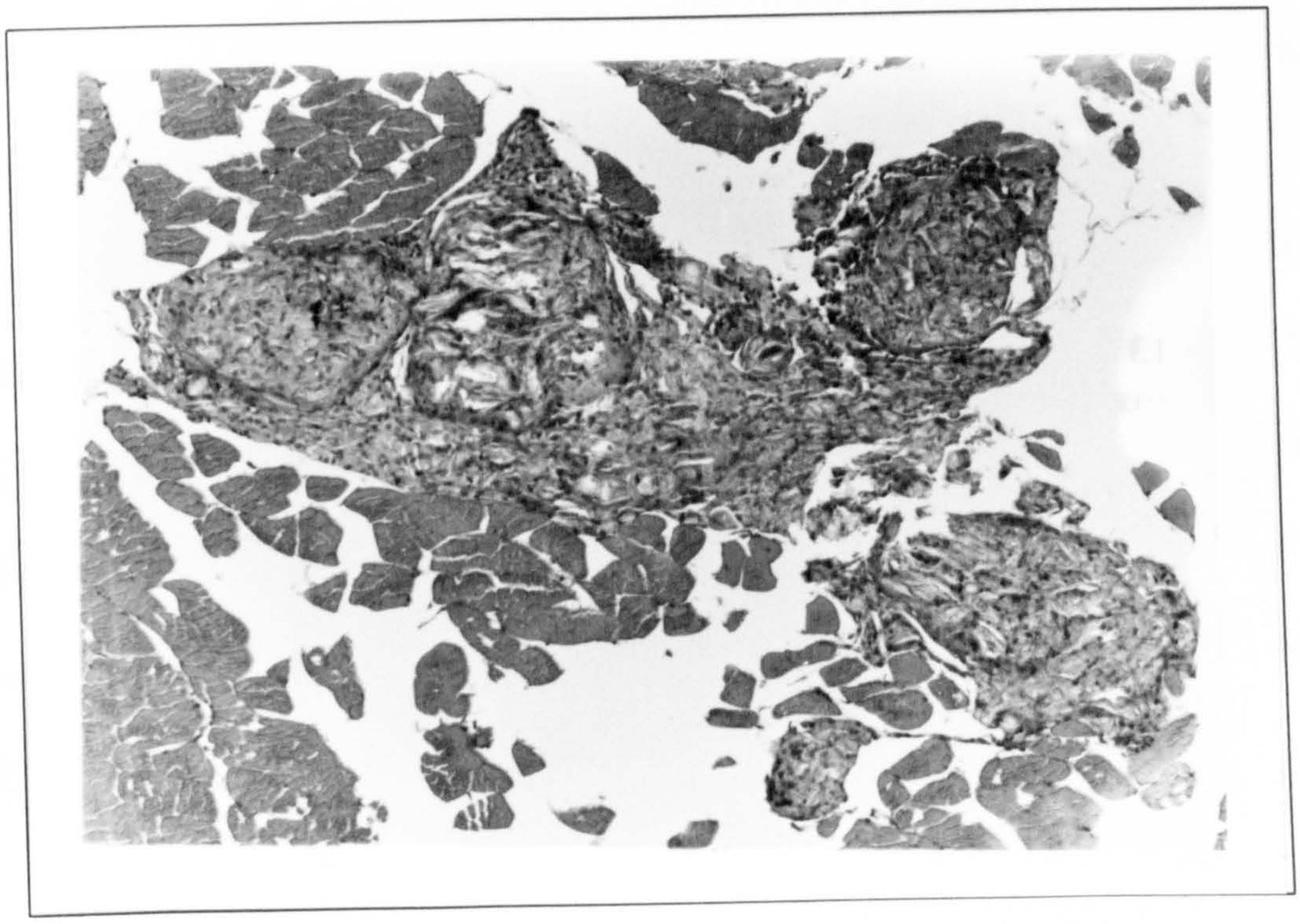

T
T
G
G

Fig. 5.13 This picture shows various granulomata between muscle bundles at 28 days p.i. Fibrous tissue interlinking granulomata is also seen. (H & E, X 110).

Fig. 5.14 The granulomata at 28 days p.i. were matured and comprised talc particles, largely necrotic materials surrounded by 5-6 layers of fibrous tissue (arrows). (H & E, X 220).



35 days

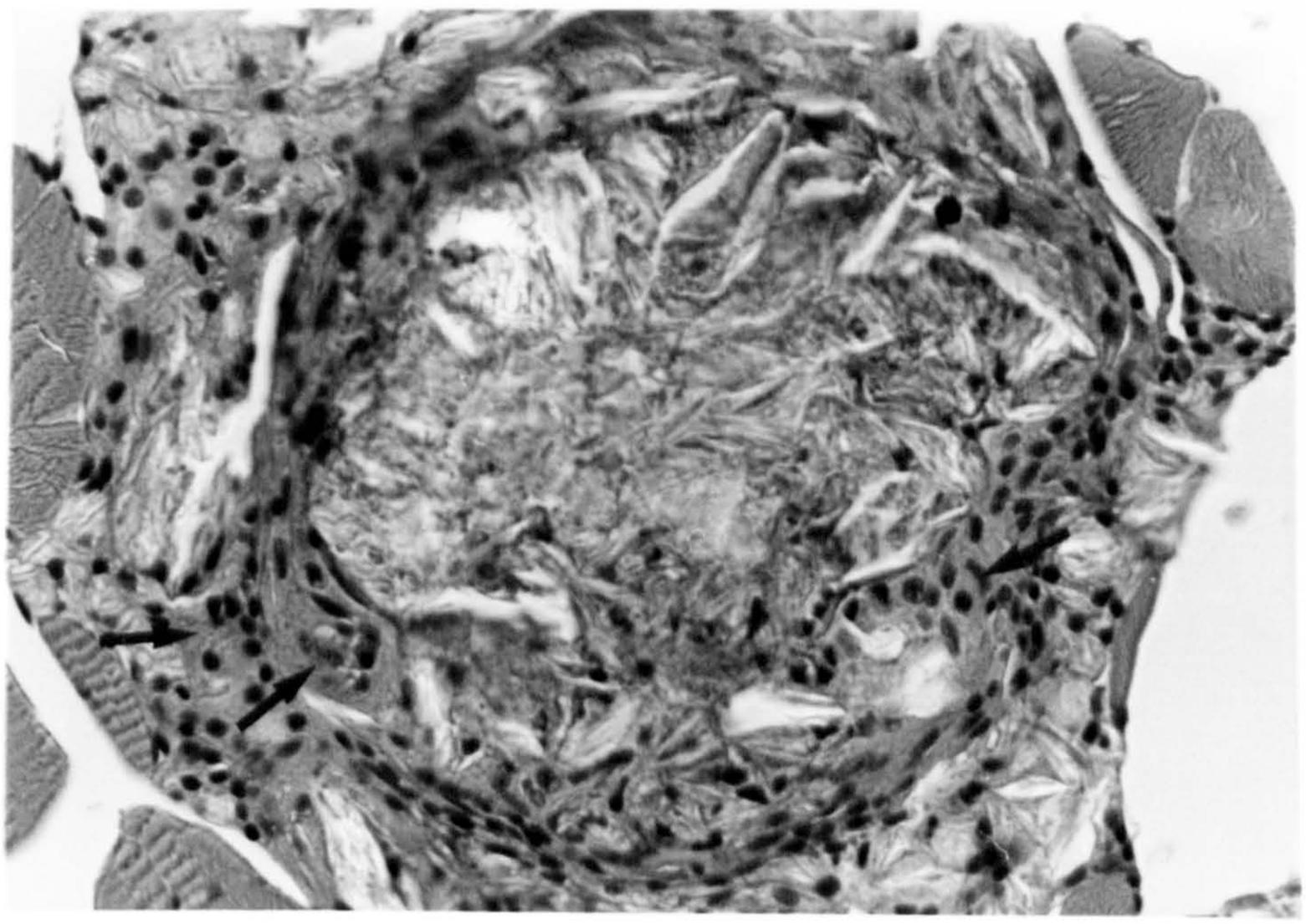
The main features of the lesion in this stage, were similar to those of 28 days after injection, but the dominant difference was more consolidation of the process of encapsulation. More layers of fibrous tissue were surrounding granulomata. The granulomata were more interlinked by fibrous tissue. Epithelioid cells in the granulomata were decreased but necrotic materials were increased (Fig. 5.15). Refractile talc crystals in the granulomata were very obvious. Some small numbers of lymphocytes, macrophages, and giant cells were seen in the lesion area, but they obviously were less than that of 28th day. A few capillaries were observed throughout the fibrous granulation tissue.

42 days

This was the final sampling period for the study and at this stage the process of encapsulation of the irritant was very advanced. Typical granulomata consisted of a relatively dense fibrous tissue at the periphery, talc particles and pink materials inside the granulomata (Fig. 5.16). Also interlinking of granulomata was obvious. The granulomata were entirely surrounded by normal muscle tissue. In some granulomata, in addition to talc crystals and pink granular deposit, some necrotic cells, sloughed materials, debris and melanin pigments occupied the granulomata. Also a few talc particles bearing multinucleated giant cells and epithelioid cells were evident. Little or no inflammatory activity in the lesion area was to be seen. Some lymphocytes were at the periphery of the lesion but macrophages were very much less evident. Melanin pigments in the fibrous tissue were seen.

Fig. 5.15 This picture demonstrates a granuloma at 35 days p.i. Epithelioid cells in the granulomata in this stage are decreased but necrotic materials are increased. Some giant cells are observed in the granulation tissue (arrows). (H & E, X 440).

Fig. 5.16 The lesion area at the last sampling period (42 days) showed that the process of encapsulation of the irritant was very advanced. Typical granulomata consisted of a dense fibrous tissue at periphery, talc particles and necrotic materials inside the granulomata, and almost no epithelioid cells remained. (H & E, X 110).



Control fish

No significant changes were detected in the fish injected with normal saline (appendix 4).

5.5 DISCUSSION

The main features of the inflammatory response in carp injected with talc (magnesium silicate) suspension in this study were similar to those of the chronic inflammatory response of teleosts fish described in plaice (Roberts & Bullock 1976; Timur, G. 1975; Timur, M. 1975; Timur, M. *et al.* 1977; Timur, M. *et al.* 1985), rainbow trout (Finn & Nielson 1971*a,b*), salmon (Roberts *et al* 1973*a,b*), snakehead (Chinabut 1989), rainbow trout and turbot (Balouet & Baudin Laurencin 1986) and to those observed in natural conditions in white sucker (Wolke & Trainor 1970).

The inflammatory cells in this study comprising macrophages, epithelioid cells, lymphocytes, fibroblasts and giant cells, which were the main components of the inflammatory response, were similar to those reported in the above mentioned fish.

In the present study, the findings at the early stages after inoculation of talc suspension, showed an acute inflammatory response consisting of hyperaemia, haemorrhage, exudate and inflammatory cells with macrophages dominated. At 6 hours after injection, myophagia began and completed by the day 4th. Presence of large number of macrophages and some PMNs at 24 hours after injection was marked. Striated refractile talc particles varying in size, in these early stages were either lying free or within the muscles but they were not found sequestrated by cells.

The cellular reaction in the present study indicated a similarity to those reported in rainbow trout at 15°C by Finn & Nielson (1971*a,b*) injected with killed bacteria, Freund's complete adjuvant (FCA), and burning. It was faster than those described in plaice injected with complete and incomplete Freund's adjuvant, *Mycobacteria*, talcum powder, beryllium (Timur, G. 1975), and carrageenin (Timur, M. 1975) at 10°C. It was also faster than in salmon reported by Roberts *et al.* (1973*a*) who used marking tag at 4-

12°C, and also in rainbow trout and turbot injected with BCG and talc at 12°C and 18°C respectively (Balouet & Bauldin Laurencin 1986). On the contrary, it was slower than that of snakehead injected with FCA at 26.9°C (Chinabut 1989).

Macrophage infiltration is an important stage in inflammatory response which encapsulates or eliminates the irritants. In this study macrophages were present and were myophagic at 6 hours after inoculation. Then they increased at 1 day and began to fuse and change into an epithelioid configuration at 2 days after injection. Thereafter the epithelioid cells started to surround and encapsulate the irritant at 3 days. The macrophages began to decrease by day 22 after injection. Also PMNs were infiltrated through the lesion at 1 day and stayed until 4 days after inoculation. Macrophage infiltration and development, in the present study was faster than had been reported 1 day in rainbow trout at 15°C (Finn & Nielson 1971*a,b*). Timur, M. *et al.* (1977) reported presence of macrophages and PMNs within 24 hours after carrageenin inoculation in plaice at 10°C, but Timur, G. (1975) showed in the same fish and at the same temperature that this happened at 3 days and 8 days in response to FCA and talc respectively. This time was 12 hours in snakehead injected with FCA at 26.9°C (Chinabut 1989).

Vascularization is an important factor in wound healing to nourish the newly developing granulation tissue which is the major element for encapsulation the irritant, isolation of the damaged tissues from the normal tissues, and repairing the damaged areas in an inflammatory lesion. In the present study, new capillaries appeared in the lesion area at the day 3 along with active fibroblasts and the development of fibroplasia at the same time. Timur, M. (1975) reported that in plaice injected with carrageenin at 10°C, with vitamin C supplement, vascularization and also first presence of fibroblasts

began at 7 days and collagen was visible at 12 days after injection. Finn & Nielson (1971*a,b*) showed in rainbow trout that fibroplasia started at 8 days after injection of FCA at 15°C. Vascularization and fibroplasia in snakehead began around the day 4th after injection of FCA at 26.9°C (Chinabut 1989). Roberts *et al.* (1973*a*) observed fibro-granulation tissue after 6 days and dense growth at 12 days in newly tagged parr (*Salmo salar*) at 12°C, whereas it was not developed until around the 18th day at 8°C, and had not even started by 20th day at 4°C. The vascularization and fibroplasia in carp (present study) therefore was faster than all of the above mentioned fish which is a striking capability in wound healing in this fish at high temperature (26.5°C).

Anderson & Roberts (1975) in their study on the effects of temperature on wound healing in a tropical and a temperate teleost showed that the rate of wound healing in an individual species is temperature-dependent. Finn & Nielson (1971*a*) described that macrophage response, clearance of necrotic muscle tissues and fibroblastic activity are dependent on the ambient temperature. Also metabolic activity and protein synthesis which effect on the three major functions of the chronic inflammation namely; macrophage infiltration, vascularization and fibrosis, are temperature dependent (Prosser 1962). Thus it is reasonable to conclude that there is a faster rate of chronic inflammation in carp (present study) in response to talc injection, compared to the species mentioned above.

Finn & Nielson (1971*a*) were the first to indicate the significant of the perimysium and intermyotomal fascia in limiting the spread of necrosis in muscle of rainbow trout. Later it was confirmed by Roberts *et al.* (1973*a*) in parr, and Anderson & Roberts (1975) in the white mountain cloud minnow (WMC) (*Tanichthyes*

albonubes) and the Atlantic salmon (*Salmo salar*). This feature was well documented in the present study.

Muscle regeneration in this study started with appearance of the basophilic small new buds at 3 days after injection. These developed in sarcolemmal tubes formation over the ensuing days. The new muscle bundles had filled the damaged area almost completely by the day 14th, but the epithelioid granulomata were still present, surrounding the necrotic materials, debris and talc fragments in the lesion area. Roberts et al. (1973*a*) did not mention the beginning of muscle regeneration in newly tagged parr but they did describe that by the 32nd day at 12°C, the severely affected muscles were completely replaced by fibro-granulation tissue, although some evidence of basophilic sarcolemmal tube formation showed that regeneration in less severely damaged muscles was still in progress. They also noted that the healing and regeneration of the muscle fibres was never particularly noticeable, and replacement fibrosis was the dominant long-term response in the muscle. Timur, G. (1975) showed that 35 days after injection of talcum powder in plaice at 10°C, occasionally some regenerating muscle fibres were seen. In Timur's, M. (1975) study in plaice, using carrageenin in the best conditions for fish which was vitamin C supplemented at 10°C, the first evident of muscle regeneration was seen 21 days after injection. Chinabut (1989) reported that in snakehead at 26.9°C, the buds of regenerating myofibres were first seen on the 4th day and had virtually replaced the fibrosis area by the 28th day after injection of FCA. From these comparisons it could be concluded that similar to the vascularization and fibrous formation, the muscle regeneration as a part of wound repair is relatively fast in carp (Present study) at high temperature (26.5°C).

It is demonstrated that different type of irritants such as complete and incomplete Freund's adjuvant, talc (magnesium silicate), beryllium oxide (Timur, G. 1975) and carrageenin (Timur, M. 1975) could produce various sizes and different types of multinucleated giant cells in plaice. Wolke & Trainor (1971) found giant cells in granulomatous enteritis in a clinical case study of a naturally infected white sucker with silicaceous diatoms. Also Balouet & Baudin Laurencin (1986) produced giant cells in rainbow trout and turbot with inoculation of talc particles and BCG. Roberts *et al.* (1973*a*) showed that using marking tag in Atlantic salmon also is able to produce giant cells within the granulomatous lesion. The first presence of both Langhans (horse shoe shape) and foreign body type of giant cells at 3 days after injection was evident in this study. Chinabut (1989) reported the presence of both types of giant cells in snakehead by day 6 at 26.9°C. Balouet & Baudin Laurencin (1986) demonstrated the presence of foreign body type giant cells in granulomatous nodules in rainbow trout (at 12°C) and turbot (at 18°C), 15 days after inoculation of talc and BCG which were more frequently found in trout than in turbot. Roberts *et al.* (1973*a*) showed that in newly tagged Atlantic salmon, foreign body type giant cells were observed at 25 days when the granulomatous lesion was matured. Timur, M. (1975) observed both types of giant cells in plaice at 10°C by day 12 while he and his co-workers (1985) reported the presence of Langhans type only in plaice at 5°C by the 85th day as the first occasion. Timur, G. (1975) in her study of giant cells in plaice at 10°C, observed both Langhans and foreign body type and also the third type named intermediate in response to inoculation of talcum powder. She reported the presence of giant cells at the 16th day and suggested that the giant cell formation was retarded in low temperature because she could not demonstrate any giant cells at any stage of her experiment in the lower temperature

(5°C). Regarding these findings, the early presence of giant cells within the lesion area at the high temperature (26.5°C) in carp, in the present study, would be expected. However, the faster formation of giant cells in carp as a part of cellular defence is a significant event in this fish particularly in surrounding and delimiting the irritants which could not be phagositised.

In the present study, lymphocytes infiltrated into the lesion area immediately after inoculation of talc suspension. They were seen along with the other inflammatory cells from the early to late stages of the inflammation process. Their number and level of activity increased by 18 days and highest level of their number and activity was at 22nd day which were also seen more leading into the lesion area. Thereafter they began to decrease significantly at 28 days but were still present at the periphery of the lesion even at the last sampling period of this study.

Timur, G. (1975) and Timur, M. *et al.* (1977 & 1985) reported the presence of plasma cells in plaice inoculated with talc at 10°C and carrageenin at 10°C and 5°C. They also showed that appearance of plasma cells is temperature-dependent. Ellis (1977) stated that many cells containing intracytoplasmic *Ig* in fish have the appearance of large lymphocytes rather than plasma cells. It is also known that lymphocytes in higher vertebrates are capable of secreting antibody before differentiation into plasma cells and it is possible that much of the *Ig* in the fishes is produced by stimulated lymphocytes which do not undergo full differentiation into plasma cells. In this study, plasma cells were not observed in the damaged area but presence of large lymphocytes, along with normal ones, especially at the peak of lymphocytes activity was evident.

Lymphocytes in birds, mammals, amphibians (Manning & Horton 1969) and also fish (Ellis 1977 & 1989; Richards *et al.* 1978) have been shown to be the executive

cells of the specific, cell-mediated immune mechanisms. In general, the presence of lymphocytes in response to some stimuli is usually considered as an indication of some form of immunological activity. Also lymphocytes are capable of increasing the activity of macrophages by secretion of chemical substances known as lymphokines or of a material called transfer factor (Spector 1977), and also macrophage inhibition factor (Timur, G. 1975). Lymphokines are soluble products of stimulated lymphocytes which could attract and immobilise macrophages and stimulate them to mature (Bloom 1971).

Plasma cells are responsible for the production of humoral antibodies. They are characterised by the presence of large amounts of intracytoplasmic *Ig* which is rapidly synthesised and secreted. Presence of the plasma cells mean that immunoglobulin is being formed. (Ellis 1977; Spector 1977). Regarding the ubiquitous presence of lymphocytes and absence of plasma cells in the present study and this fact that talc is not obviously an immunogenic substance, therefore it may be reasonable to conclude that present of lymphocytes is associated with a non-specific inflammatory reaction against inorganic irritant (talc), and also stimulating the macrophages to be more activated.

The causative role of injected talc in the formation of granuloma in carp (present study) is demonstrated by the presence of ovoid or rectangular striated refractile talc particles varying in size in the granulomata. It has also been shown in this study that talc (magnesium silicate) as an irritant, is able to provoke and induce the classical types of giant cells (Langhans and foreign body types) and the typical granulomatous inflammatory lesion in carp.

It could be concluded that the general pattern and the development of chronic granulomata in carp injected by talc (present study) is similar to that of previous studies

carried out by other workers in plaice, rainbow trout, Atlantic salmon, snakehead and turbot. The time scale of the cellular response, granuloma and giant cell formation, and wound healing in carp was much faster than those species except snakehead which had macrophage infiltration faster than carp (present study). These differences in inflammatory response could be due to type of irritant, fish species, water temperature and the possible responsibility of delayed cellular hypersensitivity.

CHAPTER 6:

CHRONIC GRANULOMATOUS INFLAMMATORY REACTION IN CARP (*Cyprinus carpio* L.) IN RESPONSE TO FREUND'S COMPLETE ADJUVANT

6.1 INTRODUCTION

One hundred years ago Bataillon *et al.* (1897) observed the first case of mycobacterial infection in fish. They named the organism *Mycobacterium piscium* (Bataillon *et al.* 1902) which is no longer a valid species (Frerichs & Roberts 1989).

Mycobacterial infections of fish are always referred to as (piscine) tuberculosis or mycobacteriosis, irrespective of specific identity of the causal organism (Frerichs 1993). It is a disease of fresh and salt water fish and is present in both tropical and temperate zones. The causative agents are Gram-positive, acid-fast, non-motile pleomorphic bacilli belonging to the genus *Mycobacterium* (Wolke 1975).

Mycobacteriosis is usually a chronic and progressive disease. The disease may or may not manifest clinical signs in infected fish, but in those fish that do show clinical signs, are variable. Where present the main clinical signs are; inability to maintain balance, emaciation, loss of scales, skin discoloration, exophthalmia, fin and tail rot, inflammation of the skin and ulceration, ascites, lordosis or scoliosis. Soft to hard greyish-white nodules of various sizes involve any organ. Classic focal granulomas composed of epithelioid cells surrounded by a wall of fibroblastic cells and occasional giant cells are prominent microscopic features of the disease. There is also an acute form which may result in death without any external signs (Wolke 1975; Dulin 1979; Austin & Austin 1987; Gomez *et al.* 1993).

The occurrence of mycobacteriosis has frequently resulted in heavy losses (Hedrick *et al.* 1987; Lawhavinit *et al.* 1988; Bragg *et al.* 1990). Also high percentages of mycobacteriosis have been found among fish populations in their natural habitats (Hastings *et al.* 1982).

From the literature it would appear that one of the most consistent inflammatory responses is that of the Freund's complete adjuvant (FCA) which is a suspension composed of mineral oil, emulsifying agent and killed *Mycobacterium butyricum*. Freund's adjuvant is used to aid production of high titres of serum antibody in experimental immunology studies (Freund 1948). Therefore FCA was used in this experiment in order to define the inflammatory response in carp (C*yprinus carpio* L.) to this immunogenic substance.

6.2 REVIEW OF THE LITERATURE

The literature about tuberculosis are used in this literature review because the majority of the available information are relevant to tuberculosis rather than the Freund's complete adjuvant (FCA). The available information from the experimental studies with Freund's adjuvant has also been used.

6.2.1 Tuberculosis (piscine mycobacteriosis)

Piscine tuberculosis is a systemic granulomatous disease characterised by the production of focal granulomata in response to bacteria of the genus *Mycobacterium* (Wolke 1975). *Mycobacterium* spp. are Gram-positive, acid-fast, non-motile, pleomorphic rods, approximately 1.5-2 x 0.25-0.35μm in size (Dulin 1975). They produce pale-cream to yellow/orange colonies on solid media. The optimum temperature for the growth varies between different strains and has been reported from 25-32°C, although some isolates will grow at 37°C (Williams & Riordan 1973; Van Duijn 1981; Chow *et al.* 1983; Frerichs & Roberts 1989). Since the first report of mycobacteriosis in common carp (*Cyprinus carpio*) by Bataillon and co-workers (1897), the disease has been recorded in 40 families and 151 species of fresh water and marine fishes and is world wide in distribution (Nigrelli & Vogel 1963). The recorded range of susceptible species has continued to increase so it is probable that any species of fish maybe infected (Dulin 1979) (table 6.1).

The mycobacteria species most frequently isolated from fish are *M. fortuitum* and *M. chelonei* from fresh water and brackish water fish, and *M. marinum* from marine fish (Post 1983; Arakawa & Fryer 1984; Nigrelli & Vogel 1963; Ashburner 1977;

Table 6.1: The occurrence of *Mycobacterium* spp. in different fish species from 1977-1996 (cited by Chen 1996).

Year	Author	Species	Country
1977	Ashburner	Chinook salmon (*Oncorhynchus tshawytscha wabaum)*	Australia
1977	Timur *et al*	Plaice (*Pleuronectes platessa* L.)	
1980	Beckwith *et al*	*Brachydanio albolineatus*	USA
1980	Bucke	Atlantic mackerel (*Scomber scombrus* L.)	England
1980	Giavenni *et al*	Marine tropical fish	Italy
1981	Majeed *et al*	Fantail goldfish (*Carassius auratus*) Goldfish (*Carassius auratus*) Kissing gourami (*Colisa labiosa*) Tetra (*Hyphessobrycon* sp.) Angel fish (*Pterohyllum* sp.)	UK
1982	Hasting *et al*	Mackerel (*Scomber scombrus*)	
1982	Santacana *et al*	Three-spot gouramies (*Trichogaster trichopterus* Pallas)	Venezuela
1983	Majeed *et al*	Carp (*Cypinus carpio* L.)	England
1987	Hedrick *et al*	Striped bass (*Morone saxatilis*)	USA
1988	Hatai *et al*	Pejerrey (*Odonthestes bonarienesis*)	Japan
1988	Mackenzie	Atlantic mackerel (*Scomber scombrus* L.)	Portugal
1990	Chinabut *et al*	Snakehead (*Channa striatus* fowler)	Thailand
1990	Shamsudin *et al*	Gold fish (*Carassius auratus*) Red eyed tetra (*Moenkhansia sanctaefilomenae*)	Malaysia
1992	Colorni	European sea bass (*Dicentrarchus labrax*)	Israel
1993	Gomez *et al*	Paradise fish (*Macropodus opercularis* L.) Siamese fighting fish (*Betta splendens* Regan) Guppies (*Poecilia reticulata* Peters) Jewel tetra (*Hyphessobrycon callistus* Boulenger) Chanchito (*Cichlasoma facetum* Jenyns) Molly (*Poecilia velifera* Regan)	Spain
1996	Gomez *et al*	Swordtail (*Xiphophorus helleri Heckel*)	Spain

Hedric *et al.*1987; Humphery *et al.* 1987; Daoust *et al.* 1989;Bragg *et al.* 1990;Shamsudin *et al.*1990; Bozzetta *et al.* 1995). Tropical fresh water and tropical marine water fishes are susceptible to *M. marinum* infection which forms the largest proportion of all reports of mycobacteria isolated from fish. The isolation of *M. fortuitum* has been less frequently documented than that of *M. marinum*, but the prevalence of infection is probably more widespread than generally suspected. Although tropical and temperate water fishes are susceptible to *M. fortuitum*, it is most frequently isolated from fresh water fishes. Infection with *M. chelonae* has so far been identified only in cold water salmonid species occurring in the fresh water hatchery environment (Frerichs 1993) (table 6.2).

The piscine tubercle is similar to that in human, with both the soft and hard forms. The soft tubercle has a centre of caseous necrosis, while the hard tubercle lacks this central zone of necrosis (Wolke 1975). The tubercle associated with piscine mycobacteriosis differs from the human tubercle in that Langhans giant cells are seldom present or rare, calcification of tubercles has not been reported and the abundant acid-fast organisms generally present in the central zone of piscine tubercle (Sutherland 1922; Nigrelli & Vogel 1963; Wolke & Stroud 1978). The predominant pathological feature of mycobacteriosis in fish is the infiltration of lymphoid cells and macrophages with granuloma formation. The typical lesions which occur in fish composed of greyish nodules located in affected organs. The nodules with varying sizes and degrees of development consist of clusters of epithelioid cells surrounded by a capsule of connective tissue of varying thickness, and areas of necrosis are often seen in the centre. The most severe granulomata are often seen located in the liver and spleen of the

Table.6.2: Mycobacteria isolated from fish (1-9 from Dalsgaard *et al.* 1992).

No.	Described species	Identical with	Accepted name
1	*M. piscium* (Bataillon *et al.*, 1902)	-	-
2	*M. marinum* (Aronson, 1926)	*M. marinum*	*M. marinum*
3	*M. fortuitum* (Nigrelli, 1953)	*M. fortuitum*	*M. fortuitum*
4	*M. platypoecilus* (Baker & Hagan, 1942)	*M. marinum*	-
5	*M. anabanti* (Besse, 1949)	*M. marinum*	-
6	*M. salmoniphilum* (Ross, 1960)	*M. fortuitum*	-
7	*M. poikilothermorum* (Amlacher, 1968)	-	-
8	*M. borstelense* (Bernstad, 1974)	*M. chelonei*	*M. chelonei*
9	*M. chelonei* (Arakawa & Fryer, 1984) subsp. *piscarium*	-	-
10	*M. gordonae* (Bozzeta *et al.*, 1995)	-	-

affected fish. The causative agent of mycobacteriosis in fish is the persistent *Mycobacterium* spp., which produce chronic inflammation. The inflammatory cells infiltrate to the infected area in response to the antigens, and among them are highly phagocytic macrophages which then differentiate into epithelioid cells, surround and isolate the antigenic agents (Noga *et al.* 1989). Amlacher (1961) stated that giant cells have not been observed in spontaneous tuberculosis but they could be seen occasionally in experimental tuberculosis. This finding was confirmed by Timur,G. *et al.* (1977) who reported presence of Langhans giant cells in plaice experimentally infected with *Mycobacterium* sp. It is possible to diagnose positive mycobacteriosis using Ziehl-Neelsen staining method for acid-fast bacteria which reveals the presence of the bacillus

in the central zone of the granulomata and within macrophages. The granulomatous tissue and surrounding areas also produce a positive reaction with periodic acid Schiff (PAS) staining method (Amlacher 1961; Noga *et al.* 1989; Gomez *et al.* 1993,1996).

Mycobacterial infections of fish are probably transmitted naturally by ingestion of contaminated food or aquatic detritus or also bacterial invasion through damaged skin or gill tissue. The fish pathogenic species of *Mycobacterium* are ubiquitous in soil and water and remain viable for 2 years or more. This means that potential sources of infective material are numerous and include the soil and water (Reichenbach-Klinke 1972). According to Nigrelli & Vogel (1963), organisms maybe released into the environment from lesions in diseased fish or from amphibians and reptiles susceptible to mycobacterial disease. Dissemination of the disease has been reported by the feeding of infected trash fish in cultured Pacific salmon (*Oncorhynchus* sp.) (Ross *et al.* 1959) and snakehead fish (Chinabut *et al.* 1990), and by the feeding of water fleas (*Daphnia* spp.) in Siamese fighting fish (*Betta splendens* Regan) (Sodjit *et al.* 1993), also via the ovarian route for the viviparous Mexican platy fish (*Xiphophorus maculatus*) (Conroy 1966), via the eggs to the F_1 generation in Chinook salmon (*Oncorhynchus tschawytscha* Walbaum) (Ashbruner 1977) and via transovarian passage in Siamese fighting fish (Kanayati *et al.* 1992).

The histopathology of the naturally developing granulomas characteristic of mycobacterial infections in fish has been studied in detail by many workers. Conroy (1970), Ross (1970), Wolke & Stroud (1978), and Van Duijn (1981) are those who investigated and produced a comprehensive descriptions of these lesions. Jakowska & Nigrelli (1953) , Finn & Nielson (1971*a,b*), Timur, G. (1975), Timur, G. *et al.* (1977)

and Chinabut (1989) reported their observations on the wounds of guppies, rainbow trout, plaice and snakehead, respectively, experimentally infected by *Mycobacterium* sp.

6.2.1.1 Spontaneous tuberculosis in fish

The first report of spontaneous tuberculosis was made in the end of 19th century by Bataillon *et al.* (1897). They isolated acid-fast bacilli from a tuberculous lesion in a common carp (*Cyprinus carpio*). It was initially assumed that human tubercle bacillus might have become adapted to fish, but the carp isolated *Mycobacterium* was subsequently identified as a distinct species and named *Mycobacterium piscium* on the basis of its isolation from fish (Bataillon *et al.* 1902).

Aronson (1926) described the first well-stabilished fish pathogen, *Mycobacterium marinum*, who isolated it from the viscera of a number of tropical marine fish species. Nigrelli (1953) isolated *Mycobacterium* from neon tetra fish (*Hyphessobrycon innesi*) and Ross & Brancato (1959) identified it as *Mycobacterium fortuitum*. Arakawa & Fryer (1984) subsequently identified six Pacific salmonid mycobacterial isolates recovered between 1964 and 1982 as *Mycobacterium chelonei* subsp. *piscarium*. Later, Arakawa *et al.* (1986) suggested that these strains be taxonomically regarded as *Mycobacterium chelonei* and it has now been corrected and known as *Mycobacterium chelonae* (Wayne & Kubica 1986).

Alexander (1913) reported the first instance of a naturally occurring case of tuberculosis in a marine fish, cod (*Gadus morhua* L.). Parisot & Wood (1960) made a comparative study of the histopathology of the mycobacteriosis condition mainly from salmonids, and proposed the name "fish mycobacteriosis" in place of "fish tuberculosis".

Ashburner (1977) studied incidence of mycobacteriosis in hatchery-confined chinook salmon (*Oncorhynchus tshawytscha* Walbaum) in Australia and reported that a large variety of external and internal signs have been noted because of the chronic nature of the disease. He found the signs of infection in gills, kidney, spleen, heart and liver. Also fish with different types of skin lesions usually show differing types of muscle involvement. The pustular material in the lesions contained acid-fast organisms. The final conclusion of this study was that this *Mycobacterium* infection has been passed on to the F_1 generation possibly either by the egg or more likely through infected ovarian fluid.

Leibovitz (1980) showed many tubercles in varying stages of development in an aquarium fish. Individual tubercles consisted of a central zone of epithelioid cells, pigment cells, acid-fast bacteria and tissue debris. An outer fibroblastic wall surrounded the central zone. Bucke (1980) in a case study of an affected mackerel (*Scomber scombrus* L.) observed fine white nodules of approximately 1mm diameter in the examination of the kidney, spleen and muscles. The systemic focal granulomatous lesions consisted of epithelioid cells and macrophages surrounded by fibrous tissue. In some granulomas the central areas consisted of necrotic material (soft tubercles) and in others the epithelioid cells were arranged in whorls without any caseous material (hard tubercles). Melanin, and Ziehl-Neelsen positive, weakly Gram positive organisms were present in the lesions but giant cell formation was not observed.

The histological examination of the lesions of the naturally infected gold fish (*Carassius auratus* L.) by Majeed *et al.* (1981) showed hard and soft tubercles throughout the visceral organs, eyes, gills and skeletal muscles. Clumps of acid-fast bacilli both in the hard and soft tubercles were shown with Ziehl-Neelsen staining.

Presence of three types of nodule, possibly representing different stages of development, was reported in naturally infected mackerel by Hastings *et al.* (1982). One type with an intact centre, surrounded by epithelioid cells and a loosely organised fibrous tissue. The second type of nodule composed of a solid caseous necrotic mass and melanin and occasionally acid-fast bacteria in centre surrounded by a layer of epithelioid cells and an outer fibrous capsule. The third type was similar to second type but the contents of fibrous and epithelioid cells capsule had a fluffy (soft and loose) appearance rather than solid. The contents of the second and third types of nodule were PAS positive, whereas mycobacteria were not observed in the nodules.

Tuberculosis in snakehead cultured in Thailand was observed by Limsuwan *et al.* (1983) and Chinabut *et al.* (1990). They found focal granulomatous lesions throughout the visceral organs and gills of infected fish similar to those previously described.

Majeed and his co-workers (1983) reported a cutaneous version of the tuberculosis occurring among carp with the absence of any frank lesions elsewhere and no macroscopic changes in the viscera. They found marked subcutaneous inflammation and oedema involving the underlying skeletal muscle, resulting myodegeneration and myositis and also consisted of mixed inflammatory cells, predominantly macrophages and fibroblastic proliferation in some of the lesions. The virtual absence of visceral involvement and lack of well-formed tubercles in their study was contrary to their previous reports (Majeed *et al.* 1981), but they demonstrated the presence of small groups or single macrophages containing acid-fast bacilli in these organs.

In a clinical study, Hatai and colleagues (1993) found *Mycobacterium* sp. infection in pejerrey (*Odonthestes bonariensis* Cuvier & Valenciennes). Many focal

granulomas were present in the viscera, but they were not observed in the lateral musculature. Infected organs contained both soft and hard focal granulomas in varying stages of development.

Gomez *et al.* (1993) studied morphological and immunocytochemical aspects of fish mycobacteriosis in naturally infected aquarium-maintained freshwater fish. Gomez *et al.* (1996) also carried out a comparative study of immunohistochemical methods to diagnose mycobacteriosis in swordtail fish (*Xiphophorus helleri*). In their first study (1993), they observed granulomas (soft tubercles) of varying sizes and degrees of development in the visceral organs. Histopathology in their second study (1996) showed granulomas consisting of epithelioid cells with few or no fibrous tissue capsules, no central area of necrosis, and varying number of pigmented cells in most of the examined fish.

In an attempt to identify *Mycobacterium chelonae* in a cichlid oscar (*Astronotus ocellatus* Cuvier), by direct cycle sequencing of polymerase chain reaction amplified 16s rRNA gene, McCormick *et al.* (1995) observed the presence of chronic granulomatous lesions and uroliths in kidney, perihepatitis and focal necrosis in the liver. The presence of acid-fast bacteria in the Ziehl-Neelsen stained sections of the kidney was also shown. They confirmed by histological examination that observed acid-fast organisms were associated with lesion formation and suggested that the disease would have had a fatal outcome if the fish not been euthanased. They also pointed out that the infected fish showed some of the typical clinical and histological signs of mycobacteriosis described by Dulin (1979) and Plumb (1994) but did not describe these signs in detail.

In all the above species of fish examined, naturally occurring piscine tuberculosis has common features. It occurs in internal organs, eyes, gills, skin and skeletal muscles with typical granulomata formation. These granulomata consist of a central zone of necrotic material surrounded by epithelioid cells and a capsule of fibrous tissue (soft tubercles) and also presence of acid-fast bacilli of *Mycobacterium* spp. in the granulomata. The presence of giant cells has not been reported by some of workers, whereas some of the other investigators observed the occasional presence of the Langhans type giant cells in the granulomatous lesions.

6.2.1.2 Experimental reaction to killed *Mycobacteria* spp.

In experimental studies, emulsified or un-emulsified Freund's complete adjuvant, or *Mycobacterium* spp. isolated from infected fish have been used to induce the conditions of chronic granulomatous inflammation.

Jakowska & Nigrelli (1953) used mycobacteria isolated and cultured from diseased neon tetras (*Hyphessobrycon innesi*) to infect female guppies (*Lebistes reticulatus*). Most bacteria were seen extra cellular during the first two hours and the eosinophils with ingested bacteria or cellular debris were greatly increased within 24 hours while a few macrophages appeared. The vacuoles in the macrophages were large and filled with a great number of mycobacteria at 48 hours, but by 72 hours the numbers of ingested micro-organisms were greatly decreased and few macrophages and eosinophils were present in 96 hours. Neither macrophages nor acid-fast bacteria were seen in the blood and haemopoietic organs of the sacrificed fish after 96 hours. The conclusion of this study was that the highly immunogenic nature of the *Mycobacterium*

may account for rapid removal of the bacteria by phagocytosis and their absence from blood and other organs. There is no further report to support this conclusion.

Finn & Nielson (1971*a,b*) used Freund's complete adjuvant as an inflammatory agent in order to study inflammatory response and effect of temperature variation on this response in rainbow trout at 15°C over a period of 16 days. They found that both PMNs and macrophages were actively phagocytic but macrophages were capable of engulfing many more bacteria than PMNs. The PMNs response was usually present after 12 or 24 hours and macrophages response after 1 day. The lymphocytes and thrombocytes were present more as a background type of cells rather than playing an active role. Fibrous tissue filled the necrotic area within 8-16 days and by 16 days the wounds had resolved into fibrous granulation tissue. They noted the presence of unspecified giant cells at 16th day in the injured skeletal muscle but did not mention the presence of epithelioid cells and granuloma formation. They concluded that inflammatory phenomena in fish were basically similar to those of mammals but less intensive and slower to appear and resolve.

Timur, G. (1975) studied the giant cells in inflammatory lesion of the plaice using Freund's complete adjuvant and *Mycobacterium* sp. as inflammatory agents. The cells found in the different stages of the granulomatous response were composed of macrophages, PMNs, epithelioid cells, lymphocytes, plasma cells, fibroblasts and giant cells (Langhans and foreign body types). Acid-fast bacteria were found within either epithelioid cells or macrophages but were never seen in the cytoplasm of giant cells. She concluded that the process of giant cell formation allowed the destruction of ingested mycobacteria. She also described that the foreign body giant cells occurring in the complete adjuvant were in response to the mineral oil rather than the mycobacteria,

because it is reported in literature that piscine tuberculous lesions did not produce foreign body giant cells.

Timur, G. *et al.* (1977) in their investigation on the experimental pathogenesis of focal tuberculosis in the plaice used *Mycobacterium* sp. isolated from caseous lesions in halibut (*Hippoglossoides hippoglossus* L.). They reported findings similar to those of Timur's G. (1975) with the exception that only the Langhans type of giant cells was observed. The peak of giant cells were at 24-28 days after inoculation, then their number fell off rapidly and by day 60 the lesion was mostly comprised of fibrous tissue, epithelioid cells and also necrosis material and bacilli in the centre of granulomata.

Chinabut (1989) reported that the cellular inflammatory response of the snakehead (*Channa straitus* Fowler) inoculated with emulsified Freund's complete adjuvant at 26.9°C, in its general pattern and in the development of chronic granulomata was similar to that of the rainbow trout at 15°C (Finn & Nielson 1971*a,b*) and plaice at 10°C (Timur, G. 1975) which were described previously. The main difference was that the rate of time scale of the cellular response in the snakehead was much faster than these two species.

From these experimental studies, it can be concluded that the main histological features of the experimentally induced granulomatous inflammatory reaction using Freund's complete adjuvant (FCA) are similar to those of the naturally occurred tuberculosis. The only exception is that both Langhans and foreign body types of giant cell could be produced with FCA inoculation, whereas in naturally occurring tuberculosis only occasional Langhans type may be observed.

6.3 PILOT STUDY

A pilot study was carried out to determine the optimum dose rate of Freund's complete adjuvant (FCA) and/or *Mycobacterium butyricum* suspension for injection and the optimum method of sampling, trimming and sectioning the tissues.

One ampoule (10 ml) of FCA and one ampoule (1 gr) of killed dried *Mycobacterium butyricum* was mixed to produce a suspension for injection. Four mirror carp were used to be injected. The fish were inoculated with 0.05, 0.1, 0.2 and 0.3 ml of suspension in the left dorsal myotome muscles using a 1 ml syringe with different gauges of needle (21 & 23 G) to determine the most convenient needle for injection.

The fish were sacrificed at 6, 24, 36 and 48 hours after injection. The blocks of dorsal muscles comprising injection areas were cut out and fixed in 10% buffered formalin for at least 24 hours before processing.

Each tissue was processed in an automatic processor using standard procedure. After processing, each tissue was trimmed and cut into two pieces, thereafter embedded into the wax. These waxed blocks of tissues were decalcified and then sectioned in 5 μm using a rotary microtome. H & E and Zeihl-Neelson (ZN) methods were used to stain the sections.

The results of the microscopic examinations of the sections using H & E staining method showed; cellular infiltration comprising macrophages, some PMNs, lymphocytes, thrombocytes, and haemorrhages, hyperaemia in blood vessels, muscle degeneration in the site of injection, and myophagia, vacuoles containing FCA with a large number of bacteria inside and near the edges of vacuoles or lying free between the

muscles. These vacuoles of FCA were surrounded by inflammatory cells. The examination of the sections stained by ZN method showed a mass of acid-fast bacilli were located at the centre of the necrotic areas and also among the muscles. Those fish were injected with a higher dose and a longer time scale, showed greater inflammatory response in microscopic examination, but they were observed to swim erratically. Also the respiration rate in these fish was high and they were lethargic and gathered near the outlet with weakness during the first day after injection.

Presence of large numbers of bacteria in the injected tissues, lethargy and lack of co-ordination movement during swimming in the injected fish, showed that using a mixture of FCA and killed dried *Mycobacterium* as an inoculum, may have caused serious problems for fish.

It was concluded that the desired inflammatory response could be achieved by using FCA alone. Therefore, it was decided to use, only, FCA as inoculum in the main study. It was determined that the injection of 0.1 ml of suspension had an ideal response and also using of 21 gauge for bigger fish and 23 gauge needle for smaller fish was more convenient for injection of FCA alone. No problems were identified in the sampling, trimming and sectioning the tissues and also the staining methods.

6.4 DEFINITIVE STUDY

The definitive study was carried out based on the results of the pilot study.

6.4.1 Materials and methods

6.4.1.1 Fish

Mirror carp were obtained from Fish Network, Upper Main Farm, Essex, England. Sixty fish were acclimatised in glass aquaria for 9 weeks prior to experimentation. The average length of fish was 14 cm (±3.5 cm). Fish were fed on a pelleted diet (Fry 02, Trouw Aquaculture, Inverbreakie, Invergordon, Ross-Shire, Scotland), once per day.

6.4.1.2 Aquaria and water system

A recirculating glass aquaria with filter system similar to that described in chapter 4 was used for stocking fish and the period of experiment. The water temperature was at an average of 24°C (±2°C). Faecal materials were removed from aquaria every day and between 10-20% of water was changed daily.

6.4.1.3 Injection procedure

Freund's complete adjuvant (FCA) (DIFCO), in 10 ml ampoules, was prepared for injection. The anaesthetised fish (0.5 ml/l water of 10% benzocaine) were inoculated with 0.1 ml of FCA intramuscularly on the left flank using a 1 ml syringe and 21 gauge needle (for bigger fish) and 23 gauge (for smaller fish). The injection was always made in line with the first ray of the dorsal fin, below the scale row, as a marker for subsequent sampling (Fig. 1.1). Control fish were injected intramuscularly on the left

flank with the same volume of sterile normal saline. After injection, the fish were returned to the aquaria until they were sampled.

6.4.1.4 Sampling and histological procedures

Two inoculated fish were sacrificed by an overdose of 10% benzocaine at 6 hours, 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after injection. Blocks of tissue, bearing injected areas, were dissected out from the dorsal muscle of sacrificed fish. These blocks were fixed, cassetted, processed, trimmed and cut, and sectioned in the same methods described in chapter 5 (appendix 1.1).

6.4.1.5 Staining procedures

A number of staining methods were used to stain microscopic sections. The standard H & E staining method (appendix 2.1.1) was used for the routine examination of sections. Also Ziehl-Neelsen (ZN) (appendix 2.1.5), Immunohistochemistry (monoclonal and polyclonal antibodies) (IHC) (appendix 2.1.4), periodic acid Schiff (PAS) (appendix 2.1.2), and Auramine O (AO) (appendix 2.1.6) staining methods were used for special purposes. The stained sections were mounted similar to those described in chapter 5.

6.4.2 Results

6.4.2.1 Gross pathology

There was no abnormality or gross lesion observed clinically in the injected fish.

6.4.2.2 Histopathology

6 hours

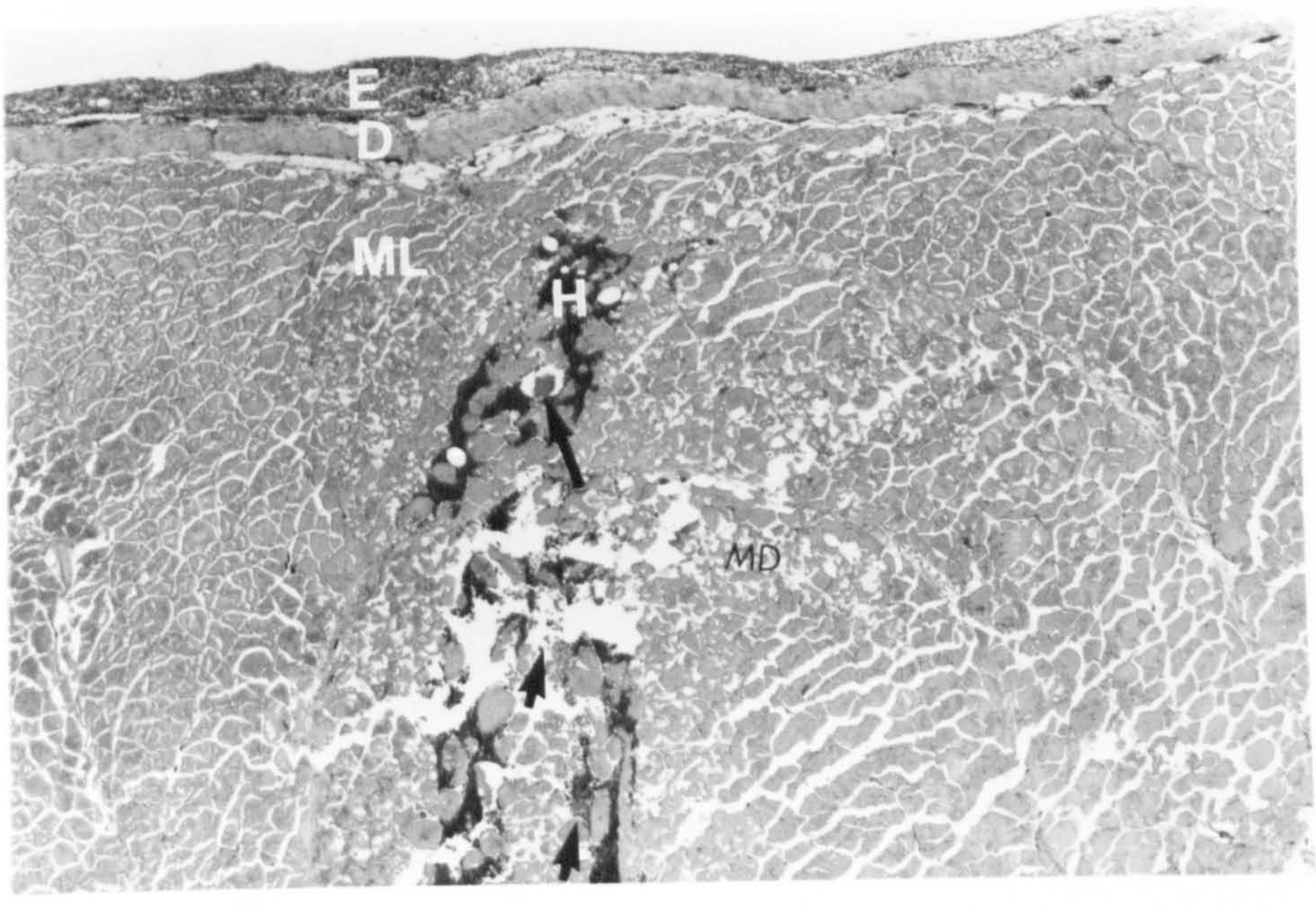
By 6 hours post-inoculation of FCA, an acute inflammatory response was observed. It was consisted of haemorrhage, inflammatory cells mostly lymphocytes and macrophages. Muscles in the injected area were degenerate and myophagia was observed (Fig. 6.1). A large number of mycobacteria were observed within the vacuoles of FCA and among the muscles in the lesion area using H & E and ZN staining methods (Fig. 6.2).

1 day

At 24 hours, there was migration of more inflammatory cells comprising lymphocytes, PMNs and macrophages into the area of lesion. Marked myopathy and muscle degeneration associated with trauma of the injection of FCA was observed (Fig. 6.3). There was active myophagia, and blood vessels were associated with white blood cells. Melanin pigments were seen around the hyperaemic blood vessels. Haemorrhage with fibrin was seen in the damaged area. Vacuoles containing oil were observed among the degenerated muscles, surrounded by white and red blood cells(Fig. 6.4). A few acid-fast mycobacteria were seen in the lesion area using ZN staining.

Fig. 6.1 The site of injection at 6 hours after inoculation of FCA showed extensive haemorrhage (H), muscle degeneration (MD), and cellular infiltration (arrow heads). A large number of mycobacteria were observed in the lesion area (arrow). (H & E, X 44). E=epidermis, D=dermis, ML=muscle

Fig. 6.2 This picture shows a large vacuole (V) containing FCA between degenerated muscle area (MD) at 6 hours after inoculation of FCA. A large number of mycobacteria (arrows) are within the vacuole. (ZN, X 440).


E
D
ML
H
MD

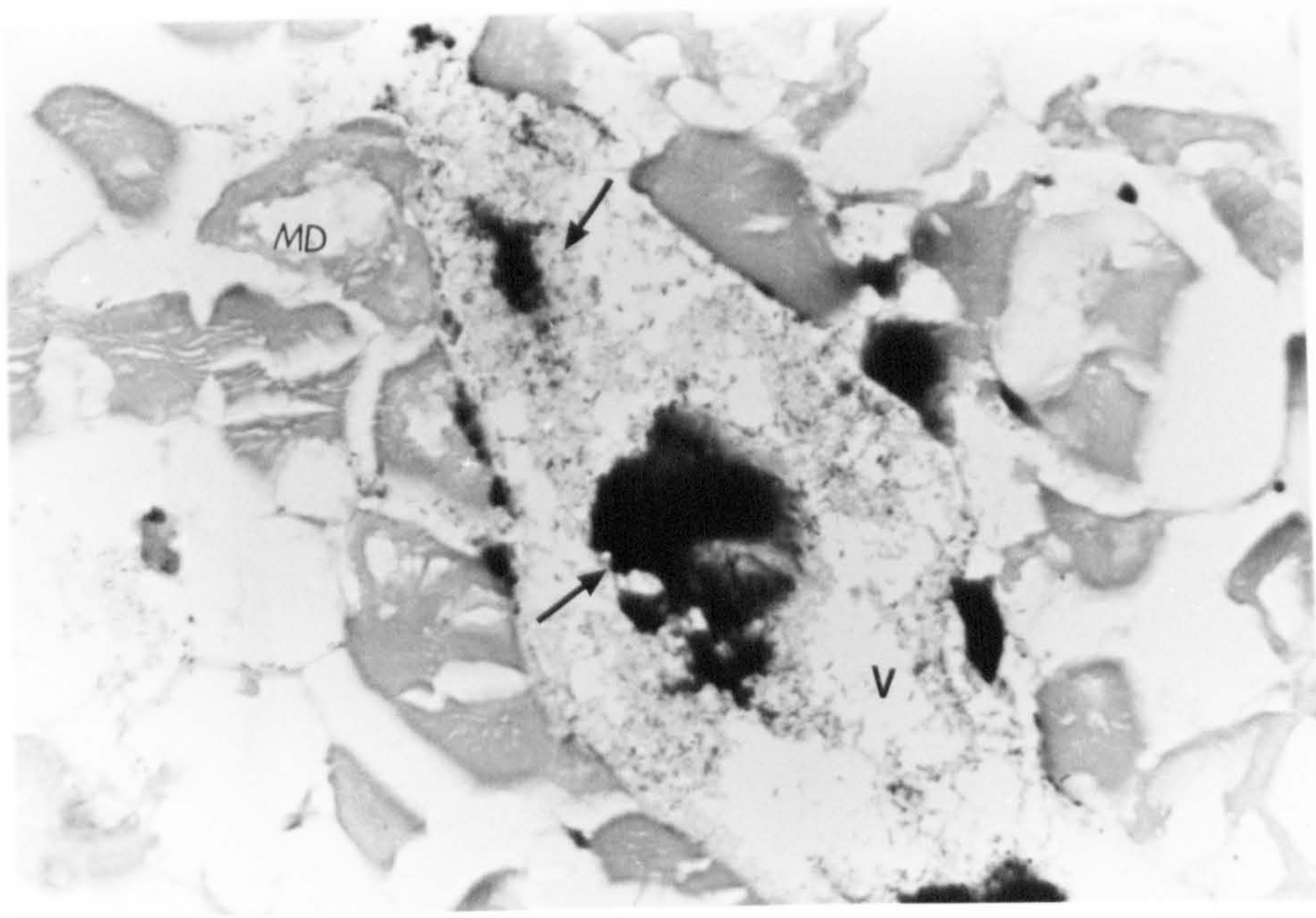
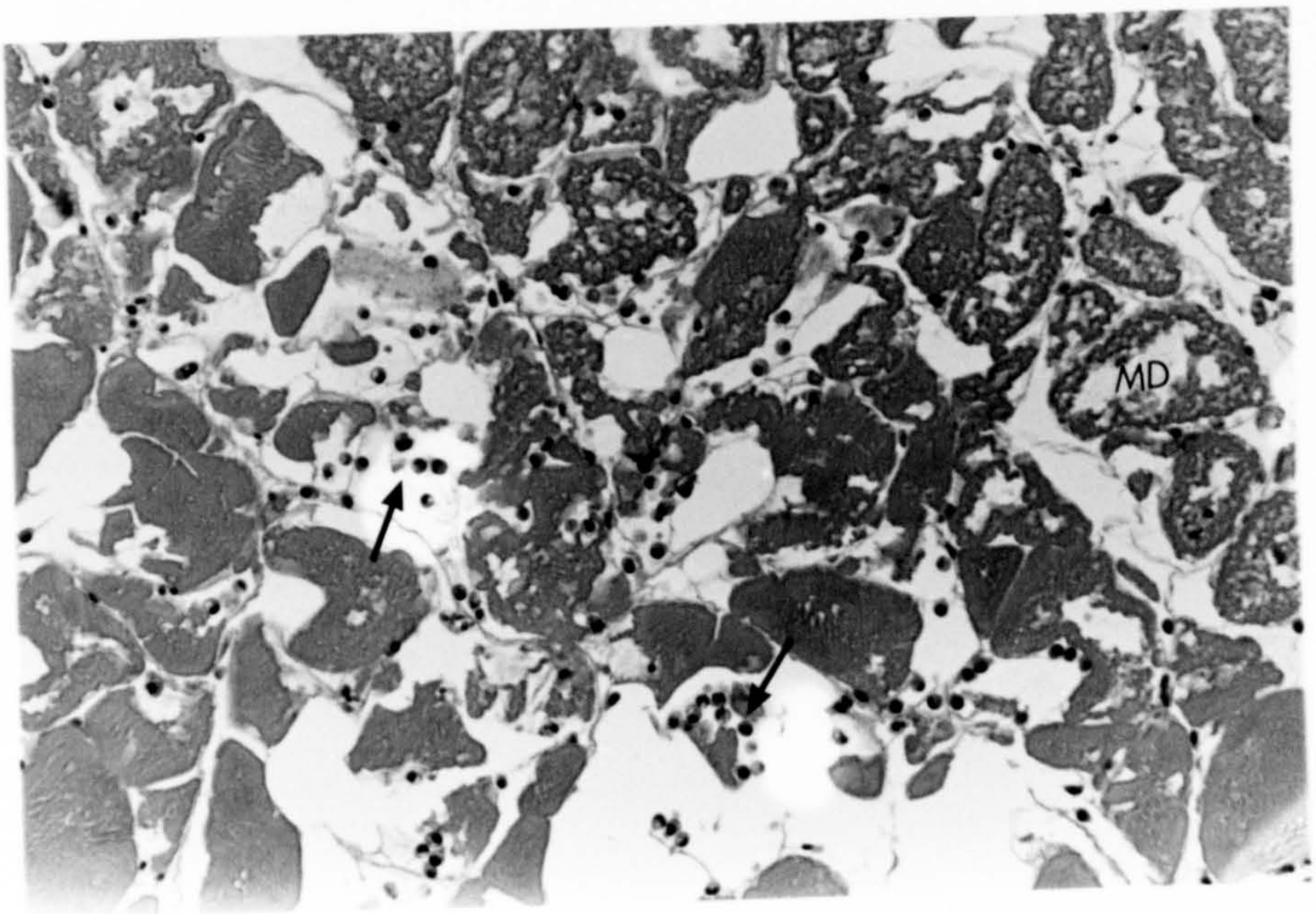
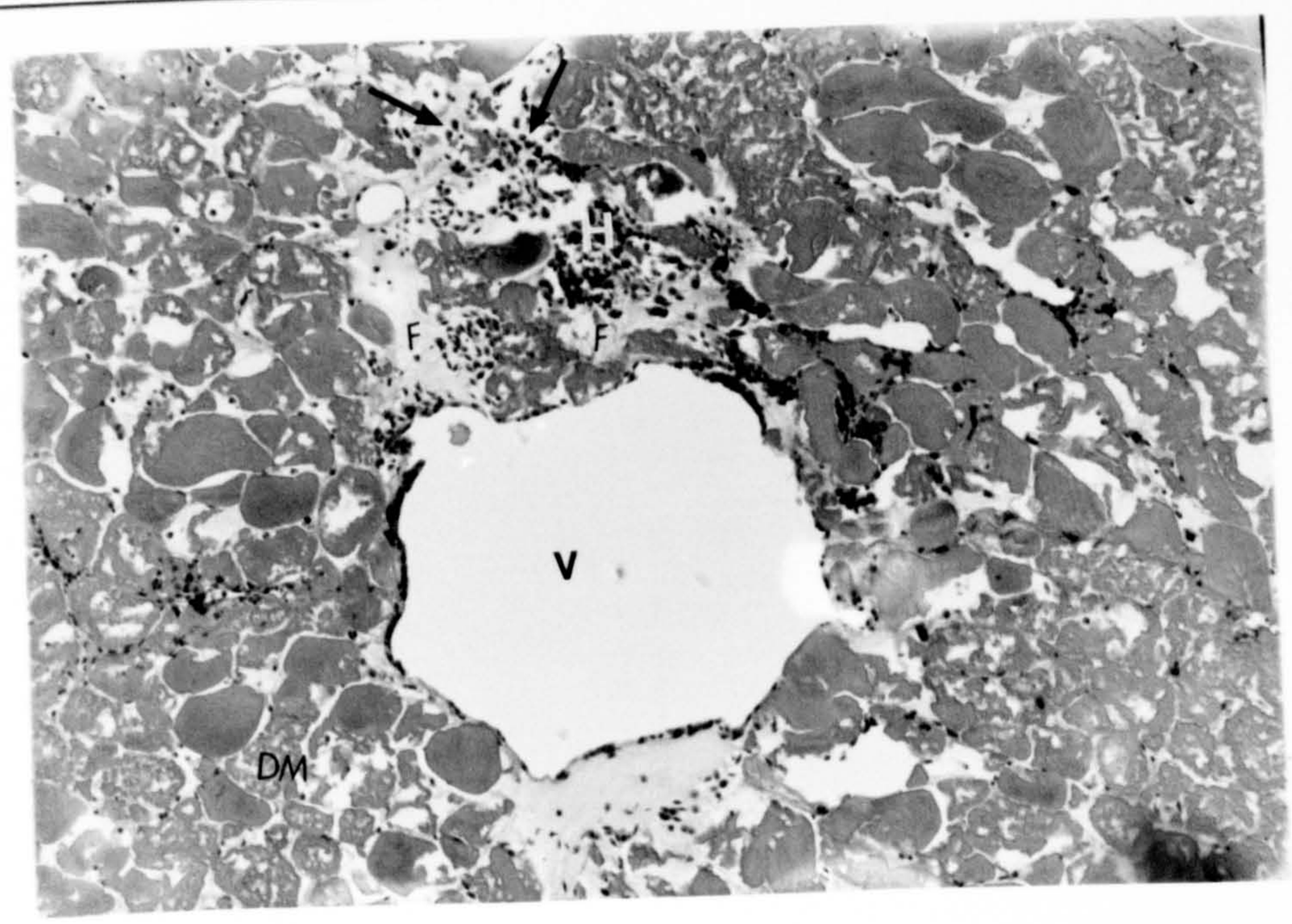

MD
V

Fig. 6.3 Marked muscle degeneration (MD) and active myophagia (arrows) were observed by 24 hours p.i. (H & E, X 220).

Fig. 6.4 This picture shows a large vacuole (V) containing FCA between degenerated muscles (DM) at 24 hours p.i. Haemorrhage (H) with fibrin (F) and inflammatory cells (arrows) are observed around the vacuole and in the damaged area. (H & E, X 110).


MD


H
F
F
V
DM

2 days

On the second day after inoculation of the FCA, mild haemorrhage, presence of the inflammatory cells, including lymphocytes, PMNs and macrophages, and also myophagia were obvious in the lesion area (Fig. 6.5). Some melanin granules were spread in the inflammatory area, especially associated with haemorrhage and hyperaemic blood vessels. Myopathy and degenerated muscles due to the trauma of the injection of FCA was more marked. New capillaries had formed in the damaged area. There were vacuoles of oil among the muscles in the site of injection, surrounded by some inflammatory cells. Some mycobacteria were seen in the lesion area using ZN staining.

3 days

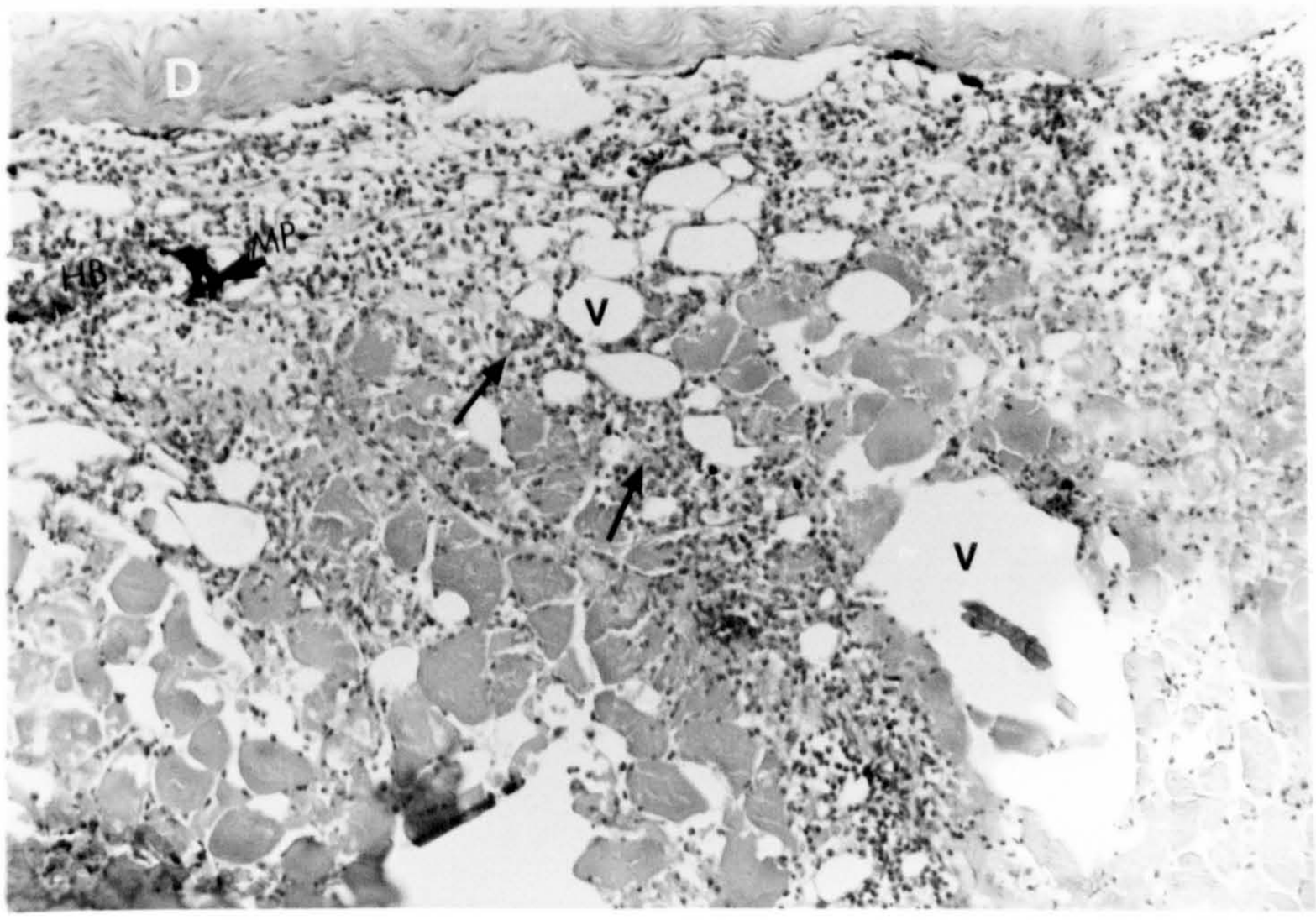
The main feature in this stage was presence of epithelioid cells, sequestrating the lacunae of FCA (Fig. 6.6). Lymphocytes, PMNs, macrophages, and melanin pigments spreading through the area were seen. Degenerate muscles were obvious and also initiation of muscle regeneration with new muscle buds were evident in the area along with new capillaries and some fibroplasia. A few mycobacteria were observed in the area of lesion using ZN staining.

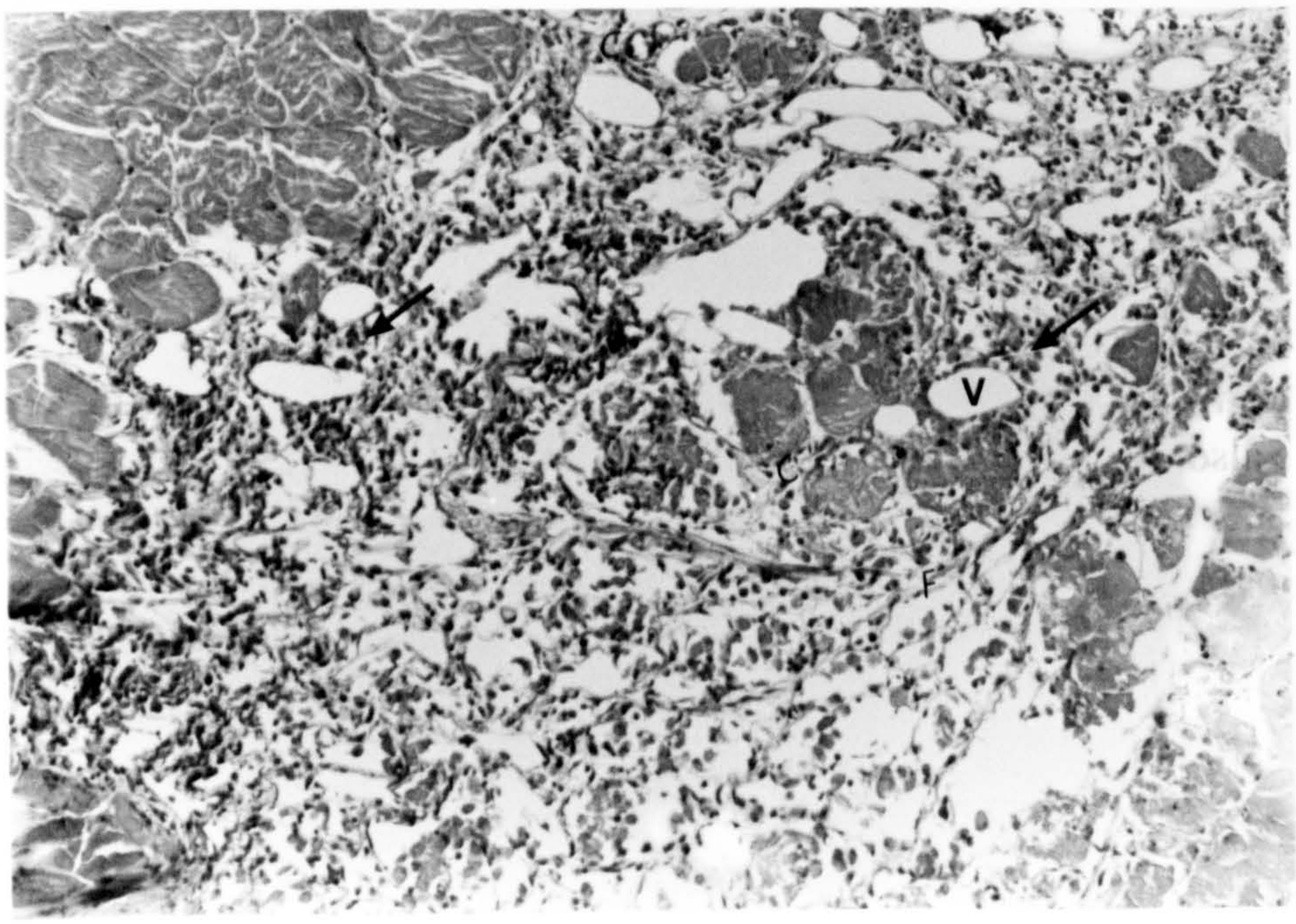
4 days

The sequestration of adjuvant by marked increasing the number of epithelioid cells around the lacunae of FCA was active. Myophagia was completed and surviving myofibrils were isolated by fibrogranulation tissue. Regeneration of muscles with

Fig. 6.5 On the 2nd day p.i. high levels of macrophage infiltration were observed. Macrophages (arrows) surrounded FCA vacuoles and were very actively myophagic in the lesion area. A hyperaemic blood vessel (HB) is obvious in the lesion area with melanin pigment (MP) around. (H & E, X 110).
D=dermis, V=vacuole of FCA

Fig. 6.6 The main feature at 3 days p.i. was presence of epithelioid cells (arrows) sequestrating the lacunae of FCA (V). Some fibroplasia (F) and new capillaries (C) were also observed. (H & E, X 110).


D
HB
MP
V
V


V
F

marked muscle fibre buds and also new muscle fibrils was obvious. Haemorrhages in the lesion area and new capillaries found in the defect area. The number of lymphocytes were increased, but PMNs and macrophages were decreased. Some melanin pigments were spread in the damaged area and fibroplasia was in progress. First presence of a few giant cells (Langhans and foreign body types) was evident among the epithelioid cells (Fig. 6.7). A few mycobacteria within the vacuoles of FCA were seen.

5-6 days

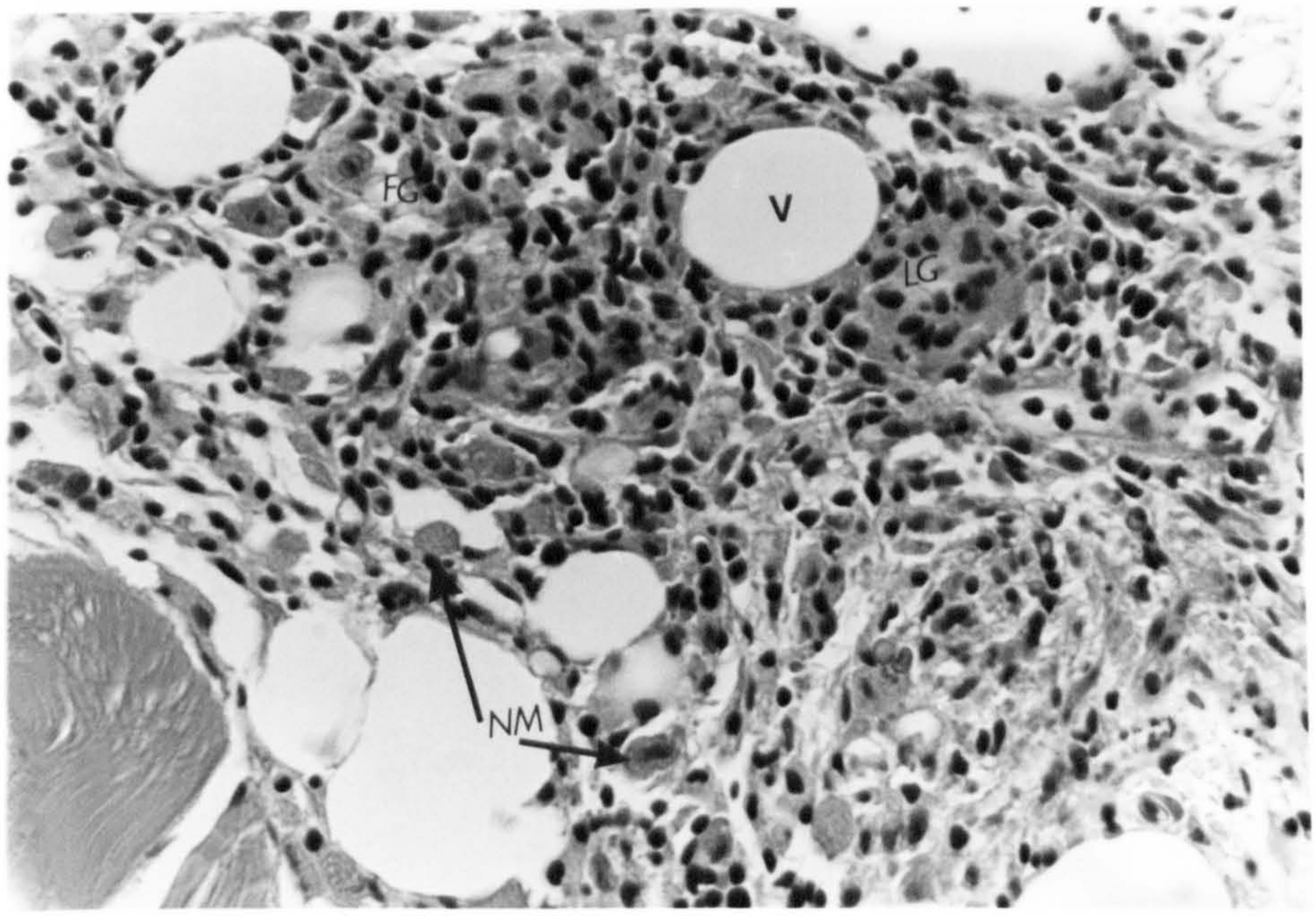
At this period, more new muscle buds, new capillaries, fibroblasts activity and inflammatory cells were seen (Fig. 6.8). An increasing in the number of lymphocytes was obvious in the damaged area (Fig. 6.9). Epithelioid cells sequestrated FCA lacunae, and presence of a few giant cells in the lesion area were observed. Fibrosis and collagen formation in the defect area was active. Presence of some mycobacteria were still evident within the vacuoles of FCA and also in the cytoplasm of some macrophages. Also a very strong reaction visible as a golden-brown cytoplasmic colour within the phagocytic cells in the granulomata was observed using IHC staining method (Fig. 6.10).

7 days

Some haemorrhages, regenerated myofibrils and vascularization throughout the injected area, presence of occasional foreign body and Langhans giant cells dispersed among the epithelioid cells, foci of lymphocytes, and oil vacuoles were obvious in this stage (Fig. 6.11). No mycobacteria were observed in the tissues.

Fig. 6.7 Sequestration of FCA by marked increasing the number of epithelioid cells around the vacuoles (V) was active by 4 days p.i. Muscle regeneration was active and new muscle buds (NM) were observed. First presence of Langhans (LG) and foreign body (FG) types of giant cells were evident. (H & E, X 440).

Fig. 6.8 More new muscle buds (arrow heads), new capillaries (arrows) and increasing in number of lymphocytes (L) were observed at 5 days p.i. (H & E, X 220).


FG
V
LG
NM

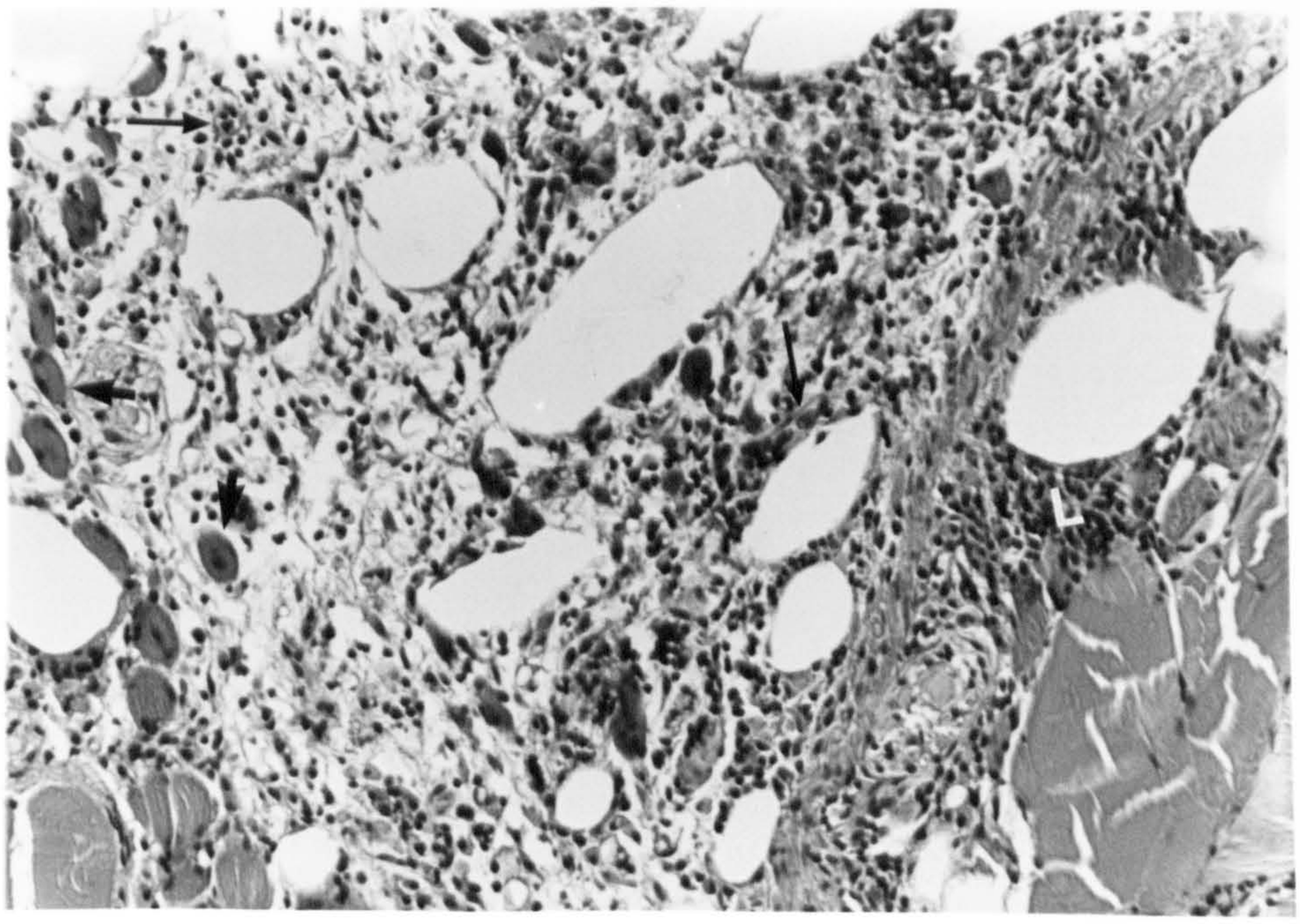
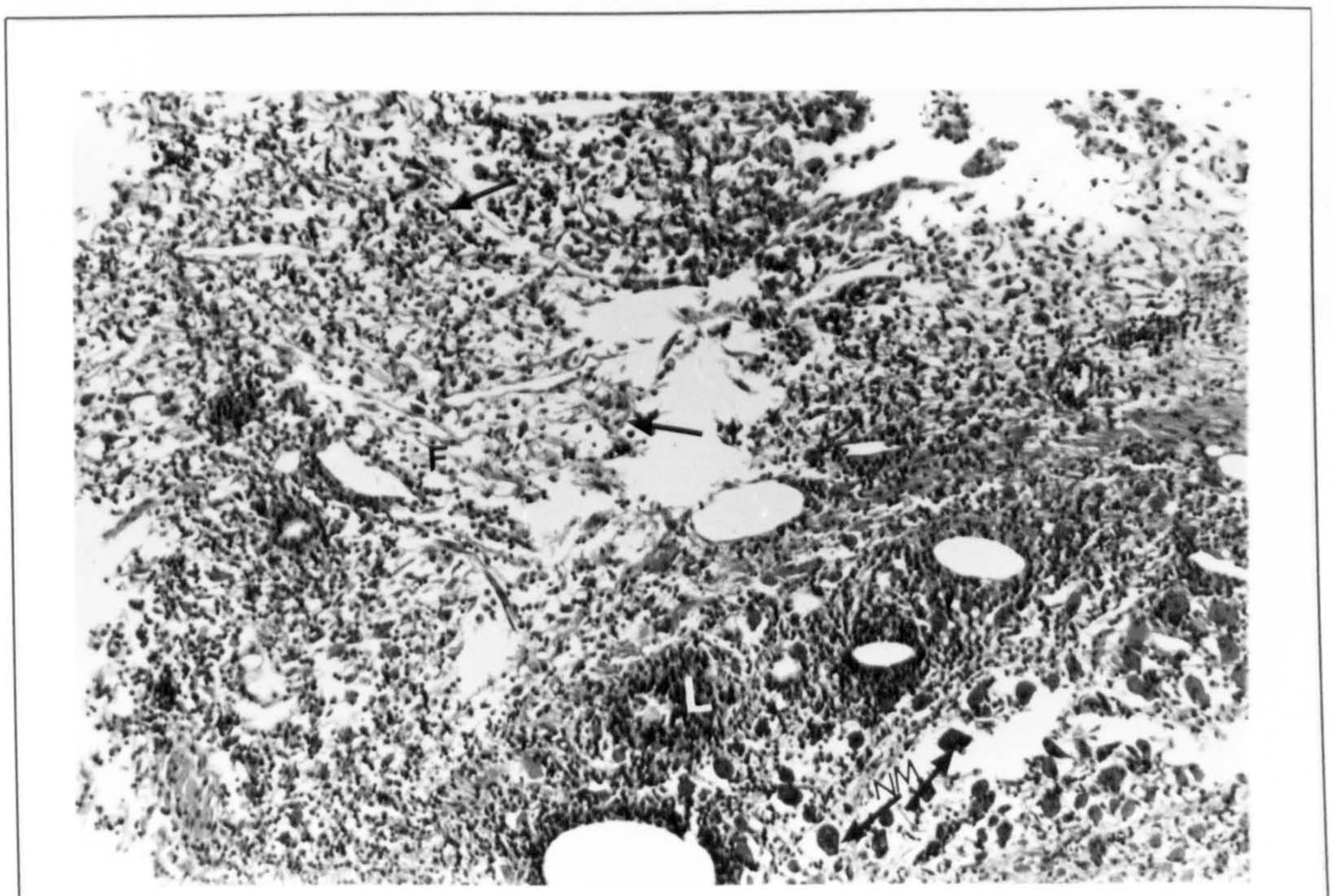
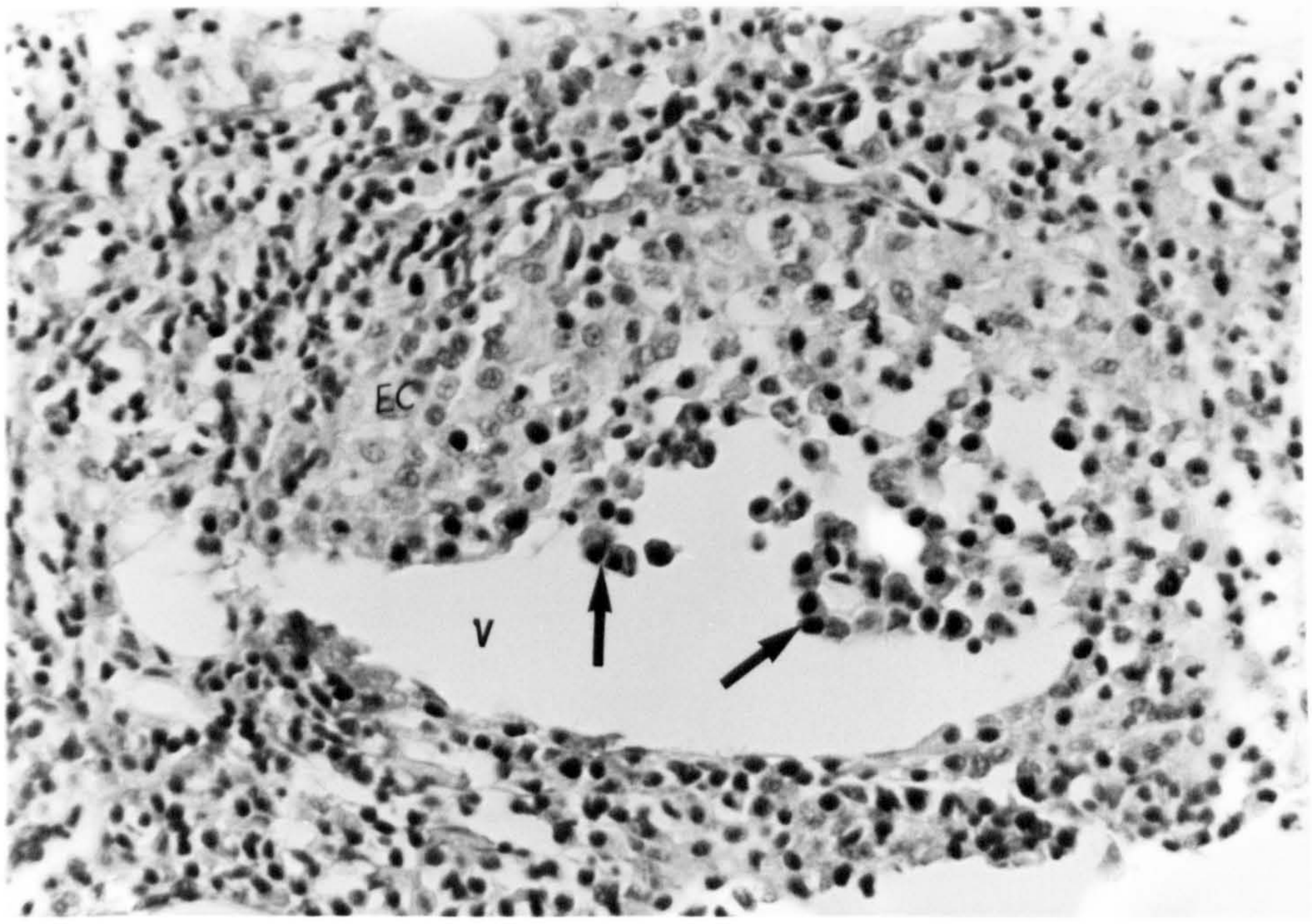

L

Fig. 6.9 Increasing in number of lymphocytes (L) in the lesion area especially around the vacuoles was obvious at 6 days p.i. Fibroplasia (F) was active and new muscle buds (NM) and new capillaries (arrows) were observed. (H & E, X 110).

Fig. 6.10 A very strong (+++) reaction was observed in tissue sections at 6 days p.i. using monoclonal antibody. This reaction was visible as a golden-brown cytoplasmic colour within the phagocytic cells (arrows) in the granulomatous areas. (IHC, X 440). EC=epithelioid cell, V=vacuole of FCA


F
L
M


EC
V

10 days

At this stage the new capillaries in the fibrosis area along with some haemorrhages and fibroblasts activity were seen. Scattered complex of fibroblasts and macrophages with some lymphocytes were observed in the lesion area where muscle tissue had been removed by myophagia. Also some giant cells were evident. Lacunae of FCA with smooth edges surrounded by epithelioid cells, were observed among the defect area (Fig. 6.12).

14 days

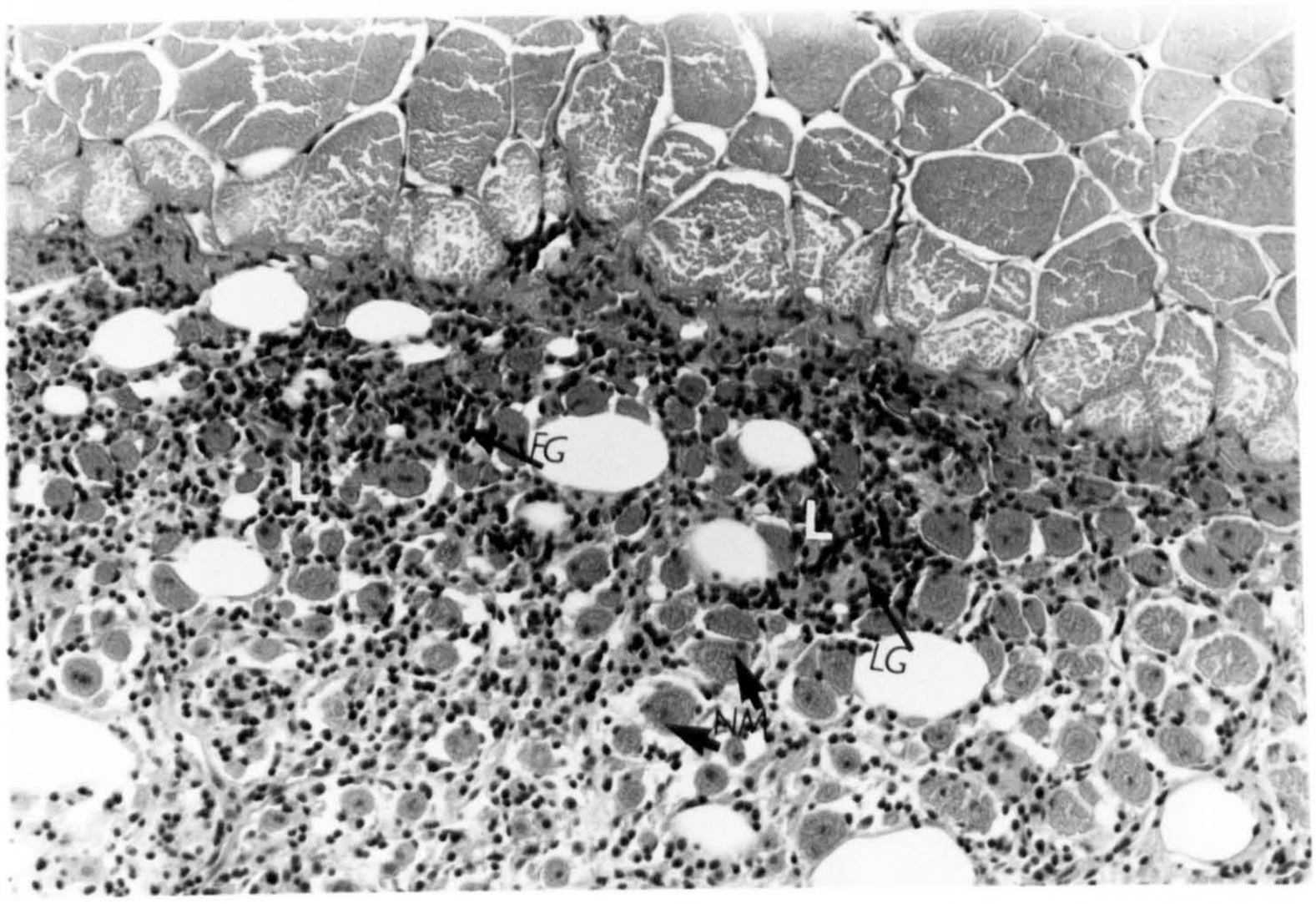
The macrophages of granulation process extended up between the intact muscle fibres, and were engaged in myophagia of the sporadic affected fibres. New capillaries and some engorged vessels associated with melanocytes and filled in with red blood cells were seen. Langhans and foreign body giant cells were obvious among the epithelioid cells in the defect area (Fig. 6.13). There was some fibrosis especially around the edges of the lesion. Lymphocytes activity and also foci of fibroblsts, macrophages and lymphocytes were seen in the lesion area (Fig. 6.14).

18 days

On the 18th day after injection, fibrous tissue tended to surround vacuoles of FCA in some sites, and in some other sites a layer of fibroblasts surrounded the vacuoles with epithelioid cells around them. This was the beginning of small and large granulomata forming in the defect area (Fig. 6.15). Giant cells, new capillaries and lymphocytes activity especially in fibrous tissue were seen.

Fig. 6.11 At 7 days p.i. regenerated myofibrils (NM) increased. Presence of occasional foreign body (FG) and Langhans (LG) giant cells was observed. Foci of lymphocytes (L) were seen around the oil vacuoles. (H & E, X 220).

Fig. 6.12 This picture shows a general view of the lesion area at 10 days p.i. Lacunae of FCA (V) sequestrated by epithelioid cells are observed in the defect area with a smooth edge (arrow heads). The inflammatory response are still more cellular (arrows) rather than fibrogranular. (H & E, X 44).
E=epidermis, D=dermis


FG
L
L
LG
AM

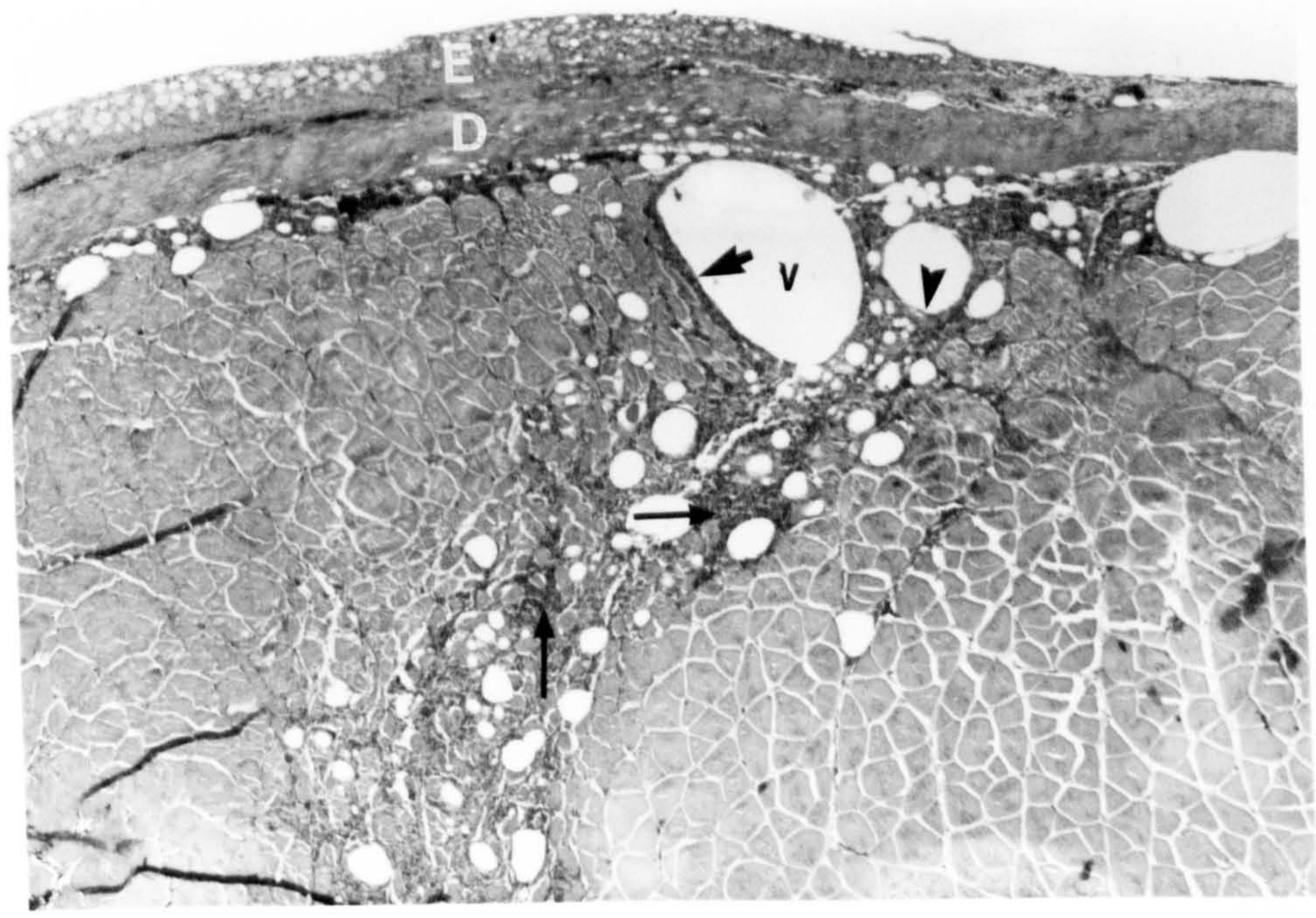
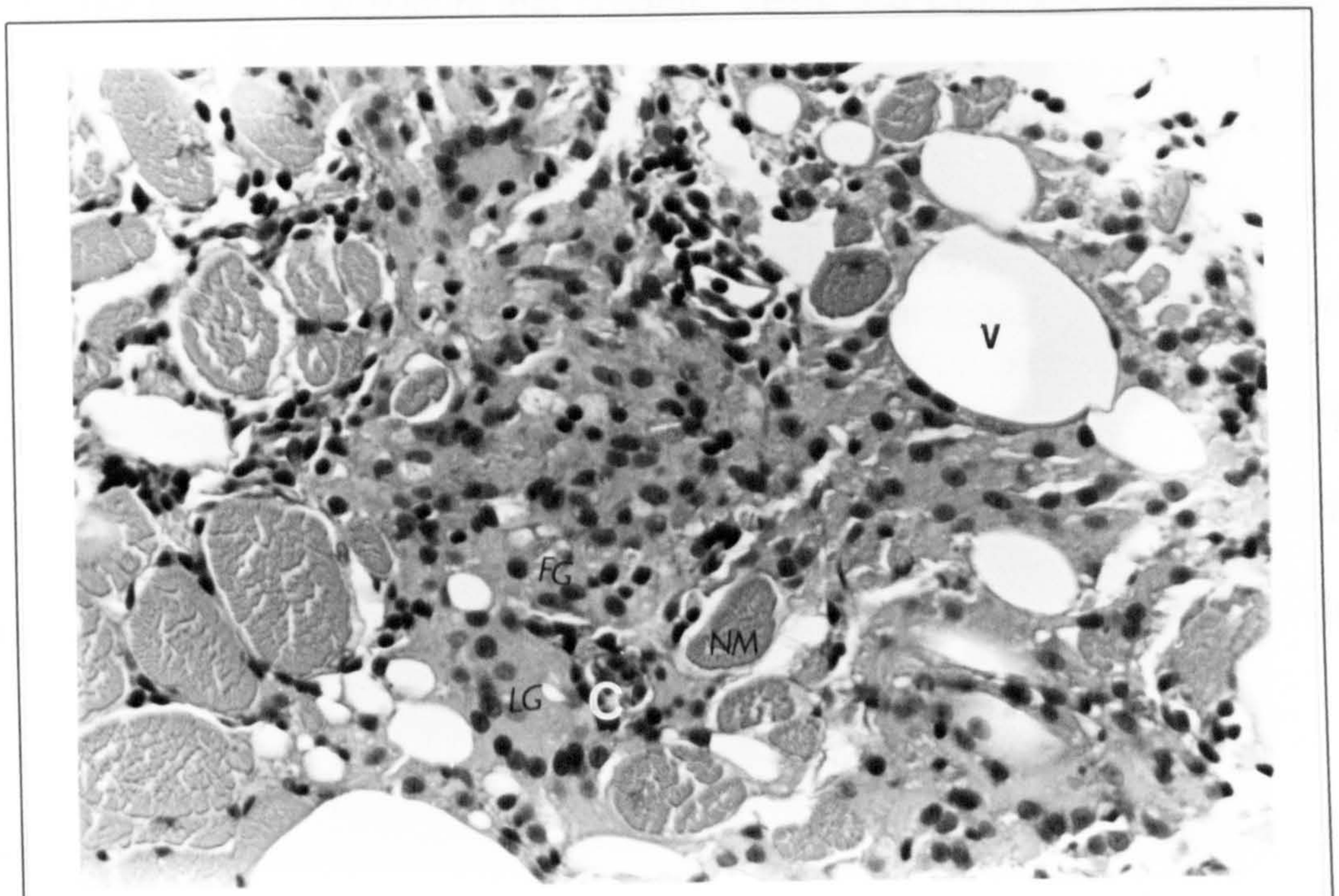
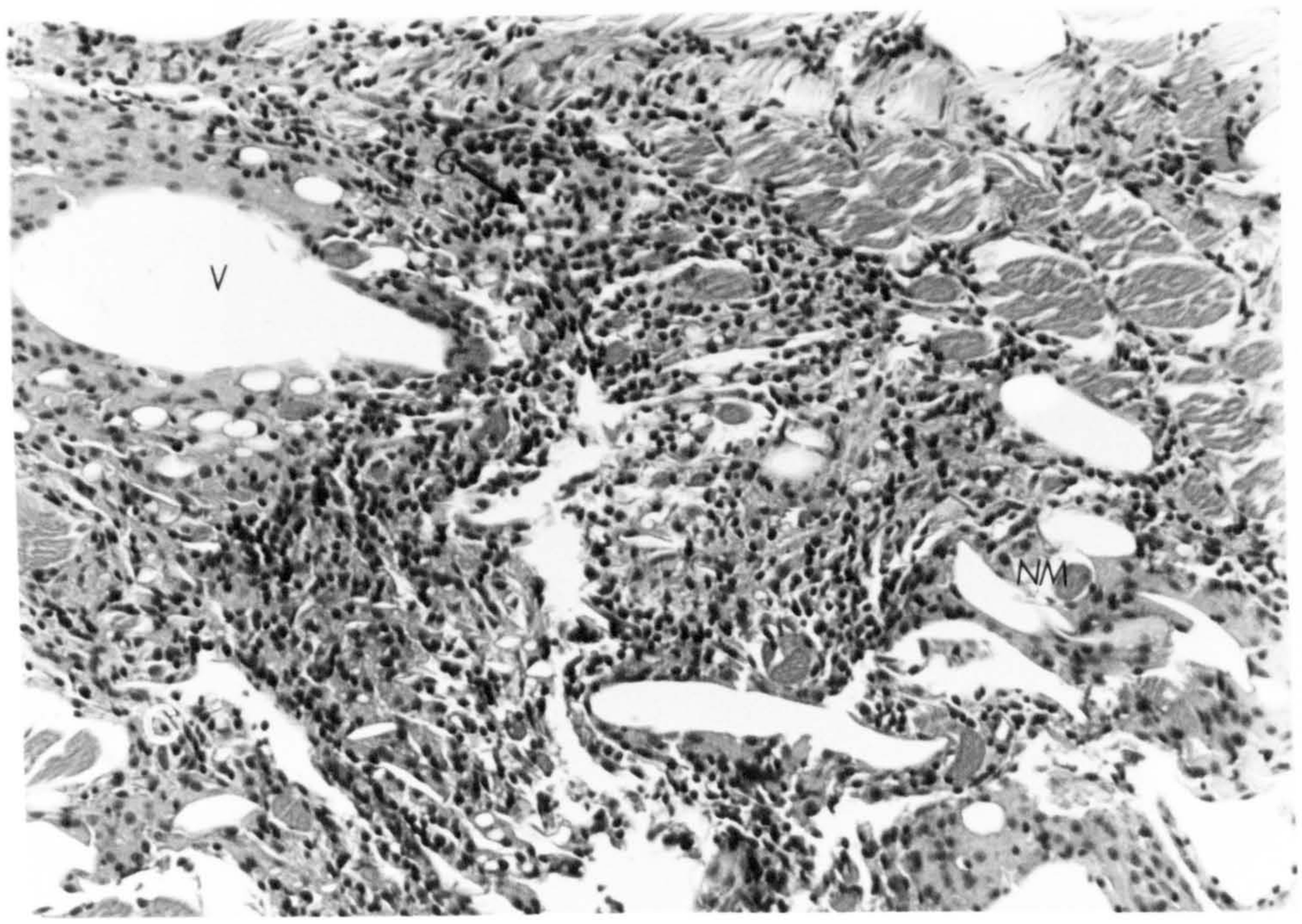

E
D
V

Fig. 6.13 This picture demonstrates a part of the lesion area at 14 days p.i. Regenerated muscles (NM), Langhans (LG) and foreign body (FG) giant cells, new capillaries (C) and vacuoles of FCA (V) are observed in the inflammation area. (H & E, X 440).

Fig. 6.14 At 14 days p.i. the lesion area was more cellular especially with large lymphocytes domination. Regenerated muscles (NM), new capillaries (C), lacunae of FCA (V) and giant cells (G) were observed in the lesion area. (H & E, X 220).


V
FG
NM
LG
C


V
NM

22 days

At 22 days after injection, the lesion was only marginally different from the 18th day where the fibrosis was mature and layered. There was small and large granulomata forming throughout the lesion area with surrounding FCA vacuoles and epithelioid cells around them, by fibrous tissue. In some granulomata layers of epithelioid cells encroached into the lumen and divided them (Fig. 6.16). Inside some of the granulomata sloughing of effete macrophages into the lumen, also pink granular deposit, with nucleic acid debris were evident. Large numbers of lymphocytes around the granulomata and within the fibrous tissues was marked. In some parts of the lesion, small foci of caseation in association with lymphocytes activity were seen. Vascularization, congestion of blood vessels along with melanin pigments around, fibroplasia, presence of macrophages, some eosinophilic granular cells (EGCs) and giant cells were observed.

28 days

At this stage more solidification of granuloma with matured and layered fibrosis around them was dominant feature. Also high level of lymphocytes activity with caseation of vacuoles was marked (Fig. 6.17). There was evidence of continuous sloughing of effete macrophages into the lumen of vacuoles. This material was a pink granular deposit, with nucleic acid debris. Macrophage bridges within the granulomata were developing, dividing the large granulomata. Vascularization was active in the lesion area. Congestion of blood vessels with melanin pigments around were seen. A few EGCs and giant cells around the granulomata were evident, but a large number of

lymphocytes were seen around them, especially within fibrous tissues and also among muscles.

35 days

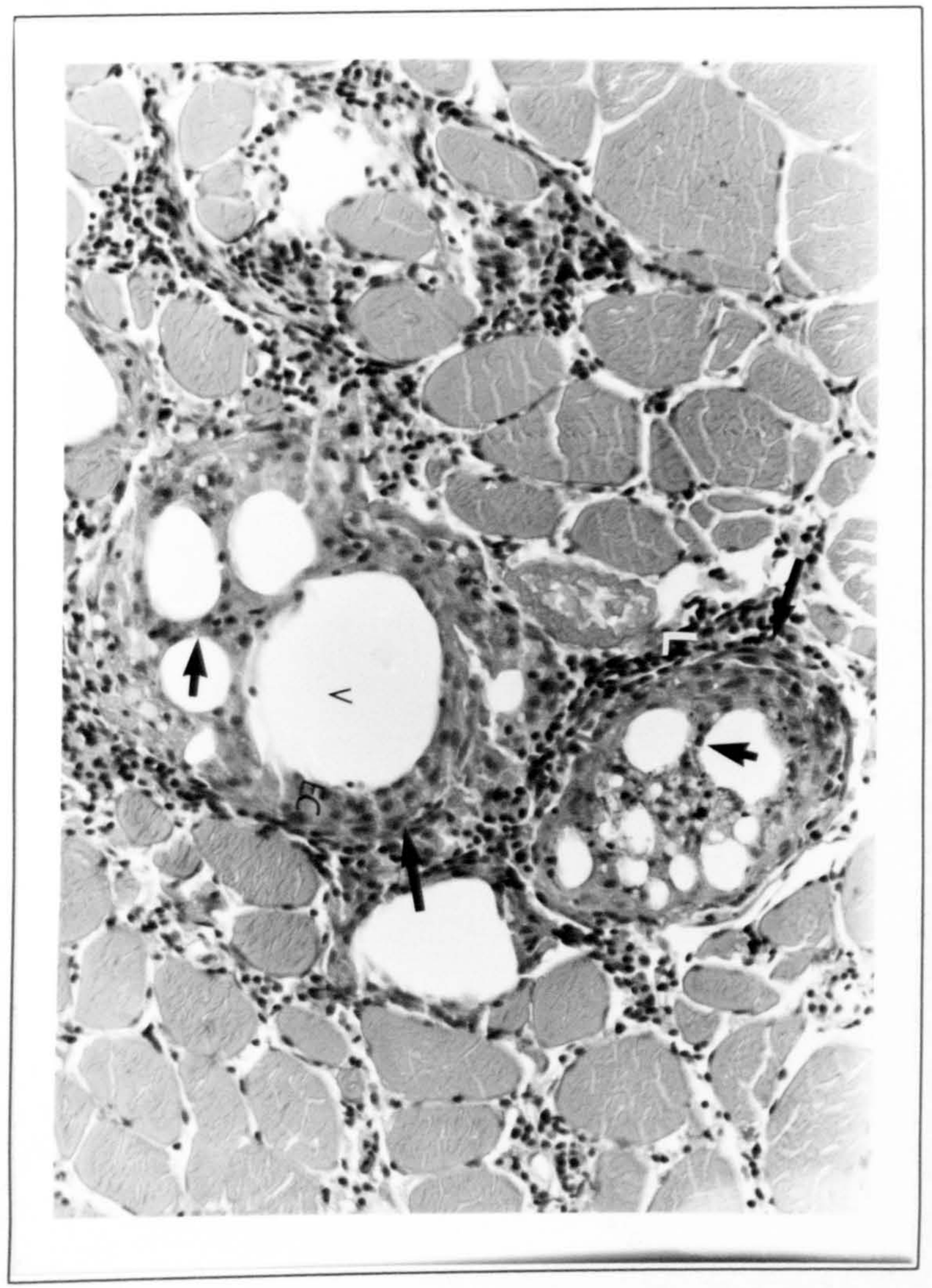
At this stage, typical granulomata were seen in various sizes in the lesion area. Similar to the 28th day, more consolidation of the process of encapsulation of the granulomata was obvious. More fibrous tissues were seen around the granulomata and in the lesion area (Fig. 6.18). New capillaries and blood vessels were very active in nourishing the area producing fibrous tissue. Continued bridging of the oil lacunae with macrophages and dividing large granulomata into smaller sizes was observed. Some large lacunae with dense epithelioid capsule, fibrous tissue around and necrotic materials and debris inside, were seen. Lymphocytes activity along with some EGCs caused caseation in some sites. Macrophages, EGCs and some vacuoles were observed in the caseaous necrotic areas.

42 days

This was the final sampling period for the study. More consolidation of the process of encapsulation, in comparison with the previous stages, was evident. All remaining lacunae being reduced and filled with necrotic epithelioid debris and some pigments and pink material. Also bridging of the large spaces inside the granulomata with necrotising tissues was very obvious (Fig. 6.19). These lacunae were surrounded by dense fibrous layers. There was also a more sloughed materials within the remaining vacuoles. New capillaries in the fibrous tissues were obvious and in high

Fig. 6.15 By day 18, fibrous tissue (arrows) tended to surround vacuoles of FCA (V), which was the beginning of small and large granulomata formation. (H & E, X 220). EC=epithelioid cell, G=giant cell

Fig. 6.16 At 22 days p.i. fibrous tissue (arrows) surrounded the vacuoles (V) and epithelioid cells (EC) around them. Layers of epithelioid cells encroached into the lumen and divided the granulomata (arrow heads). Large numbers of lymphocytes (L) were observed around the granulomata and in the lesion area. (H & E, X 220).


EC
V

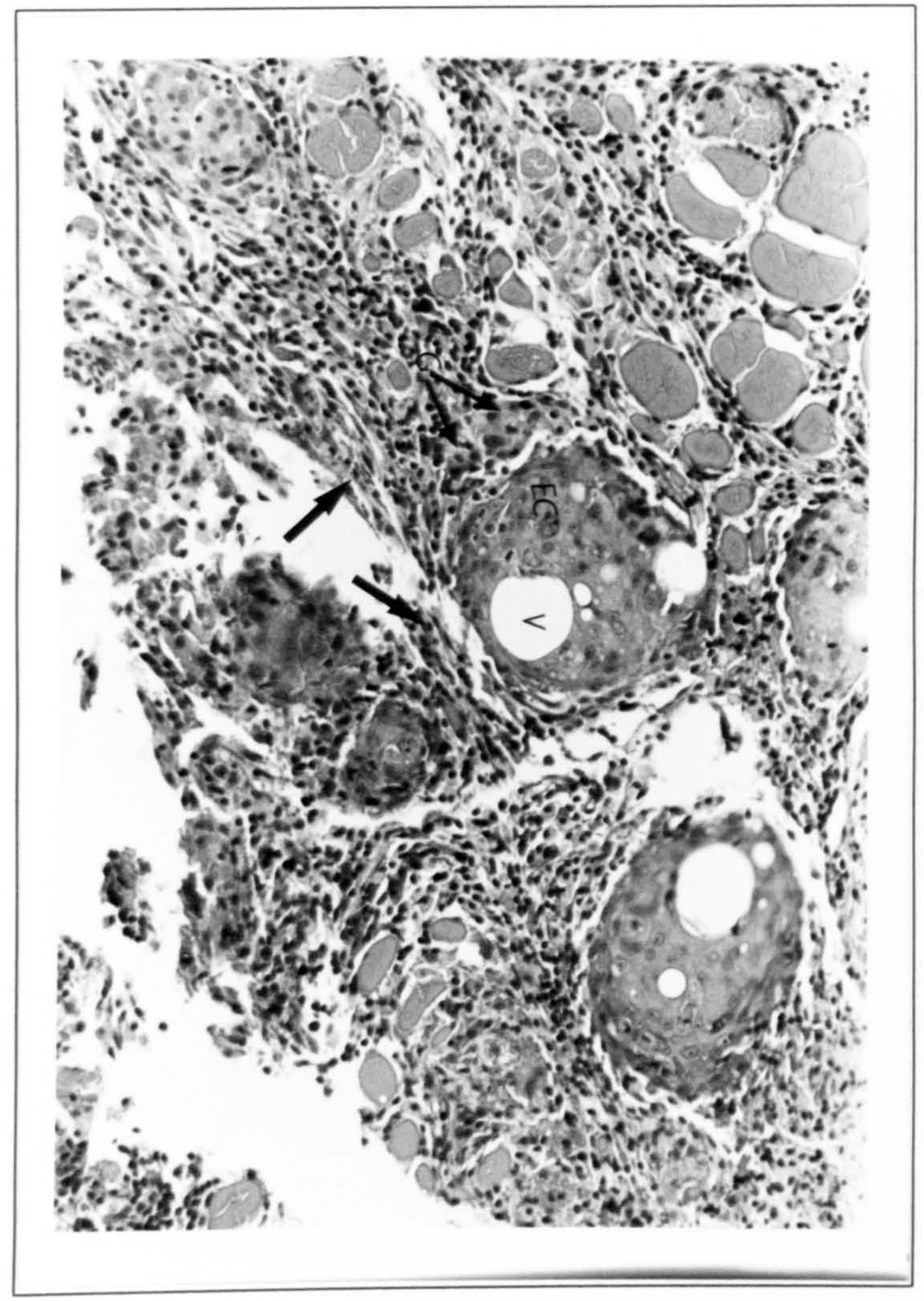
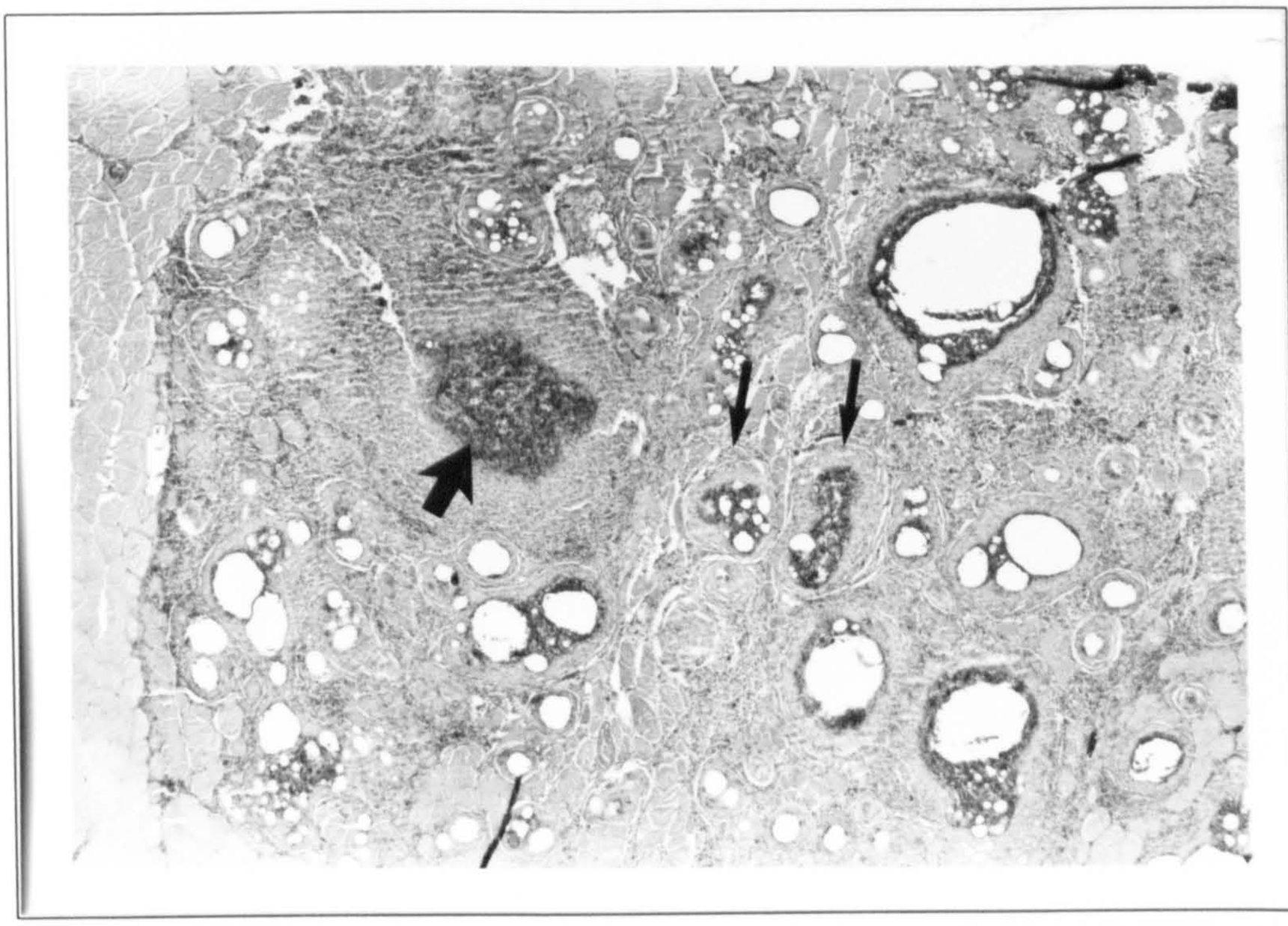

EC
V

Fig. 6.17 This picture demonstrates a general view of the injected area at 28 days p.i., which is an extensive granulomatous lesion. High level of lymphocytes activity with caseation is marked (arrow head). More solidification of granulomata with matured and layered fibrosis around and some necrotic materials inside is observed (arrows). (H & E, X 44).

Fig. 6.18 By 35 days p.i. typical granulomata (TG) were observed in various sizes in the lesion area. More fibrous tissue (arrows) were seen around the granulomata. Bridging of the oil lacunae with macrophages (arrow heads) and dividing large granulomata into smaller size was observed. (H & E, X 110).



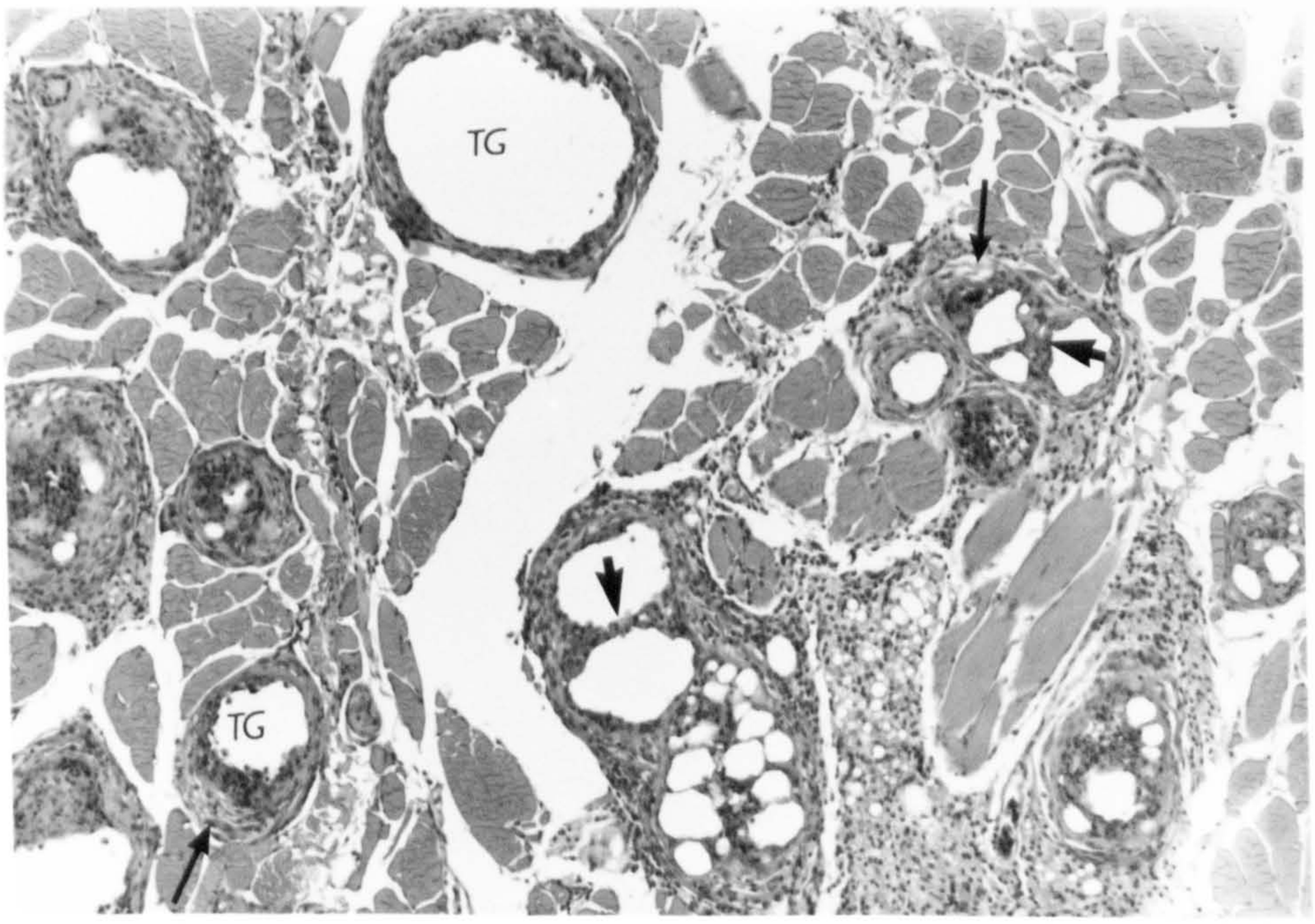
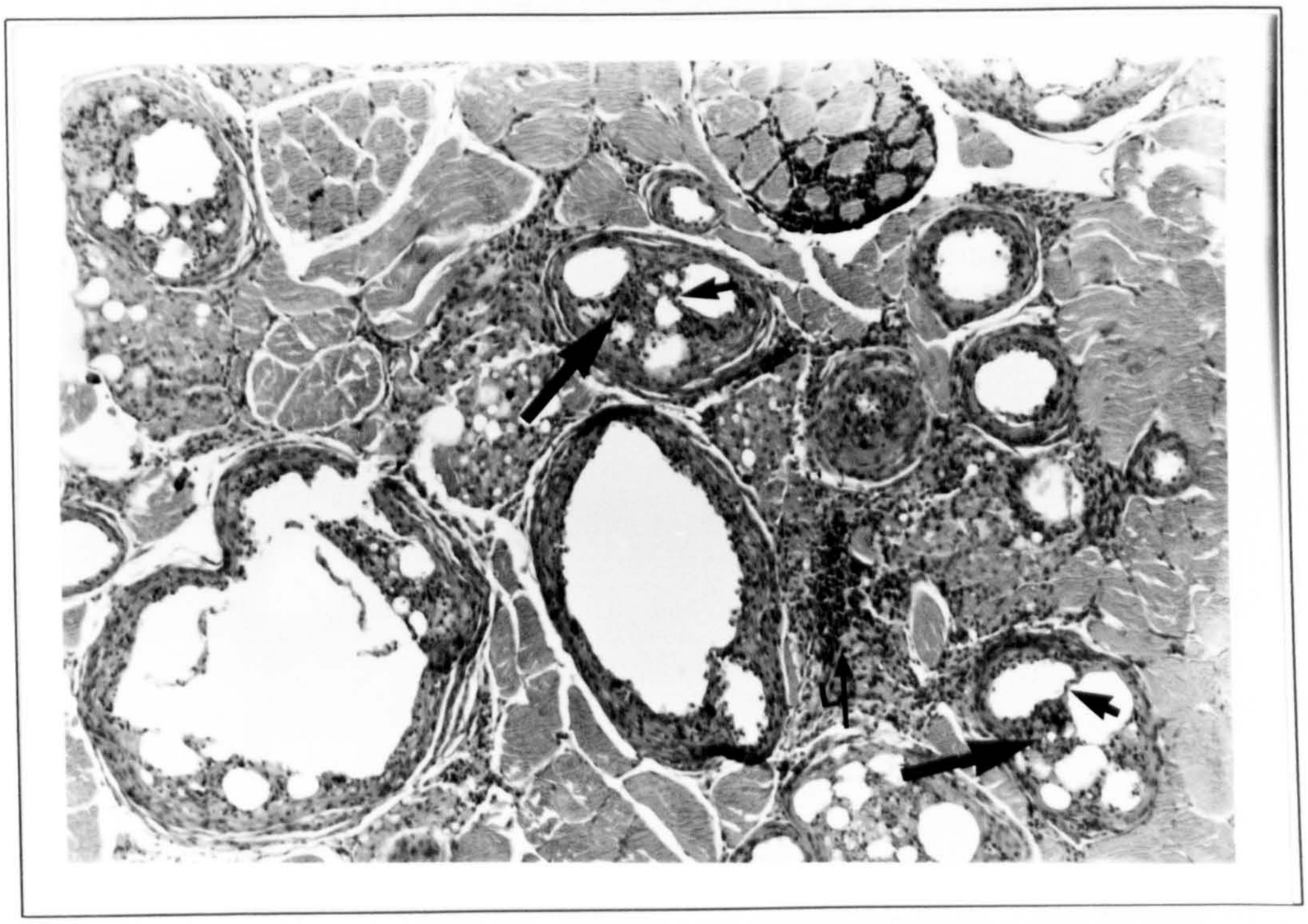
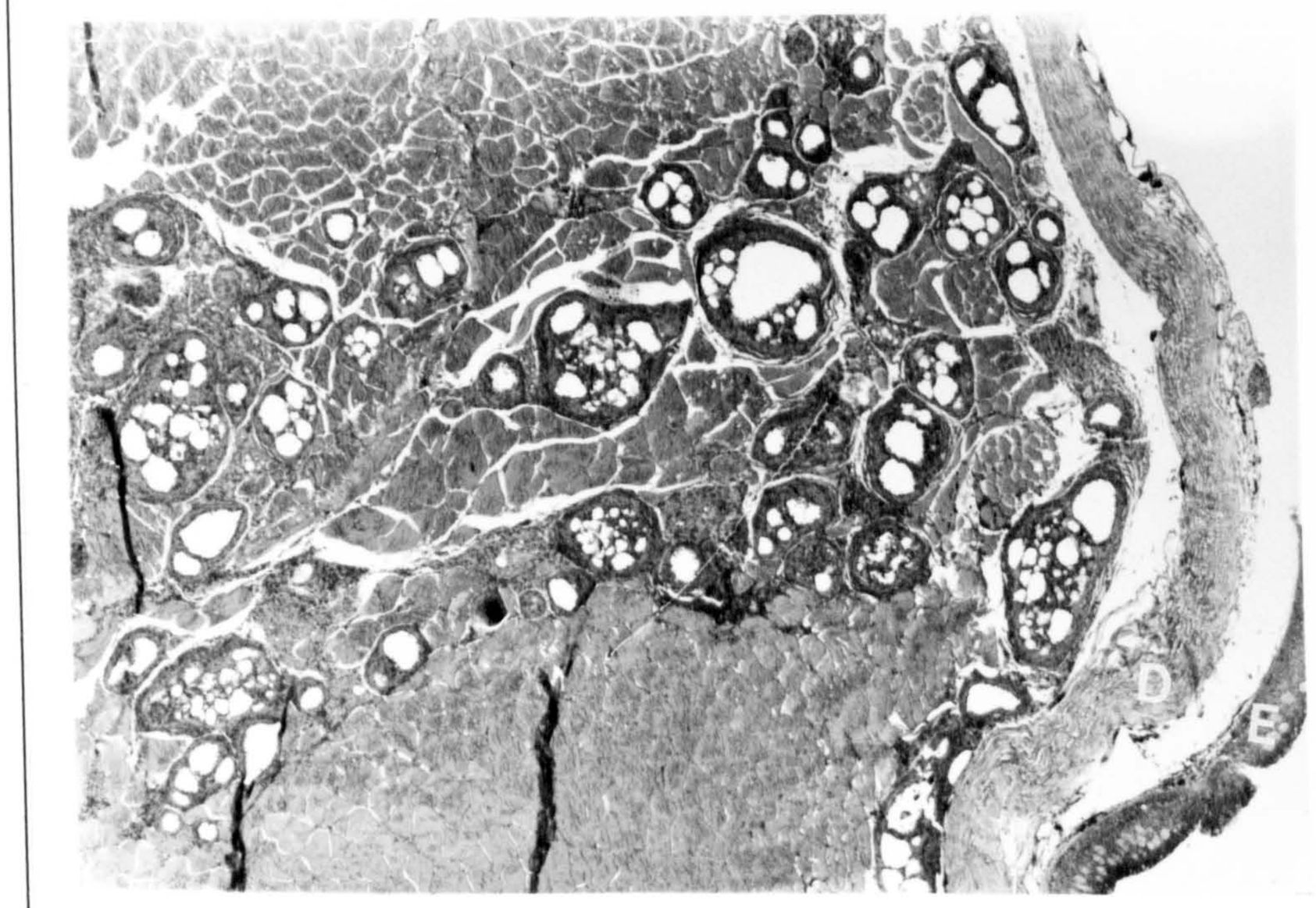

TG
TG

Fig. 6.19 All remaining lacunae of FCA at 42 days p.i. being reduced and filled with necrotic materials (arrows). Also bridging of the large spaces inside the granulomata with necrotising tissues was obvious (arrow heads). Foci of lymphocytes (L) in the lesion area and between the muscle fibres were seen. (H & E, X 110).

Fig. 6.20 This picture demonstrates a general view of the last sample at 42 days p.i. Although the complete resolution of the lesion had not occurred by this stage, all the vacuoles of FCA are surrounded completely by matured and dense fibrous tissue, with more necrotic materials within the vacuoles. Cellular components are decreased in the lesion area and granulation tissue increased. (H & E, X 44).
E=epidermis, D=dermis




D
B

activity. Also EGCs, lymphocytes, macrophages and some small vacuoles were observed in the caseaous necrotic areas. However, complete resolution of the lesion had not occurred by this stage of the experiment (Fig. 6.20).

Control fish

No significant histological changes were observed in the fish injected with normal saline (appendix 4).

6.4.2.3 Detection of mycobacteria in the sampled tissues

Attempts were made to detect the inoculated *Mycobacterium butyricum* in fish tissue in the present study. Different staining methods such as; H & E, ZN, AO, PAS, and IHC using monoclonal antibody (Mab) and polyclonal antibody (PAb), were used for histological examinations of the sections for detection of *Mycobacterium butyricum*. The first three methods detect bacteria but PAS and IHC could demonstrate the reaction of bacterial components to Schiff's reagent and antibody, respectively. The result of these examinations is summarised in table (6.3).

Table 6.3: The results of different staining methods in 6 samples of 15 samples from FCA inoculated fish.

Staining methods → / Sampling times ↓	H & E (bacilli)	ZN (acid-fast bacilli)	AO* (brilliant to gold bacilli)	IHC (golden materials / brown reaction) (MAb)	IHC (golden materials / brown reaction) (PAb)	PAS (positive materials/ reaction)
6 hours	+++	+++	−	−	−	+
2 days	±	+	−	−	−	++
6 days	+	±	−	+++	+	+++
14 days	−	−	−	−	−	+++
28 days	−	−	−	−	−	+++
42 days	−	−	−	−	−	+++

Abbreviations:

H&E : haematoxylin and eosin
ZN : Ziehl-Neelsen
PAS : periodic acid Schiff
IHC : immunohistochemistry
MAb : monoclonal antibody
PAb : polyclonal antibody
AO : Auramine O

Symbols:

− : no bacteria / reaction
± : a very few bacteria / a very weak reaction
+ : a few bacteria / a weak reaction
++ : a medium number of bacteria / a strong reaction
+++ : a large number of bacteria / a very strong reaction
* : Smear of FCA, as control slide, was positive.

6.5 DISCUSSION

The features of the inflammatory response in carp (present study) inoculated with FCA, were very similar to those of the rainbow trout (Finn & Nielson 1971*a,b*), plaice (Timur, G. 1975; Timur, G. *et al.* 1977) and snakehead (Chinabut 1989) in response to FCA. The cells involved in the inflammatory response in this study were composed of PMNs, lymphocytes, macrophages, epithelioid cells, giant cells and fibroblasts, which were similar to those found in rainbow trout, plaice and snakehead.

Macrophage infiltration, fibrosis and vascularization are characteristic features of a chronic inflammation. At 6 hours after inoculation of FCA in this study, macrophages infiltration in the lesion area and initiation of myophagia by them was observed. Then their number and myophagic activity increased and started to line up into an epithelioid configuration in the 3rd day. Active encapsulation process of the vacuoles with epithelioid cells started in 4 days after injection, and also myophagia completed at this time. PMNs were seen from 24 hours after inoculation of FCA and remained until the 4th day. These findings showed that cellular response in carp, in present study, was much faster than in rainbow trout at 15°C (Finn & Nielson 1971*a,b*) and plaice at 10°C (Timur, G. 1975; Timur, G. *et al.* 1977), but slower than in snakehead at 26.9°C (Chinabut 1989). They reported development of the PMNs and macrophages by day 1 in rainbow trout, day 3 in plaice and by 12 hours in snakehead. Vascularization plays an important role in nourishing the newly developing fibrogranulation tissue in an inflammatory lesion. In this study, new capillaries were formed in 2nd day while fibroplasia was initiated 3 days after injection. Chinabut (1989) reported initiation of vascularization and fibroplasia in the snakehead at 26.9°C, around day 4. Finn & Nielson (1971*a,b*), Timur, G. (1975) and Timur, G. *et al.* (1977),

did not state the starting time of vascularization, but they did report fibroblast activity by day 8-16 in rainbow trout at 15°C, and by day 21-28 in plaice at 10°C respectively.

Therefore it can be concluded that cellular infiltration, vascularization and fibrosis in carp (present study) were considerably faster than in rainbow trout and plaice studied by Finn & Nielson (1971*a,b*) at 15°C, Timur, G. (1975) and Timur, G. *et al.* (1977) at 10°C. In comparison with snakehead at 26.9°C, investigated by Chinabut (1989), cellular infiltration in carp was slower, but vascularization and fibroplasia were faster than snakehead.

As Anderson & Roberts (1975) showed that the rate of wound healing process in an individual species, including the rate of cellular infiltration, fibrosis and vascularization, is temperature dependent, therefore the faster occurrence of these features in carp (present study) at 24°C, would be expected. This fast rate of inflammatory reaction in this fish species could be considered as an important privilege whenever wound healing process in response to stimuli is in progress.

Finn & Nielson (1971*a*) described three important parts of the perimysium in the process of inflammatory response of fish muscle as a limiting barrier for the spread of the necrotic agents and confining the lesion, a carrier of many infiltrating leukocytes in its large vessels and also a source of fibrocytes and fibroblasts. This was later supported by Roberts *et al.* (1973*a*) in salmon and Chinabut (1989) in snakehead. The present study confirmed these important roles of the perimysium, because the lesion did not extended to the neighbouring muscles which were separated by perimysium, and blood vessels within the perimysium were a source of infiltrating leukocytes into the lesion area, and also fibroblast activity started from this connective tissue (perimysium).

The basophilic buds of regenerating myofibres were first seen on the 3rd day. The new muscle bundles gradually replaced the fibrosis area until only left typical granulomata in the defect area at the end of experiment. The muscle regeneration started mostly at the edge of the lesion which was similar to the reports of Timur, G. (1975) and Chinabut (1989). Timur, G. (1975) found the beginning of muscle regeneration around 35-42 days in plaice at 10°C which was very slow in comparison with carp in the present study, and Chinabut (1989) reported that the regenerating myofibres in snakehead at 26.9°C, were first seen on the 4th day which is 1 day slower than in carp observed in this study.

Development of both Langhans and foreign body types of giant cells first was observed in this study at 4 days. This time for plaice at 10°C (Timur, G. 1975; Timur, G. *et al.* 1977) was 16-18 days while it was 6 days for snakehead at 26.9°C (Chinabut 1989). Timur, G. (1975) failed to demonstrate giant cells in plaice at low temperature, suggested that the giant cell formation was related to temperature. The present experiment was carried out at 24°C, therefore the early formation of giant cells within the lesion area could be, to some extent, justifiable. From literature in relation to piscine tuberculosis (reviewed in this chapter) it could be concluded that foreign body type giant cells do not normally occur. Timur, G. (1975) reported that both types of giant cell were produced in both Freund's complete and incomplete adjuvant experiments. She concluded that foreign body giant cell formation in her experiments were in response to the mineral oil of adjuvant rather than the *Mycobacterium* sp. Secombes (1985) noted that giant cells are capable of some degree of phagocytosis but lower than macrophages. The findings of the present study did not support this report, because acid-fast bacteria were never seen in the cytoplasm of giant cells.

The lymphocytes infiltrated into the lesion area in the very early stages along with the other inflammatory cells and their number and activity increased gradually. At 14 days the focal aggregations of lymphocytes in the area of the lesion were observed. Foci of active lymphocytes were observed almost until the final sampling. According to Ellis (1989), lymphocytes are at the centre of specific defence mechanism and are responsible for initiation and mediating the aspects of specific immunity. Therefore the presence and activity of lymphocytes maybe related to their responsibility for the development of a cell-mediated immunity in response to the immunogenic substance (FCA) which contained *Mycobacterium butyricum*.

Timur, G. (1975) showed extensive collar of melanocytes around the degenerated blood vessels at 42 days in plaice at 10°C, while Chinabut (1989) demonstrated active and large melanin pigment containing cells within the inflammatory tissue around day 14 in snakehead at 26.9°C. She also reported a very dense and distinctive melanization around the various sized FCA granulomata and in the stroma of fibrous tissue during the late stages of the lesion. She suggested that there seemed to be a negative correlation between presence of eosinophilic granular cells (EGCs) and melanization in such a way that the melanization was a later response where the EGCs response was unsuccessful. The results of the present study did not support this hypothesis. Melanization neither around the granulomata nor within the stroma of fibrosis occurred in this study with the exception of the presence of melanin pigments around the large blood vessels and also a few of them spread throughout the lesion area in early stages of the lesion.

EGCs had been reported from many normal fishes (Chaicharn & Bullock 1967; Logan & Odense 1974; Roberts *et al.* 1972; Ellis *et al.* 1989) and parasitised fishes

(Lester & Daniels 1976; Reimschussel *et al.* 1987; Ellis *et al.* 1989). Chinabut (1989) reported the presence of numbers of EGCs in snakehead, in loose connective tissue near to areas of caseation and decreased in numbers around the 49th day after inoculation of FCA. A few EGCs were observed in the present study around the granulomata from 22 days after inoculation but their numbers did not appear to increase during the late stages of the lesion. The exact role of EGCs in fish is controversial but it has been reported that they are histaminogenic (Ellis 1981 & 1985), and maybe similar to mammalian mast cells (Barber & westerman 1978). They have also been reported as a part of fish defence mechanism (Ezeasor & Stokoe 1980; Powell *et al.* 1990) and poorly phagocytic (Barnett 1996).

Plasma cells are usually associated with immune response and observed in fish by the immunofluorescent technique (Ellis 1976 & 1977). They have also been reported in chronic lesion in plaice using Unna-Pappenheim staining method (Timur, M. 1975), but Chinabut (1989) did not observe plasma cells in snakehead in response to FCA injection. Plasma cells were not observed in the present study.

Different methods are used to detect the presence of *Mycobacterium* spp. in fish tissue. It is possible to diagnose the presence of the bacilli of *Mycobacterium* spp. in the infected tissues using the Ziehl-Neelsen staining method. The central zone of necrosis, caused by *Mycobacterium* spp., is also PAS-positive (Amlacher 1961). Dalsgaard *et al.* (1992) believed that direct microscopy and Ziehl-Neelsen staining of clinical specimens lack sufficient sensitivities and specificities. Roberts & Hamilton (1968) showed that the demonstration of mycobacteria was more successfully effected by the fluorescent Auramine O technique than by the ZN staining method. Bacilli in this staining technique varies from brilliant green to gold in colour (using fluorescent microscope),

and easily distinguished. Gomez *et al.* (1993 & 1996) reported that the immunocytochemical techniques (using polyclonal and monoclonal antibodies) facilitates the detection of mycobacterial antigens, particularly in areas where bacilli can not be observed using other methods. These techniques reveal a positive reaction visible as a golden-brown cytoplasmic colour within phagocytic cells in infected tissues. McCormick *et al.* (1995) reported that the molecular methodology, i.e. direct cycle sequencing of polymerase chain reaction (PCR) amplified 16S rRNA gene sequences, enable accurate and rapid identification of the acid-fast bacilli.

In the present study, H & E staining revealed bacilli within the lesion area and vacuoles of FCA in the early stages after inoculation which were confirmed as acid-fast bacilli using ZN staining. No bacteria were observed after the 6th day with H & E or ZN staining, while PAS-positive materials were observed in the cytoplasm of phagocytic cells and dispersed throughout the lesion area in all sections examined.

Immunohistochemial methods (using monoclonal and polyclonal antibodies produced against *Mycobacterium* sp., Chen 1996) demonstrated very strong (+++) reaction with monoclonal antibody, visible as a golden-brown cytoplasmic colour within phagocytic cells, in the granulomatous area in only the 6th day after inoculation of FCA, while there was a weak (+) response in only the 6th day with polyclonal antibody (PAb).

No bacteria was observed at any sample in AO method, using fluorescent microscope, while the brilliant green to gold bacilli were seen in the control samples using FCA smear.

The findings of this part of the present study showed that bacteria were detected up to 6 days using H & E and ZN staining while bacterial components was not

demonstrated in IHC method properly (only observed at day 6), and also no bacteria were demonstrated in the same period in AO method. These findings indicated that either the trial errors in employing the materials and methods may has been occurred or the amount of the bacteria was so low that they were not detectable in the specified slides examined.

Carp is considered as a fish with the capability to degrade and remove *Mycobacterium* spp. very rapidly (R. H. Richards, 1996, personal communication). The findings in this study could support this view, because a few mycobacteria were detected at 2 days after injection of FCA and from 6 days onwards no mycobacteria were observed in the lesion area.

The occurrence of errors during the using of materials and methods for staining, insufficient amount of bacteria present in the samples examined, using killed bacterium as a stimulus without having ability to grow and increase in number after inoculation, and ability of carp to degrade the inoculated *Mycobacterium butyricum* very quickly, might be the reasons for inability to detect the bacteria in the tissue properly, stressing the necessity of examination of all tissues in such investigation.

However, the cellular inflammatory response of carp (present study) to Freund's complete adjuvant was similar to that of the rainbow trout at 15°C (Finn & Nielson 1971*a,b*), plaice at 10°C (Timur, G. 1975; Timur, G. *et al.* 1977) and snakehead at 26.9°C, (Chinabut 1989) in its general pattern and granulomata formation. The rate and time scale of the cellular response in carp was much faster than rainbow trout and plaice and almost similar to that of snakehead, although these observed differences may be purely a function of temperature. The findings of this study also suggest that healthy

carp has a well developed capacity for dealing with the *Mycobacterium butyricum* in FCA, experimentally, and is able to rapidly remove the mycobacteria by phagocytosis.

CHAPTER 7:

THE INFLAMMATORY RESPONSE OF CARP (*Cyprinus carpio* L.) TO *Aeromonas hydrophila* INFECTION

7.1 INTRODUCTION

Bacterial diseases are responsible for high mortality in wild and cultured fish (Frerichs & Roberts 1989). The normal bacterial flora of fish is a direct reflection of the bacterial population of the water in which they swim (Horsley 1973; Sakata *et al.* 1980). These micro-organisms (the normal bacterial flora) are essentially opportunist pathogens which infect a fish made susceptible to infection by stress factors or other disease processes. The most important micro-organisms in this respect is *Aeromonas hydrophila*, which is generally considered as a facultative pathogen, invading the tissues of a fish severely stressed or physically traumatised by another agent. *A. hydrophila* is a Gram-negative bacterium which is wildely distributed in fresh water systems. It has been reported from a wide range of fish species in temperate and tropical water systems (Snieszko & Axelrod 1971; Plumb *et al.* 1976; Allen *et al.* 1983).

The organism has been identified in all countries where pond and ornamental fresh-water fishes are cultured (Frerichs & Roberts 1989). *A. hydrophila* is a motile species of aeromonads which are often ubiquitous microbial flora of aquatic animals and maybe pathogens of poikilotherms, homoiotherms and even man (Salton & Schnick 1973; Fraire 1978). In warm-water aquaculture, *A. hydrophila* is considered to be a major problem, but it is difficult to distinguish direct losses from those caused by secondary infections (Amin *et al.* 1985; Ruangapan *et al.* 1986).

Environmental factors such as crowding, low concentrations of dissolved oxygen and high organic content in the water, industrial pollution, abrupt temperature changes, physical injuries, and spawning may also contribute to infections by *A. hydrophila* (Pippy & Hare 1969; Shotts *et al.* 1972).

One of the most common fish bacterial disease syndromes is motile aeromonas

septicaemia (MAS) caused by *A. hydrophila*. An outbreak of MAS among wild or pond-raised fish is difficult to control despite corrective action by fish biologists. It appears that once the infection is established, rapid growth of the bacterium and elaboration of its toxic products may cause irreparable systemic damage which leads to death, and can occasionally cause devastating losses in both wild and farmed fish population (Brenden & Huizinga 1986; Roberts 1993).

This study was conducted to investigate the inflammatory response and the pathogenesis of the inflammatory lesion induced in healthy carp (*Cyprinus carpio*) by a strain of *Aeromonas hydrophila*.

7.2 LITERATURE REVIEW

7.2.1 Characteristics of *Aeromonas hydrophila*

7.2.1.1 Biological characteristics

The genus Aeromonas is naturally and conveniently divided into motile and non-motile groups of organisms. The mesophilic, motile group comprises *A. hydrophila* (synonym, *A. liquefaciens*), *A. sobria* and *A. caviae* (synonym, *A. hydrophila* subsp. *anaerogenes*) (Frerichs & Millar 1993). *A. hydrophila* has acquired a number of synonyms (Snieszko & Axelrod 1971; Wolke 1975), but was mostly well known and described before as *Aeromonas liquefaciens*, *Aeromonas punctata* and *bacterium punctata* (Frerichs & Roberts 1989; Angka *et al.* 1995). *A. punctata* is no longer a recognised species and isolates previously identified as *A. punctata* would now be classified as *A. hydrophila* or *A. sobria*. All three species of motile aeromonads have been recovered from fish, although *A. hydrophila* is the most frequently isolated and significant pathogen (Frerichs & Millar 1993). *A. hydrophila*, a member of the family Vibrionaceae, is Gram negative, motile, rod shaped, whose normal habitat is in soil and water, having capacity to infect cold-blooded vertebrates and mammals (Bullock *et al.* 1971; Popoff & Veron 1976; Post 1983; Ho *et al.* 1990). The first report of the isolation of what is presumed to have been *Aeromonas hydrophila* was by Sanarelli (1891), since which time the organism has been identified in many fresh-water fishes in all parts of the world (Frerichs & Roberts 1989). It is widely distributed in clean and organically polluted fresh-water, water with high sewage levels, and also in marine systems, except at the most extreme salinity (Geldreich 1973; Hazen *et al.* 1978; Heuschmann-Brunner 1978; Kaper *et al.* 1981; Newman 1982). It has also been recognised as a part of the

intestinal micro flora of healthy fresh-water or sea water fishes (Thorpe & Roberts 1972; Sakata *et al.* 1980; Newman 1982). It is also a primary and secondary pathogen of a number of aquatic and terrestrial animals including humans (Haward & Buckley 1985). *A. hydrophila* strains are characterised by active motility, achieved by means of a single polar flagellum and production of gas and acid from carbohydrates. They are straight rods measuring 0.5 x 1.0-1.5μ, facultatively anaerobic, non-spore forming, and resistant to the vibriostat 0/129. They have ability to produce 2, 3 butanediol. They are cytochrome oxidase positive, reduce nitrates, and have a G+C ratio of 57-63%. Their colonies are generally white to buff, circular, smooth and convex, formed within 24 hours at 22-28°C. The minimum temperature that *A. hydrophila* can grow is 0-5°C, the optimum temperature for culture is 25-30°C, and the maximum is very close to 45°C. They maybe readily isolated on any general purpose medium such as tripticase soy agar, brain heart infusion agar and sheep blood agar (Bullock 1961; Bullock & McLaughlin 1970; Rouf & Rigney 1971; Newman 1982; Post 1983; Frerichs & Roberts 1989; Roberts 1993).

The differentiation between the *A. hydrophila* and the other motile aeromonads (i.e. *A. caviae* and *A. sobria*) is on the basis of carbohydrate and other biochemical reactions. They are readily distinguishable on the basis of the differentiating factors defined by Popoff & Veron (1976) and Popoff (1984) (table 7.1). Tajima *et al.* (1992) suggested that "because of the confusion surrounding the conventional biochemical identification of motile *Aeromonas* spp., serological properties be investigated as a serious possible alternative of classification. Serological groupings based on possession of specific antigens have been successfully used in the classification of several bacterial species". Serological typing has proven extremely useful in various groups of

Table 7.1: Differentiation between Aeromonads species by physiological and biochemical properties (From Popoff 1984).

Characteristics	*A. hydrophila*	*A. caviae*	*A. sobria*
Motility	+	+	+
Monotrichous flagellation in liquid medium	+	+	+
Lophotrichous flagellation in liquid medium	−	−	−
Coccobacilli in pairs, chains and clumps	−	−	−
Rods in singles and pairs	+	+	+
Brown water-soluble pigment	−	−	−
Growth in nutrient broth at 37°C	+	+	+
Indole production in 1% peptone water	+	+	+
Esculin hydrolysis	+	+	−
Growth in KCN broth (Møller technique)	+	+	−
L-Histidine and L-arginine utilisation	+	+	−
L-Arabinose utilisation	+	+	−
Fermentation of salicin	+	+	−
Fermentation of sucrose	+	+	+
Fermentation of mannitol	+	+	+
Breakdown of inositol	−	−	−
Acetoin from glucose (Voges-Proskauer)	+	−	d
Gas from glucose	+	−	+
H_2S from cysteine	+	−	+

Symbols: +=typically positive, –=typically negative, d=differs among strains

organisms. This not only allow to investigate outbreaks of the disease, but also aid in the identification of primary habitats and modes of transmission. Serotyping also provides an efficient means for identifying clones with special virulence features, which are very important in clinical microbiology. It is usually limited to reference laboratories because for very important pathogens, antisera are not commercially available (Altwegg 1996). Because of the antigenic cross-reactivity between *A. hydrophila*, *A. salmonicida* and *A. sobria* (Leblanc *et al.* 1981), the specificity of serogroups has been questioned, but the core region of the lipopolysaccharide of *A. hydrophila*, of relevance to O-serogroups, is distinct (Shaw & Hodder 1978).

7.2.1.2 Antigenic structure and extracellular toxin production

The motile aeromonads are generally different antigenically. Despite this diversity of H and O antigens (Ewing *et al.* 1961; Takahashi & Kusuda 1977), an antiserum prepared against one particular strain of *A. hydrophila* was effective in removing the haemolytic activity of all other strains examined. The heat-labile extracellular protease of such strains also had common precipitins (cited by Newman 1982). The somatic or O antigens of the motile aeromonads are very heterogeneous (Eddy 1960; Liu 1961), and species specific O antigens are found on the different species (Rao & Foster 1977). It was possible to characterise twelve O antigens and nine H antigens within motile aeromonas species (Popoff 1984). The presence of K antigens have also reported by DeMeuron & Peduzzi (1979), which partly inhibit the O agglutination reaction (Popoff 1984).

Strains of *A. hydrophila* produce various extracellular toxins or enzymes that maybe virulence factors and possibly virulence determinants (Buckley *et al.* 1981).

Probably due to species and strain diversity, a wide variety of possible virulence characteristics have been suggested for the *A. hydrophila* as follows; 1) surface antigens: outer membrane proteins (OMP), S-layer protein, lipopolysaccharide (LPS) endotoxin 2) toxins: haemolysin (α & β), entrotoxins, cytotoxic factor, aerolysin 3) enzymes: nucleases (DNA & RNAase), proteases, acetylcholinesterase, gelatinase, caseinase, elastase, lipase, lecithinase 4) surface adhesines/lectins: pilli (fimbriae), haemagglutinins, adhesins (adherence to cells), pili agglutinins, leukocidins 5) iron acquisition: siderophores 6) invasiveness: plasmid 7) serum resistance (Wretlind *et al.* 1971; Scholz *et al.* 1974; Bernheimer *et al.* 1975; Nord *et al.* 1975; Annapurna & Sanyal 1977; Boulanger *et al.*1977; Donta & Haddow 1978; Cumberbatch *et al.* 1979; Atkinson & Trust 1980; Mittal *et al.* 1980; Ljungh *et al.* 1981; MacIntyre *et al.* 1981; Olivier et al. 1981; Riddle *et al.* 1981; Ljungh & Wadstrom 1982; Thune *et al.* 1982*a,b*; Janda *et al.* 1984; Barghouthi *et al.* 1986; Chakraborty *et al.* 1986; Howard & Buckley 1986; Thune *et al.* 1986; Dooley *et al.* 1986; Dooley & Trust 1988; Leung & Stevenson 1988*a,b*; Cahill 1990; Nieto *et al.* 1991; Ansary *et al.* 1992; Leung *et al.* 1995*b*; Angka *et al.* 1995; Gosling 1996; Lee *et al.* 1997). These factors are believed to work in concert, contributing to the overall virulence of this bacterium (Leung *et al.* 1996).

The correlation of extracellular toxins and haemolysin production with pathogenicity has been suggested by Hsu *et al.* (1981), Allan & Stevenson (1981), and Thune *et al.* (1982*b*). Thune *et al.* (1982*a*) demonstrated that endotoxin was not lethal to the fish, but there appeared to be two lethal factors in the exotoxin preparation, protease and haemolysin. Thune *et al.* (1982*b*) also demonstrated the lethality of two distinct proteases in a haemolysin deficient strain. Thune *et al.* (1986) found that β-haemolysin, despite its toxicity, does not play a role in the pathogenicity of *A.*

hydrophila infection. They suggested that the proteases maybe significant virulence factors. Chabot & Thune (1991) also found no apparent relationship between protease production and pathogenicity and virulence. Yadav *et al.* (1992) showed that cytotoxin-producer strains of *A. hydrophila*, were more frequently associated with epizootic ulcerative syndrome (EUS) infected fish compared to normal fish (EUS is explained in chapter 8). They also reported that haemolysin-producing strains were equally present in healthy and EUS infected fish, and haemolysin was not correlated with cytotoxin production. Shotts *et al.* (1985) found that elastase production was positively related to virulence but found no other correlation.

Nieto *et al.* (1991) and Rodriguez *et al.* (1992) showed that acetylcholinsterase (AcChE-toxin) produced typical neurotoxic symptoms in rainbow trout. Rodriguez *et al.* (1993) investigated on fish pathogenic isolates of motile aeromonads (*A. hydrophila*, *A. sobria*, *A. caviae*) for production of the fish lethal acetylcholinesterase toxin (AcChE-toxin). They found that all strains (except *A. sobria*) gave positive results with different patterns of bands using western blotting for screening the extracellular products (ECP) of these strains.

The presence of an S-layer on the surface of cells was strongly correlated with virulence in laboratory challenge (Thune *et al.* 1986). S-layer has also been reported from non-motile aeromonads. Garduno *et al.* (1992) reported that S-layer producing *A. salmonicida* survives and multiplies in rainbow trout macrophages. Leung *et al.* (1995*b*) used five of the most virulent strains of *A. hydrophila* and found that they shared a common resistance to the killing effect of naive fish serum. Other factors such as lack of auto agglutination in 0.2% acriflavine, instability after boiling, production of an S-layer, proteases and haemolysins did not correlate well with virulence. They

considered the serum resistance as a good indicator for screening virulence of *A. hydrophila* strains isolated from diseased fish in South-East Asia. Chabot & Thune (1991) have shown that strains with a characteristic S-layer on their surface were likely to be particularly virulent in catfish aeromonad infection, and Torres *et al.* (1990) have shown that strains with a particular heat-stable antigen are likely to be found in association with EUS-affected snakehead.

Entry into host cells is a specialised strategy for survival and multiplication utilised by a number of pathogens (Moulder 1985; Finlay & Falkow 1989). Besides avoiding the host immune system, intracellular localisation places the organism in an environment potentially rich in nutrients, yet devoid of competing organisms (Leung *et al.* 1996). Leung *et al.* (1995*a*) studied *in vitro* interaction of *A. hydrophila* and tilapia phagocytes, and found that all virulent and avirulent strains of *A. hydrophila* could multiply in non-activated, and Freund's complete adjuvant activated phagocytes. Activated phagocytes increased the uptake of the bacteria into cells, and the rates of intracellular replication for these bacteria were faster than in non-activated phagocytes. They suggested that virulent strains interacted with the phagocytes slightly differently from the avirulent strains, and that serum components and phagocytes may together prevent the growth of avirulent *A. hydrophila* in fish. Leung and co-worker (1996) also studied the *in vitro* interaction of *A. hydrophila* with epithelioma cells of carp (EPC). They found that all the virulent and type strains invaded and multiplied inside EPC cells, the cytopathic induced effect was attributed to growing and metabolically active bacteria. Avirulent strains did neither multiplied nor induced cytopathic effects in the EPC monolayers.

Lallier & Daigneault (1984) suggested that some virulent strains of *A.*

hydrophila have particular toxins for fish, whereas weakly virulent strains did not. The existence of resistance factor plasmids in *A. hydrophila* is also documented (Aoki *et al.* 1971, 1972, 1973; Shotts *et al.* 1976; Toranzo *et al.* 1983; Chang & Bolton 1987; Ansary *et al.* 1992).

Shotts *et al.* (1985) and DelCorral *et al.* (1990) in addition to studying proteases and other exotoxins as virulence factors in motile aeromonad infections, have studied the physical capacity of the bacterial cells to adhere to erythrocytes, on the basis that the ability to adhere to host cells is a pre-requisite for infection. They concluded that the ability of particular strains to produce disease was not directly related to adherence capacity, but that disease appeared to be related to multiple physiological and biochemical markers. They found difficult to define any single factor or group of factors which could fully account for the degree of virulence.

According to DeFigueiredo & Plumb (1977) strains of *A. hydrophila* isolated from water were significantly less virulent than the isolates from diseased fish and fresh water prawns.

7.2.2 Pathogenesis of the diseases associated with *Aeromonas hydrophila*

Disease associated with *A. hydrophila* was first recognised by Sanarelli (1891), who reported an outbreak of a disease in eels associated with what is presumed to have been *A. hydrophila*. Apart from fish, this micro-organism has also been isolated from diseased frogs, alligators, turtles, shrimps, man (Newman 1982) and snails (Mead 1969). Its pathogenicity often seems to be associated with stressed or compromised hosts. The main feature of the pathogenesis of all infections of fishes by *A. hydrophila*, is generalised dissemination in the form of bacteraemia, followed by elaboration of

toxins, tissue necrosis and the clinical disease known as bacterial haemorrhagic septicaemia. Bacterial haemorrhagic septicaemia was a non-specific name which could apply to almost all of the Gram-negative systemic infections of fishes, and is no longer used. The term motile aeromonad septicaemia (MAS) which is a much more precise and descriptive term is now being used (Roberts 1993).

A. hydrophila has been associated with the following diseases although it's role in the pathogenesis is not always clear; bacterial haemorrhagic septicaemia in common carp (*Cyprinus carpio*) (Snieszko *et al.* 1938), red sore disease in pike (*Esox lucius* L.) (Reed & Toner 1941), red spot in mullet (*Mugil cephalus* L.) (Burke & Rogers 1981), motile aeromonad septicaemia in catfish (*Ictaluris punctatus*) (Thune *et al.* 1982*b*) and epizootic ulcerative syndrome (EUS) in several species (Cahill 1987; Llobrera & Gacutan 1987; Lio-Po *et al.* 1992).

Newman (1983) reported that bacterial haemorrhagic septicaemia due to strains of *A. hydrophila* maybe transmitted through the water, via diseased and healthy carrier fish and other affected vertebrates, and also in association with external and internal parasites.

Any type of stress such as; crowding, excessive handling, high water temperatures, low dissolved oxygen levels, poor nutrition, and also seasonal fluctuations can cause healthy carrier fish to succumb to the disease (Rock & Nelson 1965; Haley *et al.* 1967; Meyer 1970; Shotts *et al.* 1972; Snieszko 1974). Due to the ubiquitous distribution of the organism, fish can be at risk at any time (Frerichs & Roberts 1989).

The source of *A. hydrophila* infection appears to be water, pond mud and latent carriers which have recorded from the disease (Wolke 1975). Infection may occur via ingestion (Amlacher 1961) or may also be transmitted by invertebrates such as leeches,

Argulus spp. and *Gyroductylus* spp. (Wagner & Perkins 1952; Griffin 1954).

Elliott & Shotts (1980*a*) suggested that although *A. hydrophila* may play an important part in the enlargement of ulcers, a ubiquitous organism such as this bacterium should not be considered to be the only causative agent of a unique and new disease such as cutaneous ulcers in gold fish.

In addition of a number of aquatic and terrestrial animals and humans (Howard & Buckley 1985), *A. hydrophila* is considered also to be the principal cause of bacterial haemorrhagic septicaemia in fresh water fish (Frerichs 1989), and has been reported in association with various ulcerative conditions (syndromes) including epizootic ulcerative syndrome (EUS) in South-East Asia (Llobrera & Gacutan 1987; Lio-Po *et al.* 1992), and red spot disease (RSD) in Australia (Cahill 1987). Clinical signs and histopathology of the diseases associated with *A. hydrophila* have been described by many workers (Wolke 1975; Bach *et al.* 1978; Huizinga *et al.* 1979; Brenden & Huizinga 1986; Ventura & Grizzle 1988; Frerichs & Roberts 1989; Roberts 1993). Snieszko & Axelrod (1971) classified disease caused by *A. hydrophila* under four categories as follows; 1) acute, rapidly fatal septicaemia with a few clinical signs 2) an acute form with dropsy, blisters, abscesses and scale protrusion 3) a chronic ulcerative form with furuncles and abscesses; 4) a latent form with no signs.

Grizzle & Kiryu (1993) categorised the infections by *A. hydrophila* complex into three groups; motile aeromonad septicaemia (MAS) (systemic infection with signs of disease), cutaneous (infection limited to skin and the underlying muscle), and latent (systemic infection but no external signs of disease). They described that hepatic necrosis was associated with all types of infections but was most consistently present and sometimes more severe in fish with MAS.

7.2.3 Experimental studies of *Aeromonas hydrophila* infection

7.2.3.1 Routes of the inducing infection

Different routes have been used by many workers to induce infection and to study different aspects of *A. hydrophila* infections in fish, experimentally. They include; subcutaneous injection (Elliott & Shotts 1980*b*), intramuscular injection (Elliott & Shotts 1980*b*; Wakabayashi *et al.* 1981; Amin *et al.* 1985; Brenden & Huizinga 1986; Peters *et al.* 1988; Chinabut 1989; Suthi 1991; Angka *et al.* 1995), intraperitoneal injection (Amin & Abdel-Kerim 1976; Elliott & Shotts 1980*b*; Amin *et al.* 1985; Nieto *et al.* 1985; Karunasagar *et al.* 1991; Esteve *et al.* 1993), immersion (bath challenge) with or without scale removal (Elliott & Shotts 1980*b* Ventura & Grizzle 1987,1988; Peters *et al.* 1988; Suthi 1991; Steve *et al.* 1993), gavage (Ventura & Grizzle 1987), and via live food medium (Gatesoupe 1991).

Amin & Abdel-Kerim (1976) demonstrated that intraperitoneal injection was a suitable route for infecting common carp. Ventura & Grizzle (1987 & 1988) found that channel catfish could develop systemic infection by immersion in a suspension of *A. hydrophila*, and the early histologic lesions were similar to lesions observed in naturally occurring systemic infections. Suthi (1991) showed in a limited bath challenge experiment in juvenile of *Puntius schwanenfeldi* and *Orechromis niloticus* that the entry of infection with and without skin abrasion is possible. Ventura & Grizzle (1987) in their study on channel catfish placed the bacteria into the fish stomach by gavage and found that a disease state did not develop readily by this technique, unless the fish were in crowded conditions (13.1 gr. fish / L) and held at a high temperature. Although *A. hydrophila* is known as a fresh-water pathogen, Gatesoupe (1990) observed

proliferation of this bacterium 9 days after hatching in turbot (*Scophthalmus maximus* L.) larvae prior to a high mortality. Gatesoupe (1991) isolated colonies of this strain of bacteria and cultured on medium prepared with brackish water at 18% and pH 7.7, to study their ability to produce infection and to cause mortality on turbot larvae when they introduce into live food. Rotifer was used as live food medium to induce *A. hydrophila* infection. The pathogenic effect of the opportunistic strain of *A. hydrophila* on turbot during initial feeding was confirmed, though the rotifers do not concentrate these bacteria and turbot seem able to resist the infection after the 10th day. He suggested that experimental infection with *A. hydrophila* before day 10, could be used as a test to investigate how to stimulate the resistance of turbot larvae against bacteria.

Takahashi *et al.* (1975) showed that *A. hydrophila* could cause characteristic ulcers when injected subcutaneously or intramuscularly into healthy gold fish, but Elliott & Shotts (1980*b*) reported that no typical ulcers developed on the gold fish (*Carassius auratus*) injected subcutaneously with *A. hydrophila*. They suggested that *A. hydrophila* is not involved in the initiation of cutaneous lesions but participates in the enlargement of the ulcers.

Esteve *et al.* (1993) reported that in their study, the disease produced by *A. hydrophila* in European eels (*Anguilla anguilla*) was induced by injection (intraperitoneally) as well as by waterborne exposure (bath challenge). The disease was acute in eels infected by intraperitoneal challenge, whereas it produced lower mortality rates and progressed more slowly when bath challenge was used. It is reasonable to conclude from the literature that injection route, especially intramuscularly, for inducing *A. hydrophila* infection achieves better results, but the different results which have been reported following injection of this bacterium may be due to strain diversity and

pathogenisity rather than the route of inoculation.

7.2.3.2 Experimental infection studies

Gaines (1972) studied *A. hydrophila* infection in channel catfish and reported oedema of the epithelial cells of the gill lamellae, and increase in the number of lymphoid cells with necrosis of epithelial of the tubules in kidney. Bach *et al.* (1978) also reported the sequestering of the *A. hydrophila* by the ellipsoids of the catfish spleen, while destruction of the endothelial and reticular cells of the ellipsoids was also an obvious consequence of the bacterial proliferation. Necrosis of the integument at the site of injection was also observed. They also reported a marked infiltration of macrophages, in the regions of localised bacteria infection in the spleen which phagocytose the bacteria and cell debris.

Huizinga *et al.* (1979) studied histopathology of red sore disease induced by *A. hydrophila* in naturally and experimentally infected large mouth bass (*Microterus salmoides*). They found different ranges of disease from a few scales to extensive chronic ulcerations. They also observed focal haemorrhage, oedema, dermal and underlying muscles necrosis, and infiltration of mononuclear and granulocytic inflammatory cells. Focal necrosis of liver and kidney and complete destruction of their structural integrity in the most severe infections was evident, while the spleen and heart did not show pathological changes even in cases with massive damage in the liver and kidney. Ventura (1985) reported a systemic infection after injection of *A. hydrophila* intramuscularly into channel catfish. Diffuse necrosis in the internal organs, presence of melanin-containing macrophages in the circulatory system, increased amounts of lipofuscin and haemosiderin aggregates in the liver and spleen were some features of

this infection.

The pathophysiological changes associated with experimental *A. hydrophila* infection in goldfish have been described by Brenden & Huizinga (1986). The only tissue showing marked changes in the early hours following inoculation of 50 µl of 1.5 x 10^7 cfu bacterial suspension of AM3 strain, was skeletal muscle at the injection site which showed haemorrhage and varied necrosis from moderate to severe. Variable degrees of diffuse degeneration hepatocytic and focal necrosis of the liver, mild necrosis of glomeruli and tubules of the mid-kidney, and diminished splenic red pulp were also the other features of the infection. The heart and intestine showed no pronounced changes.

Ventura & Grizzle (1988) studied histologic differences of the lesions associated with natural and experimental infections of *A. hydrophila* in channel catfish. They described that the naturally infected (systemic infection) fish were characterised by diffuse necrosis in several internal organs and the presence of melanin-containing macrophages in the blood. Fish with abraded skin exposed experimentally for 30 minutes to 5.2 x 10^6 cells/ml concentration of *A. hydrophila* which was obtained from a diseased channel catfish. After exposure, they had several types of concealed lesions including increased amounts of lipofuscin and haemosiderin in the liver and spleen, but most visceral organs were not necrotic. In experimentally infected fish, all lesions healed in fish that did not die, and prolonged infections limited to skin and muscle did not occur.

Chinabut (1989) found an acute inflammatory response in the snakehead after injection of 0.1ml of 1.57 x 10^8 cells/ml of the *A. hydrophila* suspension. The acute inflammation developed into an ulcerative wound after 2 days. The dominant feature of

her experiment was the presence of the polymorphonuclear cells which infiltrated into the inflammatory area 2 hours after injection. They were the predominant infiltrating cells throughout the time of major activity and contributed to the formation of abscessation in a similar way to that in higher animals. The lesion produced by *A. hydrophila* infection began to heal by day 7-10 and the healing process was completed around 28 days after injection.

Angka *et al.* (1995) reported that with the intramuscular injection of 10^4 cell/ml virulent strains of *A. hydrophila* into healthy fingerlings of catfish (*Clarias gariepinus*), they began to die at 18 hours after bacterial injection. They showed that the bacteria caused skin and muscle lesion at the injected sites and remained in their ulcerative tissues, liver and kidney, until 5 days after intramuscular injection. They suggested that the disappearance of the injected micro-organisms could be due to the development of effective immunity.

From the experimental studies in different fish species using *A. hydrophila*, it could be concluded that occurrence of an acute inflammatory response with different degree of necrosis in different internal organs is almost a constant feature of the infection. The variable inflammatory responses reported by workers may be due to the differences in virulence of strains of *A. hydrophila* and their ability to produce disease, and also bacterial concentrations used in the experiments.

7.3 PILOT EXPERIMENT

A pilot experiment was conducted in three stages using a small number of mirror carp. The main aim was to determine the optimum concentration and dose of *A. hydrophila*, required to produce a non-lethal infection.

1) Five fish were used in the first stage. These were inoculated intramuscularly in the flank with; 0.05 ml, 0.1 ml (2 fish), 0.2 ml and 0.3 ml, respectively, of a bacterial suspension adjusted to compare with McFarland standard (bioMeriéux, UK ltd.) in the first stage of making the bacterial suspension. The rate count for this bacterial suspension indicated that the suspension contained 1.2×10^8 organisms/ml. All infected fish died within 44 hours post-injection. The fish injected with higher dosages died (5.5-12 hrs. p.i.) before those injected with lower dosages. They showed a general erythematous reaction over the whole body surface, a swelling and fluctuant white patch around the injected site, lethargy and lack of movement co-ordination. Microscopic examination of the muscles revealed haemorrhage, severe degeneration and necrosis, cellular infiltration and myophagia. Gram-negative bacteria were also observed in section of the injection area. The standard H & E stain and also Gram stain were used to stain sections in this pilot study.

2) Eight fish were used in the second stage of the pilot study. Four fish were inoculated intramuscularly with 0.1 ml of a suspension containing 1.91×10^7 organisms/ml and four fish with 0.1 ml of a 1:10 dilution of this (i.e. 1.91×10^6 organisms/ml). The optical density (OD) of the 1.91×10^7 organisms/ml suspension was 0.025 at 680 nm. All four fish injected with the 1.91×10^7 organisms/ml of suspension died within 40 hours after injection. They showed typical gross pathology

and histopathological features of *A. hydrophila* infection, as described previously. There were no mortalities in those fish injected with 1.9×10^6 organisms/ml. Although no gross clinical signs of infection in these fish were evident, the microscopic examination of sections showed muscle degeneration, haemorrhage, cellular infiltration and myophagia.

3) Following the results of the initial two inoculations, it was decided to use a bacterial suspension between 10^7 and 10^6 organisms/ml to produce clinical signs of infection and greater inflammatory response in the tissue but keep the mortality at a minimum rate. A bacterial suspension containing 10^7 organisms/ml was prepared based on the OD determined in the second stage of inoculation. The viable counts of this suspension was estimated as 2.13×10^7 organisms/ml. Three different dilutions, (a), (b), (c), were prepared from this suspension. Viable counts for each dilution were as follows: (a)=4.26×10^6, (b)=8.52×10^6 and (c)=1.28×10^7 organisms/ml. Nine fish were used in this trial. Three fish were injected with 0.1ml of suspension a, three with 0.1ml of suspension b, and three with 0.1ml of suspension c. All three fish injected with 8.52×10^6 organisms/ml suspension died within 40 hours after injection, with typical gross pathology of *A. hydrophila* infection and exhibited an inflammatory response on microscopic examination, as described before. The two other groups had no mortalities during the experimental period. Those fish injected with 4.26×10^6 organisms/ml showed clinical signs of infection, but they were in a more stable condition than those fish which were injected with 1.28×10^7 organisms/ml. These two groups showed a marked inflammatory response on microscopic examination.

Mortalities and a greater inflammatory response were expected in those fish inoculated with the higher dose of suspension, but they were very similar to those which

received the lower dose of suspension. Those fish injected with medium dose were the only group which showed severe infection and mortalities. It was assumed that an error had been made in the third group injected with 1.28×10^7 organisms/ml. Therefore this group was not considered further in this pilot study.

Following these results, 0.1 ml of 4.26×10^6 organisms/ml was considered as an optimum dose for injection in the definitive experiment.

No problems in sampling, processing, trimming and sectioning the tissues, which were used as in previous experiments, were highlighted.

7.4 DEFINITIVE EXPERIMENT

The definitive experiment was designed based on the results of the pilot experiment.

7.4.1 Materials and methods

7.4.1.1 Fish

Mirror carp from Fish Network, Upper Main Farm, Essex, England were used as the experimental fish. One hundred fish were stocked in a stock tank with a recirculating water supply and aquarium water heaters. Sixty five fish were transferred from the stock tank to the glass aquaria in an isolated challenge room and kept for 7 weeks to acclimatise before the experiment began. The average length of fish was 13 cm (± 2 cm). They were fed with pelleted food (Fry 02, Trouw Aquaculture, Inverbreakie, Invergordon, Ross-Shire, Scotland) once per day.

7.4.1.2 Aquaria and water system

Recirculating glass aquaria systems described in chapter 4 were used for stocking fish, and during the course of the experiment. The average temperature of water was 27.5°C (± 1°C). Faecal materials were removed from aquaria every day and between 20-30% of aquaria water was changed as necessary. As live bacteria were used in this experiment, a drainage system with two tanks was used to disinfect the water before draining to waste. Effluent water from the aquaria was treated with sodium hypochlorite (1400 ppm) to kill pathogens present.

7.4.1.3 Preparation of inoculum

A highly virulent strain of *A. hydrophila* (T4), isolated from EUS affected Indian major carp (*Labeo rohita*) in Bangladesh (Millar 1994), was used as the inoculum. *A. hydrophila* (T4) was cultured in Tryptone Soya Broth (TSB) medium (appendix 3.1.6.2), for 24 hours at 22°C following removal from –70°C preservation. Broth culture was plated out onto Trypton Soya Agar (TSA) medium (appendix 3.1.6.1), for 24 hours at 22°C to check culture purity. Individual colonies from the TSA culture were suspended in sterile 0.85% saline. This bacterial suspension was adjusted to contain 10^6 organisms/ml using the method described in pilot experiment. Viable bacterial numbers for this inoculum were determined by making ten-fold dilutions from 10^{-1} to 10^{-8} of the saline suspension and spreading 100 ml of each dilution on separate TSA plates. Following incubation at 22°C for 24 hours, resultant colonies were counted on each plate. The number of viable bacteria for the inocula was determined as 5.3 x 10^6 organisms/ml and the optical density (OD) was measured as 0.010 at 680 nm on a WPA S105 spectrophotometer (WPA ltd. England).

7.4.1.4 Injection procedure

Sixty fish were inoculated into the left side of dorsal myotomal muscle with 0.1 ml of the 5.3 x 10^6 organisms/ml suspension, using a 1 ml syringe and 25 gauge needle. The fish were anaesthetised with 10% benzocaine prior to inoculation. The injection was made in line with the first ray of the dorsal fin, below the scale row, as a marker for subsequent sampling (Fig. 1.1). Control fish were injected intramuscularly on the left flank with the same volume of sterile saline. All infected fish and control fish were returned to the aquaria following injection for observation of clinical signs of infection

and subsequent sampling through to the end of the experiment. Fish were observed during the early hours following injection and thereafter daily. Dead fish were removed from the aquaria and a detailed record of the gross pathology obtained.

7.4.1.5 Histological sampling

The infected fish were sacrificed by an overdose of 10% benzocaine at 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48 hours, thereafter at 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after inoculation. Due to mortalities following inoculation, two fish were sacrificed in the early stages from 1 hour to 48 hours, thereafter one fish was sampled between 3 and 42 days post-injection. A block of tissue, including the lesion area, was immediately cut out from the injected site of each sacrificed fish and fixed in cold 10% neutral buffered formalin for at least 24 hours before cassetting. During the fixation time, buffered formalin was changed at least once. All the stages of processing to sectioning of these fixed tissues were similar to those described in chapter 5. The control fish were sampled, processed and sectioned using the same procedures as the experimental fish.

7.4.1.6 Bacterial isolation

A piece of lesion muscle from freshly sacrificed fish was sampled aseptically for histology, and from this, a very small area was cut out and chopped into smaller pieces using sterile blade and disinfected forceps for bacterial isolation. The samples for bacterial isolation were prepared from all the sacrificed fishes for histological examination. The chopped tissues were cultured in TSB for 24 hours at 22°C. Following this, the TSB was plated out onto TSA plates and incubated at 22°C for 24

hours to produce individual colonies. Identification of the isolated colonies was confirmed by a range of morphological and biochemical tests. First basic identification tests (primary tests) namely Gram stain (appendix 2.1.7.2), microscopic morphology, motility, oxidase, oxidation-fermentation (O/F) and 0/129 vibriostat sensitivity (appendixes.3.1.1-3.1.4) were carried out as well as determining growth at 37°C. After the basic identification of bacteria, the API 20 E microbial identification kit (bioMeriéux, UK ltd) (appendix 3.1.5), was used to determine biochemical characteristics. These tests were also carried out on the stock bacterium used as inoculum. Results for inoculum and isolates were compared to determine if both were identical. The same procedures were also used for control fish.

7.4.1.7 Staining procedures

In addition of the standard H & E staining for the routine microscopic examination of the sections, Gram staining (appendix 2.1.7.1), was used specifically to identify Gram negative bacteria in the sections. The stained sections were mounted in Pertex mounting medium (CellPath plc, Herts, England) for examination.

7.4.2 Results

7.4.2.1 Gross pathology

During the first few hours after inoculation, behaviour of the infected fish was normal. Thereafter the general findings from the study were as follows;

By 10 hours after injection, most of the infected fish were lethargic and remained at the bottom of the aquaria. They showed slight swelling, small white patches and pin-point haemorrhages (in some fish) at the site of injection.

At 12-24 hours after injection, the white patch and swelling became quite extensive and fluctuant. Severe generalised erythematous reaction was obvious all over the body surface. The scales above the site of injection showed severe protrusion and readily sloughed off. One quarter of the injected fishes died at this period.

After 36-48 hours, initial ulceration occurred. An oval or irregular white or pale ulcerative lesion was clearly seen and necrotic muscle at the centre of the lesion sloughed off, caused an open concave lesion about 1cm diameter. The fish with ulcerative lesions lost their scales close to the site of injection. Five more fish died at this stage. From 48 hours onwards, no further mortalities occurred.

After 3-4 days, the ulcerative lesion became so extensive and deep that a part of spinal column was obvious. The diameter of the lesion was approximately 1.5 cm. The edge of the ulcerative lesion was irregular and white.

By 5-10 days after inoculation, a healing process was observed. The edge of the lesion was irregular and distinct due to its dark pigmented border, and the size of the lesion decreased to about 0.5 cm.

At 14-22 days post-injection, most of the infected fish showed a black coloured

area and a dark depressed scar at the site of injection on the skin.

After 28-42 days, the remaining fish showed a dark smooth depressed scar with a black coloration and an irregular attached border at the injection area on the skin. A small number of new small scales grown on the scar were evident.

None of these changes were observed in the control fish.

7.4.2.2 Histopathology

1 hour

At 1 hour after inoculation of bacterial suspension, the principal feature was myofibrillar degeneration which started with central migration of sarcoplasmic nuclei, flocculation of sarcoplasms and loss of sarcolemmal integrity (Fig. 7.1). Large or small foci of haemorrhage along with hyperaemic blood vessels associated with melanin pigments were seen in the lesion area. Thrombocytes, lymphocytes and a few polymorphonuclear cells (PMNs) cells were the inflammatory cells within the defect area. No bacteria were evident in the sections stained by H & E or Gram staining methods, but Gram negative bacterial bacilli, *A. hydrophila*, were re-isolated from the muscle of the injection area, and identified using bacteriological tests.

2 hours

By 2 hours after the bacterial injection, the degenerated sarcoplasms were more fragmented and also muscle degeneration area was actively more extended to the adjacent tissue. Generalised and localised haemorrhages were found extensively in the lesion area, and also hyperaemic blood vessels with melanin granules around, either isolated or in dense aggregates, were obvious in the tissue. Lymphocytes, PMNs, and thrombocytes were infiltrated into the area. No bacteria were found in the tissue with H & E and Gram stain, but the gram negative *A. hydrophila* was re-isolated and identified from the muscle of the infected fish by bacteriological tests.

3 hours

At this stage, more muscle degeneration and flocculation of sarcoplasms with loss of membrane integrity and nuclear pyknosis were seen (Fig. 7.2). Localised haemorrhages and also engorged blood vessels with red and white blood cells inside and melanin granules around were obvious. There were more lymphocytes and PMNs comparing with the last stage. A few Gram negative bacilli were seen within the sarcoplasms in Gram stain. *A. hydrophila* was re-isolated from the site of injection and identified using bacteriological tests.

4 hours

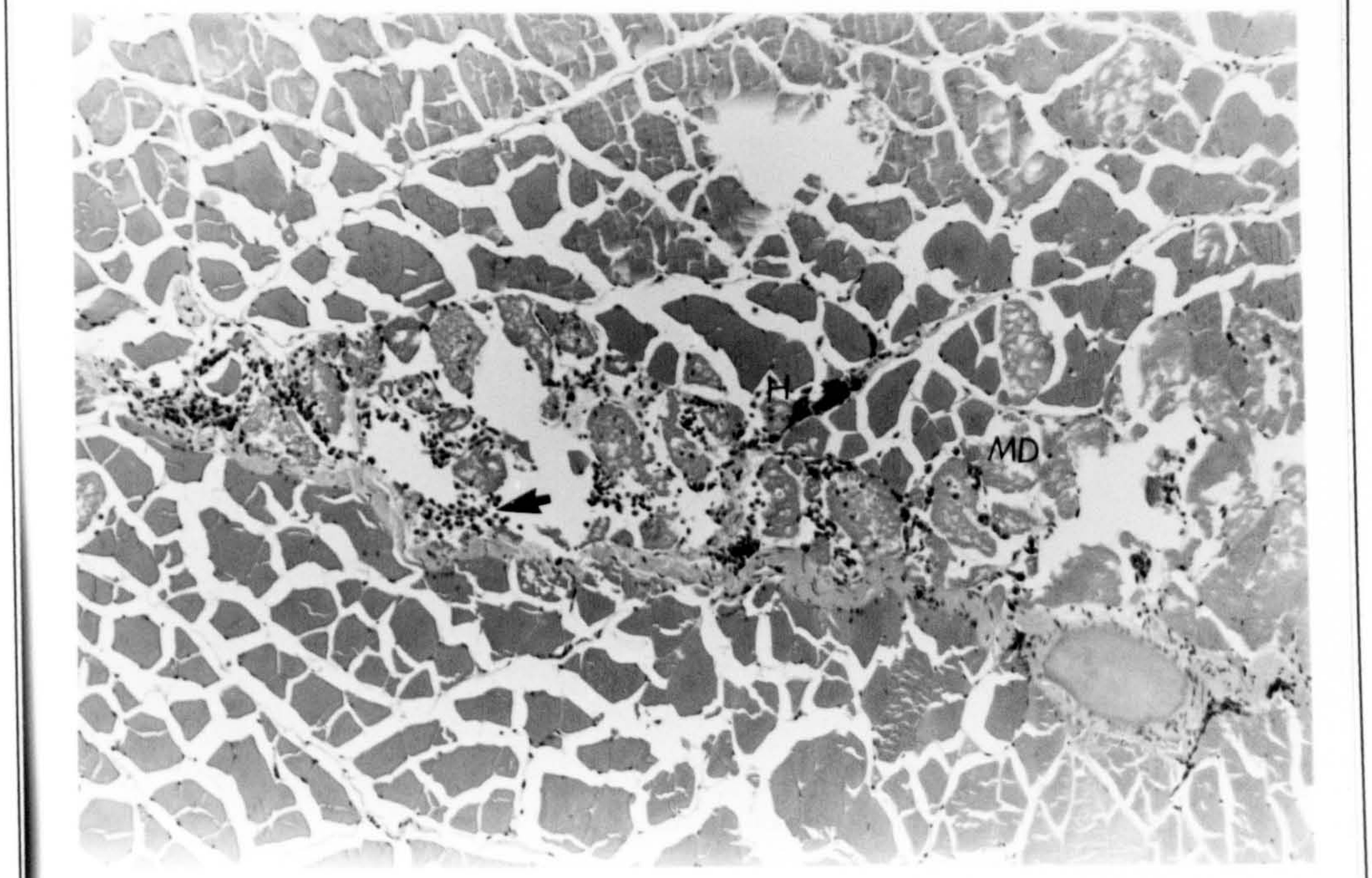
The picture of this stage of the lesion area was more cellular especially with further increase in PMNs numbers. Also for the first time some macrophages were evident in hypodermis and adjacent to the damaged muscles and also some of them, along with PMNs, were located inside the damaged muscles which could have represented the initiation of myophagia (Fig. 7.3). Flocculation of the degenerated muscles was obvious in the lesion area.

6 hours

At this stage the main feature was evidence of myophagia in the lesion area (Fig. 7.4). The dominant PMNs and some macrophages were seen within the degenerated muscles engulfing the remains of the damaged sarcoplasms. Further degeneration and flocculation of sarcoplasms was seen. Some haemorrhages and fibrin strands with thrombocytes were seen in some parts of the damaged area. Unlike macrophages, the number of lymphocytes were increased.

Fig. 7.1 At 1 hour post-injection of *A. hydrophila*, muscle degeneration (MD), foci of haemorrhage (H), and cellular infiltration (arrow head) were observed in the lesion area. (H & E, X 110).

Fig. 7.2 By 3 hours p.i. more degeneration and flocculation of muscles (MD), and haemorrhages (H) were obvious in the area of the lesion. (H & E, X 110).


H
MD

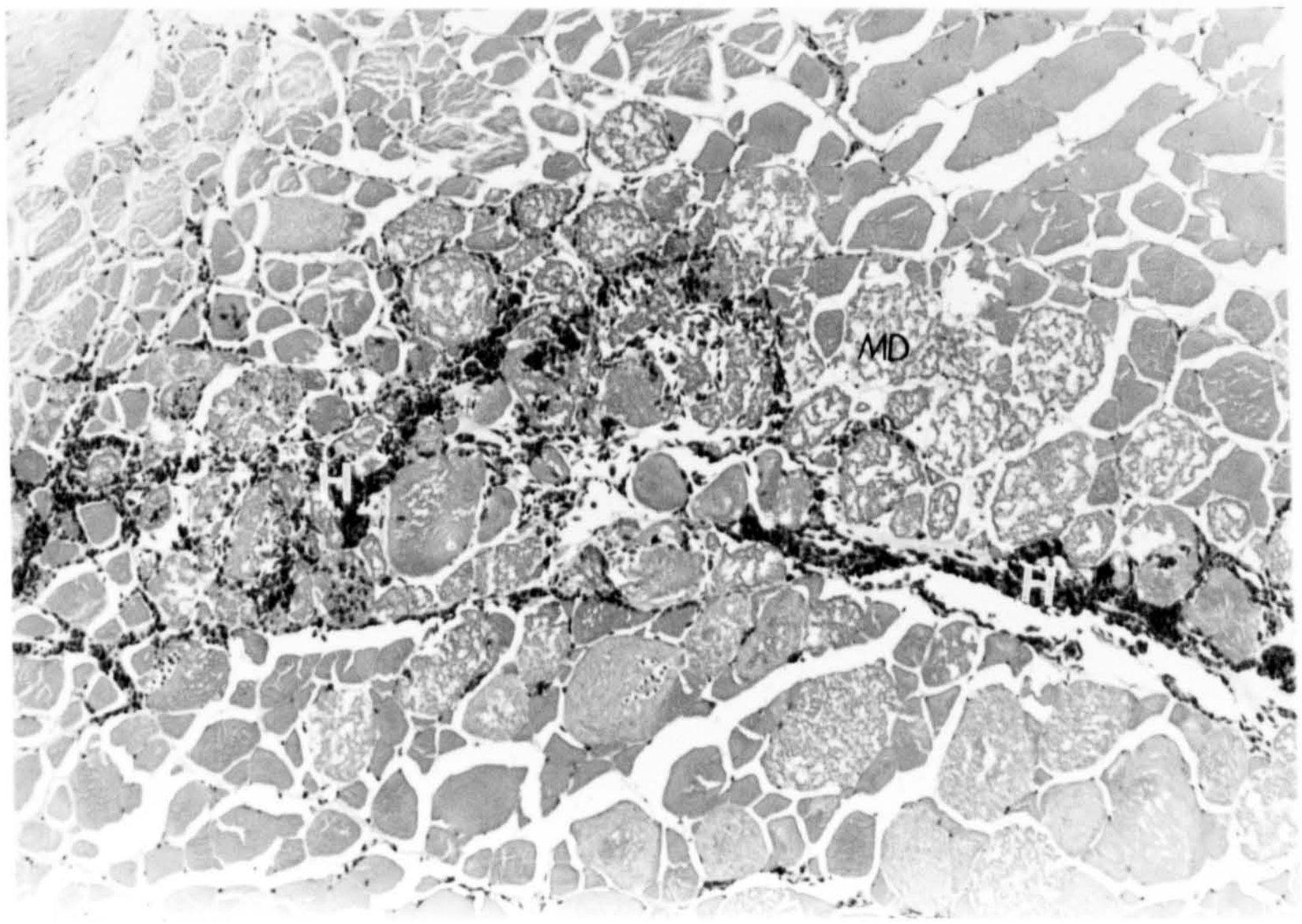
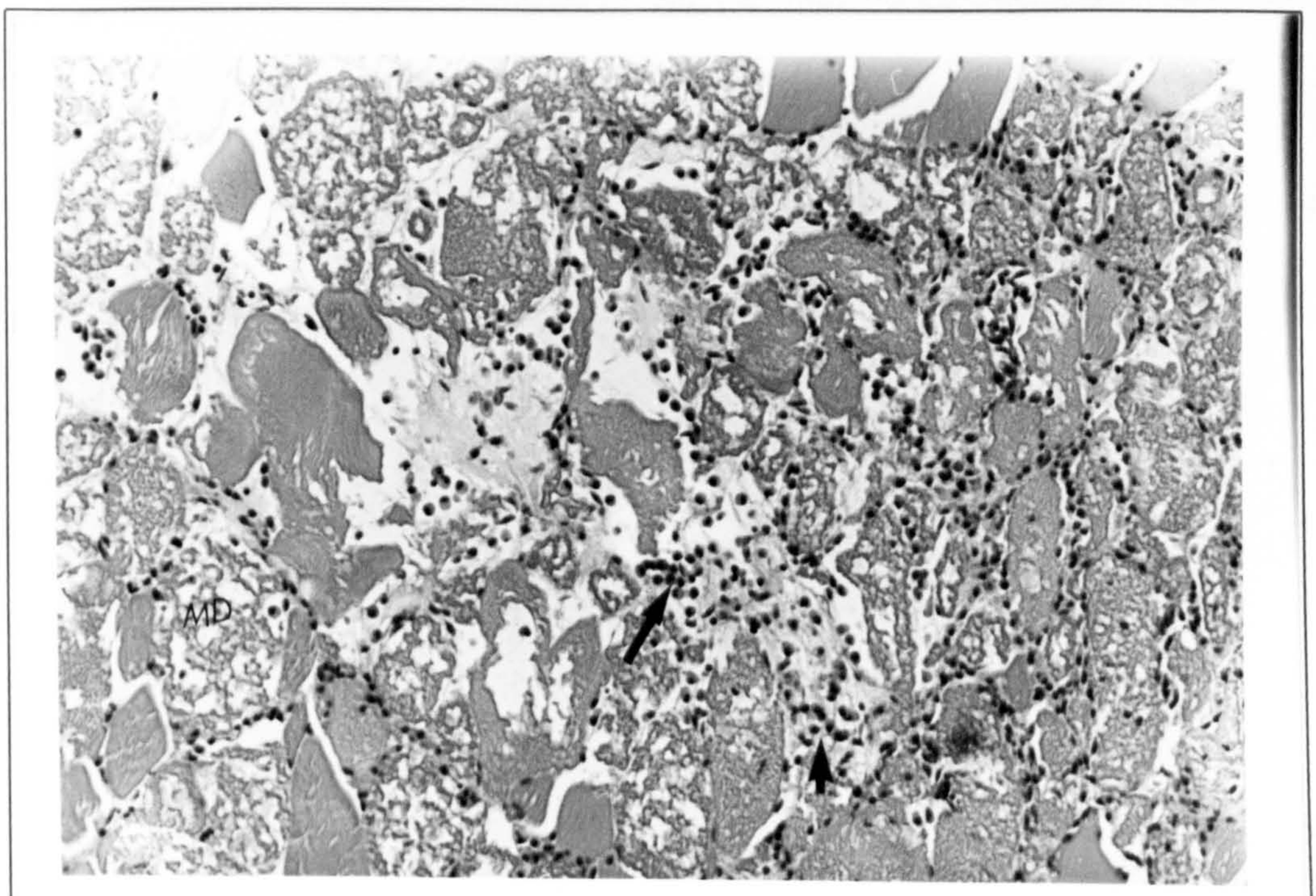
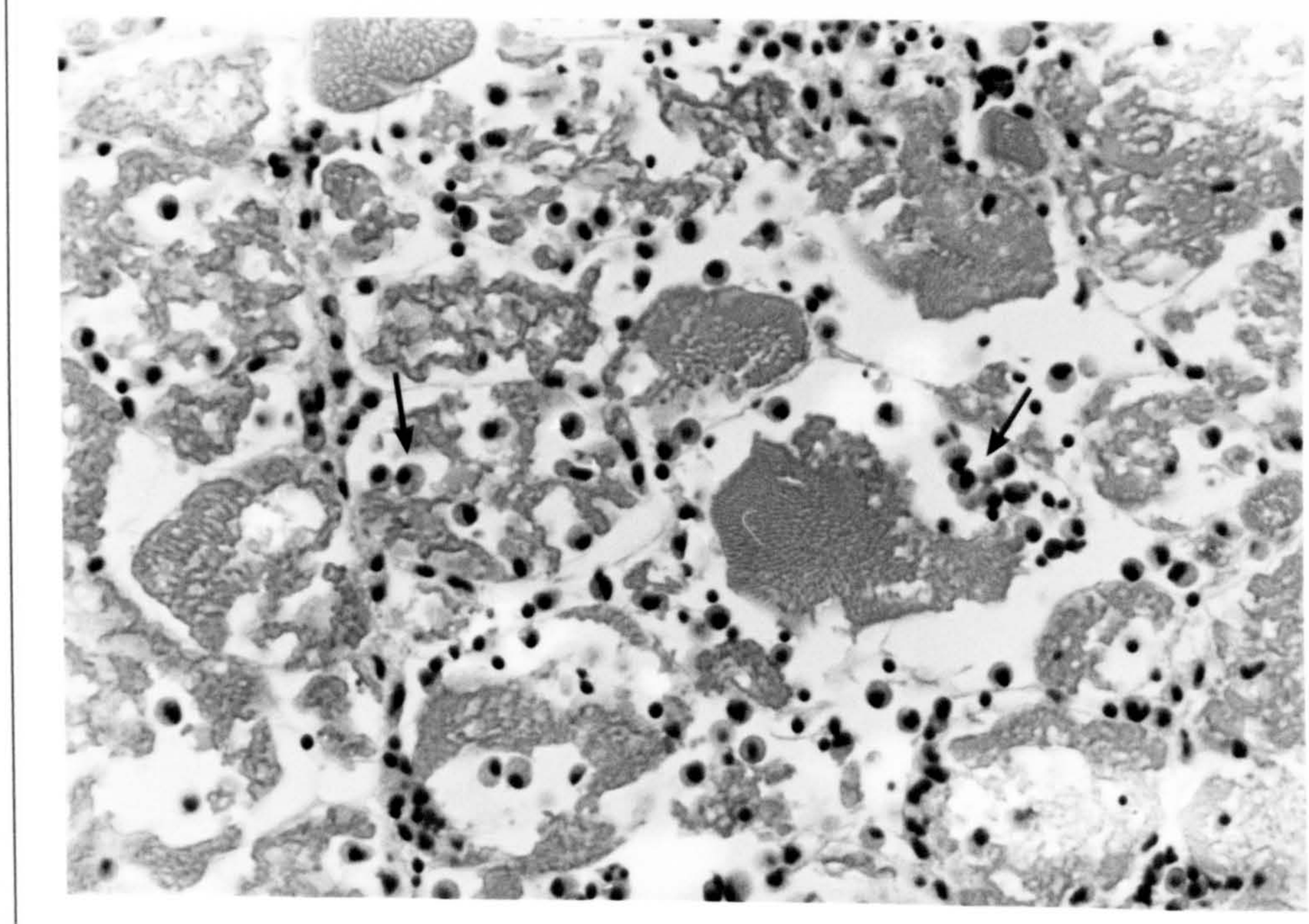

MD
H
H

Fig. 7.3 The picture of the lesion area at 4 hours p.i. was more cellular especially with further increase in PMNs number (arrow). PMNs were located inside the damaged muscles along with macrophages (arrow head). Also more degenerated muscles (MD) were observed in the defect area. (H & E, X 220).

Fig. 7.4 Evidence of the active myophagia (arrows) was the main feature at 6 hours after inoculation. (H & E, X 440).


MD



8 hours

By 8 hours, myophagia was active. The number of macrophages were increased but PMNs were still dominant cells in the area. Further degeneration of sarcoplasms which were formless was seen (Fig. 7.5). Dermis was, to some extent, oedematous and blood vessels were hyperaemic with red and white blood cells and also there were melanin pigments around them. Local and scattered haemorrhages were seen throughout the damaged area.

10 hours

At 10 hours after injection, the lesion was still dominated by PMNs activity which in some areas were very dense, aggregated, and, to some extent, degenerated along with macrophages, some amorphous suppuration, degenerated muscles and nucleic debris formed micro-abscesses (Fig. 7.6). Myophagia was in progress in the defect area. Inflammatory cells infiltration was in high level and globular sarcoplasms were obvious in the damaged area. Although the bacteria were not seen in the sections of the lesion area, *A. hydrophila* was re-isolated from the infected fish using bacteriological tests.

12 hours

The picture of this stage was mostly similar to that of 10 hours p.i. The lesion was still dominated by PMN activity. The number of macrophages were increased and myophagia was active. Large area of degenerated muscles with cellular debris was obvious. Also hyperaemia of blood vessels associated with melanin pigments was seen.

Gram negative bacteria were seen within the degenerated muscles and among them (Fig. 7.7). They were also re-isolated from the freshly sampled tissue and identified as *A. hydrophila*, using bacteriological tests.

18-30 hours

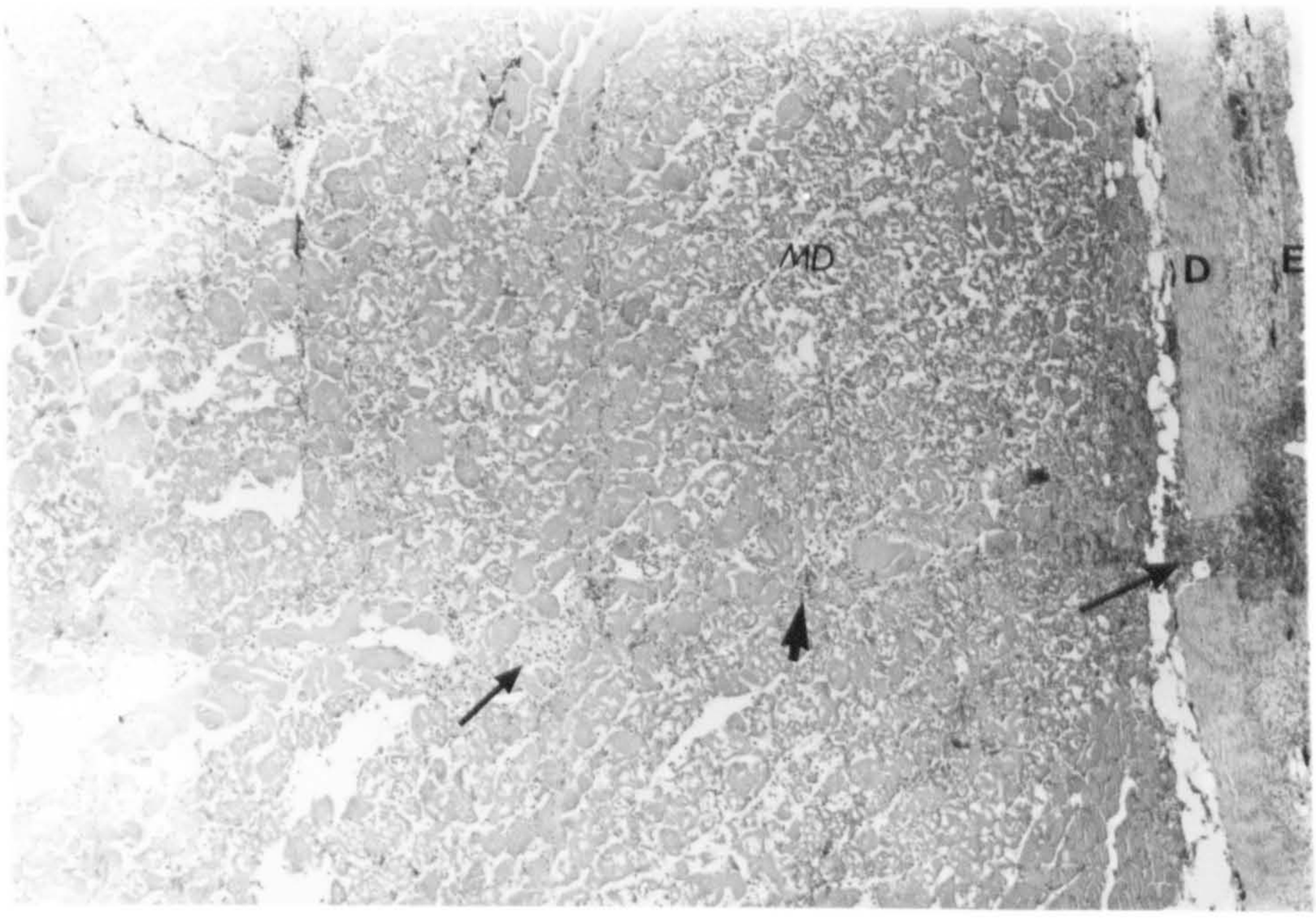
At these stages largely extended degenerated muscles were obvious in the lesion area and myophagia was active so that from the some degenerated sarcoplasms only an outline was remained (Fig. 7.8 & 7.9). The blood vessels were hyperaemic and containing inflammatory cells, and some of them were filled with fibrinogen and thrombocytes. They were also associated with melanin pigments. Inflammatory cells such as PMNs, macrophages and lymphocytes were seen in the damaged area. Masses of Gram negative bacterial colonies were dispersed within the damaged muscles and among them (Fig. 7.8). They were also observed in cytoplasm of some macrophages (Fig. 7.10). Presence of these bacteria in the injected site of the freshly sacrificed fish was also proved by re-isolating and identification them as *A. hydrophila*, using the bacteriological tests.

36-48 hours

By this time sever myonecrosis and sarcolysis was observed extending throughout the lesion area. Floccules of the degenerated muscles, degenerated inflammatory cells, debris and pyknotic nuclei showed an ulcerated area extended towards the dermis. Meanwhile the dermis was swollen due to a sever inflammatory oedema (Fig. 7.11). The presence of some pale swollen fibroblasts in the myotomal fascia and around the blood vessels showed the beginning of fibroblast activity. The

Fig. 7.5 This picture shows a general view of the site of injection in the dermal area at 8 hours p.i. which was infiltrated with inflammatory cells (arrows), and also muscle area with extensive muscle degeneration (MD), and active myophagia (arrow head). (H & E, X 44).
E=epidermis, D=dermis

Fig. 7.6 This picture demonstrates a micro-abscess (arrow head) at 10 hours p.i., comprising a very dense aggregation of inflammatory cells with PMNs dominated, some amorphous suppuration, degenerated muscles and nucleic debris. (H & E, X 220).


MD
D
E

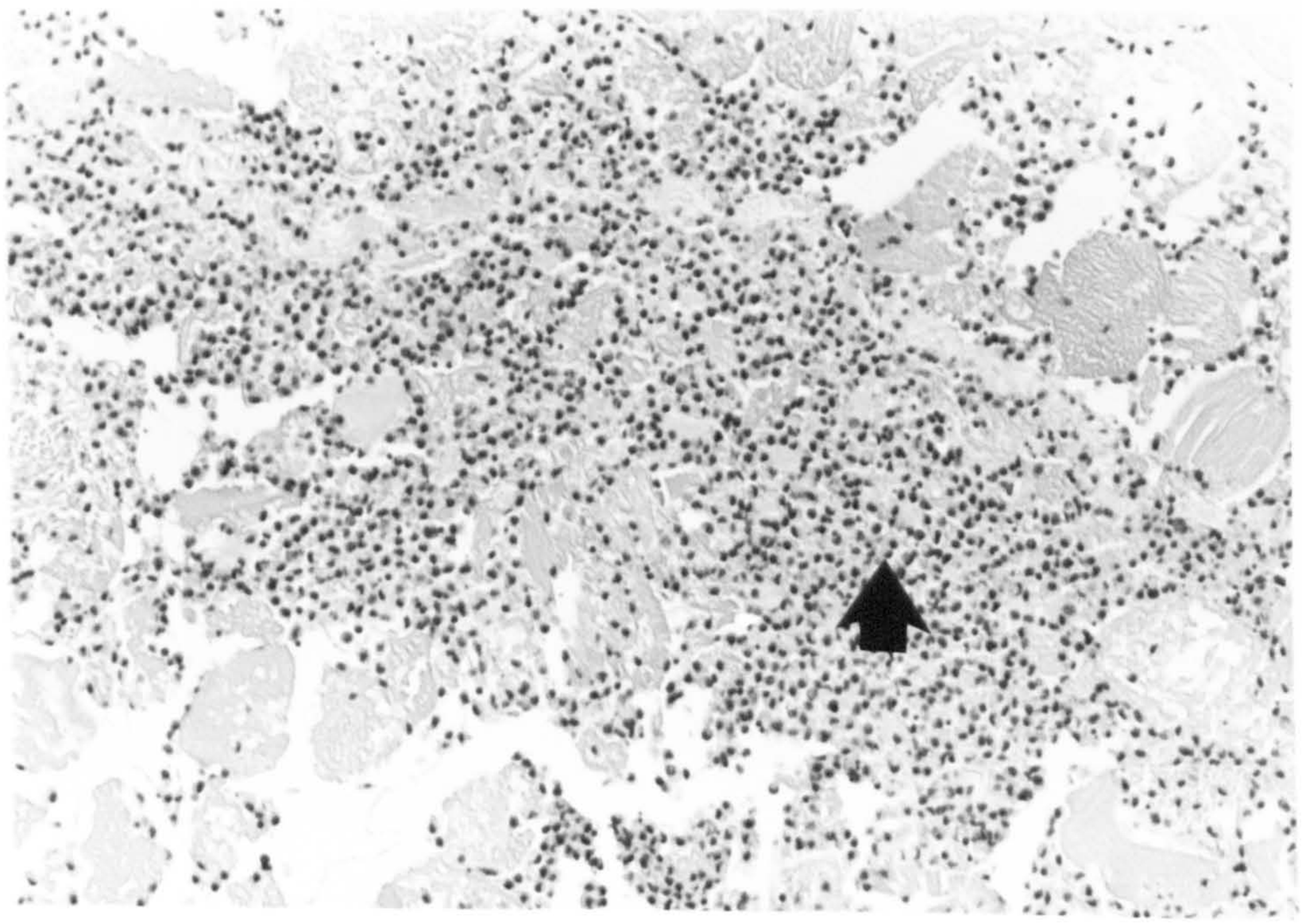
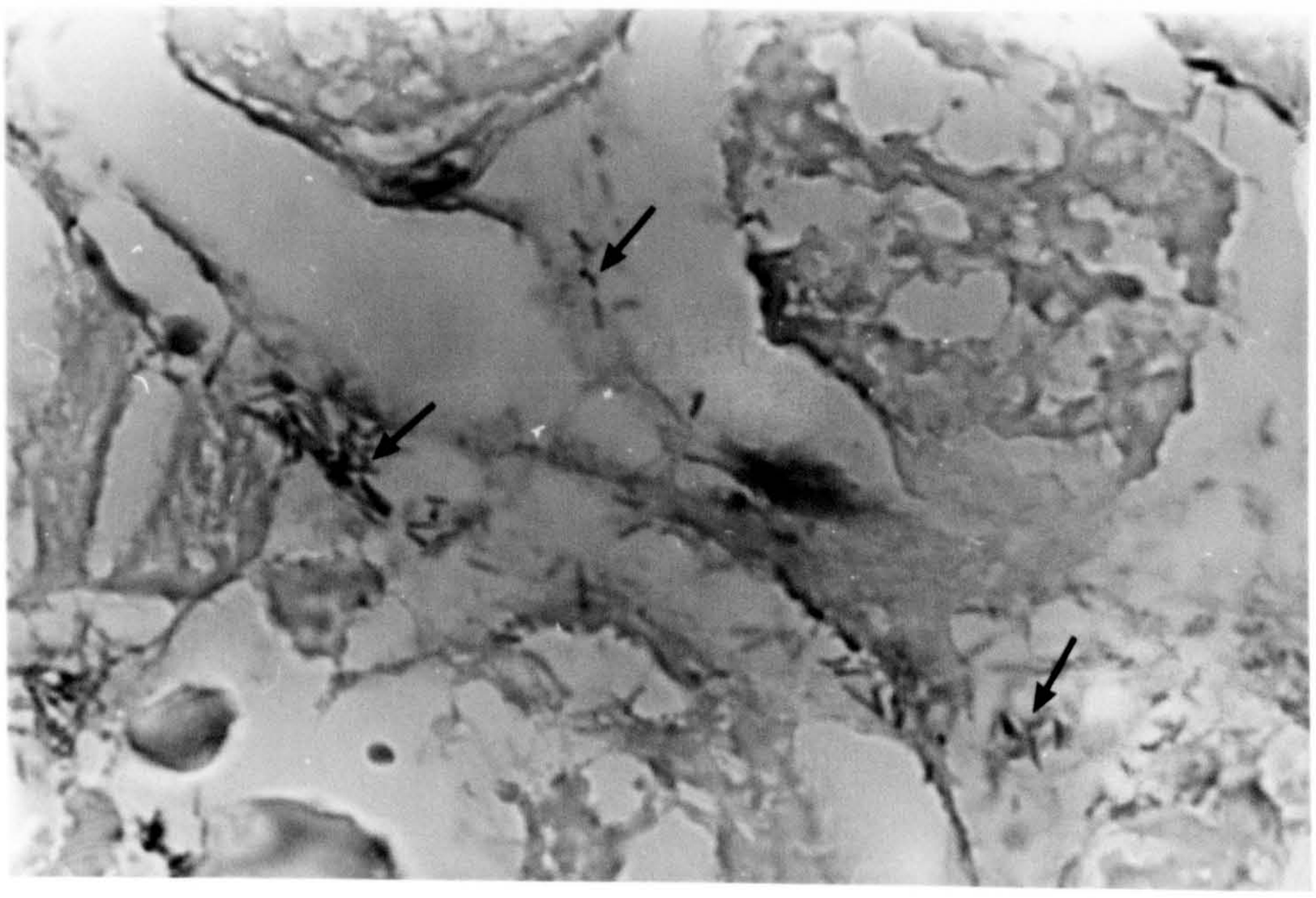


Fig. 7.7 Gram negative *A. hydrophila* were seen within the degenerated muscle and among them (arrows) at 12 hours p.i. (Gram, X 1100).

Fig. 7.8 By 18 hours p.i. largely extended degenerated muscle (MD), active myophagia (arrow heads) and masses of Gram negative bacteria (arrows) were observed in the infected area. (Gram, X 440).



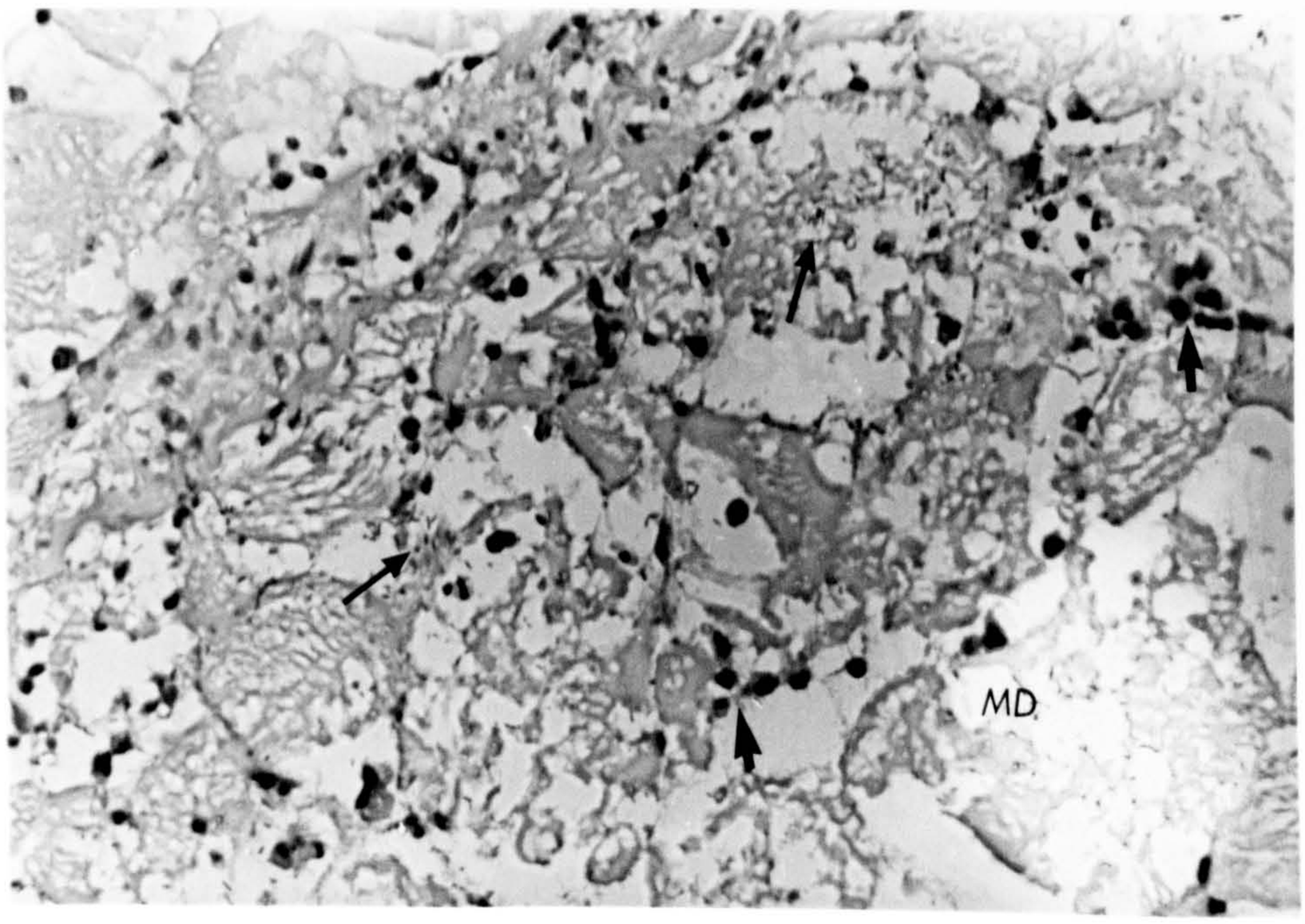
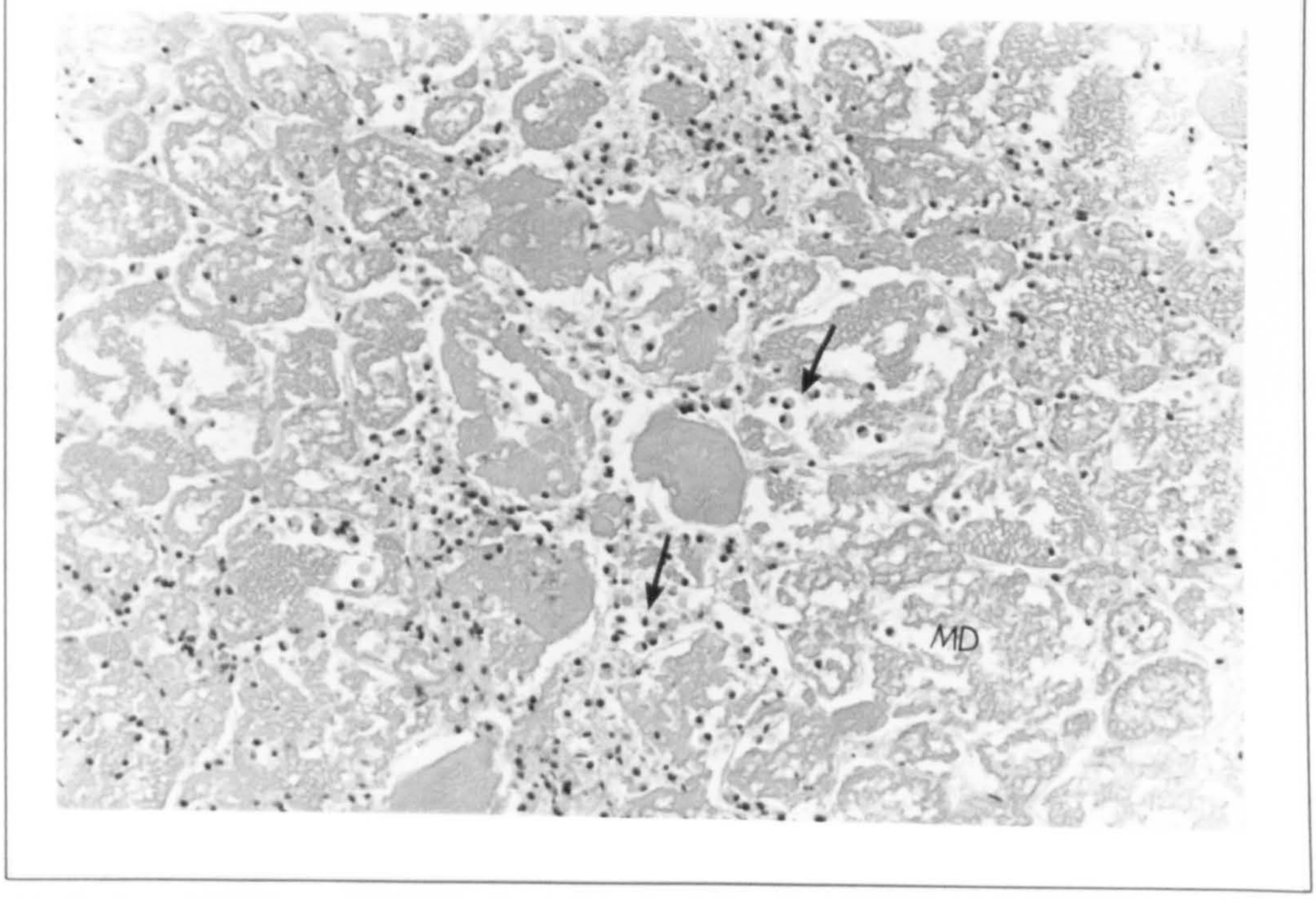
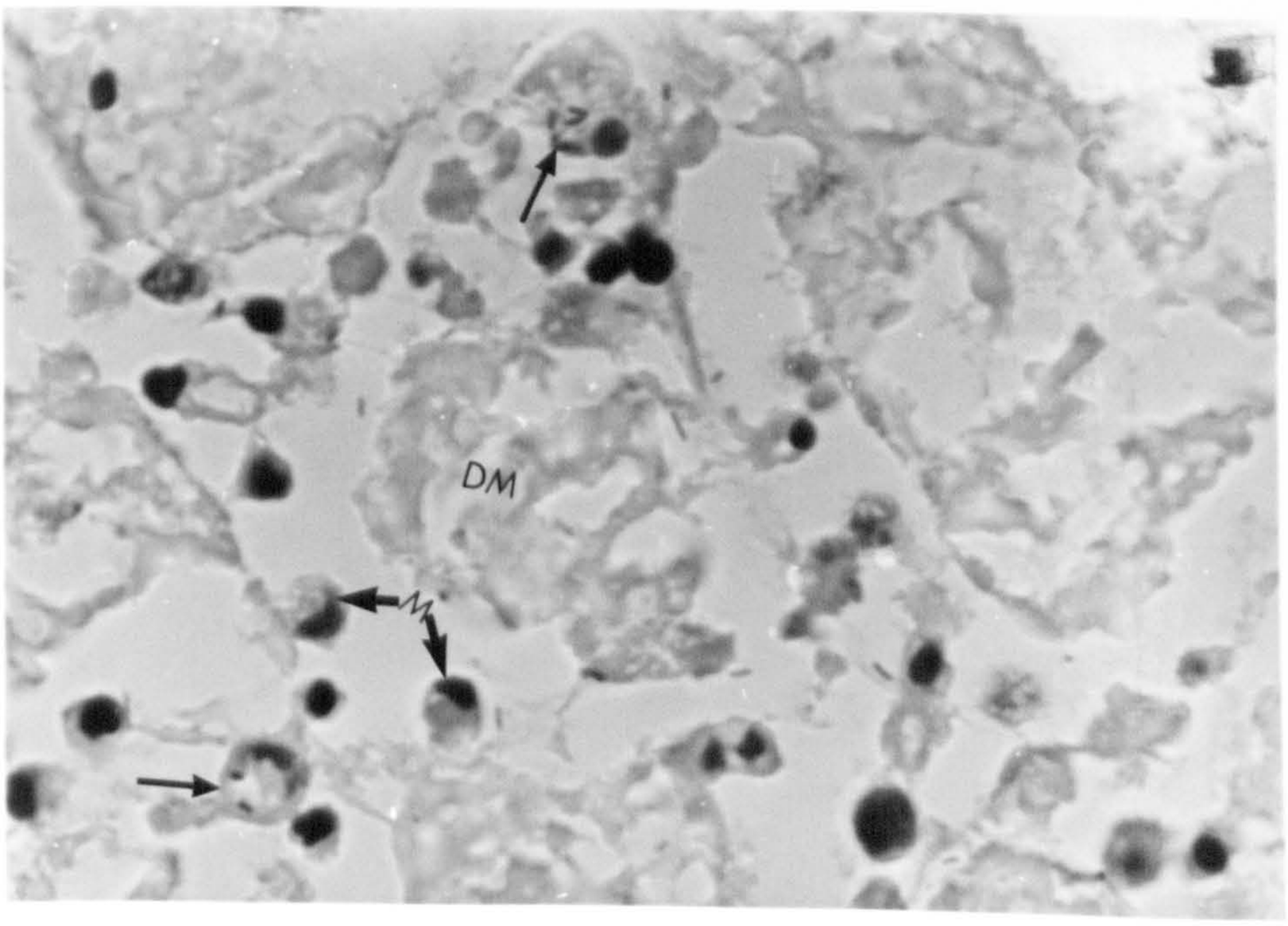

MD

Fig. 7.9 An active cellular infiltration and myophagia (arrows) with an extensive muscle degeneration (MD) were the main features at 24 hours p.i. (H & E, X 220).

Fig. 7.10 This picture shows the presence of Gram negative *A. hydrophila* within the cytoplasm of macrophages (arrows) at 24 hours p.i. (Gram, X 1100).
DM=degenerated muscle, M=macrophage


MD


DM

blood vessels in the lesion area were seen with different appearance, some of them filled with fibrinogen and thrombocytes, some with inflammatory cells and were also hyperaemic but all were associated with melanin pigments.

3 days

The picture of this stage were mostly similar to those of second day, except that the number of fibroblasts and their activity especially around the destroyed area was increased.

4 days

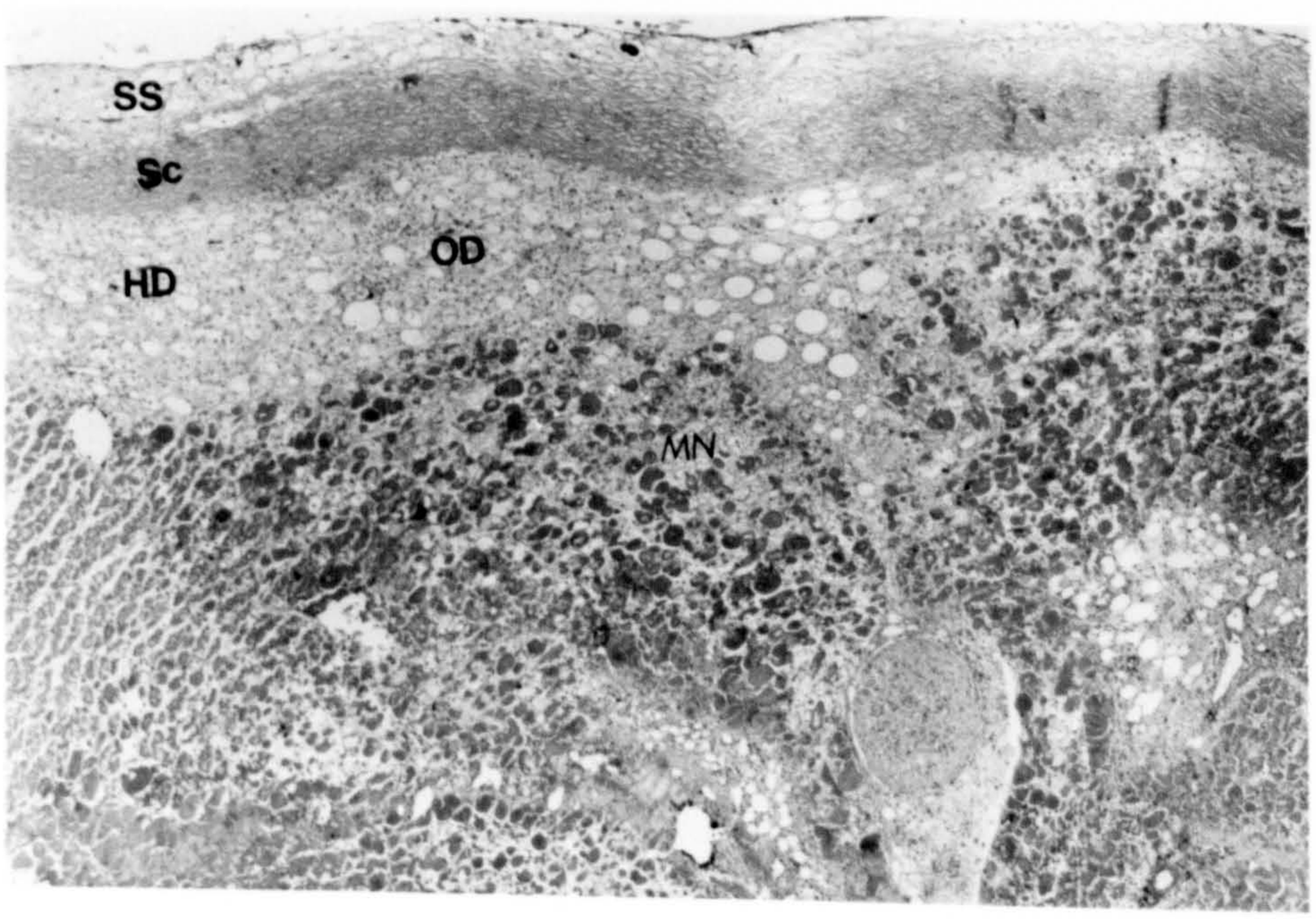
The main feature of this time was fibroplasia in the damaged area, specially in the edge of the lesion. Fibroblasts activity was increased and these cells along with macrophages which were in an epithelioid form, appeared encapsulating the necrosis area (Fig. 7.12). Also myophagia was, to some extent, in progress and removed necrotic muscles being replaced by fibroplasia. The first presence of some new small basophilic muscle buds were evident in the damaged area. Inflammatory oedema caused dermal swelling showing a large space between dermal layers.

5 days

At this stage fibroplasia and fibrosis was active and boosted by new capillaries which appeared in the fibrous tissue. Small number of macrophages was observed. New muscle buds were increased in the damaged area (Fig. 7.13). The bacteria were neither seen in the lesion area nor re-isolated from the fish by bacteriological tests.

Fig. 7.11 Severe extended myonecrosis (MN) and oedema (OD) of stratum spongiosum and hypodermis were observed at 48 hours after inoculation. (H & E, X 44).
SS=stratum spongiosum, SC=stratum compactum, HD=hypodermis

Fig. 7.12 The main feature of the lesion at 4 days p.i. was fibroplasia (F) specially in the edge of the lesion. (H & E, X 220).


SS
Sc
OD
HD
MN

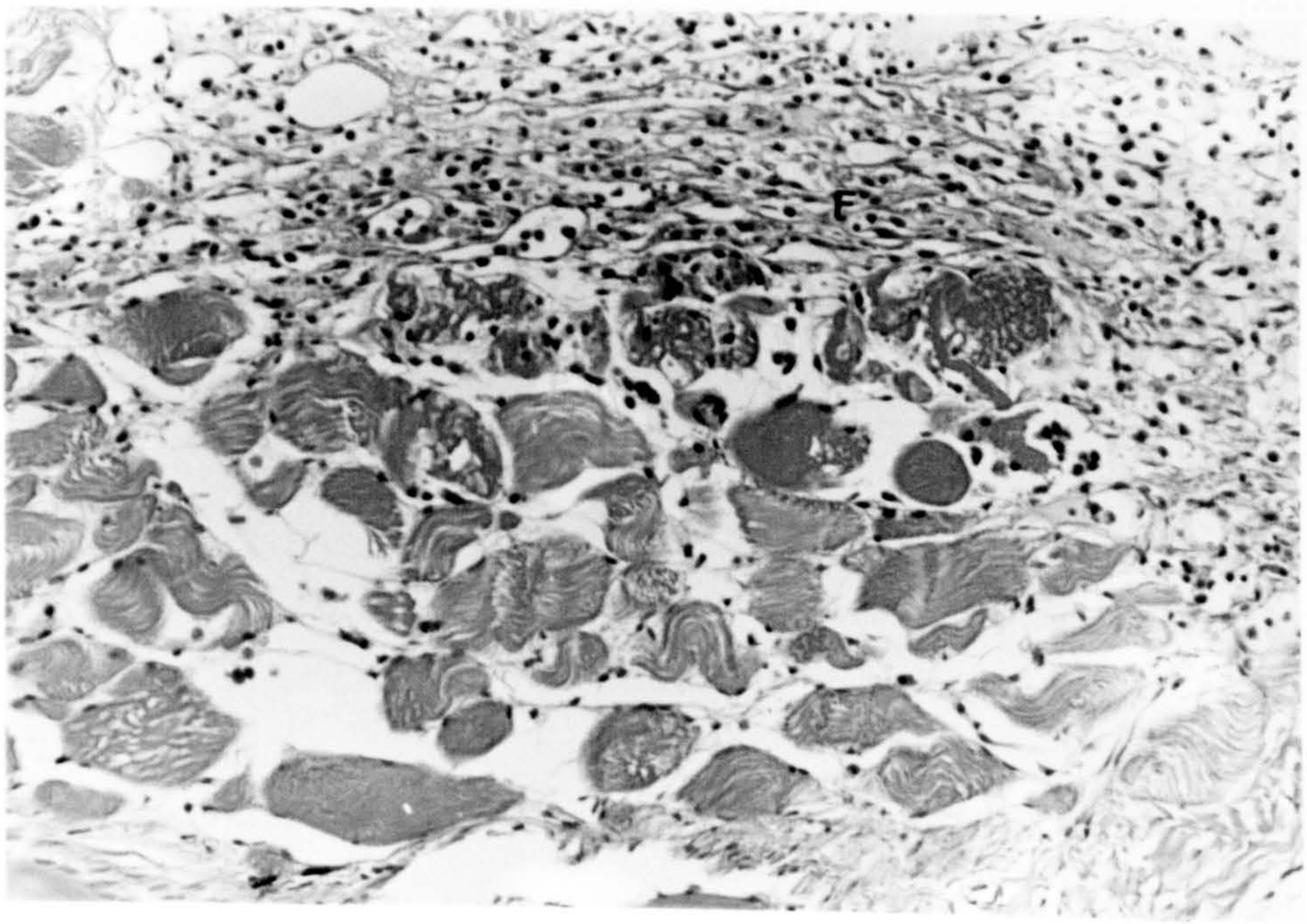
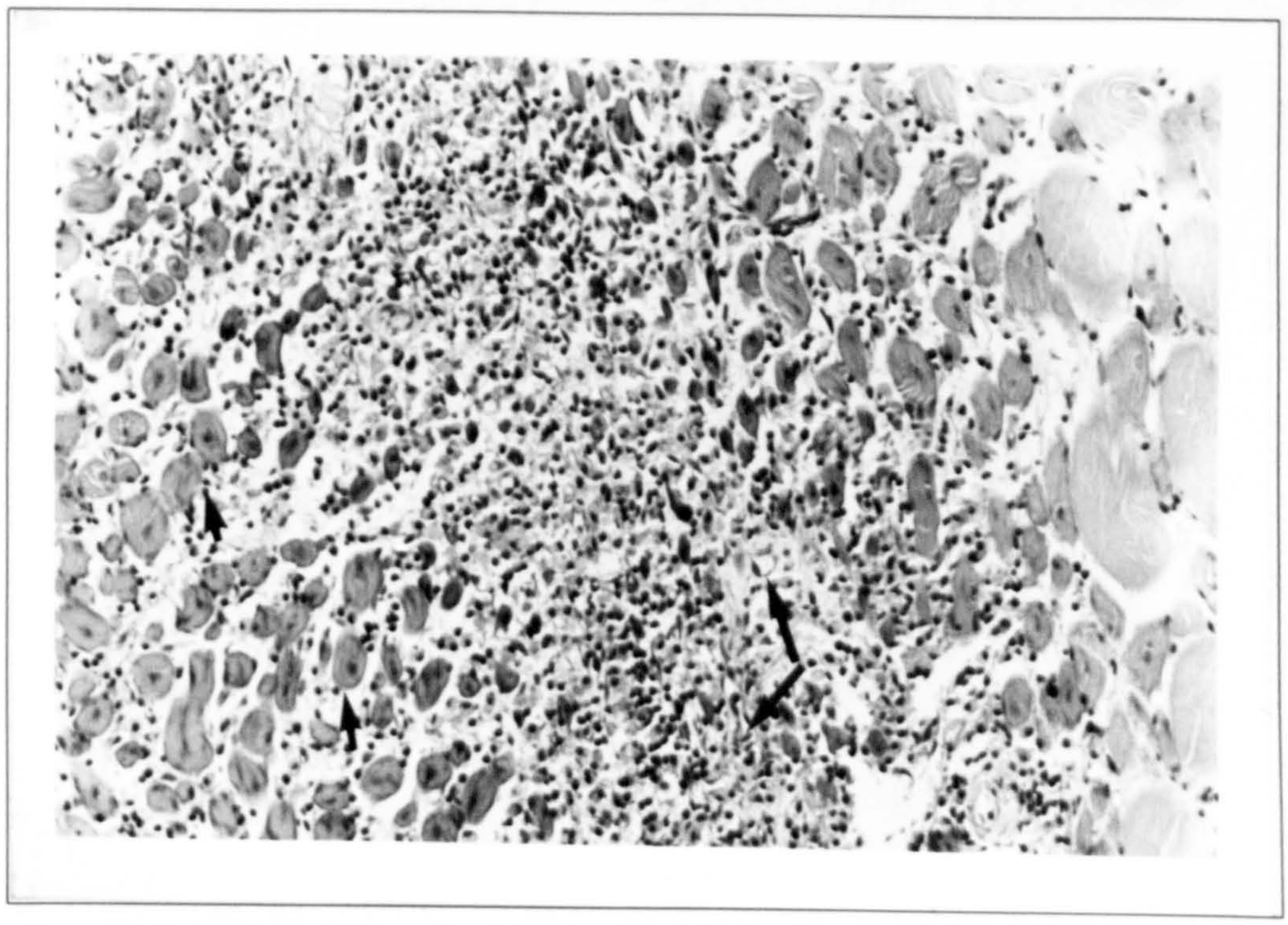
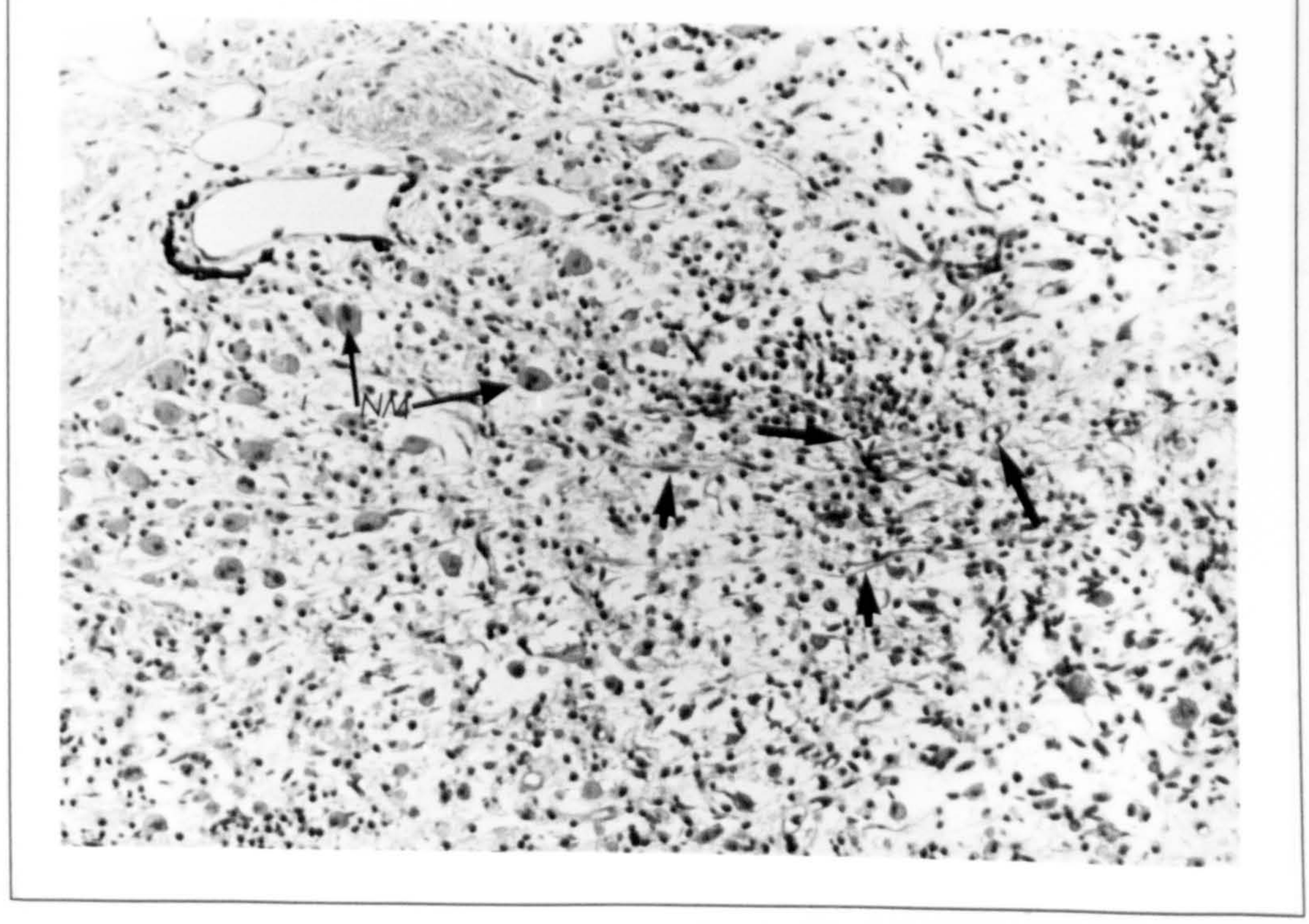

F

Fig. 7.13 At 5 days p.i. new muscle buds (arrow heads) and new capillaries (arrows) were increased in the damaged area. (H & E, X 220).

Fig. 7. 14 The dominant features at 6 days p.i. were active fibroplasia and fibrosis (arrow heads), vascularization (arrows) and new myofibrillar buds (NM). (H & E, X 220).




NM

6 days

The dominant features in this time were active fibroplasia, fibrosis and vascularization. Also new myofibrillar buds were seen extending in the damaged area (Fig. 7.14). In the centre of the lesion an area consisted of degenerated muscles and some amorphous pink staining substance with some macrophages and PMNs within, was seen surrounded by active fibrosis and muscle regeneration which was also occurring within this area. Myophagia was in progress in this part of the lesion and also in the rest of the area. New capillaries were active and nourishing the fibrous replacement area. Also some haemorrhages were scattered within the lesion area. Inflammatory cells such as macrophages, PMNs, lymphocytes, eosinophilic granular cells (EGCs), and fibroblasts were obvious in the whole lesion area. No bacteria were detected either in the sections or in bacteriological tests.

7 days

By 7 days, fibroblasts and capillaries activity, and also growth of myofibrillar elements into the granulation tissue were dominant features. Myophagia was completed. Lymphocyte activity was in a high level and macrophages in the form of epithelioid cells were seen in the lesion area (Fig. 7.15). The presence of some PMNs and EGCs was evident. No bacteria were seen in the damaged area but Gram negative *A. hydrophila* was re-isolated from freshly sampled muscle tissue by bacteriological tests.

10 days

At this stage the process of wound healing was well developed. The regenerated

muscle fibres actively developed and infiltrated into the fibrous area. There was more dense fibrous tissue replacing the damaged area and collagen forming was in a high level of activity. New epidermis was oedematous and some inflammatory cells migrated through it, and the cut edges of dermis were linked by fibrous tissue (Fig. 7.16). Capillaries were very active in nourishing the fibrosis area and also more new capillaries were seen. A large granuloma was seen comprising an amorphous pink staining substance, similar to a newly grown small scale, in the centre area, macrophages, epithelioid cells and some nucleic acid debris, encapsulated by fibrous tissue and also lymphocytes (Fig. 7.17). A large Langhans type giant cell in an area between this granuloma and oedematous epidermis was observed (Fig. 7.18). Lymphocytes accumulation, some active macrophages and some EGCs were observed in the area. In some sites a ring of fibrous tissue and inflammatory cells separated the normal muscle and damaged area. Dense melanin pigments and isolated melanin granules were scattered in the damaged dermis and replaced fibrous tissue in the muscle area and also around the blood vessels. Some haemorrhage was observed in the defect area. No bacteria were seen either in the infected area or re-isolated from the fish in bacteriological tests.

14 days

By the day 14, the progress of the dermal regeneration led to integrity in the area of the defect being almost completed. Epidermis was normal and two separated edges of dermis were completely closed and linked together. Also muscular regeneration was, to some extent, in progress but in most of the defect area regenerated muscle fibres actively developed into the fibrous area (Fig. 7.19). Fibrosis was active

Fig. 7.15 By 7 days p.i., myophagia was completed and lymphocyte infiltration (L) was increased. New muscle fibres (arrow) and new capillaries (arrow heads) were also observed. (H & E, X 220).

Fig. 7.16 This picture shows a general view of the injected area 10 days p.i. More dense fibres tissue (arrow heads) are replacing the damaged area. New epidermis (E) is oedematous and the cut edges of dermis (CD) are linked by fibrous tissue, which is associated with melanin pigments (arrows). (H & E, X 44).
L=lymphocytes aggregate


L

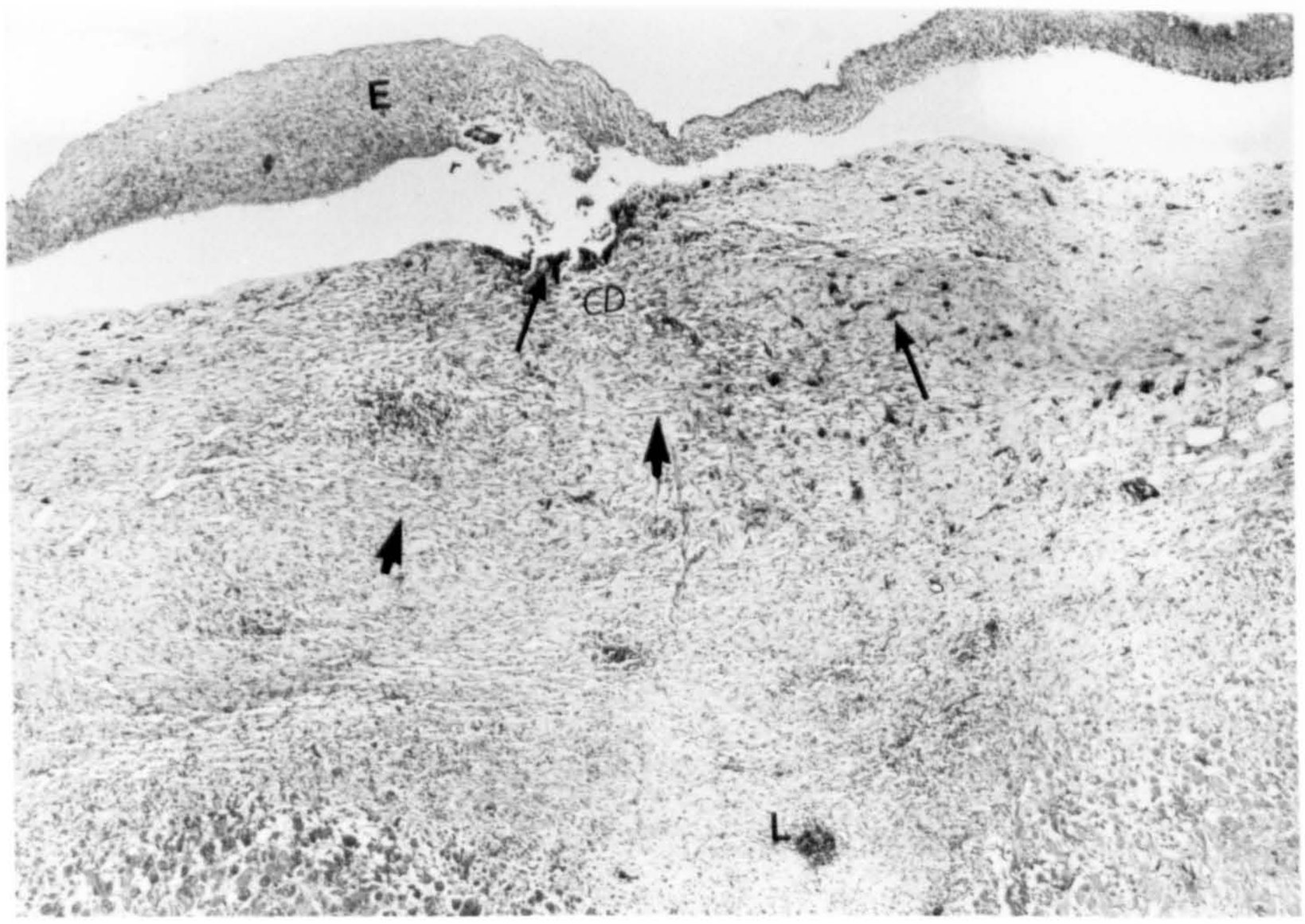
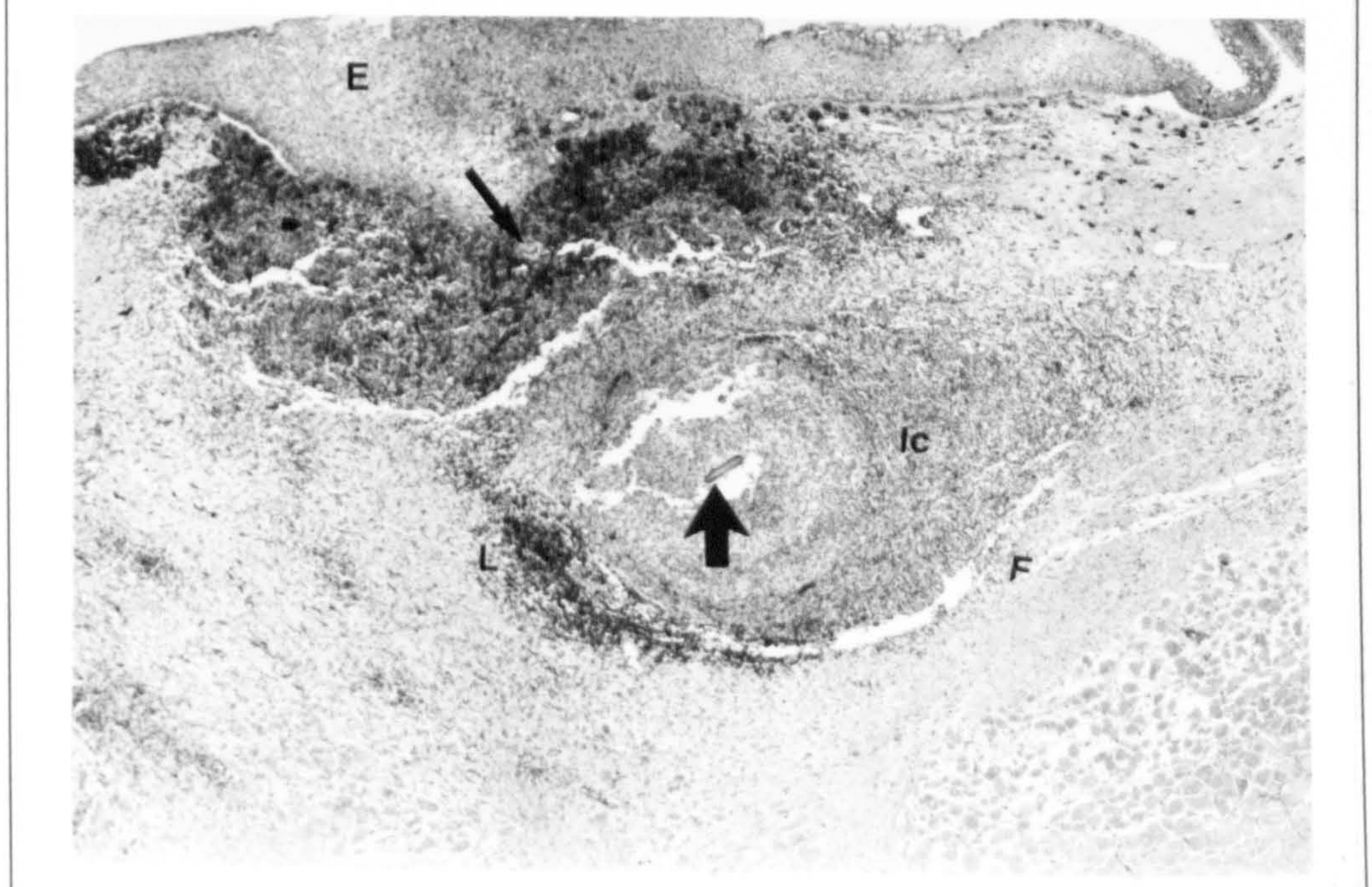

E
ED
L

Fig. 7.17 A large granuloma comprising an amorphous pink substance in the centre (arrow head), and inflammatory cells (IC), nucleic debris, fibrous tissue (F) and lymphocytes accumulation (L) was observed at 14 days p.i. Also Langhans giant cell (arrow) was seen between the granuloma and new oedematous epidermis (E). (H & E, X 44).

Fig. 7.18 The enlargement of Fig. 7.18 shows a large Langhans giant cell (arrow head) located between granuloma and epidermis (E). (H & E, X 440)


E
lc
L
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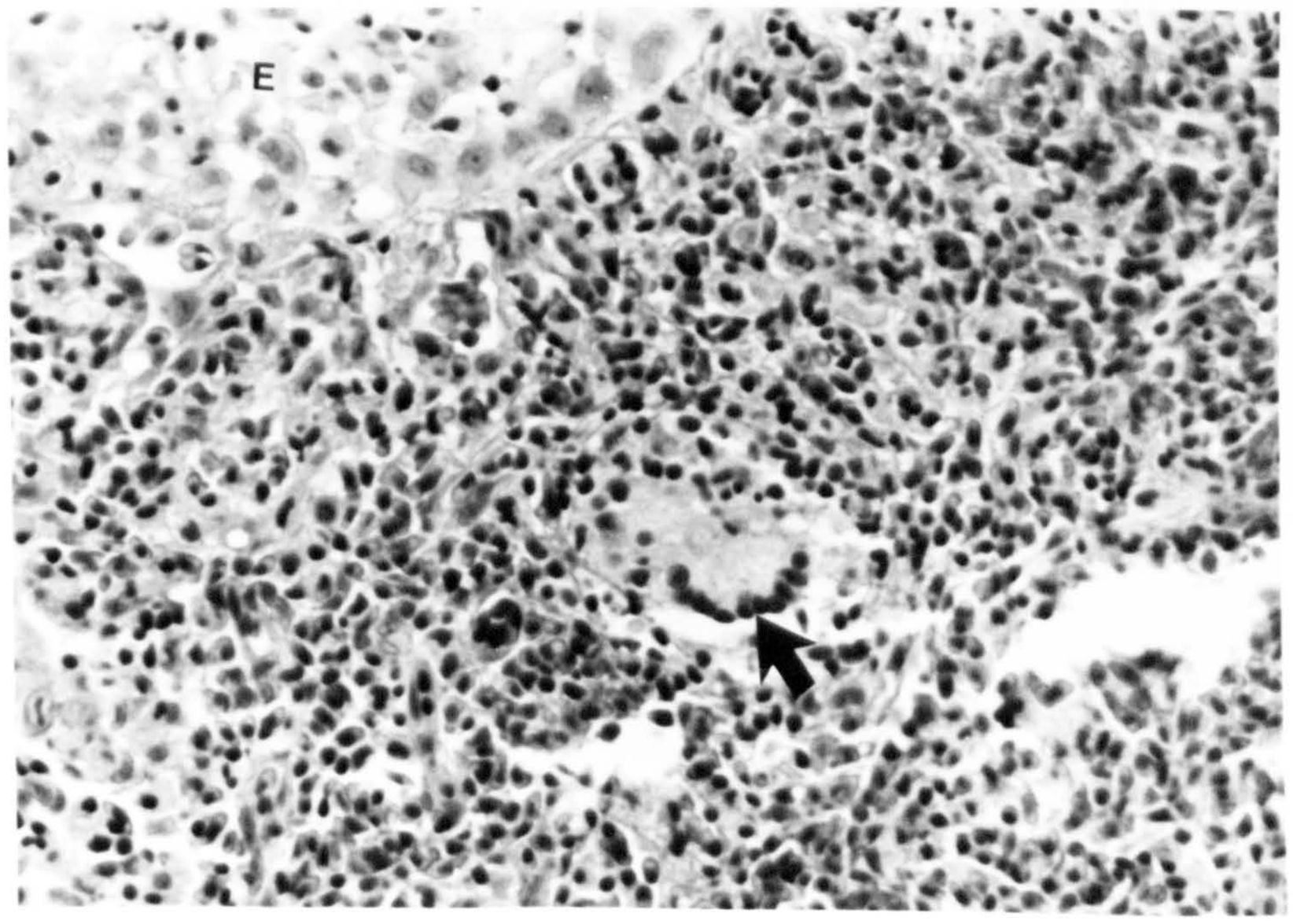

E

and well developed. New capillaries were active in the area and some scattered haemorrhages were seen. Stratum spongiosum was developing normal melanocyte structures, so that this component was scattered throughout the whole lesion area and around the blood vessels. The lesion area was still more cellular than the normal tissue. Melanin and lipofuscin containing macrophages, lymphocytes in a high level of activity, some EGCs, and macrophages in the form of epithelioid cells between the normal and regenerated muscles and also in the fibrous area, were evident. No bacteria were seen in the lesion area, and neither was *A. hydrophila* re-isolated from the infected fish using bacterial culture medium.

18-22 days

The dominant features in this period were mostly similar to those of 14th day. Muscle regeneration was still in progress and all the defect area underside the dermis was filled by new muscle bundles. Fibrous replacement with scarring of the lesion area was obvious. More cellular components, especially lymphocytes in a high level of activity, and macrophages laden with lipofuscin, melanin granules and ceroids were dominant throughout the healing lesion. Fibrosis and new capillaries were still active and melanin pigments were scattered throughout the joined dermis and fibrous replacement areas (Fig. 7.20 & 7.21). New scales were seen well formed above the newly re-joined dermis (Fig. 7.20).

28 days

At this stage a scar replaced the destroyed area by infection, but the scar tissue was reduced, and re-grown muscle bundles were increased and filled most of the defect

area. The cellular components were dramatically reduced and only a small accumulation of active lymphocytes and a small number of active macrophages were present in the healing lesion. Small blood vessels were active and nourishing the healing lesion and also some new capillaries were formed. Melanin pigments were scattered throughout the healed dermis and replaced fibrous tissue in the muscle area and around the blood vessels. Newly formed scales were growing on the damaged area within the stratum spongiosum exactly above the scar tissue (Fig. 7.22).

35-42 days

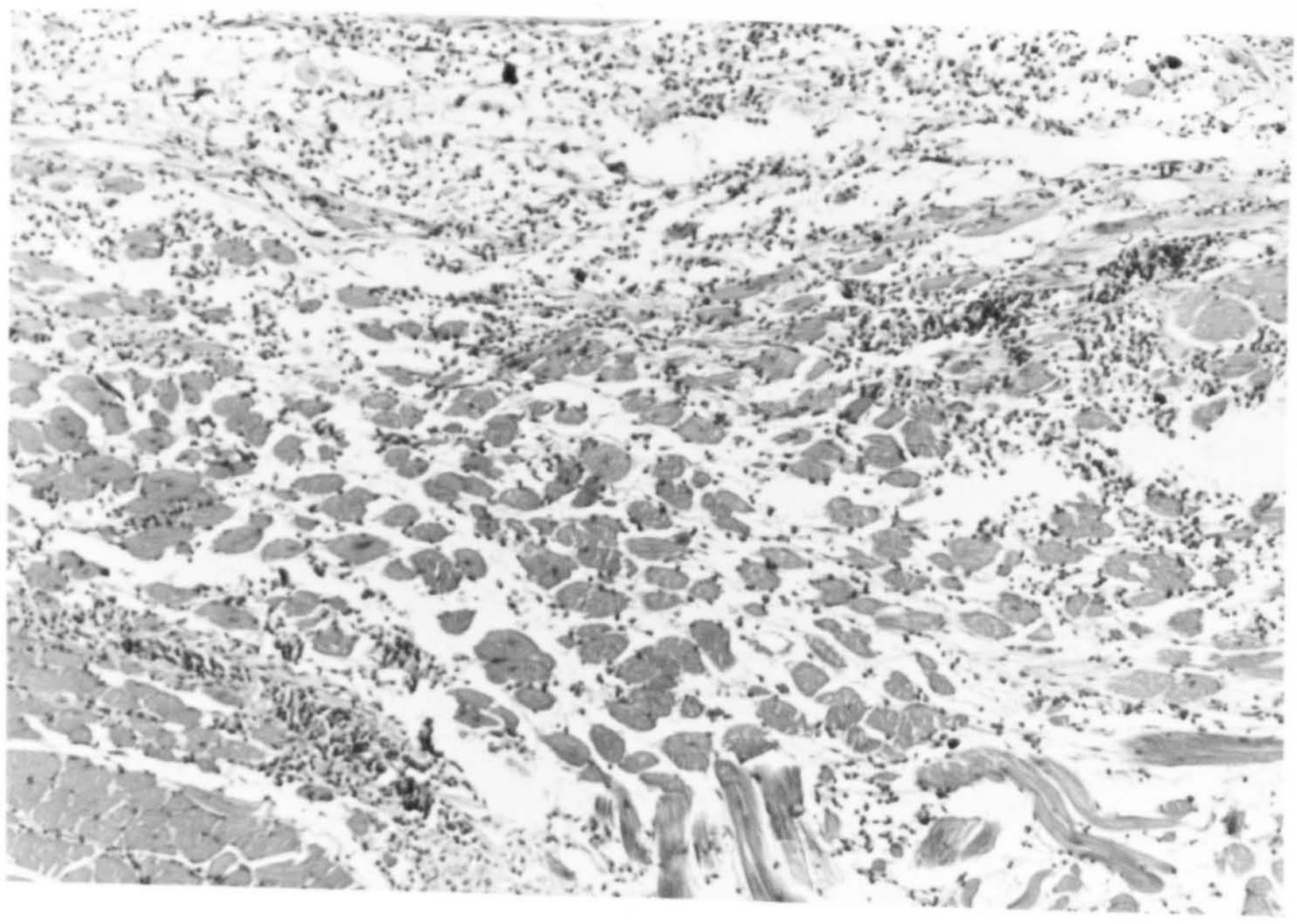
At this period the lesion area almost recovered. The scar had joined up with the dermis especially at 42 days. The re-grown muscles were indistinguishable from the normal muscles, and filled all the defect with a highly reduction of the connective tissue and cellular components. The epidermis and dermis had recovered their normal form but dense melanin pigments and isolated granules were dispersed throughout the healed area and around the blood vessels. A small number of macrophages with ceroid and melanin within their cytoplasm were still present in the scar area and adjacent to the congested blood vessels. Some new scales were seen above the healed lesion area well formed (Fig. 7.23 & 7.24).

Control fish

No histological changes were observed in tissues of control fish injected with sterile saline. Also *A. hydrophila* was seen neither in the histological sections of tissues nor isolated from the sampled tissues of control fish by bacterial tests.

Fig. 7.19 By 14 days p.i., regenerated muscle fibres actively developed into the fibrous area in most of the lesion area. (H & E, X 110).

Fig. 7.20 This picture shows the lesion area at 18 days p.i. All the defect area underside the dermis was filled by new muscle bundles (NM). Cellular components (arrow) are seen throughout the healing area, and melanin pigments are scattered throughout the joined dermis (D). New scales (arrow heads) are seen above the newly re-joined dermis. (H & E, X 44).



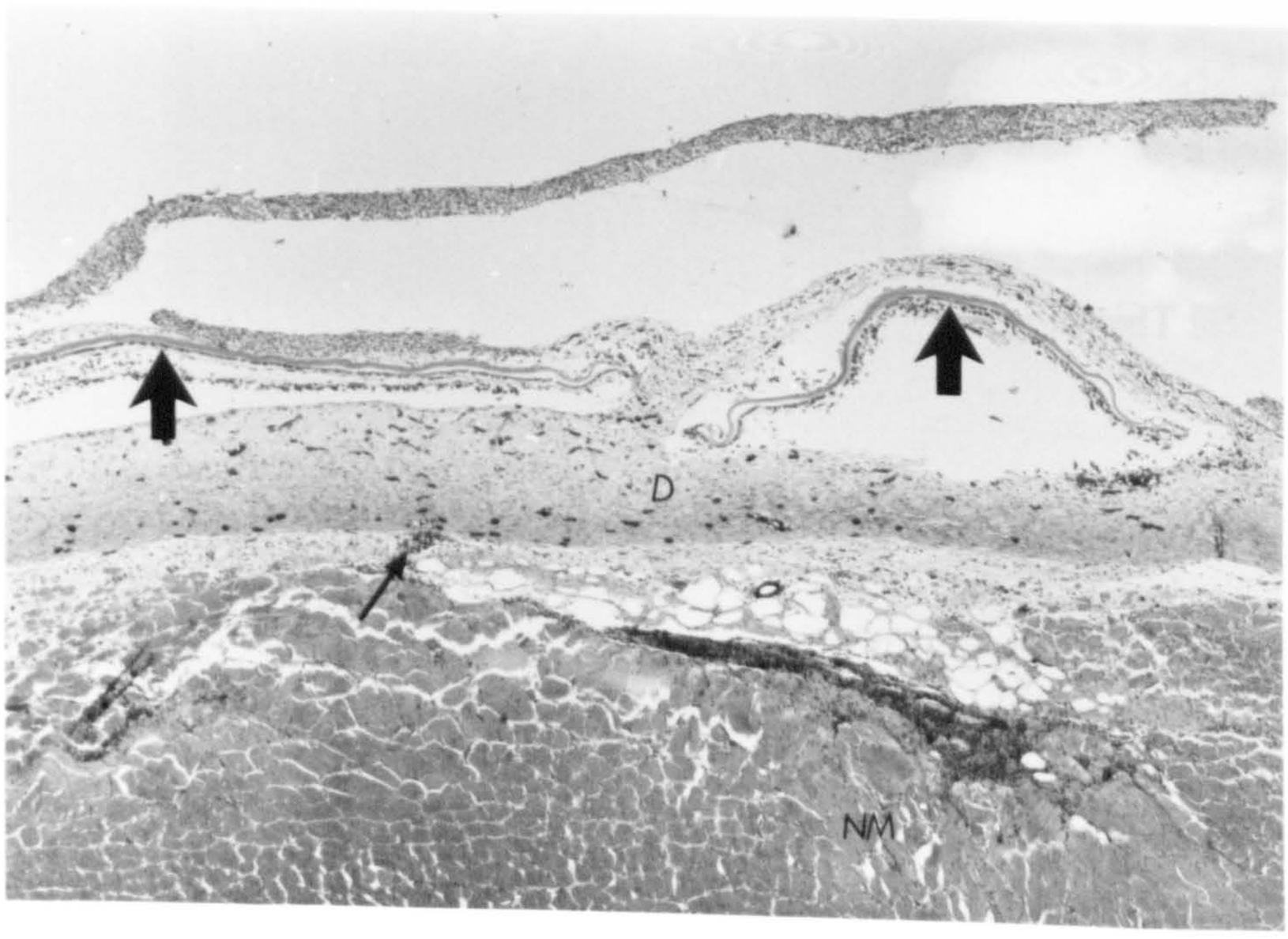
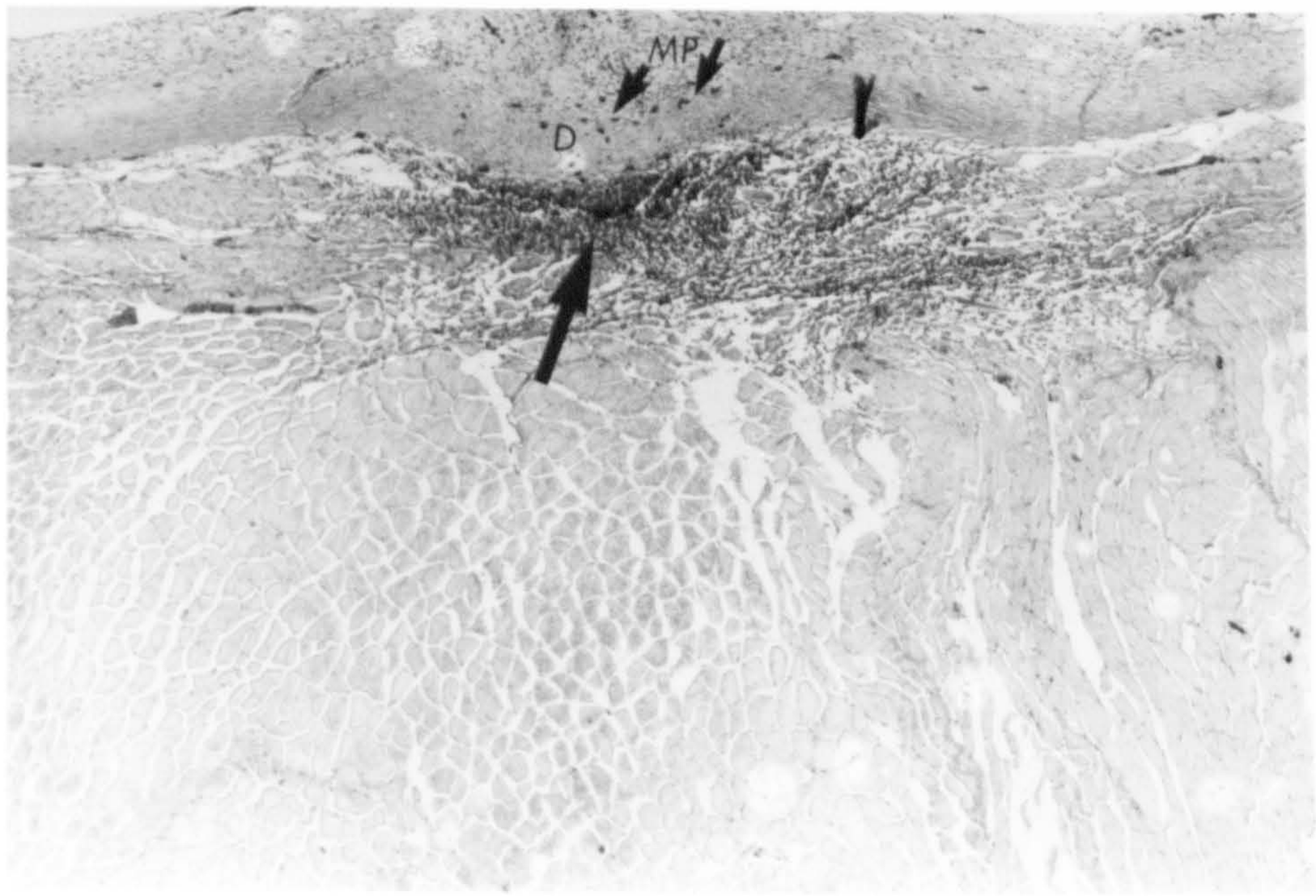

D
NM

Fig. 7.21 The healing area under the newly re-joined dermis (D) was still more cellular (arrow) at 22 days p.i. Melanin pigments (MP) were observed throughout the joined dermis and healing area. (Gram, X 44).

Fig. 7.22 This picture demonstrates a general view of the lesion are at 28 days p.i. The scar tissue is reduced and re-grown muscles filled almost the whole defect area (ML). Only a small accumulation of lymphocytes and macrophages (arrow) are present under the dermis (D) in the lesion area. A new scale (arrow head) is well grown over the scar area within the stratum spongiosum (SS). (H & E, X 44).


MP
D

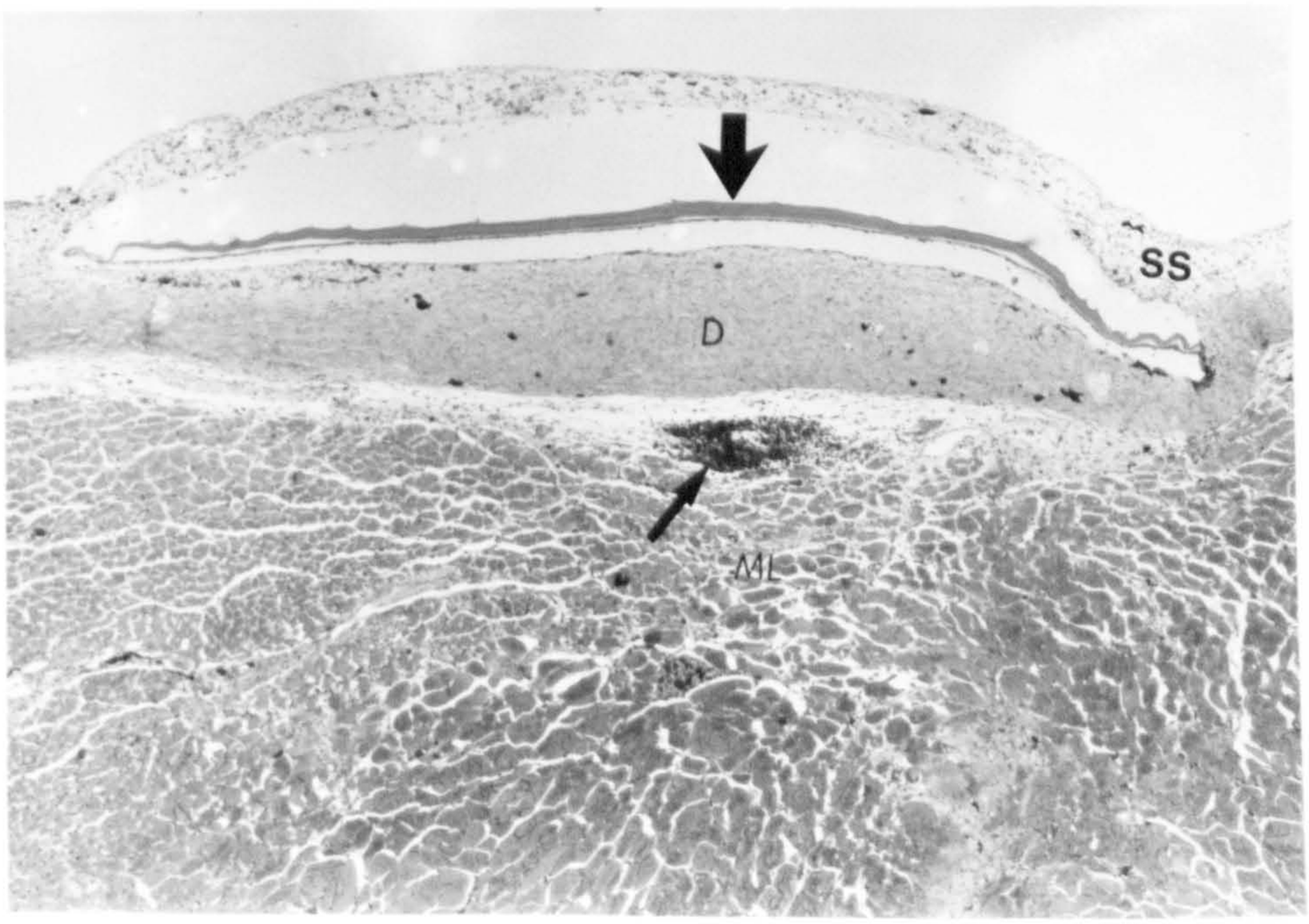
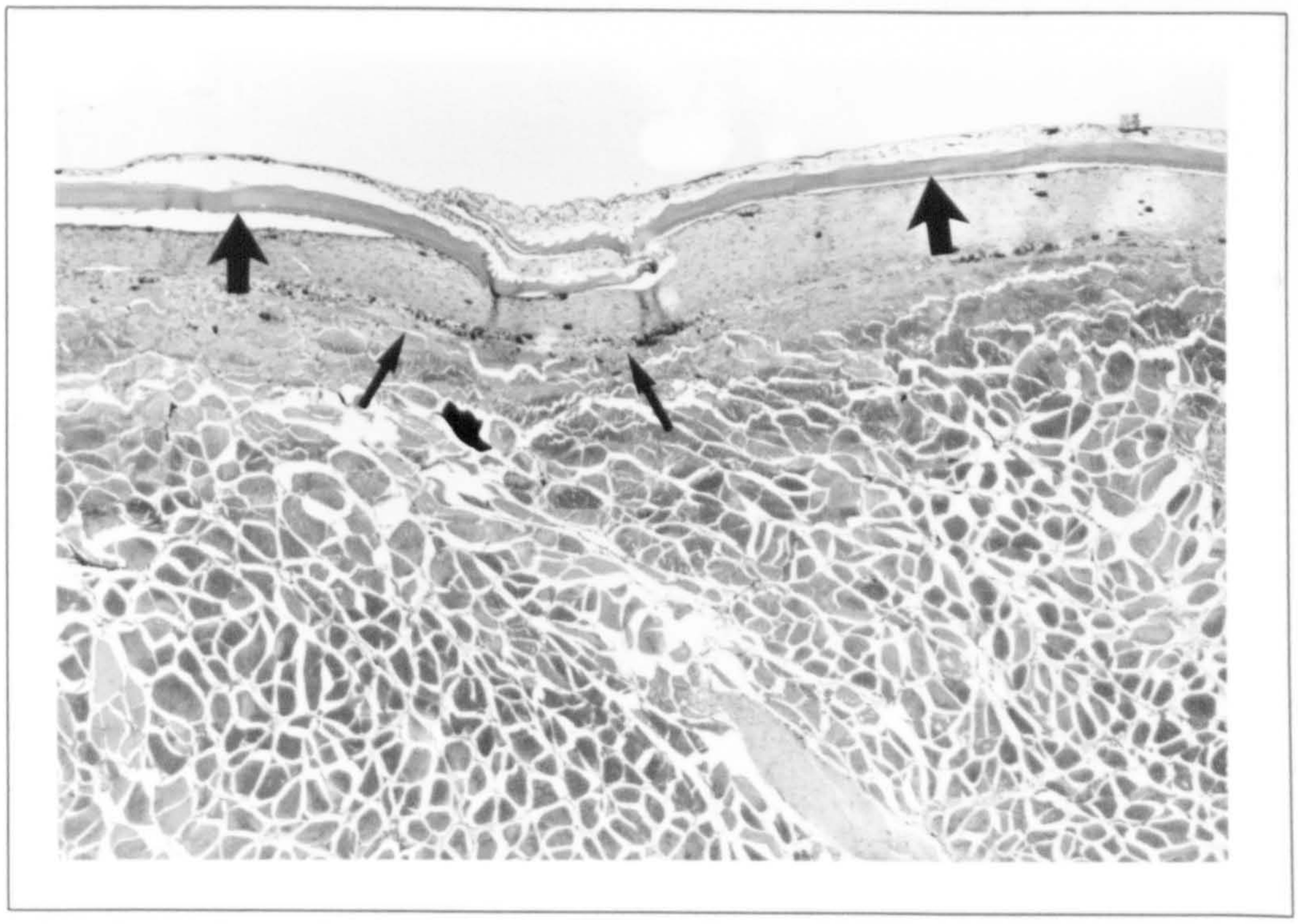
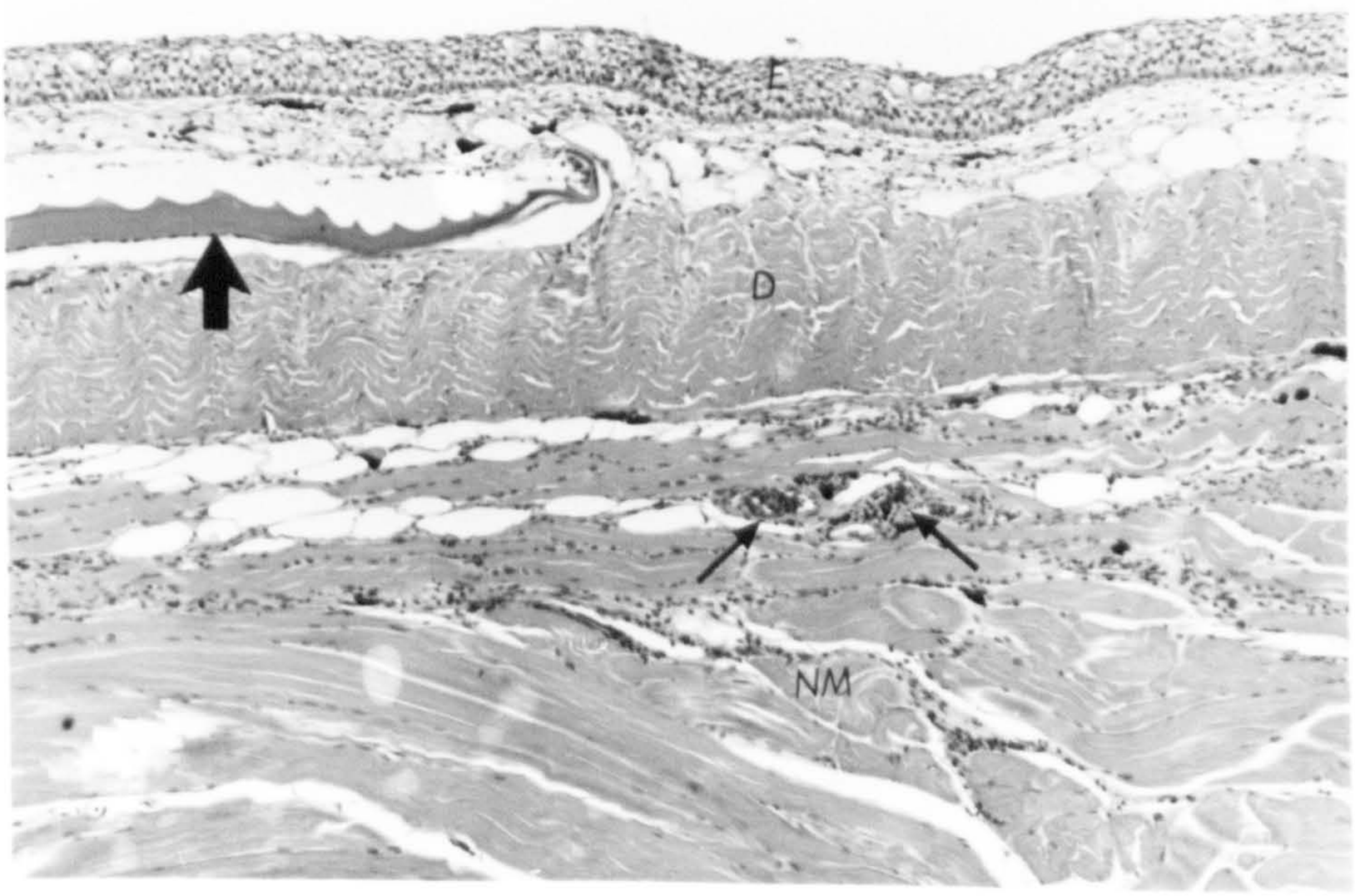

SS
D
ML

Fig. 7. 23 At 35 days p.i. the lesion area almost recovered. The scar had joined up with the dermis (arrows). Re-grown muscles were well developed and filled the defect with highly reduction of the connective tissue and cellular components. Two well developed new scales (arrow heads) are seen above the healed area of the lesion. (H & E, X 44).

Fig. 7.24 By 42 days p.i. lesion area was similar to a normal tissue. The scar completely filled with new muscles (NM) and joined with dermis (D). A few cellular components remained in the area (arrows). The epidermis (E) and dermis had recovered their normal form. A new scale (arrow head) above the healed area was obvious. (H & E, X 110).




E
D
NM

7.5 DISCUSSION

The gross pathological changes of the infected carp in this study were generally similar to those described by Brenden & Huizinga (1986) in gold fish and Chinabut (1989) in snakehead in relation to intramuscular injection of *A. hydrophila*. These changes also were similar to those described by Roberts (1993) as clinical signs of motile aeromonad septicaemia.

The first 48 hours post-inoculation of *A. hydrophila* was the most critical and fatal period in this study. During this period more than one quarter of the infected fish died with a severe generalised erythematous reaction over the whole body surface, which indicated, probably, a toxaemic condition. Haemorrhage is a conspicuous feature of *A. hydrophila* infections in poikilothermic animals (Snieszko 1975; Hazen *et al.* 1978). Haemolysin is suggested as the major active toxin and virulent factor (Rigney *et al.* 1978, Thune *et al.* 1986) which is a major lethal factor rather than protease (Allan & Stevenson 1981). Aoki & Hirono (1991) cloned and characterised haemolysin as the causal agent of haemorrhages in *A. hydrophila* infections, and Olivier *et al.* (1981) reported that haemolysin can haemolyse erythrocytes resulting in anaemia in fish. However, haemorrhage in *A. hydrophila* infections may be an outcome of involving some of the extracellular products of this bacterium together such as; haemolysin, elastase and protease.

Extensive haemorrhage and mortalities in carp (present study), therefore could be a result of the ability of *A. hydrophila* to damage the elastic and collagenous fibres of blood vessels, and also lysis of red blood cells and breakdown haemoglobin by lytic toxins properties. Death of the infected fish may also be resulted from a systemic disturbances involving the haematopoietic system and liver. The recovery of those fish

which survived following inoculation of *A. hydrophila* showed that they might have developed immunity after inoculation, since from 7 days after infection onward, presence and activity of lymphocytes increased in the lesion area. Lymphocyte cells are immunocompetent cells which are responsible for initiating and mediating the aspects of specific immunity (Ellis 1989). Baba *et al.* (1988*a*) showed that the defence mechanism in crude LPSDP (lipopolysaccharide)-immunised carp is composed of a thymus-derived sensitised lymphocyte-macrophage system. Baba *et al.* (1988*b*) also showed that the protection against *A. hydrophila* infection in carp is not dependent on humoral immunity.

This indicates the probability of differences in individual fish defence mechanism, since the bacterial strain, dose of inoculum, route of infection, the techniques used, water system and water temperature were the same for all infected fish in the present study.

Also all lesions in the surviving fish healed, and prolonged infections limited to skin and muscle did not occur. This is similar to that of channel catfish experimentally infected by *A. hydrophila* (Ventura & Grizzle 1988).

This study showed that the bacteria were present in the tissues of lesion of the fish and also re-isolated from the infected tissues after injection, but not after 5 days post-injection, which is similar to that of walking catfish reported by Angka *et al.* (1995). The disappearance of the injected micro-organisms could be due to the onset of development of effective immunity (Angka *et al.* 1995). Also clearance of bacteria from tissue has been shown to be temperature-dependent, and the time taken for clearance is much slower at low temperature (Finn & Nielson 1971*a*).

Fibroblasts activity for healing the lesion began by 2 days after injection with

presence of some pale swollen fibroblasts in the myotomal fascia and around the blood vessels. Their number and activity increased by 4 days and fibroplasia and fibrosis was active at 5 days after injection. The process of wound healing began and developed clinically and histologically by 5-10 days post-injection. Ventura & Grizzle (1988) reported that in channel catfish infected with *A. hydrophila* at 18°C, fibroblasts had proliferated in necrotic areas by day 8. Chinabut (1989) reported that in snakehead, intramuscularly infected by *A. hydrophila* at 28.5°C, wound healing began by day 7-10. She reported that healing area was distinguished grossly as being darking colour, which is the same as that seen in the healing area in the present study. The rate of wound healing in this study was faster than that of above mentioned channel catfish and snakehead, respectively.

The PMNs infiltrated into the lesion area at the first hour after infection in only a few numbers and increased gradually while the lesion progressed. They were one of the dominant inflammatory cells in the lesion area especially throughout the time of major activity, and remained until 7 days post-injection. These observations correlate with the findings of Brenden & Huizinga (1986) in gold fish (temperature not available) and Chinabut (1989) in snakehead at 28.5°C, who found PMNs at the early hours after injection and the dominant role of them throughout the sampling period. Anderson & Roberts (1975) reported the appearance of PMNs in small numbers around blood vessels at 55 hours after wounding in *Salmo salar* at 23°C, and remained for 6 days, then disappeared. They suggested that different time scale in appearance of PMNs in fishes may have been the result of either a species difference becoming evident at higher temperatures, or the presence of bacterial factors providing a powerful attraction at high temperature. They concluded that attraction of PMNs to bacterial factors appeared only

to function at high temperature. Roberts & Bullock (1976) also noted that the exact period of the presence of PMNs in an inflammatory response depending on the type of insult and the temperature. They also stated that the role of the PMNs in inflammatory response in fish is relatively minor comparing to that in mammals.

One of the roles of PMNs documented in this study was participating in myophagia along with macrophages. At 4 hours after infection some PMNs and macrophages were observed located within the degenerated muscles which could be considered as the initiation of myophagia. Thereafter they were observed within the damaged muscles engulfing the remnants of the damaged sarcoplasms. Myophagia was completed by 7 days. Ventura & Grizzle (1988) reported the presence of large numbers of lymphocytes and macrophages as early as 24 hours after injection of *A. hydrophila* in channel catfish at 18°C,. Chinabut (1989) reported the presence of small numbers of macrophages in snakehead at 28.5°C in the damaged muscle area by 4 hours which increased by 6 hours. She stated that they were not principally attracted to the bacteria, but she did not mention the presence of PMNs along with macrophages in the damaged muscles.

PMNs in mammals are a major phagocytic cells which appear rapidly at the inflammatory area (Ellis 1977), while there are different comments about their function in fish. Roberts & Bullock (1976) believed a relatively minor role of the PMN leukocytes in fish response as a significant difference from the mammalian response. Klontz (1972) and Ellis (1976) reported that plaice and rainbow trout PMNs appeared not to be phagocytic towards carbon particles or bacteria. While Finn & Nielson (1971*a,b*) reported PMNs in rainbow trout as a phagocytic cell in a bacterial infection. Also it has been reported a phagocytic role for gold fish PMNs towards thorotrast

(Weinreb & Weinreb 1969) and bacteria (Watson *et al.* 1963) and for three-spined stickleback towards carbon (Phromsuthirak 1977).

The other role of PMNs seen in this study, was contributing in micro-abscess formation. A dense aggregation of PMNs in some areas of the lesion along with some amorphous substance, macrophages, degenerated muscles and nucleic debris formed a micro-abscess. Although the bacteria were not seen in the sections of some cases, *A. hydrophila* was re-isolated from the infected fish. As described by Anderson & Roberts (1975), the presence of bacteria could be a powerful attraction for PMNs to infiltrate, dominate and aggregate in the lesion area producing micro-abscess, especially considering the average ambient temperature (27.5°C) in this study. It also could be concluded from this study that the nature of the lesion in *A. hydrophila* infection, especially in early stages is suppurative. Chinabut (1989) also reported micro-abscess formation in snakehead in response to the *A. hydrophila* infection. She described micro-abscess as the most unexpected finding in her study which was exactly similar to that in higher animals, and mentioned that it is most uncommon in fish lesions. Roberts & Bullock (1976) stated that abscessation does not occur spontaneously in fish, although aggregates of PMNs which could be described as micro-abscesses, are very occasionally seen. Roberts (1989) described that liquefactive necrosis is a result of rapid enzymatic digestion of cells in tissue. He stated that liquefaction is produced either by enzymes released from host cells such as PMNs or by lytic toxins released by infecting bacteria.

The pathological changes at the site of injection occurred mainly in the muscular layers and also in the dermis because of their first contact and encounter with the bacteria, therefore the lesion could be considered as a dermal and hypodermal lesion. As the lesion developed, there was an accumulation of necrotic tissue and tissue fluid

exudate with some inflammatory cells under the dermal layer, extending to the deeper muscular layer causing a swollen lesion to the outside. This swollen and fluctuant lesion ultimately ruptured and released exudate, containing breakdown products of necrotic tissue and bacteria into the water. The epithelial layer at the point of injection and around, was also necrotic and destroyed. The early lesion showed massive muscular degeneration with less infiltration of inflammatory cells indicated a toxic condition.

The histopathological changes at the site of injection were similar to those reported by Thorpe & Roberts (1972) in rainbow trout, Brenden & Huizinga (1986) in gold fish, Ventura & Grizzle (1988) in channel catfish, Chinabut (1989) in snakehead, and Suthi (1991) in *Pontius* sp. and *Oreochromis niloticus*.

Kaper *et al.* (1981) reported that severe damage to the muscle at the area of injection may have been caused by the enterotoxin produced by *A. hydrophila*. Brenden & Huizinga (1986) suggested that the progressive skeletal muscle necrosis at the site of inoculation maybe due to elastase and protease which are produced *in vitro* by *A. hydrophila*, and elaboration of these enzymes *in vivo*. Scholz *et al.* (1974) suggested that the unusually low inflammatory cells in some necrotic tissues maybe caused by leukocidins produced by *A. hydrophila*.

Anderson & Roberts (1975) reported that scale regeneration in wounded salmon began at the wound edge at 10 days, and within the wound by 22 days after wounding at 23°C, while Chinabut (1989) reported the first appearance of the scale buds 5 days after wounding of snakehead at 28°C, and were well formed within 14 days. Protrusion of the scales close to the lesion area due to the dermal inflammatory oedema was begun at the early stages of inflammation in the present study. Some of these scales sloughed off

as the lesion developed. From the 18th day after inoculation onwards, some small newly formed scales were seen growing above the newly re-joined dermis in the stratum spongiosum. They were later seen above the healed lesion on the edges of the scar, well formed and arranged in an overlapping pattern in the same position as the original scales. These small new scales were formed and grew in the area of the healed lesion where originally no scales grow. It is necessary to mention that experimental fish in this study (mirror carp) has only one row of scale on the flank close to the dorsal fin along the lateral line. The relation between healing the lesion and these new scales was not investigated in this study. It could be a matter for further investigation, especially considering that in integumental wound healing (chapter 4) no new scale grew around the scar of the healed skin of mirror carp.

At 10 days after inoculation, in one of the sampled fish a typical foreign body granuloma was found in the lesion area. It was consisted of a limited amorphous pink staining substance, similar to a newly grown small scale, located in the centre of the granuloma and macrophages, epithelioid cells, nuclear debris which was encapsulated by fibrous tissue and lymphocytes. A distinct horse shoe shaped Langhans giant cell was also observed around this granuloma. Roberts (1989) suggested that if an acute inflammatory lesion does not resolve quickly, then chronic inflammation with proliferation of neighbouring support tissues develops. He also explained that chronic inflammation, with development of a proliferative lesion progressing to fibrosis is called granuloma which maybe caused by different foreign bodies and may have different colour and consistency. Roberts & Bullock (1976) described that certain types of particularly resistant foreign bodies within the skin, produce a very distinctive chronic inflammatory response characterised by presence of epithelioid cells around the

stimulus and also subsequent formation of giant cells by fusion of epithelioid cells. Also Timur, G. (1975) reported that the Langhans type giant cell is frequently seen in foreign body granulomata due to a variety of irritants. Considering these explanations, acute inflammation occurred during the early hours after inoculation of the fish in this study and then developed into chronic inflammation characterised by fibroplasia and fibrosis in the lesion area. The foreign body granuloma formation in the lesion area with presence of a giant cell is an unusual response to *A. hydrophila* infection which to this author's knowledge has never been reported before. It could be described that the limited amorphous, pink staining substance in the centre of the granuloma, maybe a newly grown scale which penetrated into the hypodermal area, considering that from the 18th day new scales were evident in the lesion area. If it is a new scale, it is difficult to explain and remained unclear how the newly grown scale penetrated into the subdermal area. However, whatever it is, the defence mechanism of fish considered it as an irritant which is resistant and could not be phagocytised by phagocytic cells, so it was surrounded by granuloma and giant cell formation to prevent irritation of the tissue.

The macrophages loaded with melanin granules, lipofuscin and ceroids were commonly found at late stages in the fibrous replacement area throughout the healing lesion. Melanin is often associated with lipid residues from the breakdown of cell membranes (Edelstein 1971).

Lipofuscin is a visceral melanin (Cappell & Anderson 1971), a peroxidised and polymerised breakdown product of lipid catabolism and tissue destruction (Miquel *et al.* 1977; Ventura & Grizzle 1988). It maybe the by product from the oxidation of tissue lipids during an inflammatory response as a consequence of peroxidase, released by PMNs (Roberts 1975*a*). One of the main sources of lipofuscin maybe degenerating

mitochondria, with their high content of polyunsaturated fatty acids (Agius & Agbede 1984). Lipofuscin in association with bacterial and viral diseases can indicate phagocytic clearance of necrotic lesions (Wood & Yasutake 1956). Undigested lipid-rich substances are usually accumulate as lipofuscins (Goudie 1988). Any metabolic disorder could make lipids difficult to digest and, in consequence, the lipids would accumulate in the fish tissue (Lamas *et al.* 1996). Under normal conditions, lipofuscins are endogenous pigments which accumulate in several organs with age and are yellowish-brown (Woolf 1986). However, depending on the original lipid and where the oxidation takes place, it can be a highly coloured product (Pearse 1985). Oxidised lipids are toxic for fish (Roberts 1989). Usually, these lipids are acid-fast, and in consequence are identified as ceroids. Ceroids are considered to be typical lipofuscins in an early stage of oxidation (Pearse 1985). Ceroid usually accumulates in the liver, spleen and kidney of fish fed with rancid lipids or which are vitamin E deficient (Smith 1979; Roald *et al.* 1981; Moccia *et al.* 1984).

The acid-fast pigment ceroid is commonly associated with metabolic disorders in laboratory animals (Wood & Yasutake 1956). It has been reported that fish with their high content of unsaturated fatty acids are particularly susceptible to formation of ceroid (Yasutake *et al.* 1965).

Muscle regeneration began at the day 4 after infection with the presence of some new small basophilic muscle buds, along with fibroplasia in the lesion area, and their growth progressed as the lesion developed. At the last stage of sampling, the regenerated and regrown muscles were well developed and filled all the damaged area of the lesion so that they were indistinguishable from the normal muscles.

Chinabut (1989) reported that the regeneration of myofibrillar buds in snakehead

infected by *A. hydrophila* at 28.5°C, was seen around day 6 in the area around the centre of the lesion and considered it as a distinctive feature which has not been described elsewhere in other species. However, the muscle regeneration in carp at 27.5°C, (present study) was faster than in snakehead.

It is described in literature that physiological and anatomical differences between fish species, different species defence mechanism, virulence of the bacterial strains, route of the introducing infection, and accuracy of the techniques used, are major factors in the degree of bacterial infection and subsequent effects on fish. In this study, it was determined that difference between individuals of a species was also another major factor.

Intramuscular route of infection was used in this study to inoculate the bacterial suspension. It could be said that the intramuscular injection is a suitable route for induction of the experimental *A. hydrophila* infection, considering that the normal route of infection in the wild is more likely to be via trauma or other damages to the skin, and not by the vascular, intraperitoneal or oral (via stomach) routes.

The results of this study indicated that the rate and degree of infection by the (T4) strain of *A. hydrophila*, used in this experiment, in healthy carp, depends on the individual defence mechanism of fish, and those fish which survive from the infection by 48 hours after infection, have a well developed capacity for dealing with bacteria. Wound healing, and muscle regeneration in these fish were faster than those infected experimentally by *A. hydrophila* mentioned before.

CHAPTER 8:

THE INFLAMMATORY RESPONSE OF CARP (*Cyprinus carpio* L.) TO *Aphanomyces invaderis* INFECTION

8.1 INTRODUCTION

Mycotic infections of fish by fresh water Oomycetes can develop at all stages of the fish's life cycle and as such are of considerable economic significance to the intensive fish cultivation industry (Pickering & Willoughby 1982). Fungi are distinguished principally by their complete lack of chlorophyll. They are responsible for a range of serious and economically important diseases of teleosts. Absence of chloroplasts means that they can not make use of photosynthetic pathways for energy production and therefore are bound to live a saprophytic or parasitic existence (Roberts 1989). There are at least 100,000 species of fungi, which show great diversity in their morphology. In relation to fish diseases, they are conveniently divided into two large grouping, those with cross cell walls, the septate fungi, and those which are aseptate or without cross walls. Fungi with aseptate mycelium are placed in grouping *Phycomycetes*; these include *Saprolegnia* spp., probably the most important fish pathogenic genus (Willoughby 1994).

Fungal infections in fish may arise as primary infections or as secondary invasion of tissues which are already damaged by viral, bacterial or other mechanical agencies (Khulbe & Sati 1981). Mycotic diseases of fish have been reported in both fresh and salt water environment (Wolke 1975).

Most living organisms under certain circumstances become subject to the attack of fungi, and fishes are no exception. It is well known that whenever freshwater fishes are roughly handled or even slightly bruised, fungus infection and a high rate of mortality are likely to follow. In addition, under both natural and hatchery conditions, almost all fish eggs are susceptible to fungus attacks (Scott 1964).

The family Saprolegniaceae contains the majority of fungi that have been associated with diseases in fish and shellfish (Willoughby 1994). Members of family Saprolegniaceae which have been recorded as parasitic in fish are *Saprolegnia*, *Achlya*, *Aphanomyces*, *Dictyuchus*, *Pythium*, *Leptolegnia*, and *Leptomites* (Alderman 1982). The most important genera of the family Saprolegniaceae in terms of pathogenicity, are *Saprolegnia, Achlya* and *Aphanomyces* (Willoughby 1994).

Slow-growing *Aphanomyces* strains have been isolated from fish suffering from red spot disease (RSD) in Australia (Fraser *et al.* 1992), mycotic granulomatosis (MG) in Japan (Hatai *et al.* 1977), and epizootic ulcerative syndrome (EUS) in South East Asia (Roberts *et al.* 1993). Lilley and Roberts (1997) showed that a single species of *Aphanomyces* is involved in RCD, MG and EUS. Willoughby *et al.* (1995) have proposed the name *Aphanomyces invaderis* for this species. (soon to be named *Aphanomyces invadans*. J.H Lilley, 1997, personal communication).

EUS caused by *Aphanomyces* has been reported in over 100 species of fish (Frerichs *et al.* 1988; Lilley *et al.* 1992). It has been shown that *Aphanomyces invaderis* can produce haemorrhagic lesions and high mortalities during the outbreaks of EUS (Chinabut *et al.* 1995), but there is no report to indicate that *Aphanomyces invaderis* can breach the fish skin integrity, primarily.

The present investigation was conducted to study the inflammatory response of healthy mirror carp (*Cyprinus carpio*) to *Aphanomyces invaderis* which is known as a causative agent of EUS.

8.2 REVIEW OF THE LITERATURE

8.2.1 Saprolegniaceae and Saprolegniasis

The family Saprolegniaceae belongs to order Saprolegniales within the class Oomycetes. Almost all of the significant fish pathogens are within this family. They are water moulds possessing a profusely branching non-septate mycelium, appearing like cotton-wool tufts in water. The hyphae vary considerably in form between species, but all contain cellulose. Although the hyphae are non-septate, the reproductive structures are separated from the somatic hyphae by means of a septate zoosporangium containing biflagellate zoospores (Roberts 1989). The Saprolegniaceae is the largest family of the order with approximately 150 species (Dick 1973). Some species are found in brackish water but salinity higher than 2.8% limit their distribution (Te Strake 1959). All of the genera of the Saprolegniaceae occur throughout the world, but their incidence and importance does seem to vary on a geographical basis (Willoughby 1994).

Most genera of the family Saprolegniaceae can be identified from hyphal characteristics, the nature of the zoosporangium, and subsequent spore release, but identification of the different species has traditionally depended on characteristics of the oogonia and antheridia (Alderman 1982).

Saprolegniasis is a term frequently used to describe fungal infection of skin and gill. It may involve any of a wide variety of fungi of the orders Saprolegniales, Peronosporales, and Leptomitales, characterised by dermal ulceration, and necrosis of muscle in conjunction with an inflammatory response (Wolke 1975; Alderman 1982).

Basically the Saprolegniaceae function as saprophytes, is living on dead materials, but they also have the capacity to attack live fish material under favourable circumstances such as a very high local challenge inoculum of spores and mycelium. They usually occur externally on fish and are present in limited patches on the body surface of the host or are restricted, at least initially, to one or more of the fins (Willoughby 1994). This seems to be a consequence of crowded conditions leading to fin damage and then fin rot, culminating in fungus obtaining entry into the damaged host tissues (Richards & Pickering 1978). Also dead eggs soon attract spores of Saprolegniaceae, if they are present in the water supply, and establish growing centres of the fungi (Willoughby 1994).

Fungal infections (mycosis) frequently occur in wild and farmed fish as a consequence of stress and sexual maturity as well as effecting eggs and juvenile fish. Within this taxonomically diverse group there are examples of secondary as well as primary causative agents of disease. The former, generally infect the body surface and gill often covering large areas, and the later, usually infect and multiply within the host tissue (Bruno 1989).

8.2.2 Diseases and experimental studies associated with Saprolegniaceae

There is a considerable literature on the diseases associated with fungi in aquatic animals which indicate that they are a serious problems in both wild and cultured fishes.

The identity and significance of the mycotic component of Saprolegniaceous infections has been the subject of serious study and debate for over 100 years (Neish 1977). The first clinical demonstration of any vertebrate fungal infection was illustrated by Anderson in 1748 who studied Oomycete infection in roach (Ainsworth 1976).

Buckland *et al.* (1880) also recognised Saprolegniaceous infections of a salmon (*Oncorhynchus* sp.). A comprehensive study dealing in any detail with the aetiology and pathology of Saprolegniaceous infections among fish is that of Rucker (1944) who reported that the fungus, *Saprolegnia parasitica*, caused a secondary infection and would not typically initiate an infection. This assumption was questioned by many workers who investigated on different species of fish, and suggested that Saprolegniaceous fungi could be the major, and perhaps the only infectious component of the diseases with which they are associated (Neish 1977).

Wolke (1975) described that the gross lesions of Saprolegniasis are quite characteristic. These include focal, epidermal, white to brownish cotton-like masses randomly distributed on the gill and surface of the fish. Advanced lesion are frequently ulcerated with the underlying musculature exposed. He also stated that the histological examination reveals mycelial masses overlying zones of necrotic epithelium or overlying zones of frank ulceration. The mycelial masses are weakly eosinophilic and PAS-positive, very irregular, and often contain unidentified debris. Individual hyphae may be seen extending into the dermis and also may be between muscle fibres with a slight inflammatory response. Necrosis of muscle may be present, and there is a lymphocytic and macrophagic infiltration of the affected area.

Shanor and Saslow (1944) investigated a serious outbreak of fungal disease on aquarium fish and showed, using microscopical examination cultural studies, that aseptical *Aphanomyces* was responsible for the disease. The fungus usually developed most extensively in the dorsal region and its activity in the musculature seemed to be responsible for spinal curvature, then the hyphae began to extend out from the skin. None of the fish that became infected recovered, and usually within a week after lesions

developed, the fish died. They were unable to identify the species of *Aphanomyces*, but suggested that it is a facultative parasite which may become destructive under suitable conditions.

Neish (1977) in his observations on Saprolegniasis of adult sockeye salmon (*Oncrohynchus nerka* Walbaum) suggested that the *Saprolegnia* spp. are pathogens capable of invading living tissue and, in some cases, the fungus maybe the primary infectious agent causing the lesions. He also showed that the fungus was not tissue specific.

Willoughby and Pickering (1978) described that one characteristic of the sexual dimorphism, which exists in the structure of the skin of mature brown trout, is a reduction in the number of mucus-producing goblet cells in the epidermis of the male fish during the spawning period. They suggested that changes in structure of the epidermis of sexually mature, male rainbow trout may increase its vulnerability to *Saprolegnia* infection. Willoughby and Pickering (1977) have described before that mucus may help to protect the fish from fungal infection by removing viable spores from the body surface.

Richards and Pickering (1979) showed that fungal infection of both wild and hatchery-reared sexually mature brown trout (*Salmo trutta* L.), caused a significant reduction in concentration of the major ions in the serum of infected fish. They suggested that primary cause of death was osmoregulatory breakdown resulting in a lethal haemodilution.

Noga and Dykstra (1986) reported that they could not find evidence for a primary bacterial aetiology for the lesions in ulcerative mycosis (UM) in menhaden (*Brevoortia tyrannus* Latrobe). They found Oomycete fungi in over 95% of all the

lesions, actively contributing to the pathogenesis of the epidermal lesions and maybe responsible for initiating the ulcers. In another investigation, Noga *et al.* (1988) described this disease (UM) in the same species as a granulomatous disease associated with infection by Oomycete fungi, which probably began as a skin infection, then involved underlying muscle and viscera. The consistent presence of fungus in the smallest detectable lesions suggest that it played a prime role in initiating the infection.

Aphanomyces has been most commonly cultured from UM, but *Saprolegnia* has also been isolated (Dykstra *et al.* 1986). Dykstra *et al.* (1989) also showed that the predominant fungi isolated from all the menhaden lesions examined belong to the genus *Aphanomyces.*

Callinan *et al.* (1989) in their study on pathology of RSD in sea mullet (*Mugil cephalus* L.) described necrotising dermatitis as a severe, locally extensive, granulomatous lesion associated with invasion of dermis and underlying skeletal muscle by numerous, non-septate, fungal hyphae. They also described erythematous dermatitis as a mild to severe, focal, chronic active dermatitis without fungal involvement, and mentioned that lesion intermediate between these two forms, with small to moderate numbers of fungal hyphae in dermis and skeletal muscle also occur. They concluded from their findings that erythematous dermatitis lesions and intermediate-type lesions subsequently resolve while necrotising dermatitis lesions consistently develop into dermal ulcers, with associated severe necrotising granulomatous myositis. They failed to identify the invasive fungus which caused the RSD. Later, Fraser *et al.* (1992) showed that all fungus isolates belong to a single species within the genus *Aphanomyces* which is a constant finding in the dermal ulcers of RSD.

A histopathological comparison was made by Wada *et al.* (1996) between ayu (*Plecoglossus altivelis*) and carp (*Cyprinus carpio*) artificially infected with *A. piscicida* as the causative fungus of MG. They observed typical pathology of MG in ayu, while no gross signs of inflammatory responses were observed in carp. Carp responded histologically to the fungus more quickly and intensively than ayu with suppressing the fungal activity by the granulomatous inflammatory response. They concluded that lesions produced by fungus in carp were restricted to a small area, while it was extended to neighbouring tissue in ayu because of weaker defence mechanisms.

Shanor and Saslow (1944) described *Aphanomyces* sp. infection of the skin and muscle of several freshwater fish species maintained in an aquarium. Miyazaki and Egusa (1973) and Noga *et al.* (1988) showed that UM and MG have many pathological similarities to RSD. Later, Noga (1994) also reported that UM of fish of the Western Atlantic maybe part of the same syndrome as EUS. Lilley and Roberts (1997) in their study confirmed reports of similarities in the histopathology of EUS, RSD and MG, but they could not find any evidence of the invasive nature of the UM fungus as described by Noga *et al.* (1988).

EUS is the term generally used to describe a serious epizootic condition of wild and cultured freshwater fishes which has spread throughout South East Asia and extended into the Indian sub-continent (Tonguthai 1985; Roberts *et al.* 1992). The disease affects many species, but losses are most obvious in the snakeheads, *Channa spp.*, *Puntius spp.*, and in culture of the Indian major carp. It produces high levels of mortality in fish of all ages over a very short time scale, and it effects a wide range of species at the same time (Roberts *et al.* 1986). Subasinghe (1993) pointed out that the causative agent(s) of EUS are capable of horizontal transmission. Roberts *et al.* (1993)

found during the isolation studies, that fungi of the *Aphanomyces* group were an important component of the lesion flora in EUS. They also revealed in direct microscopic examination of lesions of affected fish that an obvious mycelium of a branched, aseptate fungus was present in all cases.

Karunasagar *et al.* (1994) in their study on mycological aspect of EUS in India showed that *Aphanomyces* was one of the most common fungi associated with the ulcerative condition. The fungal isolates could not infect uninjured EUS susceptible fishes in the laboratory, but when placed under the scales or skin by damaging the intact skin, lesion developed which subsequently healed without treatment. They suggested that EUS in the natural environment is of complex aetiology involving perhaps more than one pathogen.

Hatai *et al.* (1994) identified fungi isolated from the lesions of a disease in ornamental fish, dwarf gourami (*Colisa lalia*), as a member of the genus *Aphanomyces*. They stated that this fungal infection closely resembled in pathology the MG which is known in cultured ayu (*Plecoglossus altivelis*) in Japan, and suggested that the causative fungus was a new species of the genus *Aphanomyces*. Wada *et al.* (1994) also continued the study on histopathological features of this mycotic disease and reported that the disease was characterised by systemic granulomatous lesions associated with *Aphanomyces* sp. They compared the features with those of other mycoses, such as; MG, UM, RSD and EUS, and concluded that the infection in dwarf gourami caused by *Aphanomyces* sp. shared similar histopathological features including mycotic granulomatous lesions, with those of earlier reported mycoses.

Callinan *et al.* (1995) showed that the *Aphanomyces* isolates from EUS in the Philippines and RSD in Australia, were morphologically and culturally similar. They

concluded that their findings combined with epizootiological and pathological similarities between EUS and RSD, suggested the two syndromes were identical and a single *Aphanomyces* sp. may have been the primary infectious cause.

Very recently, Lilley and Roberts (1997) compared pathogenicity and culture studies of the *Aphanomyces* involved in EUS with other fungi. They infected snakehead (*Channa striata* Bloch) experimentally, with zoospores of 58 fungal isolates comprised *Aphanomyces* isolates from EUS-affected fish, saprophytic *Aphanomyces*, *Achlya* and *Saprolegnia* spp. from infected waters, and further Saprolegniaceous fungi involved in other diseases of aquatic animals. They found that only *Aphanomyces* strains isolated from EUS-affected fish, and RSD reported from Australia, and also MG described from Japan, were able to grow invasively through the fish muscle and produce the distinctive EUS lesions. They concluded from their findings, as a further evidence, that a single species of *Aphanomyces* is responsible for much of the characteristic pathology of EUS, and that the same species is involved in RSD and MG. This fungus is *Aphanomyces invaderis* (Willoughby *et al.* 1995).

8.2.3 Temperature and fungal infection

The significant effects of temperature on the development of fungal infections has been shown by many workers. Scott (1956) has shown that the motility of the primary zoospore in *Aphanomyces patersonii* could be controlled by variation in temperature, and Salvin (1941) has claimed that below 10°C, the primary zoospore did not behave in a normal manner.

It has been shown that Saprolegniasis of eels, ceased when the water temperatures rose above 18°C (Hoshina & Ookubu 1956; Hoshina *et al.* 1960). Roth

(1972) reported that fungal infection in experimental white suckers generally occurred when temperature was lower than 10°C.

Neish (1976) reported that both hyphal growth and zoospore production by the fungus decrease with decreasing temperature, but it would seem that despite this lower growth and reproduction rate of the fungus, low temperatures reduce the resistance of the host so much that even small numbers of zoospores can initiate infection.

Pickering and Willoughby (1982) described that marked differences in the tolerance of fungi to high and low temperatures are found and these seemed to be indicative of ecological adoption. The temperature response characteristics of pathogenic fungi isolated from teleost fish seem to reflect, to a considerable degree, the thermal preferences of the host fish.

Sohnle and Chusid (1983) in their study on filamentous fungal infection in rainbow trout showed that the fungi of the family Saprolegniaceae normally grow much more rapidly at temperatures higher than those usually encountered by trout. They also reported that healthy rainbow trout were able to resist infection after inoculation with Saprolegniaceae fungi, even at very cold temperature (2-4°C).

Roberts (1989) described that temperature has a significant effect on the development of *Saprolegnia* infection. Whilst infection following trauma may occur at any temperature compatible with fish life, most epizootic occur when temperatures are low for that fish species.

Fraser *et al.* (1992) observed small increases in mean colony diameter at 24 hours after incubation of *Aphanomyces* sp. at all temperatures. Subsequently, no growth occurred at 3 or 37°C. There were significant differences between isolates in their subsequent growth at the other temperatures. They reported maximum growth for the

bream and mullet isolates occurred at 31°C, and for the whiting isolate at both 22°C and 25°C.

Bly *et al.* (1992) reported that occurrence of "winter kill" syndrome in channel catfish ponds maybe correlated with rapid decrease in water temperatures known to induce *in vivo* immunosuppression in channel catfish held in the laboratory (Bly & Clem 1991) and with the presence of a ubiquitous fungus identified as a member of the genus *Saprolegnia*.

Roberts *et al.* (1993) showed that temperature-growth relationships of putative pathogen *Aphanomyces* and saprophytic *Aphanomyces* isolated from snakehead, grown on GP agar, were different. At temperatures between 10 and 31°C, linear growth rates of the putative pathogen *Aphanomyces* were only a third to a half of those of the saprophytes. They also stated that the saprophytic *Aphanomyces* isolates from water or sediments grow well at 37°C whereas the putative pathogens die immediately. This could be a most useful criterion to identify the *Aphanomyces* pathogens. Later Willoughby and Roberts (1994*a*) also reported that the pathogenic isolates of *Aphanomyces* showed slow growth rates at all temperatures compared with saprophytic isolates of *Aphanomyces*. They suggested that the basic temperature-growth relationships for EUS *Aphanomyces* seem to be a common pattern, irrespective of the geographic origin.

Saprolegnia parasitica is less aggressive when water temperatures are higher or after a sudden fall in the temperature. This, probably, acts as a physiological stress on the fish, making them particularly vulnerable (Willoughby 1994).

Chinabut *et al.* (1995) infected snakehead (*Channa striatus* Bloch) experimentally with the specific pathogenic *Aphanomyces*, isolated from fish affected

with EUS. The infected fish were held at three different temperatures; 19, 26 and 31°C. The inflammatory response and mycotic granulomatosis was marked in fish kept at 26 and 31°C. Mortalities in the fish kept at 19°C were considerably higher than those kept at 26 and 31°C and all stages of the inflammatory process were slower to develop and reduced in extent at the low temperature. They concluded that their findings help to explain why the mortalities from EUS occur when water temperatures are low.

Khulbe *et al.* (1995) showed that high temperature (>28°C) retarded the disease process of zoosporic fungi (*Achlya* spp., *Aphanomyces* sp., *Saprolegnia* spp., *Pythium* sp.) in adult fishes of the species *Mastacembelus armatus, Mystus vitatus, Nandus nandus, Tor putitora* and *Tor tor* of Naini Tal district in India.

8.2.4 Fungus toxin and other properties

Neish (1977) reported that to date there is no evidence to suggest that the fungi can produce any systemic toxins. The damage done by the fungus can be directly related to the tissue destroyed in the immediate area of the hyphae. Pickering and Willoughby (1982) also stated that there is no evidence that pathogenic *Saprolegnia* strains produce any toxins that might be transmitted systemically. Buttrell (1974) suggested that enzyme and other toxins are commonly present in plant-pathogenic fungi.

Peduzzi and Bizzozero (1977) suggested that proteolytic enzyme activity could allow certain *Saprolegnia* species to convert from a saprophytic form of existence to a necrotrophic mode of nutrition feeding on fish tissue.

Alderman and Polglase (1986) reported that many micro-organisms which are in competition with each other, especially fungi, produce antimicrobials, for example the

well-documented members of the genus *Penicillium*. Bly *et al.* (1992) also confirmed in their preliminary studies that *Saprolegnia* sp. produces an, as yet unidentified, antimicrobial agent(s) which inhibits the growth of other micro-organisms. The damage due to *Saprolegnia* fungi can be directly related to a chymotrypsin-like proteolytic enzyme activity (Peduzzi *et al.* 1976; Peduzzi & Bizzozzero 1977). Shafer *et al.* (1990) also stated that *Aphanomyces* seems to have special proteins in its mycelium, which gives it enhanced salinity tolerance.

However, assuming the fungus is the only pathogen, the time of death of infected fish will be a function of the growth rate of the fungus, the initial site of the infection, the type and quantity of tissue destroyed, and the ability of the individual fish to withstand the stress of the disease (Neish 1977).

8.2.5 Biological aspects of the *Aphanomyces*

Shanor and Saslow (1944) described a long time ago that *Aphanomyces* species are more widely known as plant parasites or as parasites of such invertebrates as the European crayfish and of some smaller fresh water *crustacea*. They also suggested that adults as well as young fish are attacked and the virulence of the organism on both age groups are equally severe. Scott (1961) stated that de Bary in 1860 established a new genus of the family Saprolegniaceae, *Aphanomyces,* as a result of his continued study of the aquatic fungi and algae. Hatai (1989) described that *Aphanomyces* spp. are well known as common fungal pathogens of fish and shell fish. Scott (1961) reported that the genus *Aphanomyces*, comprising approximately 30 species, is among the smaller and less frequently encountered genera of the Saprolegniaceae. A list of *Aphanomyces* spp. is as follows (Scott 1961; Dick 1973):

A. stellatus	*A. astaci*	*A.exoparasiticus*	*A. hydatinae*	*A. apophysii*
A. scaber	*A. helicoides*	*A. raphani*	*A.acinetophagus*	*A. daphniae*
A. laevis	*A. parasiticus*	*A. cochlioides*	*A. americanus*	*A. patersonii*
A. phycophilus	*A. ovidestruens*	*A. cladogamus*	*A. amphigynus*	*A. bosminae*
A. norvegicus	*A. euteiches*	*A. camptostylus*	*A. sparrowii*	*A. irregulare*

None of these *Aphanomyces* which Scott (1961) listed in his monograph on *Aphanomyces*, are fish pathogens (Willoughby *et al.* 1995).

Members of the genus *Aphanomyces* differ from other Saprolegniaceous fungi in the formation of long, delicate zoosporongia, undifferentiated from the vegetative hyphae. The zoospores are formed in a single row within the sporangium and encyst at the mouth immediately upon emergence. Species distinctions are based on the nature of the sex organs (Scott 1964).

Collected data recorded for water samples indicates that members of the genus *Aphanomyces* as well as most other genera of this family are abundantly represented in fresh waters. Isolates of *Aphanomyces* spp. have appeared more frequently from water samples containing algae, or samples rich in various plant and animal debris. Environmental factors such as temperature, and the amount and type of water may have a marked effect on the growth and morphology of this fungus. The isolates of *Aphanomyces* survive for long periods only at temperatures of 12-20°C (Scott 1961).

With few exceptions, members of the genus *Aphanomyces* maybe cultivated on artificial media. Species occurring in nature as parasites of algae, aquatic animals and phanerogams may be treated similarly and consequently are to be considered only facultative parasites. The macroscopic appearance of *Aphanomyces* in water culture is that of a very delicate, almost imperceptible, halo of filaments which develop radially

from the submerged substratum and extend outward into the surrounding water (Scott 1961). Saprophytic *Aphanomyces* strains are abound in tropical waters, readily contaminate the surface of lesions of affected fish and grow rapidly, but the delicate *Aphanomyces* of EUS is slow-growing at all temperatures up to 37°C (Willoughby *et al.* 1995).

The dimorphic species of Saprolegniaceous fungi include forms in which there are successively formed two morphologically distinct zoospores. The primary zoospore is generally pyriform, apically biflagellate, and actively motile. In the genus *Aphanomyces* and related genera this first stage is suppressed within the zoosporangium and the primary zoospores normally encyst at the orifice following discharge. The later bodies are termed "primary zoospore cysts" and ultimately germinate directly to produce the secondary zoospore. After a period of encystment, the contents of the primary zoospore cysts emerge as typical reniform, biflagellate zoospores, the secondary zoospores swim actively. The rounding up and encystment of the secondary zoospores following a period of active swimming and the germination of these secondary zoospore cysts by means of a single germ tube to form a branched mycelium, completes the cycle of asexual reproduction (Scott 1961). Usually, within a short period following the cessation of asexual reproduction or, less frequently, simultaneously with zoospore formation, sexual reproduction organ appear on the vegetative filaments. Morphologically, the sexual reproductive organs are of two types, the female organ or " oogonium ", and the male organ or, " antheridium ". Oogunia are variable in shape, size and in the ornamentation of their walls. The type of oogonial wall ornamentation is the primary characteristic used for the identification of the species of *Aphanomyces* and the

separation of these species into three large groups. However, the identification of *Aphanomyces* spp. relies on the morphology of oogonia and antheridia (Scott 1961).

Aphanomyces is distinguished from *Achlya* by its narrow, delicate mycelium and by its narrow sporangia containing a single row of spores. Asexual reproduction in *Aphanomyces* is variable (Webster 1980).

Pickering and Willoughby (1977) in their study on epidermal lesions and fungal infection on the perch (*Perca fluviatilis* L.) showed that several different fungi may occur together and colonise lesions on the body surface of a single fish. They stated that with the enlargement of the lesion and progressive growth of the fungi it was clear that *Achlya*, *Aphanomyces* and to a lesser degree *Saprolegnia* also became established and an *Aphanomyces* isolate produced oogonia at 20°C which was identified as *A. laevis* de Bary.

Although members of the genus *Aphanomyces* have been found growing parasitically on fish, they are not generally regarded as playing a major role in fish mycoses in fresh water. The most important role of *Aphanomyces* in aquatic animal pathology is in the crustacea, where *A. astaci* is responsible for mortalities of feral crayfish known as crayfish plague. It grows in the host's exoskeleton (Alderman 1982).

Hatai (1980) reported that a member of the genus *Aphanomyces*, which he believed it to be a new species, has been identified as responsible for MG in pond cultured ayu (*Plecoglossus altivelis*) and refer to as the MG-fungus.

Dykstra *et al.* (1989) reported that studies comparing the growth and sporulation capabilities of the test *Aphanomyces* demonstrated that the river water was the best medium for growth and sporulation. Also estuarine water is not inimical to growth of *Aphanomyces* and maybe stimulatory. They also showed that bacterial growth routinely

prevented isolation of *Aphanomyces* sp. When fish muscle was contaminated by bacteria due to poor aseptic technique, *Aphanomyces* did not grow effectively.

In Japan, *Aphanomyces piscicida* was isolated from MG affecting wild and cultured fresh water fishes (Hatai *et al.* 1977; Hatai *et al.* 1984). In the south-eastern United States, Dykestra *et al.* (1989) associated one or more *Aphanomyces* sp. with an ulcerative mycosis affective several estuarine fish species, including the commercially important Atlantic menhaden (*Brevoortia tyrannus* Latrob).

Dykstra *et al.* (1986) isolated *Aphanomyces* and *Saprolegnia* from characteristic lesions of UM on Atlantic menhaden and other fish. The cultural studies of one of the *Aphanomyces* isolates revealed that the fungus was stimulated to increased vegetative growth and improved zoosporulation by low levels of NaCl. The salt tolerance of this fungus was higher than the known recorded limits for Oomycetes in regard to zoosporogenesis. They suggested that there could be a complex interaction between the fungus, possibly pre-stressed fish, and the salinity of the water systems.

Hearth and Pedgett (1990) have demonstrated that spores of an *Aphanomyces* isolate from ulcerated menhaden will germinate in sea water in the presence of exogenous nutrients. They found from respiration during prolonged salinity exposure as well as hyphal morphology, that the degree of salinity tolerance generally was greater than previously reported for this or other Saprolegniaceous fungi. Later, Fraser *et al.* (1992) showed that at all salt concentrations, small changes in mean colony diameter of the three isolates of *Aphanomyces* occurred after 24 hours of inoculation. Further growth declined progressively as salt concentration increased, and no isolate made further growth on media containing 12, 16 or 20% NaCl. However, they concluded that

the *Aphanomyces* isolate would not grow at salinity more than 12% and would not sporulate at more than 2%.

Whiffen (1945) established that the Saprolegniaceae have no vitamin requirements, and Roberts *et al.* (1993) found it difficult to maintain *Aphanomyces* isolates on glucose-peptone (GP) agar. Therefore they held the *Aphanomyces* isolates in GP plus $1gl^{-1}$ yeast extract, which was used as a broth (GPY).

Aphanomyces in culture is generally much finer with narrower hyphae than is the mycelium observed within the tissues of EUS affected. *Aphanomyces* sp. isolated from the depths of the lesions of the EUS affected fish could produce a very delicate distinct, *Aphanomycete*, which is physiologically and morphologically distinct from the numerous saprophytes of the lesion and when introduced into the tissue of normal fishes this fungus is highly invasive (Roberts *et al.* 1994).

Aphanomyces astaci has been reported as a parasite of European crayfish (*Astacus astacus*) (Sparks 1985). *A. piscicida* was reported as a pathogen of MG in ayu in Japan (Hatai & Egusa 1978; Hatai 1980). UM in Atlantic menhaden in the USA is also caused by *Aphanomyces* sp. (Noga *et al.* 1988). *Aphanomyces* sp. was reported as a causative agent for RSD in sea mullet (*Mugil cephalus*), yellow fin bream (*Acanthopagrus australis*) and san whiting (*Sillago ciliata*) in Australia (Fraser *et al.* 1992). Epizootic ulcerative syndrome (EUS) is also thought to be caused by *Aphanomyces* sp. (Lilley *et al.* 1992; Roberts *et al.* 1993). The infection mode, the histopathology and the aetiology of these diseases reported by various authors are closely similar to each other, which pathogens grow in the tissues rather than on the surface of aquatic animals (Hatai *et al.* 1994).

Willoughby and Roberts (1994*b*) showed that zoospores of an *Aphanomyces* from an ulcer on an EUS-affected snakehead fish (*Channa* sp.) could lose their motility following physical shock (agitation with glass fragments), or chemical (ionic) shock with 100 mg Ca/l, but motility was regained after 4.5 hours, without an intervening encystment phase. They suggested this as an indication that, in this fungus at least, zoospores might be transported, immobile, by water currents and encyst only when they alight (or perhaps are moved against) a suitable surface for encystment.

8.2.6 *Aphanomyces invaderis*

The species *invaderis* of the genus *Aphanomyces* was the fungus species used in the present study as the pathogen. Willoughby, Roberts and Chinabut (1995) were first who isolated this species from the tissues of EUS infected fish, identified as a new species and named it "*Aphanomyces invaderis*". They described its detailed biological characteristics as follows;

"An aquatic fungus with wide, aseptate mycelium, 11.7-16.7μm across, *in situ* in the tissues of EUS diseased fish, but mycelium more narrow in culture, only 8.3 μm across in young advancing hyphae. Undulating or wavy mycelium occurs when the fungus dies in culture or in EUS diseased fish. The fungus is very slow-growing in culture gaining only 4 mm radius a day on glucose-peptone agar at 24°C; the fungus grows at 31°C but dies at 37°C. The mycelium may bear sporangia, which are no wider than the background mycelium, about 10 μm across. Sporangia are terminal on mycelia and often complex, with primary zoospores emerging laterally and encysting immediately; the encysted spores are held together, in balls. Complex sporangia with four lateral points of emission of zoospores are 630-930 μm long; sporangia with three

lateral points of emission of zoospores are 430-540 μm long; sporangia with only two or one lateral points of emission of zoospores are only 330-470 μm long. Primary zoospores cysts, 6.7-10 μm diameter, subsequently release secondary, motile zoospores, with two flagella. Following dehiscence a sympodial branch of mycelium develops just below the empty complex sporangium. Mature zoospores which fail to escape from dehisced sporangia retain good viability and germinate readily, often as a large multiple unit, evidently derived from several fused zoospores. These germination units may possibly be the organs of dissemination, since chlamydospores are problematical at present and oogonia so far unknown. Pathogenic in various freshwater fish, especially the snakehead (*Channa* spp.), but also the half-beak (*Xenetodon cancila*), and the rohu (*Labeo rohita*) in Thailand, Bangladesh and the Philippines".

It has recently become apparent that the name *A. invaderis* may be changed to *A. invadans* in the forthcoming edition of the Index of Fungi (J.H. Lilley, 1997, personal communication.).

Lilley and Roberts (1997) confirmed that *Aphanomyces invaderis* is responsible for the fungus-related lesions in EUS-affected fish, and stated that *A. invaderis* is also involved in RSD and MG. They also showed that *A. invaderis* is potentially lethal at 22°C, but is unable to infect noble crayfish (*Astacus astacus*), which is one of the natural hosts for *Aphanomyces astaci*.

Although the ecology of *A. invaderis* is a mystery as no isolates have been obtained from natural water bodies, early MG and RSD reports indicate that its geographical range probably extended from Japan to Australia by the early 1970s (Lilley & Roberts 1997).

The failure of all the EUS, RSD and MG isolates to grow at 37°C on GPYA is characteristic of *A. invaderis* (Lilley & Roberts 1997) which confirms the description of Willoughby *et al.* (1995), using glucose-peptone (GP) agar, for *A. invaderis*.

Thompson *et al.* (1997) investigated the antibody response of snakehead fish (*Channa striata*) to *Aphanomyces invaderis*, in an attempt to develop a vaccine against EUS. They found similarities in the banding patterns in SDS-PAGE (sodium dodecyl sulphate polyacylamid gel electrophoresis) protein profiles between the extracts of *A. invaderis* isolated from EUS-affected snakehead fish in Thailand, *A. piscicida* isolated from MG infected Ayu in Japan, and *Aphanomyces* sp. obtained from grey mullet infected with RSD in Australia. They also found that fish infected with *A. invaderis* (strain PA7) produced antibodies against on 10kDa band. When fish were immunised with fungal extract, known to contain this band, they do not produce antibodies. They concluded that the fungus has to be alive and growing to elicit an immune response against the 10kDa band, which is a serious implication for the development of vaccines against EUS.

Lilley and Inglis (1997) investigated susceptibility of 54 isolates of various fish-pathogenic and saprophytic fungi to some of the antibodies, fungicide and disinfectants. *Aphanomyces invaderis* associated with EUS, other *Aphanomyces* isolates from RSD and MG, and also crayfish plague fungus, *Aphanomyces astaci*, were more sensitive to most of the chemical agents than the other fungi tested. They also showed that *A. invaderis* can be clearly distinguished from saprophytic *Aphanomyces* sp. tested by its greater susceptibility to malachite green, sodium chloride and sodium hypochlorite.

Lilley *et al.* (1997) showed, using RAPD-PCR, that *A. invaderis* from EUS-affected areas and *A. piscicida* from MG outbreaks are not only the same, but probably

constitute a single clonal genotype. They also showed in their study, using 20 isolates of *A. invaderis* from several locations in Bangladesh, Thailand, Indonesia, Philippines, Australia, and Japan, that all the *A. invaderis* isolates had identical nucleotide sequences within the 18S nuclear rRNA gene region. The results of their study indicated an extreme lack of genetic diversity between all *A. invaderis* isolates.

Chinabut *et al.* (1995) reported that *Aphanomyces invaderis* can produce advanced haemorrhagic lesions and high mortalities during the outbreaks of EUS, and Lilley *et al.* (1997) also confirmed that *A. invaderis* can produce severe pathological changes in several European salmonid species.

8.3 PILOT STUDY

A pilot study was carried out using a small number of mirror carp as experimental fish and rosy barbs (*Puntius conchonius*) as control fish. This pilot study was conducted to determine the following:

1) To assess the procedures by which the susceptibility of mirror carp to infection with *A. invaderis* could be compared to that of rosy barbs.

2) To evaluate the method of fungus culture and zoospore production so that zoospore suspension could be reliably prepared and used as the inocula for injection.

3) To determine the optimum dose of inoculum, needle gauge and sampling time.

4) To practise the procedure for trimming, processing and sectioning the samples.

5) To choose the best staining method for observing fungus in tissues.

Puntius spp. are known to be very susceptible to EUS (Lilley *et al.* 1992; Khulbe *et al.* 1995) and infection with the EUS-associated fungus, *Aphanomyces invaderis* (Roberts *et al.* 1994). Therefore, given the easy availability of the ornamental species rosy barb, this was chosen as the positive control in this trial.

A zoospore suspension as the inoculum for injection was prepared using the routine method of the mycology laboratory in the Institute of Aquaculture (this method will be explained later in the definitive study). This suspension was examined under the phase contrast microscope to evaluate the presence of motile zoospores.

Five mirror carp were inoculated with 0.05, 0.1 (two fish), 0.2 and 0.3ml of spore suspension in the left dorsal myotome muscle using a 1ml syringe and needle gauge 25.

Six rosy barbs were injected with 0.1ml of the same inoculum at the same site of the dorsal muscle and the same time, using the same syringe and needle size.

One mirror carp was sacrificed at 6 hours, 1, 3, 5 and 7 days post-injection. Tissue blocks containing the injection site were cut out and fixed in 10% buffered formalin for at least 24 hours prior to processing. Each tissue block was processed using routine histology methods. The tissues were trimmed and cut down the middle into two pieces after processing, to be assured of finding the right orientation of the injection site and having the injection area in the sampled tissues. Thereafter the tissue pieces were embedded into wax, decalcified and sectioned at 5 μm.

For comparison one rosy barb (control fish) was sacrificed and sampled, fixed, processed, trimmed and cut, embedded into wax, decalcified and sectioned at the same time and also using the same methods as the experimental fish.

H & E, immunohistochemistry (IHC) using polyclonal rabbit anti sera raised against *Aphanomyces invaderis* (isolate PA7) mycelial extract [extract prepared as described by Thampson *et al.* (1997)], and Grocott staining methods were used to stain the sections.

One rosy barb was left for 17 days after injection when it died due to severe fungal infection.

No gross pathology was observed in either experimental or control fish during the period of the pilot experiment. One rosy barb that had been left after this period showed lack of activity, weakness, loss of appetite, imbalance in swimming and redness at the site of injection on the 16th day after inoculation. On day 17 post-injection this fish died and a lump of fungus mat was seen on the surface of the body in the injection area.

Microscopic examination of the sections stained by H & E showed that in carp, an inflammatory response in the tissues around the injection site had occurred at the early stages after inoculation and resulted in granuloma formation surrounding the fungal pathogen in the late stages of the pilot experiment. Briefly, the characteristic features of a chronic inflammation process namely: infiltration of the inflammatory cells, vascularization and fibrosis, were observed in the carp. Fungus was observed in the injection area using both IHC and Grocott staining methods. However, in control fish, which were known to be susceptible to infection with *A. invaderis*, the inflammatory response was much lower and slower than in carp. Only in the late stages were minimal macrophage infiltration, myophagia, fibrosis and vascularization observed. The dominant feature in control fish was muscle degeneration and regeneration in the lesion area. However, fungus was not seen in the lesion area of the control fish by 7 days p.i. using both IHC and Grocott staining methods. But massive amounts of fungus were seen in the injection area and also in kidney and liver of the control fish which died at day 17 after injection.

The pilot study achieved the following results which would be of use in the main experiment:

The method for producing zoospores and preparing spore suspensions as routinely used by the mycology laboratory in the Institute of Aquaculture was shown to be quite satisfactory for the main study.

It was determined that the best dose rate for injection is 0.1ml of suspension. Less than this dose did not cause a great inflammatory response for scientific examination and higher doses cause serious effects on normal behaviour and physical activity without having a greater inflammatory response.

The needle gauge 25 was ideal for injection because the suspension was watery and easy to inject through this needle.

In addition to the standard H & E staining, other staining methods were used for staining the sections. It was decided to use Grocott staining in the definitive study because every tiny spore or fungus hyphae could be stained and recognised readily and clearly using this staining method.

8.4 DEFINITIVE STUDY

Definitive study was designed following the findings of the pilot study.

8.4.1 Materials and methods

8.4.1.1 Fish

Mirror carp were obtained from Escot Aquaculture Ltd., Parklands farm, Devon, England. Sixty eight fish were kept in glass aquaria for acclimation for 3 weeks before the experiment began. Fish were fed on a pelleted diet (Fry 02, Trouw Aquaculture, Inverbreakie, Invergordon, Ross-Shire, Scotland) once per day.

8.4.1.2 Aquaria and water

The aquaria and water system used in this experiment were the same as those described in chapter 4. The water temperature was at an average of 27°C (±1.5°C). Faecal material was removed from aquaria every day and between 10-50% of the water was changed as necessary.

8.4.1.3 Preparation of inoculum

An *Aphanomyces invaderis* isolate (PA7) was obtained from the collection maintained at the Institute of Aquaculture, University of Stirling. The culture was grown in petri dishes containing glucose-pepton-yeast (GPY) broth ($3gl^{-1}$ glucose, $1gl^{-1}$ peptone, $0.5gl^{-1}$ yeast, $0.128gl^{-1}$ $MgSo_4.7H_2o$, $0.014gl^{-1}$ KH_2Po_4, $29.3mgl^{-1}$ $CaCl_2.2H_2o$, $2.4mgl^{-1}$ $FeCl_3.6H_2o$, $1.8mgl^{-1}$ $MnCl_2.4H_2o$, $3.9mgl^{-1}$ $CuSo_4.5H_2o$, $0.4mgl^{-1}$ $ZnSo_4.7H_2o$). After 3 days at 20°C, mycelial mats were washed in five changes of

autoclaved pond water (APW: 1 part filtered pond water, 2 parts distilled water). Thereafter mycelial mats were incubated overnight at 20°C to allow for production of secondary zoospores. After the incubation period, mycelia were examined under phase contrast microscope to confirm the presence of motile secondary zoospores. A suspension of these zoospores was used as inoculum for injection.

8.4.1.4 Injection procedure

Thirty two mirror carp with an average length of 13cm (±2cm) were injected intramuscularly on the left flank with 0.1 ml of the spore suspension. The fish were anaesthetised with 10% benzocaine (0.5 ml/l) before injection. Inoculation was made using a 1ml syringe and 25 gauge needle. The injection was made in line with the first ray of the dorsal fin, below the scale row, as a marker for subsequent sampling (Fig. 1.1). Control fish were injected intramuscularly on the left myotome with the same volume of sterile normal saline. After injection, the fish were kept in the aquaria until they were sacrificed.

8.4.1.5 Histological sampling procedures

Two injected fish were sacrificed by an overdose of 10% benzocaine, at 6 hours, 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after inoculation. Blocks of tissue encompassing the injected site were immediately cut out and fixed in cold 10% buffered formalin for at least 24 hours before processing. Formalin was changed and refreshed at least once during the fixation period. The fixed tissues were then cassetted, processed, cut, decalcified, and sectioned similar to those procedures described in chapter 5 (appendix 1.1).

8.4.1.6 Staining procedures

The standard H & E staining method was used for the routine examination of sections. Special stains were used for observing the fungus in tissue sections namely Grocott (appendix 2.1.3), and periodic acid Schiff (PAS) (appendix 2.1.2). The stained sections were mounted in Pertex mounting medium (CellPath plc, Herts, England) with a cover glass for microscopic examinations.

8.4.2 Results

8.4.2.1 Gross pathology

There was no gross signs visible of any change indicating inflammatory responses in the surface of the body of the injected fish with *Aphanomyces invaderis*, during the course of the study.

8.4.2.2 Histopathology

6 hours

At 6 hours after inoculation of the spore suspension of *Aphanomyces invaderis*, there was sarcoplasmic degeneration throughout the area of injection. Also initiation of myophagia was evident. There were haemorrhages in the damaged area and some hyperaemic blood vessels with melanin granules around at the edges of the lesion. Infiltration of inflammatory cells such as PMNs, lymphocytes, macrophages, and thrombocytes with fibrin strands in the lesion area and between the necrotic myofibres was obvious (Fig. 8.1). Swelling of some fibroblasts in the intermyotomal fascia adjoining the effected myotome at this stage indicated the initiation of fibroblast activity.

1 day

By this time, muscle degeneration was increased and spread throughout the injected area. Myophagia was active. Macrophages, lymphocytes, PMNs and thrombocytes with fibrin strands had increased in the inflammatory area. Some new

swollen fibroblasts, with pale staining nuclei, were evident in the intermyotomal fascia and around the blood vessels (Fig. 8.2).

2 days

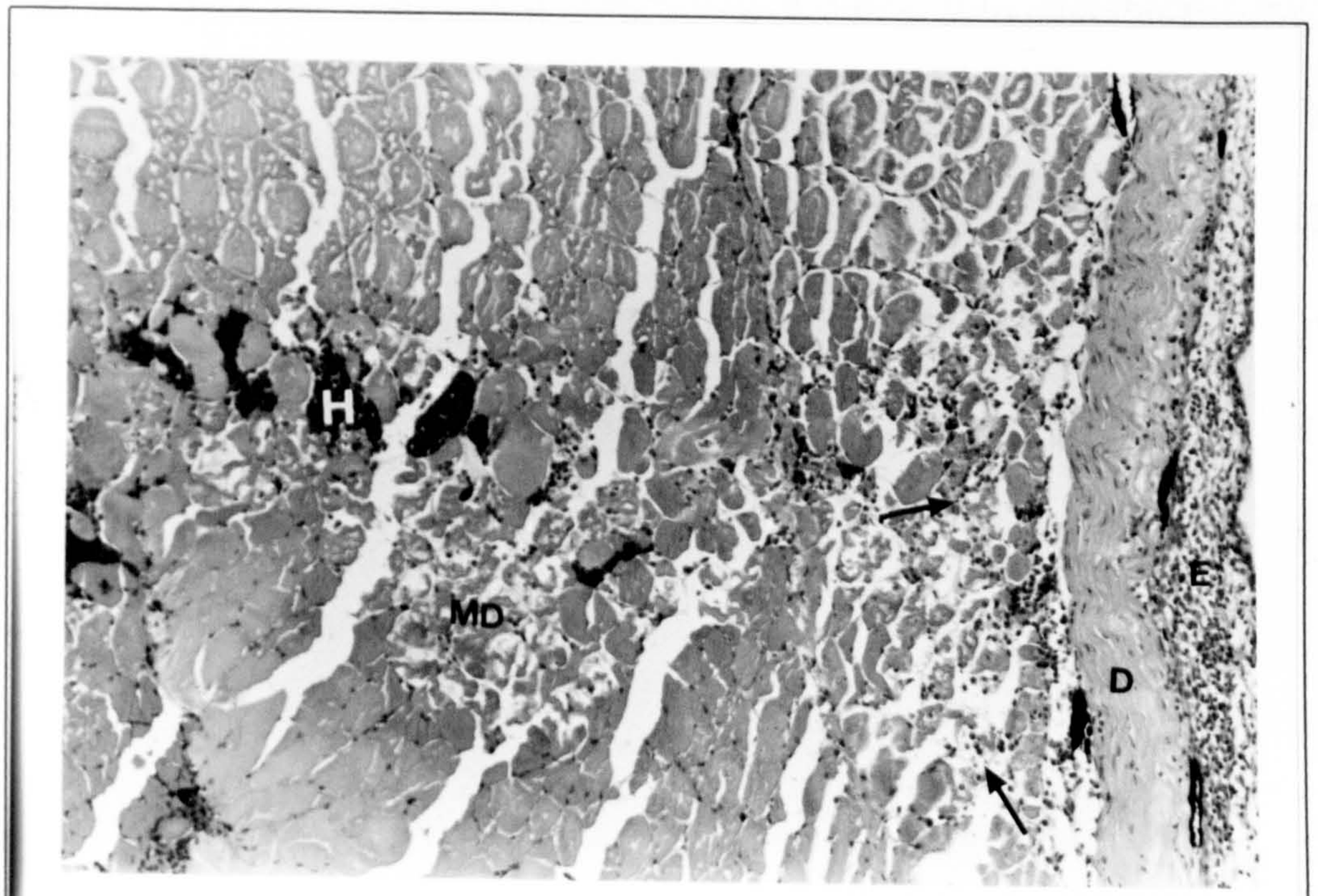
The dominant feature at this stage was myophagia and presence of active macrophages. The necrotic myofibrils were spread throughout the lesion area. Also small number of fibroblasts had migrated in the defect area.

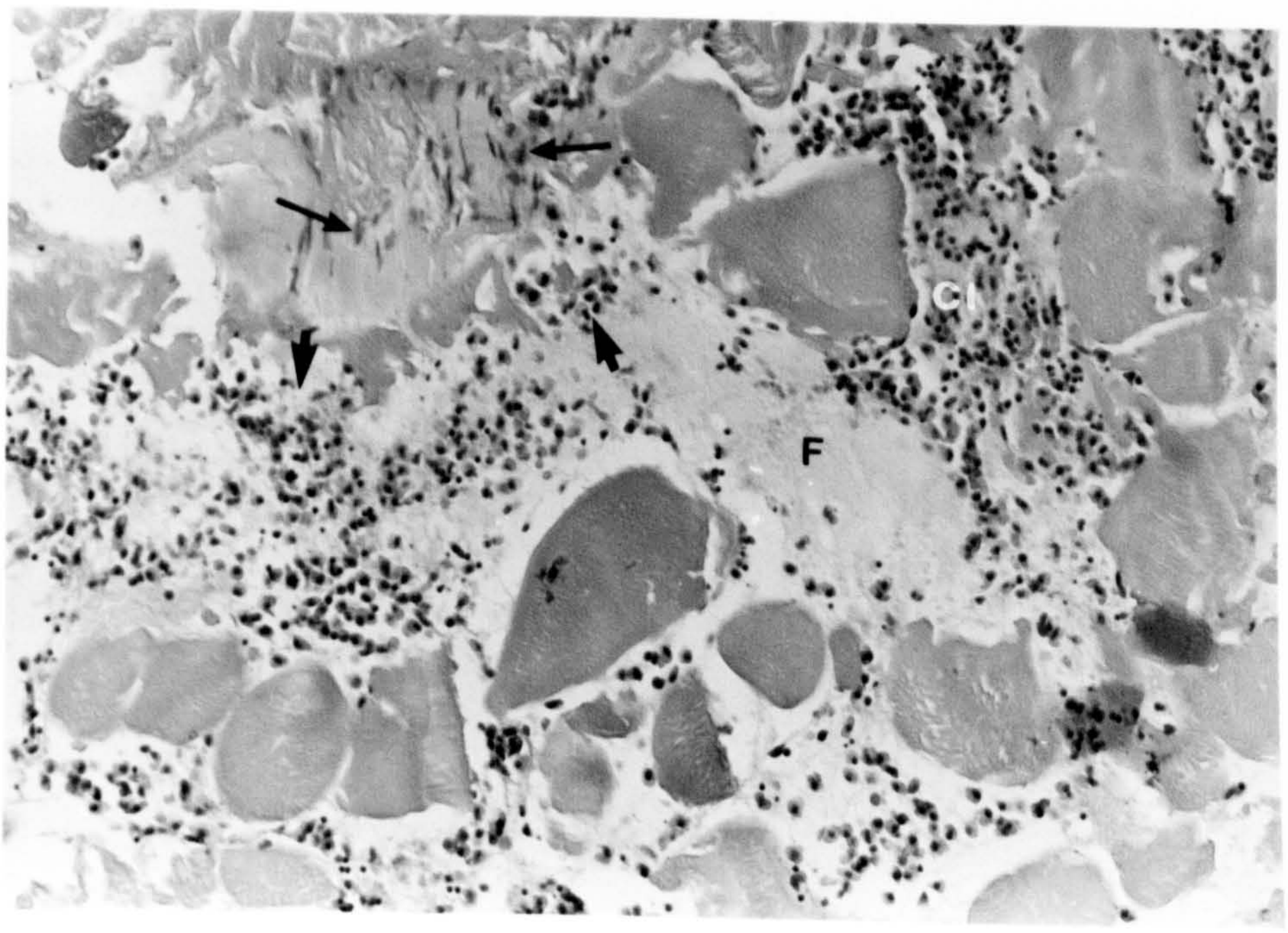
3 days

By the 3rd day, macrophages were present in a whorling epithelioid cells pattern in the lesion area and around the fungus. Also myophagic macrophages were active and myophagia was still in progress in some areas. Degenerated muscles had been removed by myophagia and were replaced by epithelioid cells and some fibrous tissues. Fibroplasia was active along with vascularization. Blood vessels and new capillaries were hyperaemic and some local haemorrhages were seen in the lesion area. Initiation of muscle regeneration with redeveloping peripheral nucleation in myofibrils which had been slightly damaged and also with presence of some new basophilic small buds of muscle was evident. First presence of giant cell types (Langhans and foreign body types) in the defect area was clearly evident. Also giant cells of intermediate type were seen. There was some fungus hyphae in the infected area which were surrounded by epithelioid cells (Fig. 8.3 & 8.4 & 8.5).

Fig. 8.1 At 6 hours post-inoculation of *Aphanomyces invaderis*, muscle degeneration (MD), haemorrhage (H) and myophagia (arrows) were observed in the damaged area. (H & E, X 110).
E=epidermis, D=dermis

Fig. 8.2 By 24 hours p.i., myophagia was active (arrow heads) and cellular infiltration (CI) with fibrin (F) was increased. Also some swollen fibroblasts with pale staining nuclei (arrows) were observed. (H & E, X 220).


H
MD
D
E


F

4 days

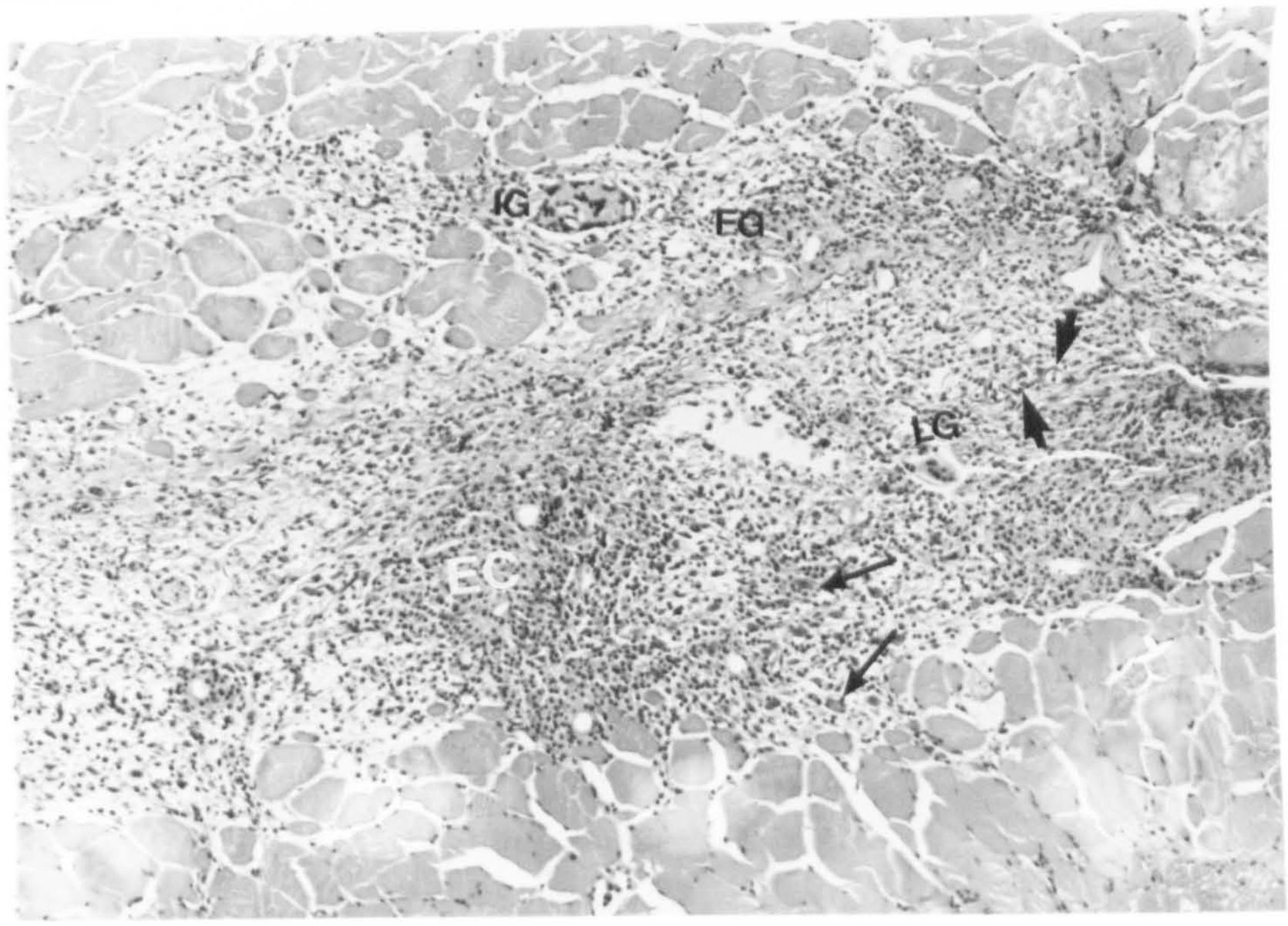
At this stage the most defect area was filled with epithelioid cells which was dominant feature of the lesion. Myophagia was still in progress in the small area of necrotic muscle fibres. Myofibrils regeneration and also some new muscle buds were evident near the damaged muscle areas and in the fibrous tissue. Fibroplasia and fibrosis were obvious in the area, along with formation of new capillaries. Hyperaemic small blood vessels and some haemorrhages were present in the area. Lymphocytes, macrophages and epithelioid cells were inflammatory cells present in the lesion. More giant cells which mostly engulfed the fungal hyphae, and also were surrounded by epithelioid cells was evident. Fungus was obvious in the infected area surrounded by epithelioid and giant cells (Fig. 8.6).

5-7 days

At the beginning of this period myophagia was completed and no necrotic muscle fibres were seen in the lesion, while the regeneration of muscle fibres was active. Presence of the epithelioid cells and granulation tissue was dominant picture in this period which filled all of the defect. Some layers of tightly bound epithelioid cells, surrounded by fibrous tissue, in some areas were evident. More giant cells comprising Langhans, foreign body and intermediate types, were evident in the area of the lesion. Fibrosis with active collagen formation were seen accompanied by forming new capillaries, while the previous formed new capillaries were active and nurishing the fibrosis area. Fungus was seen engulfed by giant cells and whorling epithelioid cells (Fig. 8.7-8.11).

Fig. 8.3 This picture shows a general view of the lesion area at 3 days p.i. Macrophages are seen in a whorling epithelioid cells pattern (EC). Three types of giant cell, Langhans (LG), foreign body (FG) and intermediate (IG), and also new capillaries (arrow heads) and new muscle buds (arrows) are observed in the lesion area. (H & E, X 110).

Fig. 8.4 In addition of Langhans and foreign body type giant cells, intermediate giant cells (arrow head) were observed at 3 days p.i. Fibroplasia (F), new capillaries (C) and new muscle buds (NM) were also seen. (H & E, X 440).


IG
EG
LG
EG

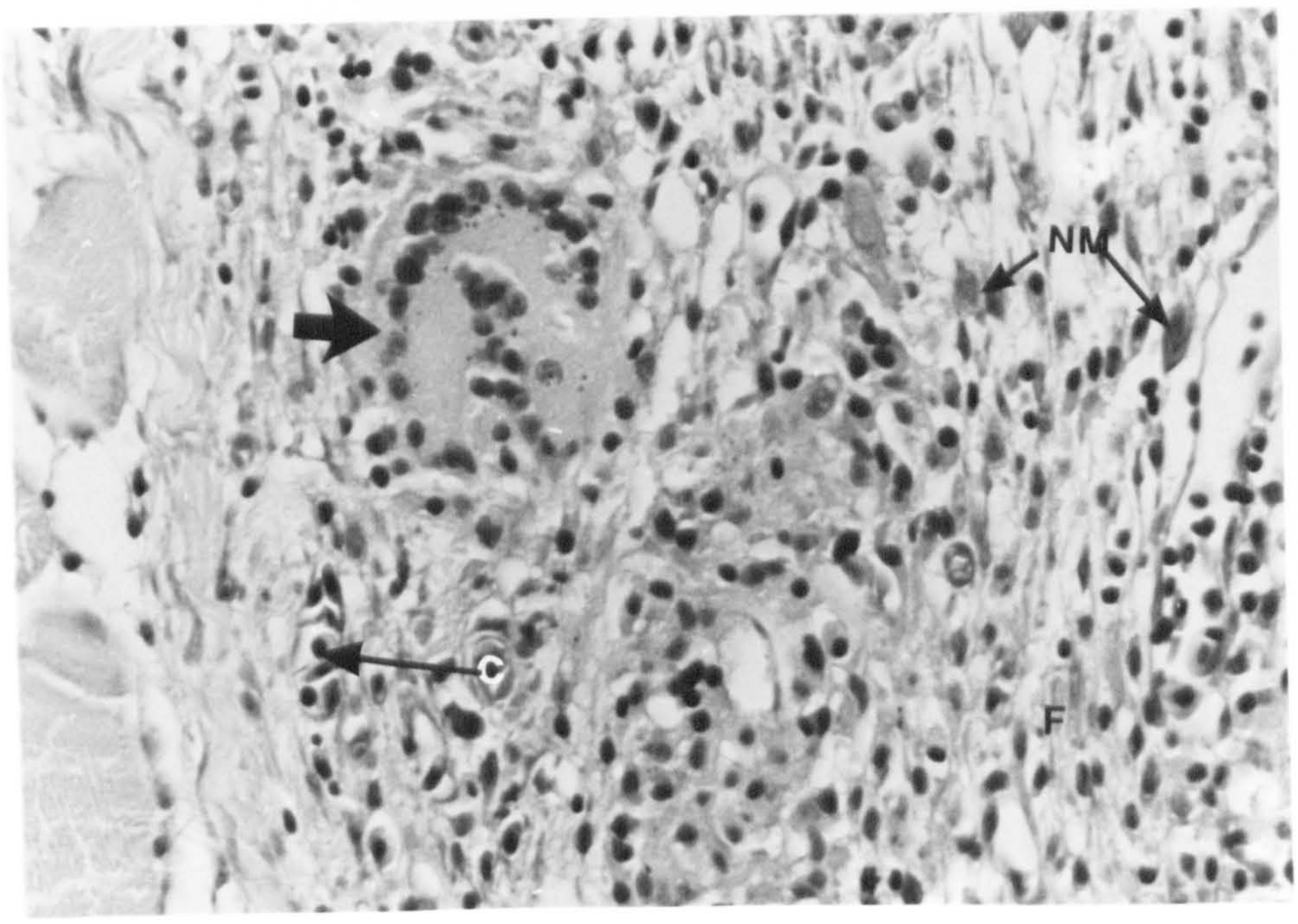
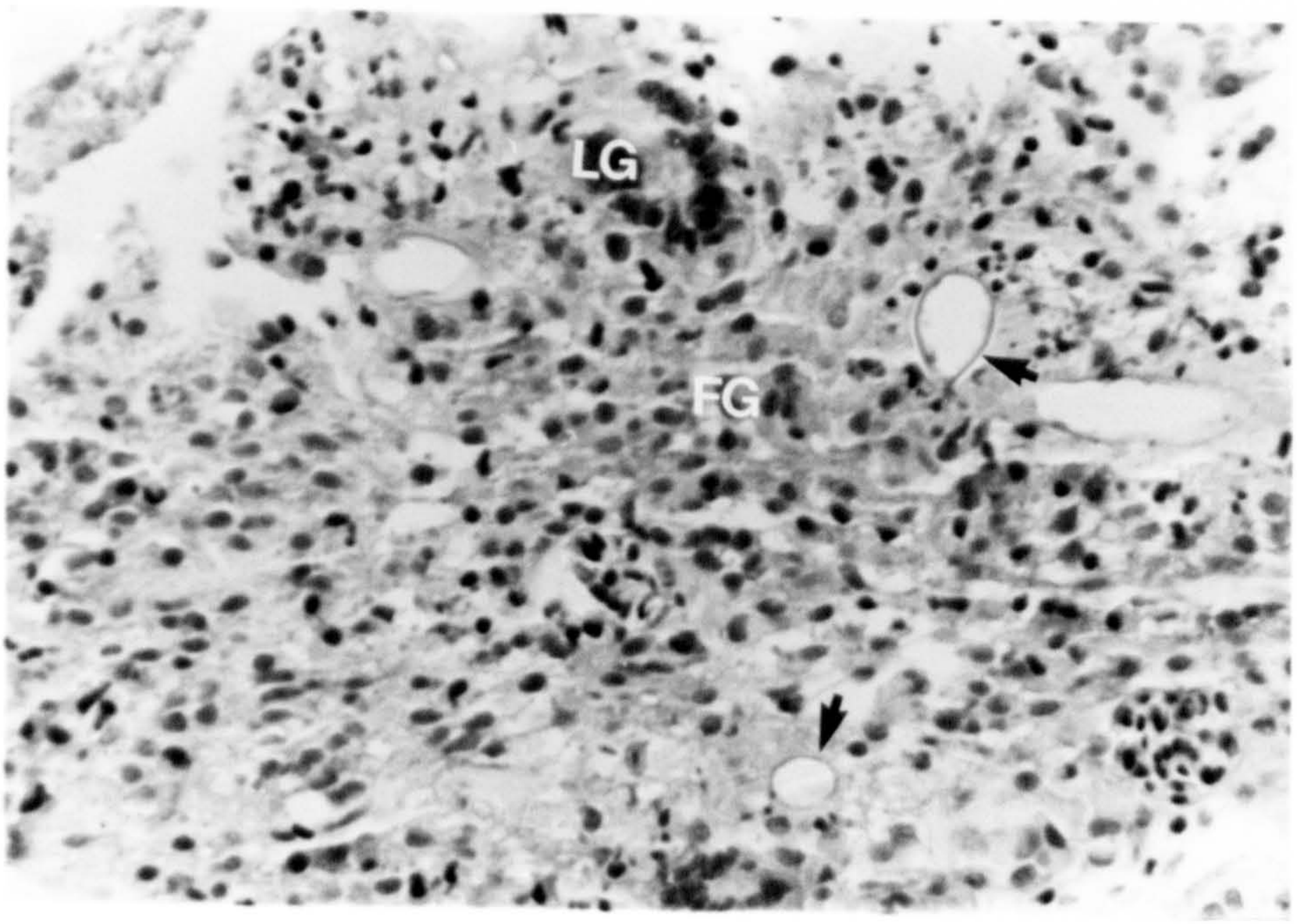
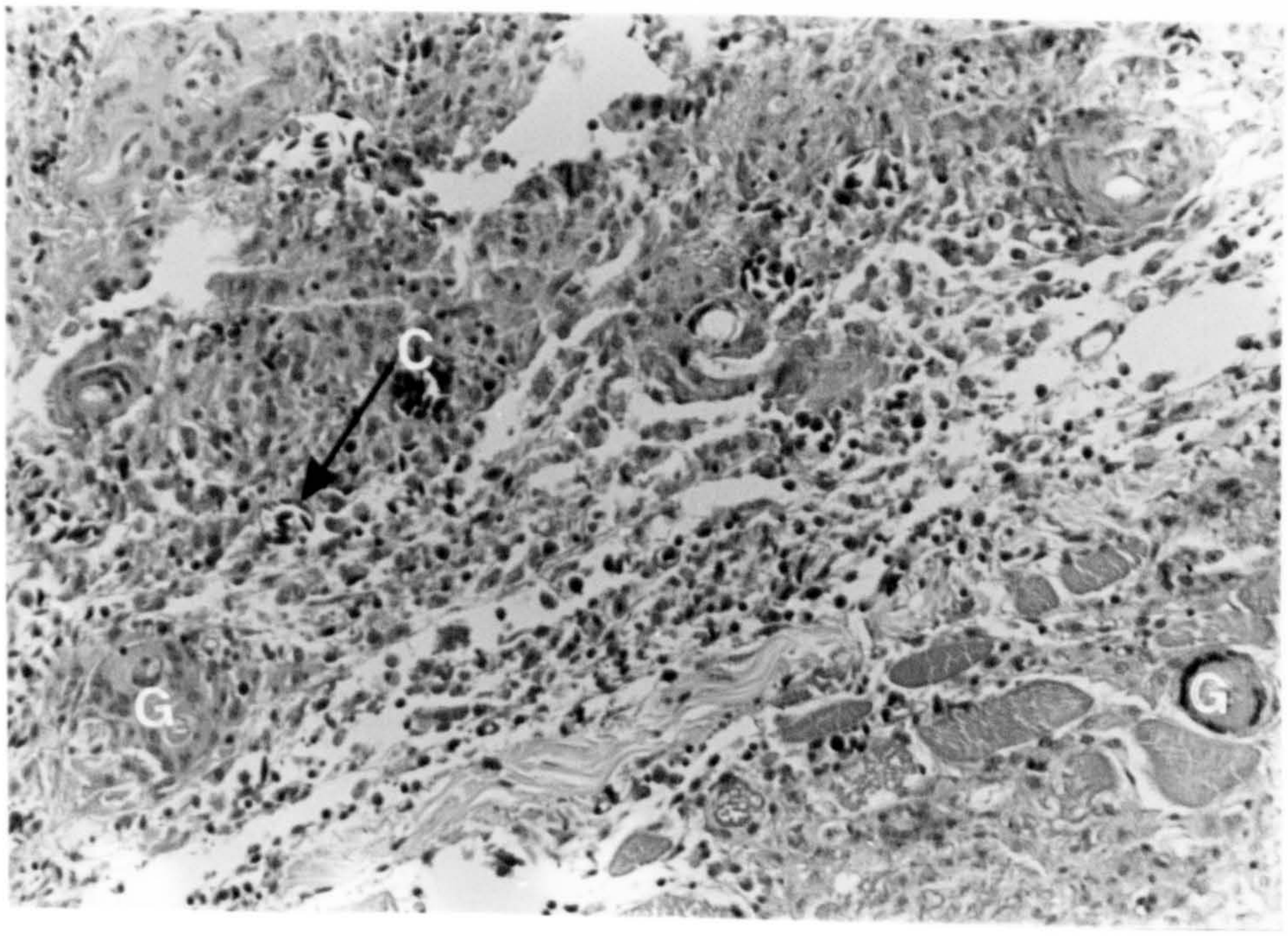

NM
C
F

Fig. 8.5 This picture demonstrates some fungus hyphae (arrow heads) surrounded by epithelioid cells. Langhans (LG) and foreign body (FG) giant cells are observed in the lesion area. (PAS, X 440).

Fig. 8.6 At 4 days p.i. most of the defect area was filled with epithelioid cells. Giant cells (G) which mostly engulfed the fungus hyphae, and also new capillaries (C) were observed in the area of the lesion. (H & E, X 220).


LG
FG


C
G
G

10 days

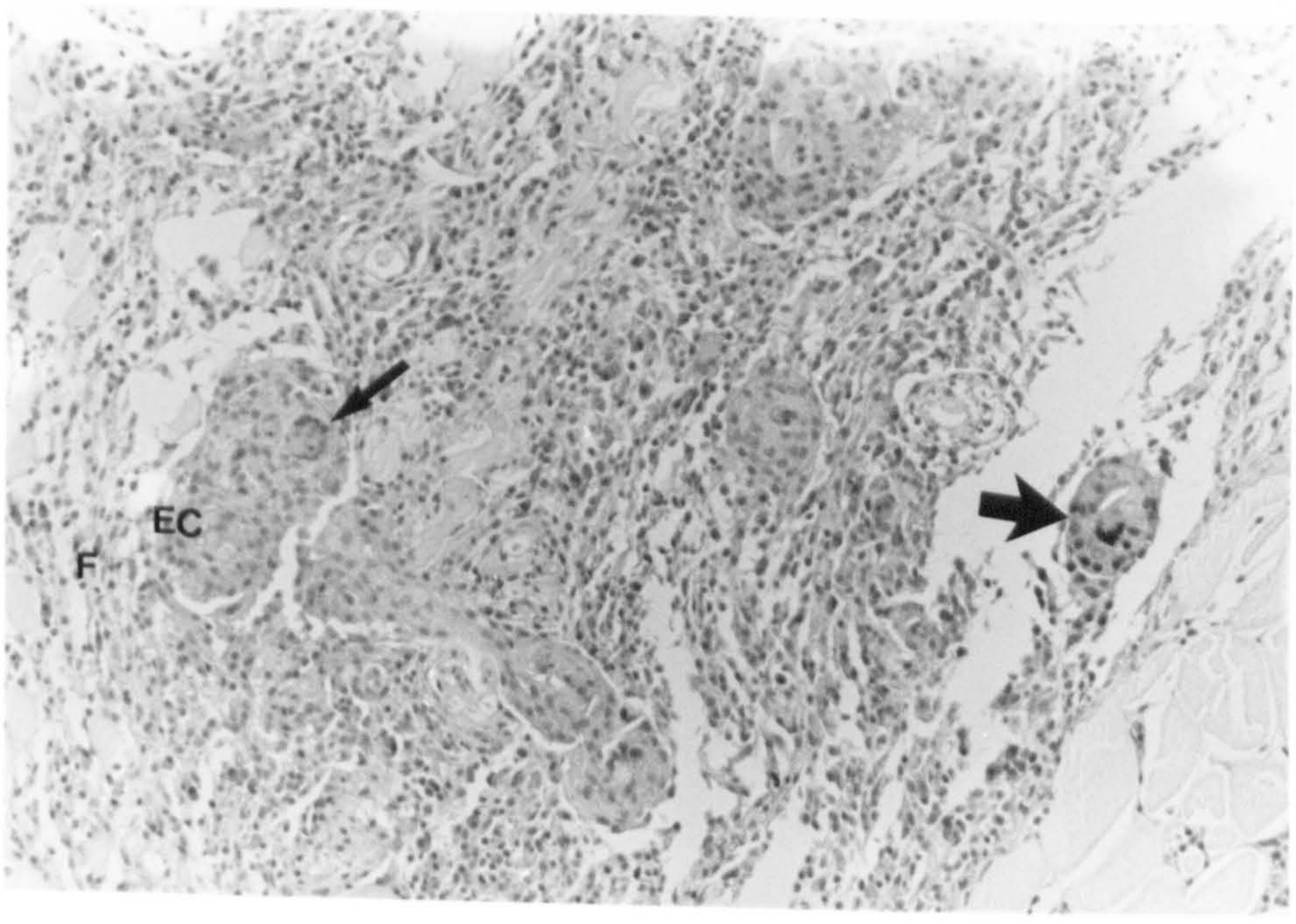
After 10 days of inoculation, the main feature in the lesion area was granulation process. This process was well in progress and epithelioid cells accompanied by fibroblasts filled the whole defect area where muscle tissue had been largely removed by myophagia, this also extended up between the intact muscle fibres (Fig. 8.12). Some granulomata, consisting of dense layers of epithelioid cells which surrounded fungus, pink materials, nuclei debris, and also in some area degenerated giant cells were obvious in the lesion, surrounded by fibrous layer. Fibrosis with collagen formation was active along with marked new capillaries. More active giant cells which had engulfed fungus were observed between epithelioid cells. Repairing of certain damaged muscles was still in progress in some areas. New formed capillaries and small blood vessels were very active in the area of fibrosis, also large blood vessels were associated with melanin pigments. Fungi were seen distributed in the whole area of the defect but were surrounded by giant cells and epithelioid cells.

14 days

By 14 days post-inoculation, well developed small and large granulomata were dominant, composed of layers of bound epithelioid cells surrounded by variable thicknesses of fibrous encapsulations and often with a central area of necrotic materials. Also fusion of some granulomata had began in the area (Fig. 8.13). Foci of lymphocytes aggregation, accompanied, to some extent, by macrophages and fibroblasts were seen in the fibrous tissue and around the granulomata. The number of lymphocytes and the level of their activity was considerable. Also macrophages with

Fig. 8.7 By 5 days p.i. myophagia was completed and presence of tightly bound epithelioid cells (EC) with surrounding fibrous tissue (F) were observed. An intermediate giant cell engulfing a fungus hypha (arrow head), and another one within a granuloma of epithelioid cell (arrow) are seen in this picture. (PAS, X 220).

Fig. 8.8 At 5 days after injection, vascularization was very active and new capillaries (arrows) were hyperaemic. A giant cell is observed in this picture engulfing fungus hyphae (arrow head), and another Langhan giant cell (LG) with no engulfed fungus. (H & E, X 110).


EC
F

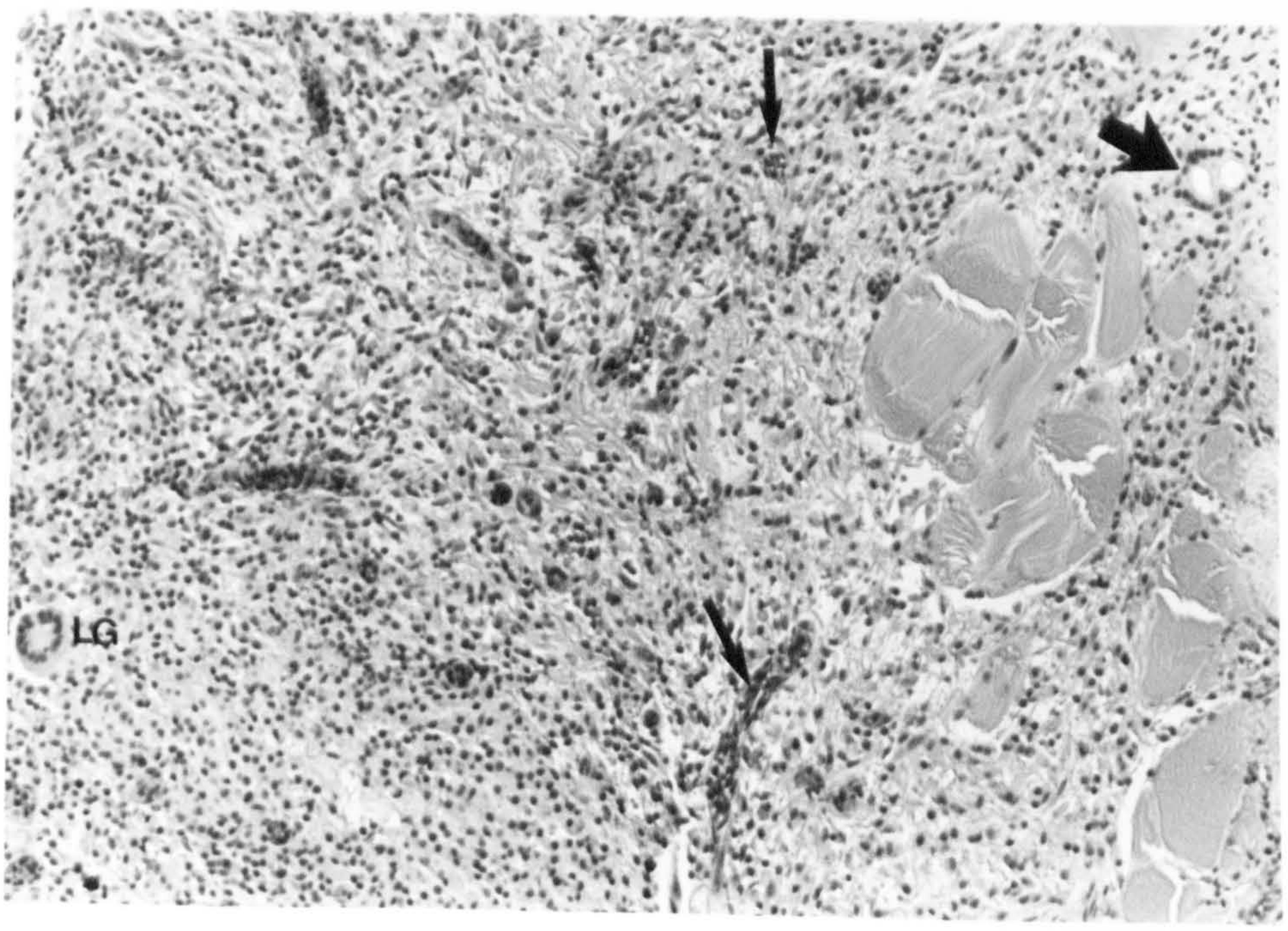
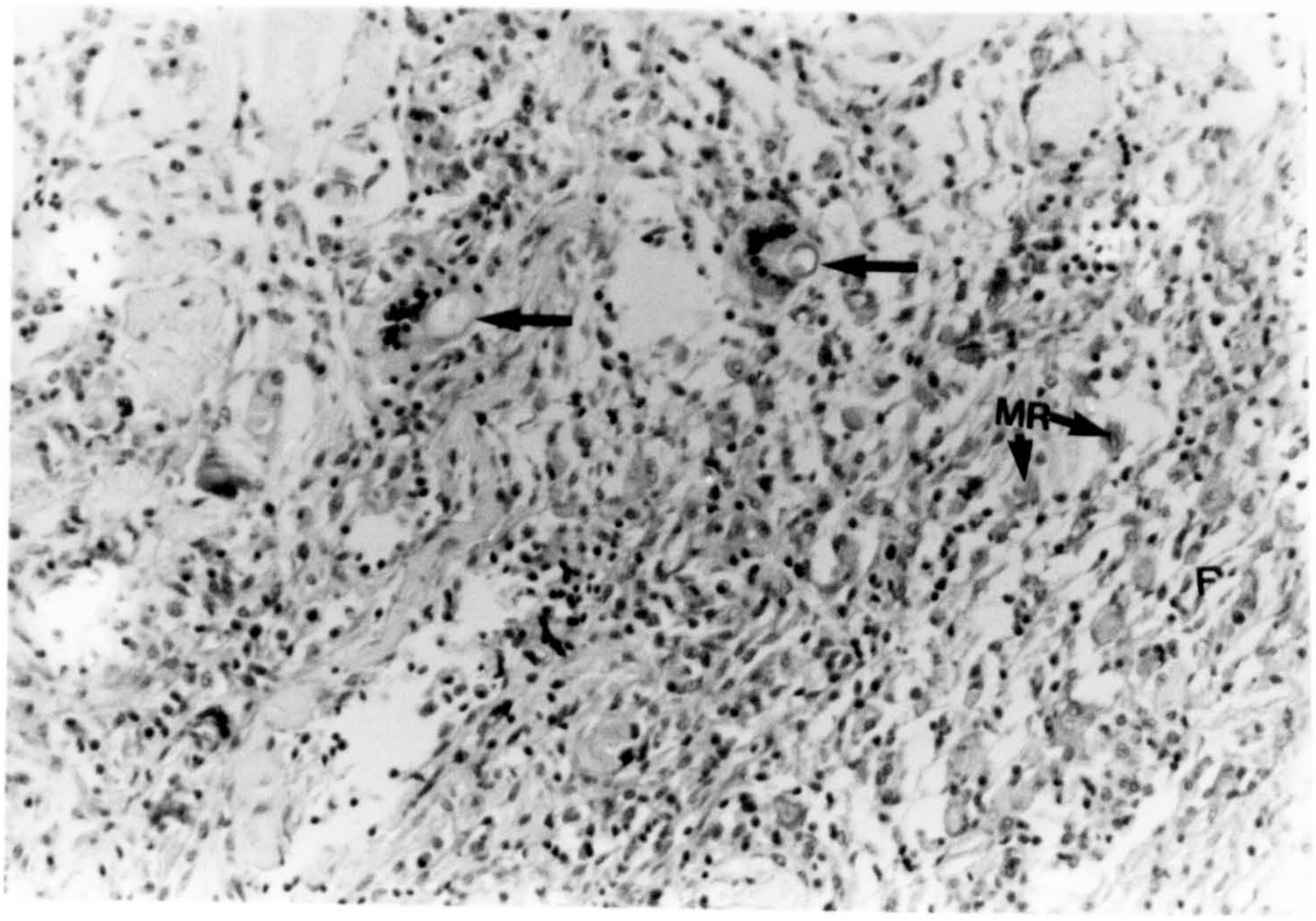

LG

Fig. 8.9 Fibroplasia (F) and muscle regeneration (MR) were active at 6 days p.i. Fungus hyphae are seen in this picture engulfed by giant cells (arrows). (PAS, X 220).

Fig. 8.10 This picture shows a general view of the lesion area at 7 days p.i. comprising epithelioid cells (EC), giant cells (G), capillaries (C) and fungus hyphae (arrow heads) sequestrated by epithelioid cells. (H & E, X 110).



-1

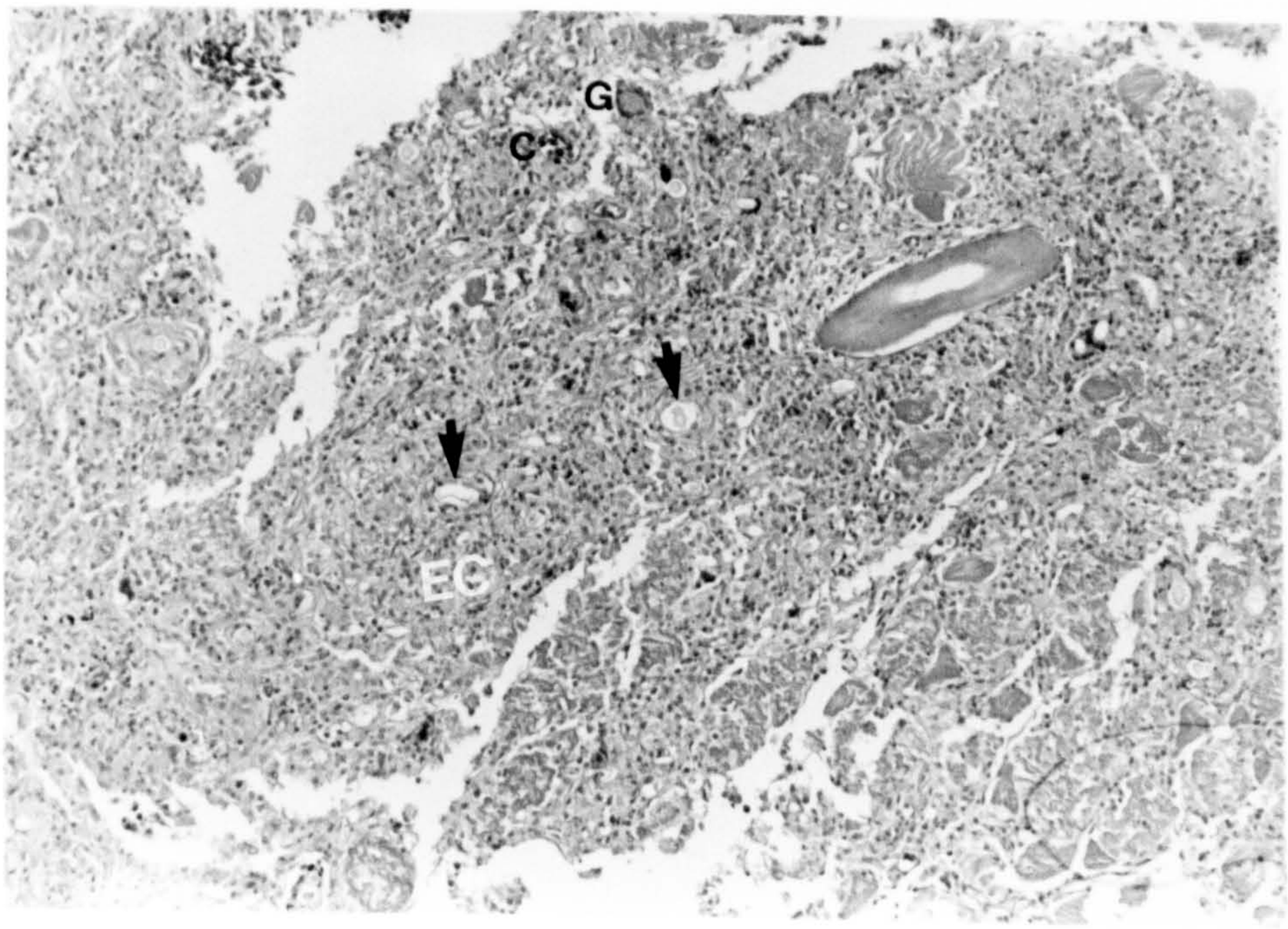
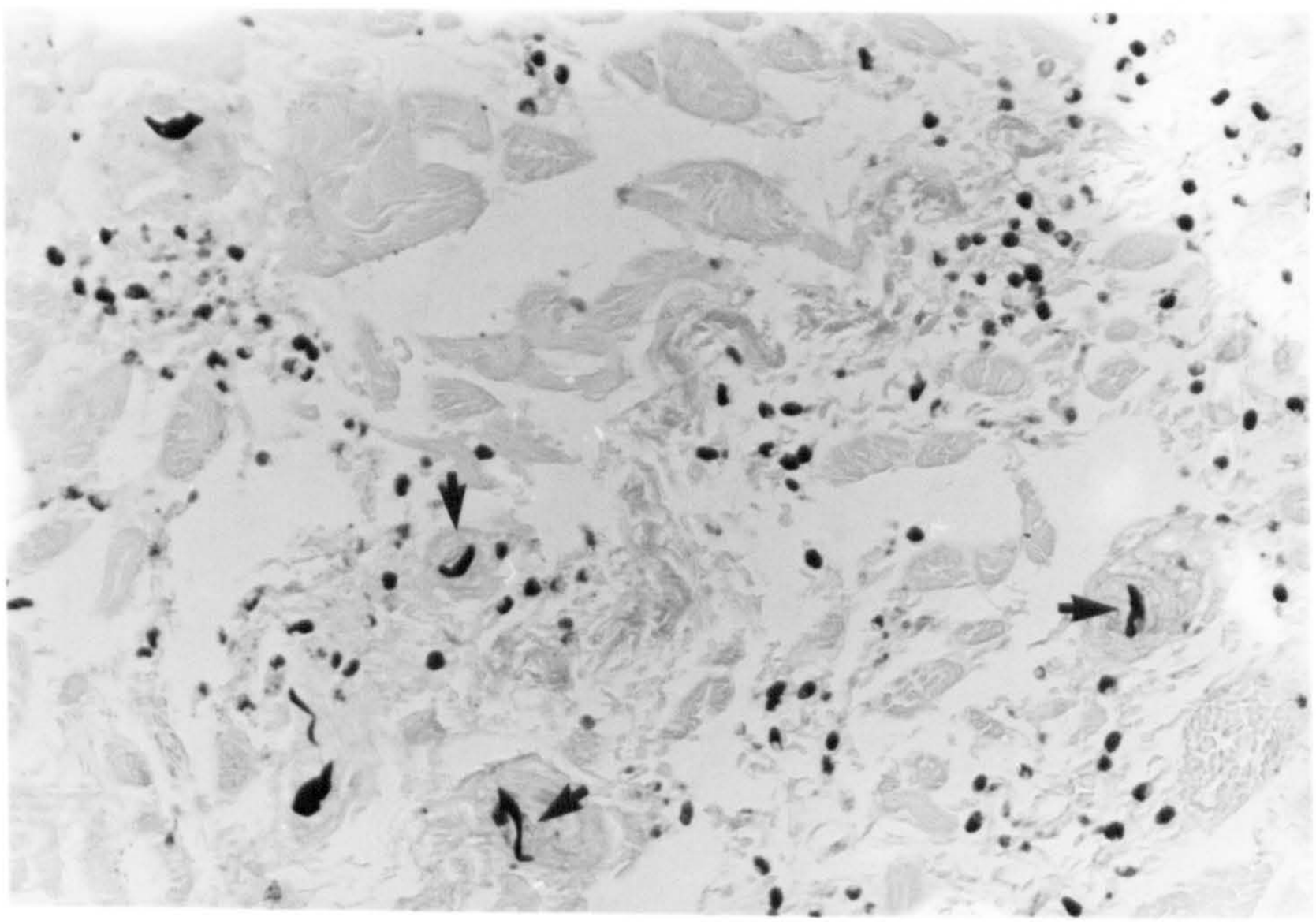
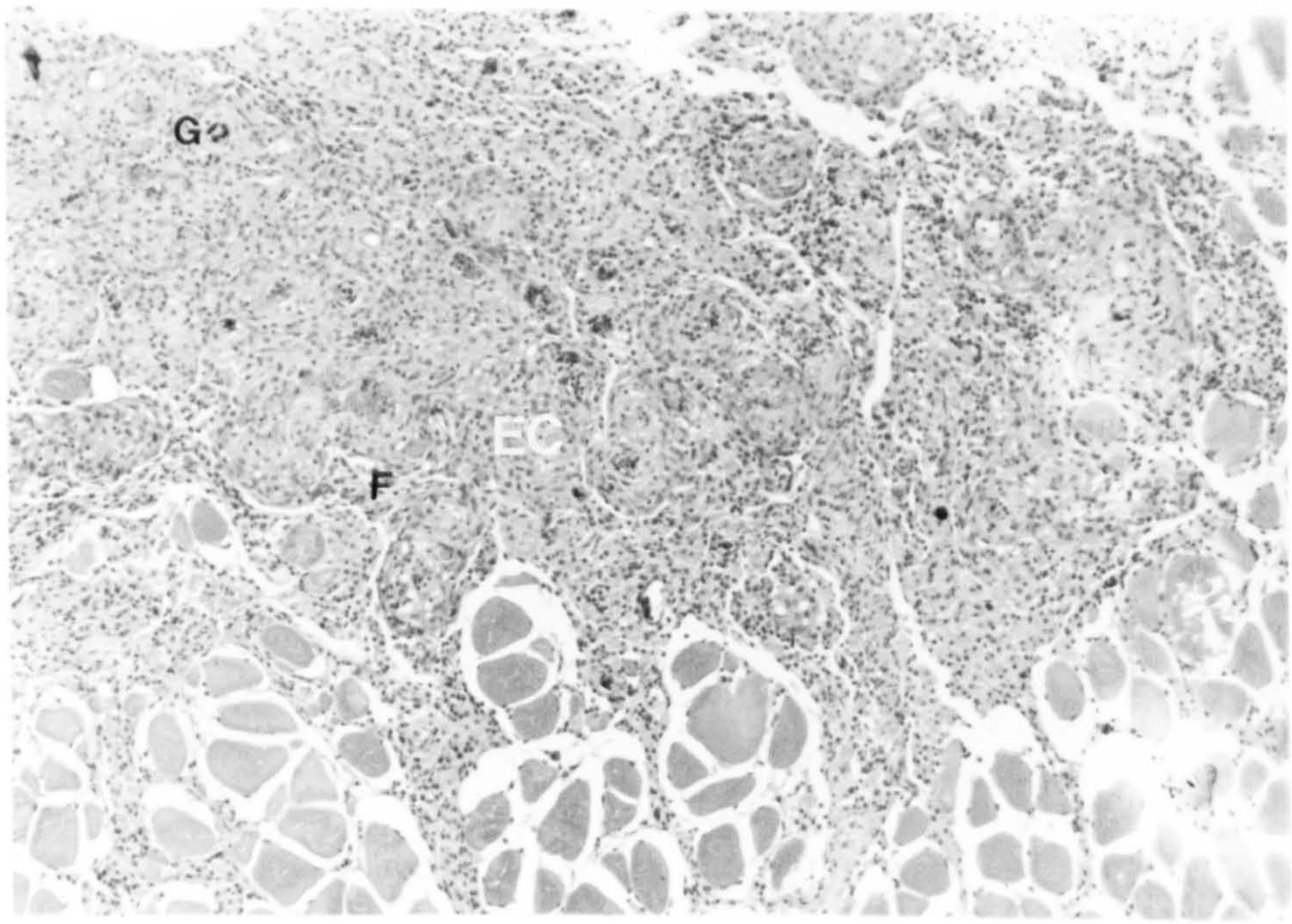


Fig. 8.11 Fungus hyphae (arrow heads) sequestrated in the lesion area at 7 days p.i. are observed in this picture. (Grocott, X 220).

Fig. 8.12 At 10 days p.i. granulation process was well in progress and epithelioid cells (EC) accompanied by fibroblasts (F) filled the whole defect area. Giant cells (G) also were observed. (H & E, X 110).




EC
F

lipofuscin in the cytoplasm were seen throughout the defect area. New capillaries were obvious, and small blood vessels were active in the area. An extreme reduction in the number of giant cells was evident as the lesion healing progressed, and a very few, if any, giant cells present in the area were surrounded by granulomata. Fungus was seen in the centre of granulomata.

18-22 days

During this period the dominant feature was developing granulation process with more solidification. Fully mature granulomata of different sizes from small to large, separated or fused were evident and filled the whole defect area (Fig. 8.14). The epithelioid layers in granulomata were reduced, while fibrous layers around them increased. The centre of these granulomata was filled by effete macrophages, nucleic acid debris, pink deposit materials, and fungus which was degenerate in some granulomata (Fig. 8.15). Fusion of small granulomata was obvious and these fused granulomata were encapsulated by layers of fibrous tissue. High levels of lymphocytes activity, with increasing in the number and foci of lymphocytes aggregation especially around the granulomata and in the fibrous tissue, was obvious. Macrophages with lipofuscin in cytoplasm were seen in the area. Melanin pigments were distributed throughout the healing lesion area (at 18 days p.i.) and around the blood vessels. Some new capillaries were formed in the fibrous tissue and all the blood vessels were congested along with local haemorrhages (at 18 days p.i.) in the lesion area. No giant cells was evident at this period in the lesion area.

28-35 days

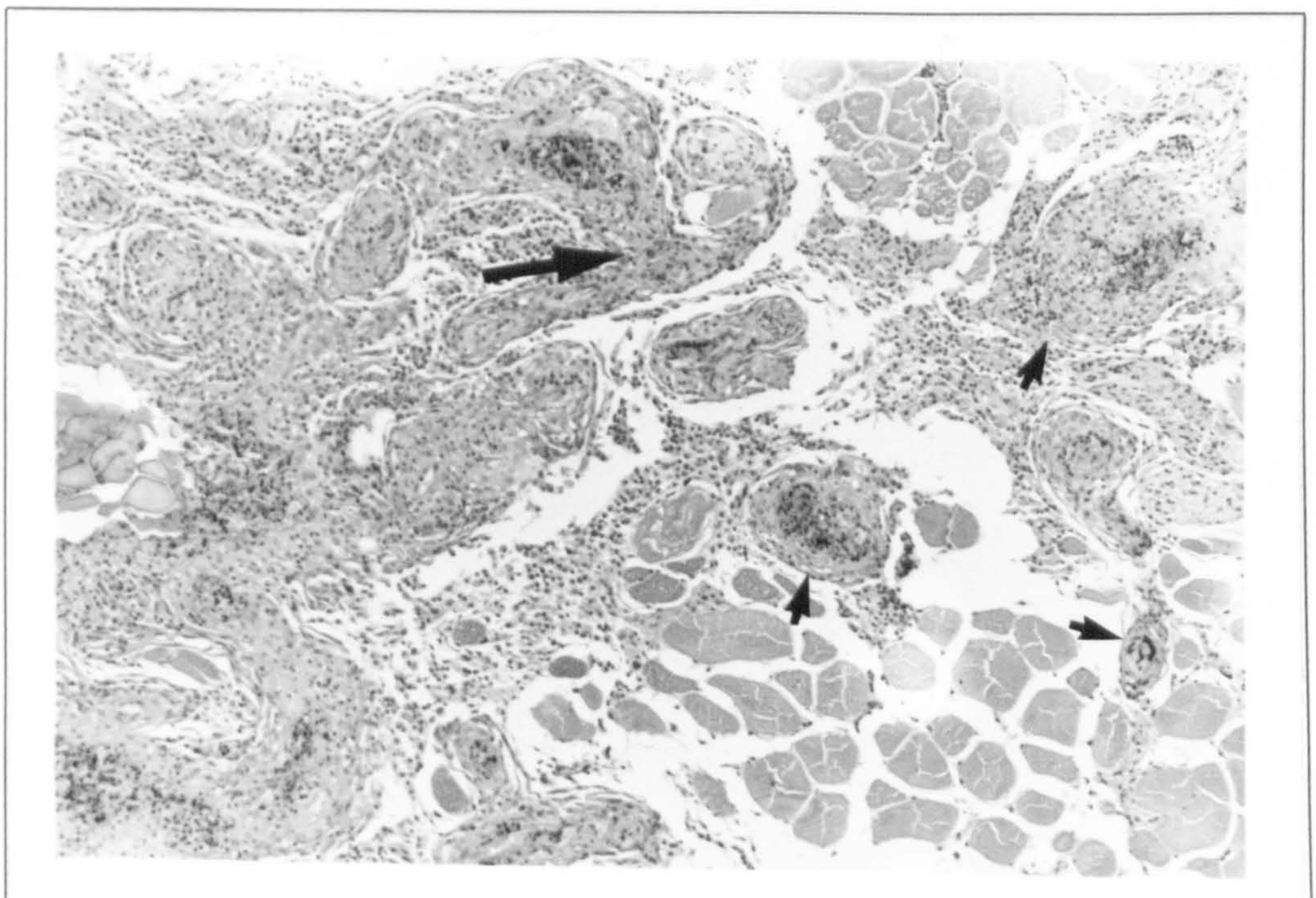
During this period, presence of the typical granulomata was the most obvious feature in the defect area (Fig. 8.16). The granulomata consisted of a masses of necrotic materials in the centre and a thick fibrous layer around. Almost no epithelioid cell remained intact in granulomata, and those that were observed were degenerate. Granulomata of different sizes, mostly small sizes, were fused together in different areas, forming larger granulomata surrounded by layers of fibrous tissue (Fig. 8.17). Macrophages with lipofuscin, melanin granules or ceroid in cytoplasm, and also foci of lymphocytes were obvious in the lesion area especially close to the granulomata. Some capillaries were seen in the fibrous tissue, and blood vessels in the area were congested, with surrounding melanin pigments. Degenerate fungus was observed in some granulomata.

42 days

This was the last sampling time in this experiment. At this time more consolidation of the process of encapsulation was evident. The lesion was healed and regardless of the presence of granulomata in the area, the structure of the tissue was almost similar to a normal tissue. The granulomata were only composed of a massive amount of necrotic materials such as; epithelioid debris, pink materials, nucleic acid debris and degenerate fungus in the centre, and dense fibrous layers around. Most of granulomata in different areas fused together, formed large granulomata with surrounding dense fibrous layers (Fig. 8.18). Although the cellular components were reduced in the area, macrophages with lipofuscin, melanin and ceroid in cytoplasm, and

Fig. 8.13 By 14 days p.i. well developed small and large granulomata (arrow heads) were dominant in the lesion area. Also fusion of some granulomata was began (arrow). (H & E, X 110).

Fig. 8.14 Mature granulomata of different size separated or fused (arrow heads) filled the whole defect area at 18 days p.i. Increasing in number of lymphocytes was obvious (arrows). Blood vessels were associated with melanin pigments (MP). (H & E, X 110).



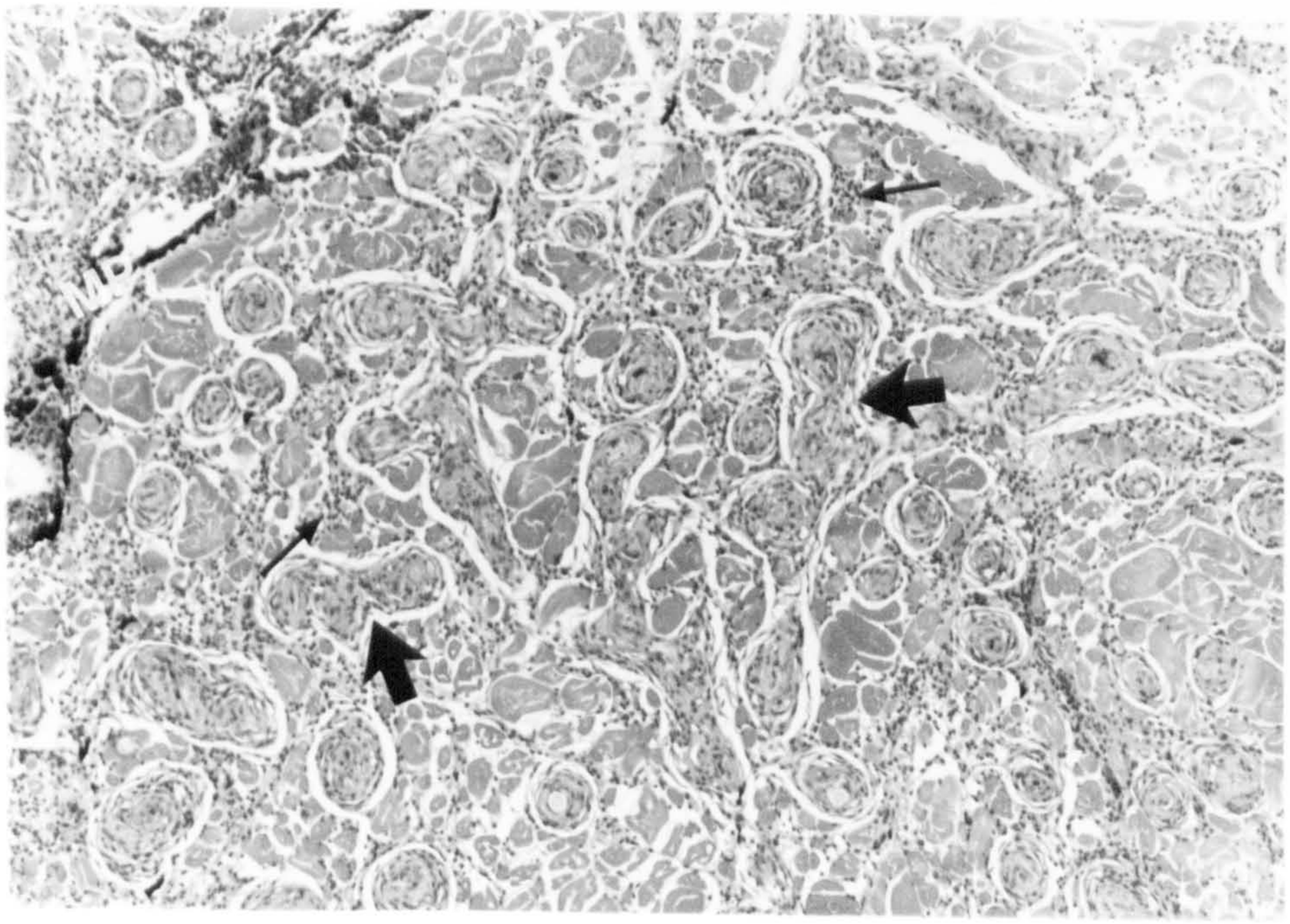
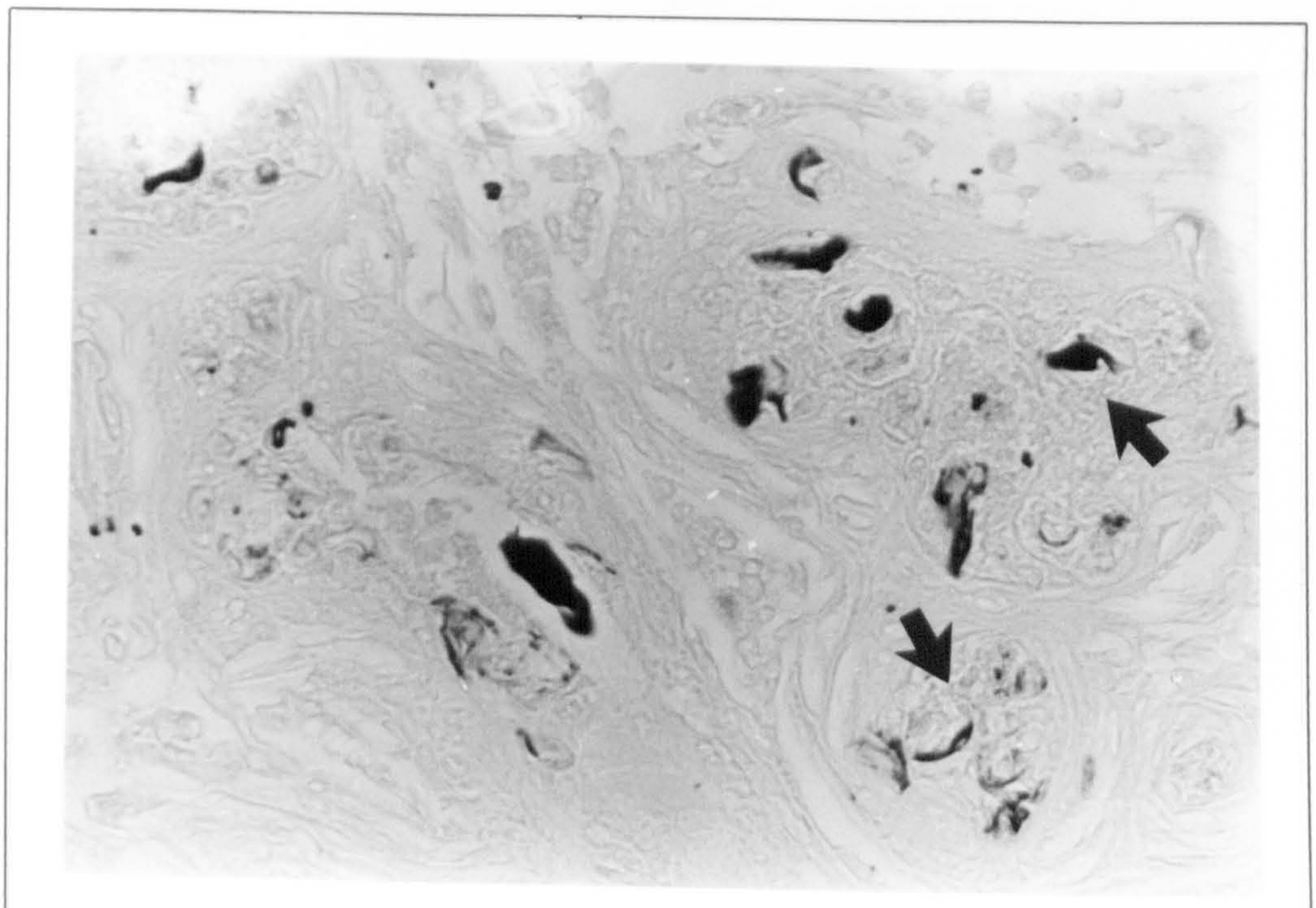


Fig. 8.15 In addition to necrotic cells, nucleic acid debris and pink deposit materials, sequestrated fungus hyphae (arrow heads) were also observed at 22 days p.i. (Grocott, X 440).

Fig. 8.16 Presence of the typical granulomata (TG) among the normal muscles was the most obvious feature at 28 days p.i. A focus of cellular aggregation with lymphocytes domination is obvious in the picture (arrow head). (H & E, X 110).



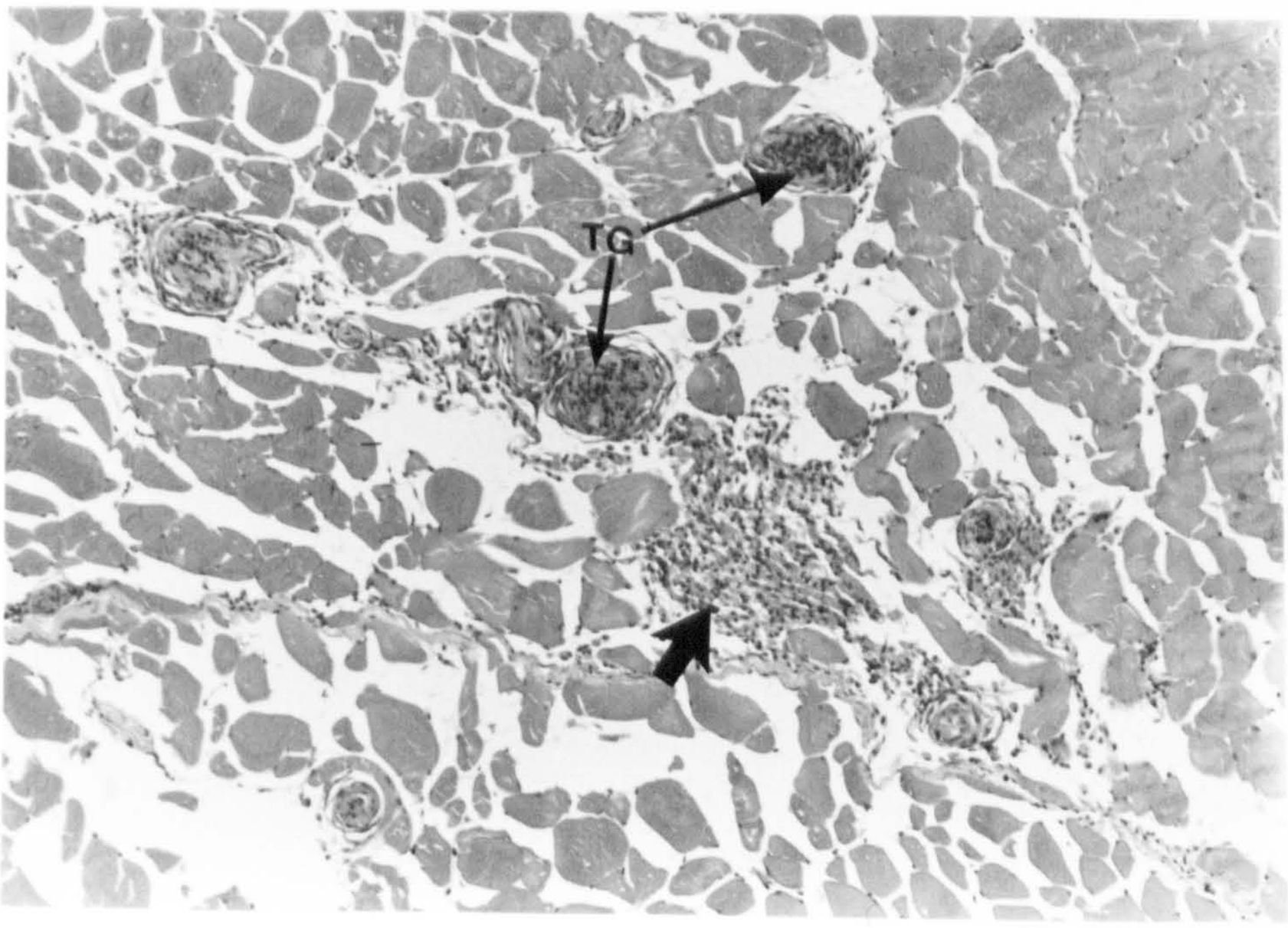
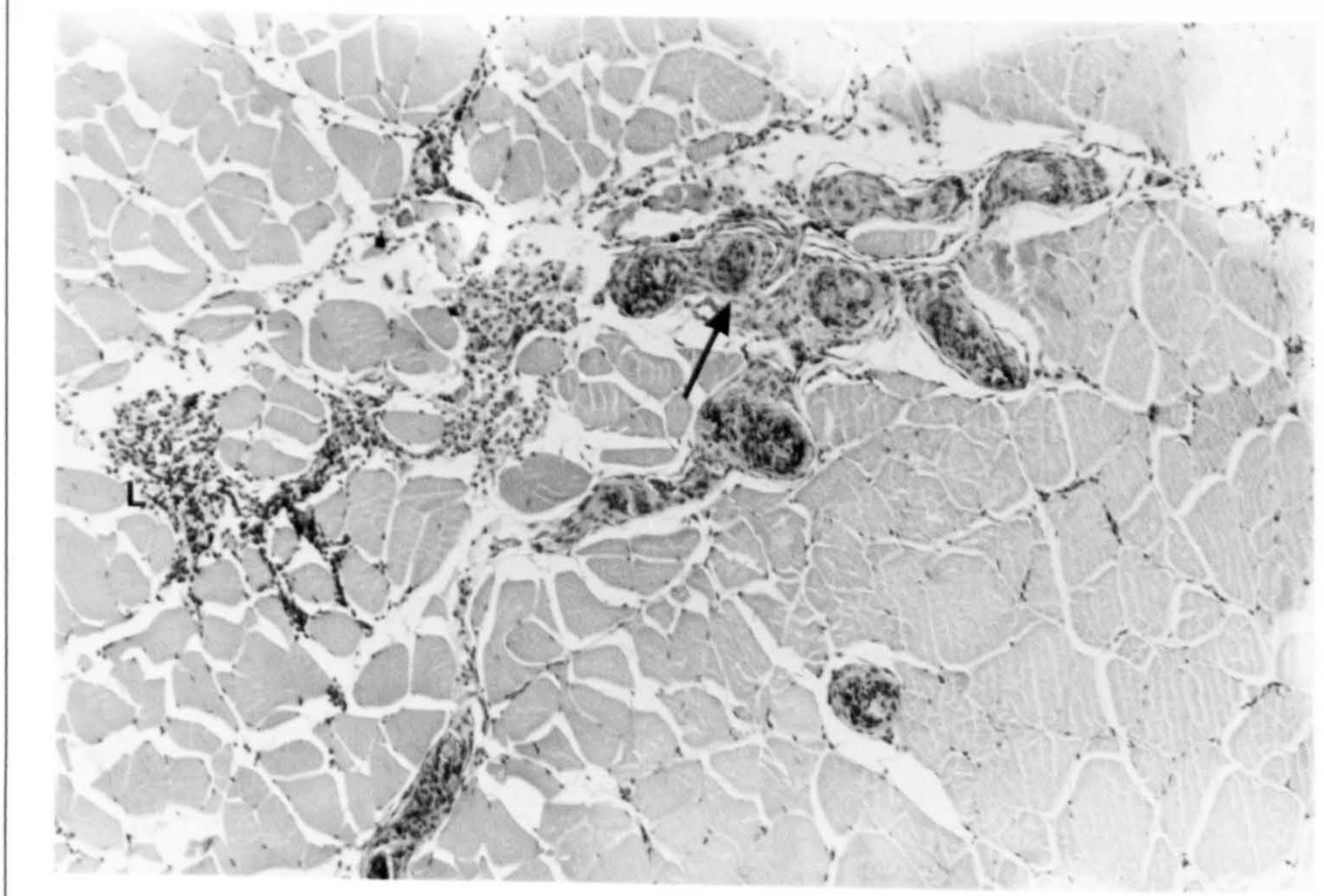
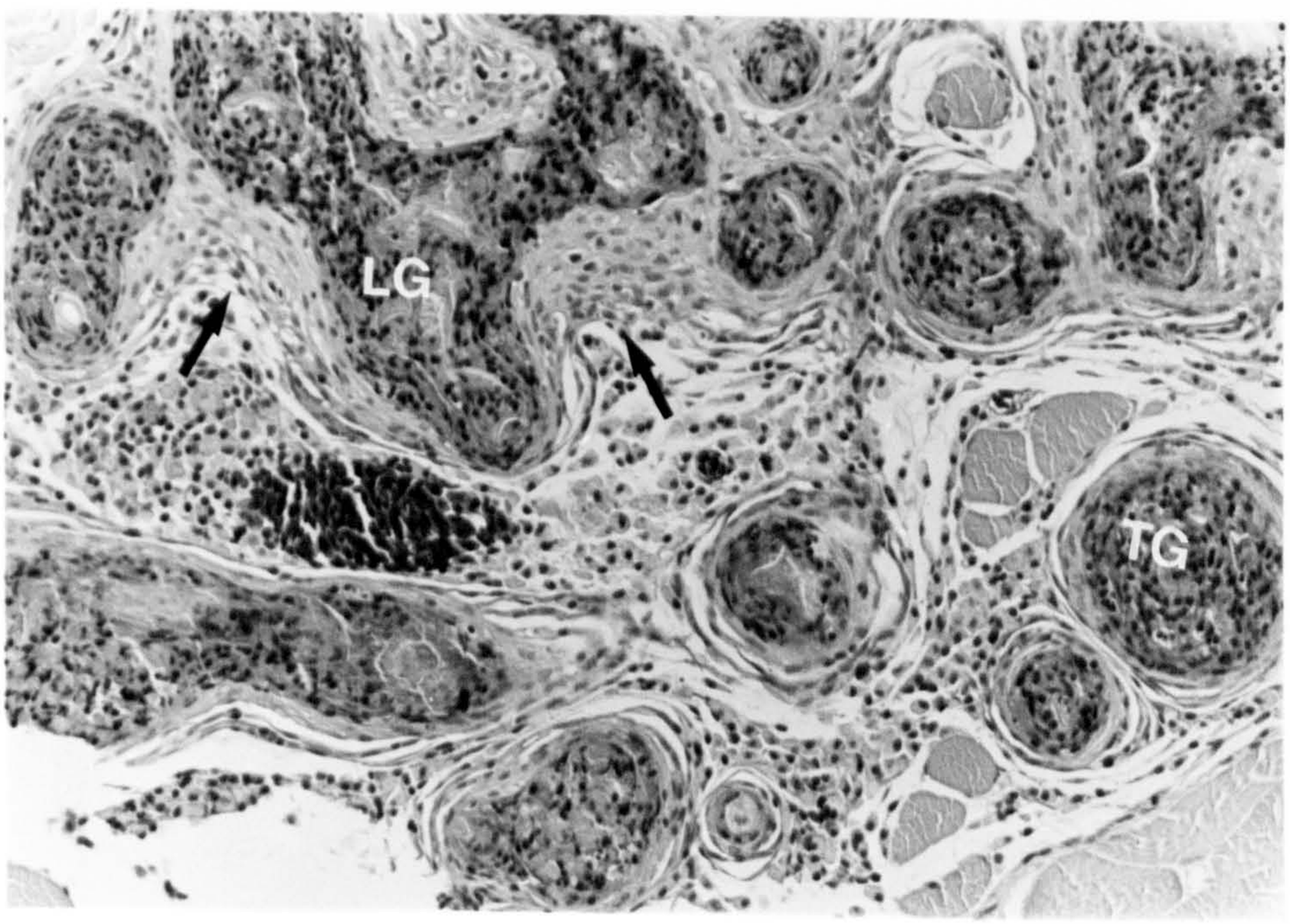

TG

Fig. 8.17 At 35 days p.i. granulomata of different sizes were fused (arrow), forming larger granulomata. Foci of lymphocytes (L) were seen in the lesion area. (H & E, X 110).

Fig. 8.18 This picture shows the lesion area at the final sampling time, 42 days p.i. Typical granulomata (TG) are mostly composed of a massive amount of necrotic materials and surrounding dense fibrous layers. Fusion of granulomata (arrows) resulting formation of large granulomata (LG) is obvious. (H & E, X 220).


L


LG
TG

also lymphocytes were present especially around the granulomata. Some capillaries were found in the fibrosis areas. All these capillaries and larger blood vessels were hyperaemic and some larger blood vessels were associated with melanin pigments. Degenerated fungi were seen in some granulomata.

Control fish

No significant changes were detected in the fish injected with normal saline (appendix 4).

8.5 DISCUSSION

Observations of the inflammatory changes in carp, in the present study, after intramuscular inoculation of *Aphanomyces invaderis*, indicated the occurrence of the characteristic features of a chronic inflammation. The main features of this chronic inflammation were cellular infiltration, fibrosis and vascularization in the lesion area.

Floccular and cloudy degeneration of the muscle fibres, and also moderate haemorrhage associated with the line of injection produced at the early stages of the infection, maybe traumatic damage caused by the passage of the needle inserting the inoculum, and the zoospore activity.

The main components of the inflammatory response were the inflammatory cells comprising macrophages, lymphocytes, epithelioid cells, giant cells and fibroblasts. Corbel (1975) stated that the cellular response to mycotic infection in fish is quite varied, ranging from granulomata formation to complete absence of response. Along with myofibrilar degeneration and presence of the pathogen in the tissue, inflammatory cells began to infiltrate into the lesion area resulting the initiation of myophagia at 6 hours after inoculation in this study. Myophagia was completed at 5 days post-injection. Initiation of myophagia in carp in this study was faster than 12 hours for early myophagia in experimentally infected snakehead with *A. invaderis*, at all temperatures used (i.e. 19, 26 and 31°C) (Chinabut *et al.* 1995).

Perimysium and inter-myotomal fascial were observed limiting the spread of necrosis in muscle in the present study which supports the findings of Finn and Nielson (1971*a*) in rainbow trout.

Some PMNs infiltrated into the lesion area at early stages and remained less than 2 days while the number of macrophages which had migrated to the necrotic area by 6

hours, was increasing. These times for infiltration of PMNs and macrophages were faster than that reported by Chinabut (1989) in snakehead infected with *Achlya debaryana* at 28°C.

Lymphocytes were observed in the lesion area from the early stages. Their number and activity increased along with developing healing process and remained in the area until the final sampling time. This long-term presence and gradually increasing in the number and activity of lymphocytes, could be an immunological reaction related to responsibility of lymphocytes for the development of cell-mediated immunity (Ellis 1977 & 1989). However, as a result of such effective cellular defence mechanisms against the invading fungus, limited growth of the fungus inside tissues occurred which was then halted rapidly by fish defence mechanisms.

First presence of giant cells in the lesion area was observed 3 days after injection. Thereafter their number and activity well increased by 10 days post-injection. With improvment in lesion healing, the number of giant cells was extremely reduced at 14th day, so that no giant cells were evident from the 18th day after injection onwards. In addition to two very well known types of giant cells, Langhans and foreign body types, intermediate giant cell type was also observed. Intermediate giant cell was described by Timur, G. (1975) in plaice, injected with talcum powder. These cells have their nuclei at periphery of the cytoplasm and also scattered in the middle of the cytoplasm.

Production of giant cells was reported neither in snakehead, experimentally, infected with *A. invaderis* (Chinabut *et al.* 1995), nor in snakehead, infected experimentally with *Achlya debaryana* (Chinabut 1989). Neither was it observed in dwarf gourami infected by *Aphanomyces* sp. (Wada *et al.* 1994).

Presence of giant cells in UM in Atlantic menhaden was reported by Noga *et al.* (1988). Also it has been found in MG in freshwater fishes, goldfish and Ayu (Miyazaki & Egusa 1972 & 1973). Miyazaki and Egusa (1972) defined two types of giant cells, one type with engulfed hyphae were always within the granulomas of muscle tissue, and another type without hyphae usually appeared in the loose connective tissue of the dermis or lamina propria. In this study, giant cells containing fungus in their cytoplasm were seen located either within the stroma of epithelioid cells or within the granulomata. Also giant cells with no engulfed fungus were either seen lying free in the lesion area, or in the granulomata.

Richards *et al.* (1978) suggested that giant cells appear to be common in fungal granulomas of fish, and Secombes (1985) demonstrated that they are capable of phagocytosis. The findings of the present study supports these views.

It is obvious from literature (reviewed in chapter 6) that only Langhans type giant cells are produced in piscine tuberculosis and foreign body types do not normally occur. Timur, G. (1975) found both types in plaice in response to Freund's complete adjuvant and concluded that foreign body types were in response to the mineral oil of adjuvant rather than the *Mycobacterium* sp. Both Langhans and foreign body types and also intermediate type were produced in the present study. It could be suggested that in addition of Langhans type, foreign body type giant cells could also be produced either in response to inert materials such as mineral oil (Timur, G. 1975), and talcum powder (chapter 5), or even biological materials such as fungus as used in this study.

Fibrosis and vascularization are two important components of a chronic inflammation which allow the lesion to heal. In the present study, new fibroblasts began to appear in the fascia and around the blood vessels by the first day after

injection. Actual fibroplasia, which is a major element for encapsulation the irritant and repairing the damaged areas, along with vascularization was active by 3 days post-injection which is similar to that of snakehead infected by *Achlya debaryana* at 28°C (Chinabut 1989), but much faster than 8 days in *A. invaderis* infected snakehead at 26 and 31°C, reported by Chinabut *et al.* (1995). Connective tissue proliferation in this study resulted in joining and repairing the damaged and separated parts of the lesion area, and also encapsulation of the spores. Formation of new capillaries in the lesion area is an important source to nourish the newly developing granulation tissue. Their number and activity in the lesion area was gradually increased by the day 18th, as healing progressed. Thereafter they began to decrease but never totally disappeared by the end of the sampling time. New capillary buds were observed within the supporting stroma around the granulomata in snakehead, 2 days p.i. of *A.invaderis* at 31°C (Chinabut *et al.* 1995).

Mycotic granulomata formation as a defence mechanism of fish to wall off and kill the pathogen began by surrounding the fungus by epithelioid cells at 3 days post-inoculation. Then, along with an increasing layers of tightly bound epithelioid cells and their activity, granulation tissue began to surround dense layers of epithelioid cells, resulting in the formation of the well developed granulomata at 10 days after injection. Thereafter the number and the rate of consolidation of the typical granulomata increased. As the healing process developed (i.e. 18-22 days p.i.) the epithelioid layers in granulomata decreased, while fibrous layers increased so that in the late stages of the lesion, granulomata were cystic and consisted of only a necrotic core of basophilic and eosinophilic debris, and remnants of fungus, with thick fibrous layers around. Chinabut *et al.* (1995) reported that mycotic granulomata were well developed in snakehead

infected with *A. invaderis* and kept at 26 and 31°C, by 4 days p.i., and extensive mycotic granulomatosis was observed by 8 days. From the findings of this study it could be suggested that fibrous proliferation and intense granulomata formation in response to *A. invaderis*, might be an important factor inhibiting fungal growth.

Willoughby and Roberts (1994*b*) showed that motility of *Aphanomyces* zoospores could be inhibited temporarily by physical or chemical shock, but resumed after 4.5 hours without an intervening encystment phase. Therefore they might be transported by water currents, immobile and encyst only on a favourable surface. It is likely that most of the injected zoospores in the lesion of carp tissue (present study) gradually ceased their motility when were sequestrated by epithelioid cells and giant cells, but thereafter they could not regain their motility and consequently lost their viability when granulomata formed and developed. Meanwhile a limited growth of some of the zoospores during the early stages after inoculation and before epithelioid cells formation, was also completely ceased by defence system of fish.

From the results of the present study it could also be suggested that in addition to physical encounter of the fish tissue, and the fungus as a foreign body that could not be phagocytised, (surrounding the fungus and granuloma formation), carp might inhibit and suppress biological activities of the fungus by some unidentified physiological, biological and/or immunological factors, which requires further study.

Muscle regeneration in carp (present study) initiated at 3 days p.i. with redeveloping peripheral nucleation in those myofibrils with slight damage along with appearance of new basophilic muscle buds mostly at the edges of the lesion. Then these muscle buds developed into sarcolemmal tubes and thereafter into muscle bundles. As the healing process developed, the new muscle bundles and regenerating muscle fibres

filled the whole damaged area and replaced the fibrous tissue by about 14 days after injection. Chinabut *et al.* (1995) did not report the time at which muscle regeneration commenced in snakehead infected experimentally with *A. invaderis*, but did state that in the late stages of infection, regenerating muscle fibres were observed replacing the fibrous tissue, and from 14-28 days p.i., the healing process became well established in fish kept at higher temperatures (i.e. 26 and at 31°C) which is similar to the observations in the late stages of this study. Muscle regeneration was recognised by day 4 in snakehead infected with *Achlya debaryana* at 28°C, and the rate of repair of the lesion was very rapid so the infected site became normal by around day 6 p.i. (Chinabut 1989).

The delicate *A. invaderis* of EUS is slow-growing at all temperatures, but dies at 37°C (Willoughby *et al.*1995). The spores of the EUS-specific *Aphanomyces* can grow in muscle of snakehead fish at temperatures ranging from 19 to 31°C. Mortalities from EUS occur when water temperatures are low (Chinabut *et al.* 1995). Low temperatures reduce the resistance of the fish so much that even small numbers of zoospores can initiate infection (Neish 1976). In addition to the activity of the spores, the temperature effects on wound healing (Anderson & Roberts 1975), macrophage response, clearance of necrotic muscles, fibroblasts activity (Finn & Neilson 1971*a*), metabolic activity and protein synthesis (Prosser 1962), and immune response of fish (Ellis 1989). All stages of the inflammatory process are slower to develop and reduced in extent at low temperatures (Chinabut *et al.* 1995), and also the immune response of fish is considerably reduced at low temperatures (Rijkers 1982; Ellis 1989). Therefore opportunities exist for any of a wide variety of viral or other stressors, which might increase the susceptibility of fish to invasion (Chinabut *et al.* 1995). Regarding these

explanations and as a result of effective defence mechanisms of carp (present study) against the fungus, the inoculated fungus showed no continuing viable growth in carp tissue. Therefore such resistance against fungus infection and the fast rate of chronic inflammatory response and wound healing at 27°C (average water temperature in this study) would be expected. As the warm water carp is immunologically suppressed at temperatures below 15°C (Ellis 1989), further study is necessary to determine the effects of *A. invaderis* on carp at low temperatures to find whether this fungus is able to infect carp severely and cause mortalities as in snakehead fish.

Corbel (1975) described that cellular response of fish to mycotic infection varied, ranging from granulomata formation to complete absence of response. The results of the present study agree and support the granulomata formation component of Corbel's findings.

It is apparent from the results of the present study that healthy carp resist *A. invaderis* infection at high temperatures (27°C) by an active defence mechanism and employing a strong inflammatory response. The general pattern and development of the chronic inflammatory response was similar to those described earlier (chapters 5 & 6) and also stated by previous workers.

CHAPTER 9:

GENERAL DISCUSSION

DISCUSSION

All the experiments in the present study conducted to stimulate, and to define the range and type of inflammatory responses of healthy carp (*Cyprinus carpio*) against different types of stimuli. The various stimuli which have been used in this study were; superficial traumatic wound without secondary infection, inert material (talcum powder i.e. magnesium silicate), killed bacterium [(*Mycobacterium butyricum* in Freund's complete adjuvant (FCA)], live bacterium (*Aeromonas hydrophila*), and fungus (live spores of *Aphanomyces invaderis*). These are standard stimuli which the response of other fish species against their naturally or experimentally occurrence, was already defined. The experiments carried out in these ambient temperatures; traumatic wound at 27°C, talc at 26.5°C, FCA at 24°C, *A. hydrophila* at 27.5°C, and *A. invaderis* at 27°C.

The responses of the healthy fish in laboratory conditions may not be the same as that of fish affected naturally with the disease, but it is an important component of understanding the basic responses that can take place in a normal fish.

The histological studies of the inflammatory response of carp against the above mentioned stimuli in this investigation, demonstrated a similarity in general patterns of inflammation. The time scale of the inflammatory responses and the final results in the experiments were different depending on the types of stimulus.

Acute inflammation occurred in response to all stimuli used in this study, in the early stages. The main features of the acute inflammation were; increased blood flow into the lesion area and haemorrhage, fibrinogen exudate, migration of the inflammatory cells such as PMNs, macrophages, lymphocytes and thrombocytes throughout the area. Since the acute inflammation did not resolve quickly after surgical wound, and also irritation of

the tissue continued with other stimuli used, the acute inflammation progressed into chronic inflammation in all the experiments.

Chronic inflammation in traumatic wound and *Aeromonas hydrophila* infection ultimately resolved by scar formation. There was no irritant agent in traumatic wound, and also the bacterium *Aeromonas hydrophila* has been degraded and phagocytised, therefore no typical granulomata occurred in response to these two stimuli. Granulomata formation occurred in response to the irritants such as talc particles, fungus spores and mineral oil of FCA (not *Mycobacteria* sp.), which could not be phagocytised by phagocytic cells. These granulomata in early stages consisted of tightly bound epithelioid cells and giant cells which surrounded the irritant. As the lesion developed in late stages, the layers of epithelioid cells reduced and giant cells disappeared. The ultimate feature of the response consisted of regenerated muscles and granulomata comprising a central zone of necrotic materials and the irritating agent, surrounded by thick layers of fibrous tissue.

Surgical wounding, injection of talc, FCA and fungus did not cause ulceration in carp, but ulceration was a characteristic feature of the carp injected with *Aeromonas hydrophila* in the present study.

The appearance of the darkened skin scar was a gross sign of the final stage of lesion healing process in surgical wound and *Aeromonas hydrophila* infection in carp. The scar in carp wounded by a sharp blade was small and only a straight line at the site of incision, while it occupied a larger and wider area in *A. hydrophila* infected carp which ulcerated following inoculation of the bacteria. No gross sign of scar formation was observed in the fish injected with talc, FCA and fungus throughout the time of experiments. The reason for this difference in darkened skin scar formation is that the needle insertion for

injection of the talc, FCA or fungus spores suspension did not cause much damage to the dermal layers and the melanin-containing cells, but the blade for making incision cut the dermal layers and damaged the melanin pigment-containing cells. Also the destruction and lysis of the tissues by *A. hydrophila* caused either cutting and separating edges of the dermis, which healed by fibrous replacement and scar formation, or severe damage to the melanin-containing cells, which caused the dispersion of melanin pigments throughout the lesion area. The ultimate result of this was the appearance of the darkened skin scar on the site of injury.

Increasing activity of the melanin-containing cells throughout the damaged area after destruction of dermal layers by the traumatic wound or by *A. hydrophila* in this study, could be a neural reaction (Sage 1970; Roberts 1975*a*; Amiri 1991) to protect the damaged and newly repaired skin against irritants or even UV-irradiation (Bullock 1988; Roberts 1989). In fish, the epidermis generally has no protective keratinised layer, and dividing cells which are particularly vulnerable to UV-radiation are found at all levels in epidermis (Bullock *et al.* 1978). Fish skin is therefore more vulnerable to damage because it lacks an inert outer layer, mitotic division in cells takes place at all levels, and normally there are not protective pigments within epidermis since, in general, all pigments are sub-epidermal (Roberts & Bullock 1981).

There was no major damage or destruction of the epithelium in fish injected with talc, FCA and fungus, but there only was a small traumatic injury from passing the needle through the dermal layers which healed very rapidly and no histological sign remained, but in the surgical incision and *A. hydrophila* infection a major damage to the epithelium occurred. The damage caused by *A. hydrophila* was more severe with a large open wound

which may have been due to the toxin and extracellular products of *A. hydrophila*. However, re-epithelialization in the wounded fish by sharp blade, was completed by 4 hours after wounding, which is relatively fast in comparison with many other fish species (table 4.1). Initiation of linking between cut edges of dermis in surgical wound was at 6 days, and were completely linked together and closed at 16 days after wounding. The time scale for wound healing in *A. hydrophila* infection was different from the surgical wound. The exact time of re-epithelialization was not recorded because of the severe damage to the skin in which caused a large lesion. However, at 10 days p.i. new oedematous epidermis was observed, and also linking of the cut edges of dermis with fibrous tissue was recorded. At 14 day, epidermis was normal and dermis was completely linked and closed.

One of the most important responses of fish against injury or pathogenic infection is macrophage infiltration in the damaged area. Macrophages act as protectors of the body and also as scavengers. They digest foreign bodies and necrotic debris (Ellis 1977), and are considered to be the effector cells in non-specific immunity because they are active against a wide array of micro-organisms including viruses, bacteria, fungi and protozoa (Allison 1978). Macrophages are the dominant inflammatory cells in most cellular inflammatory responses. They are capable of developing into epithelioid cells and also giant cells (Roberts 1989). The results of the present study indicate the important role of macrophages in carp in defence mechanism, and removal of damaged tissues which facilitates repair of the damaged area. In this study, macrophages infiltrated the lesion area varying times after damaging the fish tissue by incision, inert and biologic materials. They were found within 2 hours after surgical wounding, and 4 hours after injection of *A. hydrophila*. The sampling time for three other insults was begun at 6 hours after inoculation, hence it was not possible

to detect and record the first appearance of macrophages in very early stages of the lesion. The myophagic and phagocytic phase of macrophages in all insults used, was at 6 hours after induction, except in *A. hydrophila* which was 4 hours post-injection. Macrophages changed into epithelioid cells and formed multinucleated giant cells against talc, FCA and fungus which could not be phagocytised. This phenomenon did not occur in either surgical wound or *A. hydrophila* infection.

PMNs infiltrated in the lesion area at 2 hours after surgical wounding and remained for 8 days. The time scale for appearance of PMNs in the lesion area, as a part of inflammatory response, after injection of talc and FCA was 24 hours and they remained in the area of the lesion until 4 days. These times in fungal and bacterial infections were different. The first sampling time in fungal infection was 6 hours p.i. and PMNs were found in the damage area at this time. They maybe found in the stages earlier than 6 hours but it was not possible to tell from this experiment. They remained in the area for 1-2 days. In the *A. hydrophila* infection, the appearance of PMNs in the defect area was at the first hour p.i., which was faster than that of the other insults used in this study. They were observed in the area for 7 days, which was longer than that of talc, FCA and fungus, and almost the same as in surgical incision.

No obvious role and activity was observed from PMNs in the lesions caused by talc, FCA and fungus, but they were seen around and, to some extent, within the degenerated muscles in the tissues wounded by blade. They seemed to take part in myophagic activity along with macrophages. The greatest numbers of PMNs in carp, in the present study, were observed in those fish infected with *A.hydrophila*. They were one of the dominant inflammatory cells in the area of the lesion throughout the time of major activity. They

participated in myophagia along with macrophages, and also contributed in micro-abscess formation. They were also one of the important factors along with *A. hydrophila* toxins, which resulted in the suppurative nature of the lesion.

Anderson and Roberts (1975) suggested that one of the reasons of different time scale in appearance of PMNs in fishes could be the presence of bacterial factors which provide a powerful attraction at high temperatures. The type of insult and the temperature are also two factors noted by Roberts and Bullock (1976) which affected the exact period of the presence of PMNs in an inflammatory response.

Although the PMNs in mammals are a major phagocytic cells, the phagocytic aspect of the PMNs in fish is still controversial and a debatable concept (Chapter 7). If their role in phagocytosis, especially in bacterial infection, is accepted, therefore their active presence as a phagocytic cell along with macrophages inside the degenerated muscles would be expected. If they are not phagocytic cells, their presence from the very early stages after infection in the area of the lesion and throughout the critical time of the inflammatory response against the pathogen, and inside the degenerated muscle requires an alternative explanation.

Contribution of the PMNs in micro-abscess formation in bacterial infection was documented in experimentally infected carp in this study. It is generally assumed that PMN leukocytosis, suppuration and abscessation do not occur in fish, and Roberts and Bullock (1976) suggested that aggregates of PMNs, so-called micro-abscesses, are very occasionally seen. Chinabut (1989) reported typical suppuration and abscessation as a very marked feature of the response of the snakehead to *A. hydrophila* infection. However, the micro-abscess formation in carp in this study, could be considered as a part of the inflammatory

response of this fish species at high temperature (27.5°C), at least against experimental bacterial infection.

Melanin-containing cells were found in the surgical wounding, and also after the injection of different materials in this study, generally around the blood vessels. Eight days after surgical wounding they started to develop a layer below the new and delicate epidermis and underside fibrous tissue. Melanin was also observed in the fibrous tissue and inside the some of the granulomata produced after injection of talc. Melanization was seen neither around the granulomata nor within the stroma of fibrous tissue after inoculation of FCA. In the late stages of bacterial infection melanin pigments were found throughout the fibrous area and below the new epidermis. They were found generally scattered around the blood vessels in fungus-infected fish.

These findings indicated that the melanin-containing cells had a high level of activity whenever the epidermis was severely damaged as in the wounded fish, and also in the fish infected with *A. hydrophila*. In this situation they appeared in a dendritic shaped throughout the damaged area, especially under the new epidermis. This seems to be a protective reaction for skin, controlled by nervous system (Sage 1970; Roberts 1975*a*; Amiri 1991), when the new delicate epidermis is vulnerable to traumatic damage of many kinds (Roberts 1975*a*). Timur, G. (1975) and Finn and Nielson (1971*a,b*) did not report occurrence of melanin pigments in the granulomatous lesions produced in plaice at 10°C and rainbow trout at 15°C, while Chinabut (1989) found dense melanin pigmented cells around the granulomata in the snakehead at 26.9°C, especially in the late stages of the lesion. She did not found melanin in fish infected with *A. hydrophila*.

Multinucleated giant cells were not found in surgical wound and *Aeromonas* infection in this study, but they were observed at 3 days after inoculation of talc and fungus, and 4 days after injection of FCA. Roberts (1989) stated that giant cells usually develop in association with material which is not readily digested. It is obvious that phagocytic cells could not digest talc particles or fungus spores, but they can phagocytose bacteria, and degenerated materials and debris produced by the traumatic wound. Production of giant cells in FCA-injected fish, as suggested by Timur, G. (1975), maybe in response to the mineral oil of adjuvant rather than the *Mycobacterium*. However, in this study, giant cells were produced in response to inert (talcum powder), biologic (fungus spores), and also a mixture of inert and biologic materials (FCA). Two well known types of giant cells, Langhans and foreign body types, were seen in the injected fish with three above mentioned irritants. A further type so-called intermediate was also found in fish infected with fungus. It seems that there is no relationship between the cause of granulomata formation and the giant cell type, and even provoking the production of giant cell itself. For example, Timur, G. (1975) found intermediate giant cell in plaice in response to injection of talc, while it was not found in talc-injected fish in this study. Furthermore, intermediate giant cell was observed in the fish infected with fungus *Aphanomyces* in the present study, whereas Chinabut *et al.* (1995) did not report any giant cells in snakehead infected with *Aphanomyces*, but Noga *et al.* (1988) found giant cells in UM in Atlantic menhaden, and Miyazaki and Egusa (1972 & 1973) reported it from GM in gold fish and Ayu. However, these differences maybe due to the differences in individual physiological, biological and/or immunological characteristics of the fish species, and also differences in ambient temperatures. It is reported by Secombes (1985) that giant cells are capable of

phagocytosis, but lower than macrophages. This was, to considerable extent, observed in this study.

In addition to cellular infiltration in an inflammation, vascularization, muscle regeneration and fibrosis development are other very important factors and fundamental necessaries for fish to heal the wounds produced by stimuli. These characteristic features of the inflammatory response were very active and rapid in carp in response to all of the stimuli used in the present investigation. The ambient temperatures of the experiments in the present study averaged 26.4°C, which could be a very important factor in strengthening of the inflammatory response and quickening of the wound healing in carp, compare to other previously mentioned species. The affect of temperature on wound healing is well studied and documented by many workers (Chapter 4).

The findings from this investigation indicated that the healthy carp is capable of producing a most effective and rapid inflammatory response against various stimuli used in this study. Localisation of the irritants which could not be phagocytised and removed from the damaged tissues, and also healing of the lesions caused by stimuli, were distinct features of the inflammatory response observed in the present investigation. Further study needs to be undertaken to determine whether the inflammatory response of carp to these stimuli in lower temperatures is similar to the findings of this study, or different.

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APPENDIXES

APPENDIXES

APPENDIX 1

1.1 Histology

The methods used in the present study are those are being used as routine methods in the histology laboratory of the Institute of Aquaculture, University of Stirling as follows;

1.1.1 Fixation

The aim of fixation is;

1) to prevent autolysis, bacterial decomposition and putrefaction.

2) to coagulate the tissues so to prevent loss of easily diffusable substances e.g. glycogen.

3) to safeguard the tissue against the damaging effects of processing.

4) to leave tissues in a condition which facilitates differential staining with dyes and other reagents.

Material for histological examination should be placed in fixative for at least 24 hours prior to cassetting. Ten percent (10%) neutral buffered formalin is normally used. One volume of tissue per twenty five volumes of fixative is recommended. Formalin is an irritant of eyes, nose and skin. it should be handled only in a fume cabinet and disposable gloves should be worn whilst handling and dissecting samples.

1.1.1.1 10% neutral buffered formalin

sodium dihydrogen phosphate (monohydrate)	4.0g
disodium hydrogen phosphate (anhydrous)	6.5g
formaldehyde	100ml

distilled water 900ml

Other commonly used fixatives include gluteraldehyde, bouin's, carnoy's and davidson's.

1.1.2 Cassetting

Tissue samples should be trimmed to a suitable size and must not be overcrowded in cassettes as this will lead to ineffective dehydration and ultimately difficulty in sectioning. Small samples (smaller than the size of the pores in the cassette) should be wrapped in tissue paper before placing in the cassette. Soft tissues (e.g. kidney, spleen, digestive system, heart, brain etc.) and hard tissues (e.g. muscle, skin, gills and eyes) should be kept separate. Cassetted samples should not be allowed to dry out and must be left in a bowl of water or fixative until loading onto the processor.

1.1.3 Processing

The aim of processing is to impregnate the tissue with an embedding medium which will give support to the tissue during section cutting. The most satisfactory embedding material for routine histology is paraffin wax.

1.1.3.1 Dehydration

Dehydration is the first stage in the processing of fixed tissues and involves the removal of water by immersion in a graded series of alcohol.

1.1.3.2 Clearing

This term relates to the appearance of tissues after they have been treated by chloroform which removes the dehydrating agent. The essential requirement of the clearing agent is miscibility with both the dehydrating agents and the embedding agent.

1.1.3.3 Wax impregnation

The function of wax impregnation is to remove the clearing agent from the tissues and then complete permeation of the tissues by paraffin wax.

1.1.3.4 Processing schedule

The above mentioned procedures are carried out by placing the cassetted tissues into a basket which is clipped onto an automatic tissue processor. The basket is moved round automatically at the appropriate time intervals as follows;

1) 50% methylated spirit	1 hr
2) 80% methylated spirit	2 hrs
3) 100% methylated spirit	2 hrs
4) 100% methylated spirit	2 hrs
5) 100% methylated spirit	2 hrs
6) 100% ethanol	2 hrs
7) 100% ethanol	2 hrs
8) chloroform	1 hr
9) chloroform	1 hr
10) molten wax	2 hrs
11) molten wax	2 hrs
12) molten wax	2 hrs

1.1.4 blocking out

Cassettes are removed from the processor and placed in the auxiliary wax bath on the histoembedder. The metal lid is removed and the appropriate size of basemould selected to give an adequate margin of wax around the tissue. The base is filled with

molten wax by pushing back gently on the dispenser handle. The mould is then placed onto the coldplate and the tissues are orientated into the solidifying wax using warmed forceps. Orientation of the tissue depends primarily on the type of section required. Tissues such as skin should be embedded so that the skin edge is uppermost in the block. This makes sectioning easier as the soft tissue underneath is cut through before the hard skin surface. The empty cassette is placed on top of the mould and topped up with wax. By placing the mould on the coldplate the wax solidifies and the sample is held in position. Once solidified (approximately 5 min) the block is removed from the base mould. The reusable basemould is then returned to the appropriate position, in the tray, on top of the histoembedder.

1.1.5 Microtomy

1.1.5.1 Trimming-in

The surface layer of wax has first to be removed to expose the complete surface of the specimen. this is carried out on the microtome using an old blade. The rate of advancement of the block towards the knife is determined manually at this stage.

1.1.5.2 Section cutting

Before sections can be obtained from blocks of hard tissue it is often necessary to surface decalcify them. This is carried out by placing the trimmed blocks face down in a vessel containing a layer of rapid decalcifying solution for approximately one hour. Blocks of soft tissue which have been hardened excessively during processing can be soaked in water prior to cutting for approximately 30-60 min depending on tissue. Blocks are cooled using a coldplate prior to sectioning. Specimens are clamped into the block holder, which is automatically advanced by five microns on every rotation of the operating wheel. When a "ribbon" of sections is obtained this is removed using forceps and floated out onto the surface of the water. The best section is selected and picked up

on a clean glass slide. The case number is marked onto the bottom of the slide using a diamond pen and the slide is rested face down against the runners on the hotplate. Slides are then placed into racks with the sections all facing in the same direction (this ensures sections are not wiped off whilst coverslipping after staining). Racked slides are then dried in an oven at 60°C for at least one hour before staining. This fixes the section onto the slide and prevents it floating off whilst staining.

APPENDIX 2

2.1 Staining

The staining methods used in this study are those routinely being used in the histology laboratory of the Institute of Aquaculture, University of Stirling. To prepare this appendix, Carleton's Histology Technique revised and rewritten by R.A.B Drury and E.A. Wallington (1980), also has been used.

In order to examine sections effectively under the microscope they require to be stained. Many different staining techniques can be used. The most common being haematoxylin and eosin (H & E).

2.1.1 Haematoxylin and eosin

2.1.1.1 Preparations

Haematoxylin	1gr
Sodium iodate	0.2gr
Potassium alum	50gr
Citric acid	1gr
Chloral hydrate	50gr
Distilled water	1000cm^3

2.1.1.2 Technique

xylene (dewaxing)	5 min
absolute alcohol (i)	2 min
methylated spirit	1.5 min
wash in tap water	
haematoxylin	5 min
wash in tap water	

acid alcohol	3 quick dips
wash in tap water	
Scott's tap water	1min
wash in tap water	
eosin	5 min
wash in tap water	
methylated spirit	30 sec
absolute alcohol (ii)	2 min
absolute alcohol (i)	1.5 min
xylene (clearing)	5 min
xylene (coverslip)	

Slides must not be left out of xylene as they will dehydrate. Sections are coverslipped after the last xylene using Pertex mounting medium.

2.1.2 The periodic acid-Schiff (PAS) reaction

This staining method is used to demonstrate acid mucopolysaccharides (mucin) and glycogen. PAS with tartrazine in cellosolve counterstain is also used for the demonstration of carbohydrates.

2.1.2.1 Technique

sections to water	
oxidise in freshly prepared 1% aqueous periodic acid	5 min
wash in running water	5 min
rinse in distilled water	
place in Schiff's reagent	20-30 min
wash in running water	

stain nuclei with mayers haematoxylin	5 min
wash in running water	
dip 4 times in acid alcohol	
wash in running water	
Scott's tap water	1 min
wash in running water	

If the sections need to be counterstain with tartrazine, they should be counterstain with 1% tartrazine in cellosolve for about 5 min in this stage. Then the sections should be blot dried on filter paper.

dehydrate, clear and mount in Pertex

Results

In PAS staining, PAS-Positive substances are red or magenta and nuclei are blue. Also fungi are red/purple.

In PAS with tartrazine staining, PAS-Positive substances are red, nuclei are blue, and cytoplasm, muscles and connective tissue are yellow.

2.1.3 Grocott's modification of gomori's hexamine silver method

2.1.3.1 Preparation

1) Stock solutions

5% sodium tetraborate (borax) in distilled water	
5% silver nitrate in distilled water	5 ml
3% hexamine in distilled water	100 ml

A white precipitate will form, but this dissolves on shaking.

2) Working solutions

Mix 2ml of the borax solution with 25ml of distilled water. Add 25ml of the hexamine-silver nitrate stock solutions. Use freshly prepared.

2.1.3.2 Technique

take sections to water	
oxidise in 5% chromic acid	60 min
wash in running water	
rinse briefly in 1% sodium bisulphite	
wash in tap water	5 min
wash in at least three changes distilled water	
place in preheated working solution(56°C) (the section should be yellowish brown)	30-60 min
rinse in six changes of distilled water	
tone in 0.1% gold chloride solution	2-5 min
rinse in distilled water	
place in 2% sodium thiosulphate(hypo)	2-5 min
wash thoroughly in water	
counterstain with light green solution	30 sec
dehydrate, clear and mount in Pertex	

<u>Results</u>

Fungi are seen sharply outlined in black, mucin grey, and background pale green.

2.1.4 Immunohistochemistry

The methods of immunohistochemistry most commonly used are with either an antibody conjugated to HRP or fluorescen isothiocyanate (FITC) as the secondary antibody. The enzyme method takes longer than the fluorescence method, but it gives a

permanent record of the staining and allows counterstaining of the section. The fluorescence method is quicker although the staining fades after a short time.

Cytospin, cell smears or tissue sections (fresh or formalin-fixed) can all be used for immunohistochemistry. Cryostat sections, cytospin and smears which are not stained immediately can be stored for several months at -20°C. Allow the slides to warm to room temperature prior to staining.

2.1.4.1 Technique for Immunohistochemistry using formalin-fixed sections with HRP staining.

-Prepare paraffin-embedded tissue sections from control and infected fish.

-Dewax sections in xylene (2 x 5 mins), 100% ethanol (5 mins), 70% ethanol (3 mins) then rinse in distilled water.

-Mark rings around the tissue section using a wax PAP pen (BDH Ltd). This allows small volumes of sera or culture medium to be added onto the section.

-Block exogenous peroxidase activity by incubating the slides for 10 mins at room temperature with 10% v/v hydrogen peroxide in methanol.

-Wash the slides three times with Tris buffered saline (TBS, 0.05M Tris, 0.15M NaCl pH. 7.6)

-Block non-specific binding sites with normal goat serum diluted in 1/10 in TBS for 10 mins at room temperature.

-Pour off the goat serum and dry the slides by tapping the edges on paper towels. Use immunised mouse (rabbit) sera (1/100 in TBS) (as a positive control) and normal mouse (rabbit) sera as a negative control. Incubate the slides for 60 mins at room temperature in a moist chamber.

-Wash slides as above.

-Add goat antimouse (rabbit)-HRP conjugate (1/50 in TBS) to the slides for 30 mins.

-Wash excess HRP off of the slides as previously described.

-To visualise a reaction, incubate the slides for 10 mins with 3'3 diaminobenzidine tetrahyrochloride (DAB) in the presence of hydrogen peroxide (H_2O_2). To prepare the solution add 100µl of 1% H_2O_2 to 0.5 ml (1.5 $mgml^{-1}$ DAB) and 5 ml TBS.

-The reaction is stopped by immersing the slides in tap water.

-The slides are counterstained with haematoxylin for 3-4 mins. They were then dehydrated in 70% ethanol for 3 mins, 100% ethanol for 10 mins and rinsed twice in xylene for 5 mins. Slides were mounted using glycerol/PBS (50:50).

N.B. Do not allow slides to dry out after fixation.

Result

Positive tissue appears brown in colour under a light microscope.

2.1.5 Ziehl-Neelsen method for acid-fast bacilli

2.1.5.1 preparations

1) Carbol-fuchsin:

Basic fuchsin	1 gr
Absolute alcohol	10 cm^3
5% phenol in distilled water	100 cm^3

2) Acid-alcohol:

1% hydrochloric acid in 70% alcohol

3) Counterstain:

Methylene blue	1 gr

Glacial acetic acid	1 cm^3
Absolute ethyl alcohol	20 cm^3
Distilled water	80 cm^3

2.1.5.2 Technique

take sections to water

xylene	5 min
absolute alcohol	2 min
methylated spirit	1.5 min

cover section on slide with filter paper

Drop carbol fuchsin onto slide with pipette until a small pool of stain is formed.

In a fume cupboard heat the stain over a low bunsen flame until stain starts to steam.

Remove slide from heat and leave for ten minutes.

Wash in running tap water for at least 5 min.

Differentiate in acid alcohol, until section becomes pale pink and no more colour comes away- approx. 30 sec to 1 min.

Rinse in running tap water.

Counterstain in 1% acidified methylene blue for 1 min.

Rinse in running tap water.

Differentiate counterstain in alcohol	approx. 30 sec to 1 min.
Xylene 2 (clearing)	5 min
Xylene 3 (coverslip)	1 min

Coverslip using Pertex mounting medium.

Results

Acid fast bacilli are red, other bacteria blue, cells and their nuclei blue and red blood corpuscles should retain a slight red colour.

2.1.6 Auramine stain

Auramine stain is a popular, simple and sensitive method for the detection of acid fast bacilli, particularly *Mycobacterium tuberculosis* and *M. Leprae.*

2.1.6.1 Preparation of Auramine solution (Truant's solution)

Auramine O (Cl 4100)	1.5 gr
Rhodamine B (Cl 749)	0.75 gr
Phenol	10 ml
Distilled water	50 ml
Glycerol	75 ml

2.1.6.2 Technique

1) Deparaffinized tissue section or smear of sputum, etc.

2) Stain with 3 ml of Auramine solution for 15 min at room temperature.

3) Wash with tap water.

4) Rinse repeatedly with 0.5% Hcl-alcohol, and check after each rinse under the fluorescence microscope until removal of non-specifically staining materials is recognised.

5) Dip into 0.5% Hcl-alcohol for 1-3 minutes.

6) Wash with tap water for 30 sec-1 min.

7) Stain with 0.5% potassium permanganate for 2 minutes.

8) Wash with water.

9) Mount

10) Fluorescence microscopy.

Result

Acid fast bacilli stain orange or yellow.

2.1.7 Gram staining

2.1.7.1 Gram's stain for bacteria in sections

Take sections to water	
Crystal violet	2-3 min
Wash with iodine	
Flood slide with iodine	2-3 min
Decolourise with acetone	
Wash quickly with water	
Neutral red	2-3 min
Rinse rapidly in water	
Blot dry with filter paper	
Xylene and coverslip	

Results

Gram positive bacteria are purple

Gram negative bacteria are pink

2.1.7.2 Gram's stain for bacteria in smears

The morphology of bacteria is difficult to observe in wet, unstained preparations and these are not permanent. It is usual to stain thin films of organisms in order to examine them. The Gram's stain is a differential stain as it demonstrates bacteria of different types.

This staining procedure is the most important and most widely used in bacteriology for it divides nearly all bacteria into one of two categories.

Gram Positive: Resist decolourisation by ethanol or acetone and stain blue/purple.

Gram Negative: Are decolourised by ethanol or acetone and are stained red/pink.

This difference in colour reaction is due to the different chemical composition of the cell wall and membrane. This procedure is used to show the general morphology of the bacteria as well as demonstrating their Gram reaction.

2.1.7.2.1 Preparations

1) Crystal Violet Solution

Crystal violet (C.I. No. 42555)	2g
95% Ethanol	20ml
Ammonium Oxalate	0.5g
Distilled Water	80ml

Dissolve Crystal Violet in Ethanol

Dissolve Ammonium Oxalate in Distilled Water

Mix these two solutions, allow to stand for 24 hr then filter

2) Iodine Solution

Iodine	1g
Potassium Iodide	2g
Distilled Water	300ml

Dissolve the potassium iodide in as small a volume of water as possible then add the iodine and allow it to dissolve. Then add the remaining water. Allow to stand for 25 hr then filter.

3) Safranine Solution

Safranine (C.I. No. 50240)	0.25g
95% Ethanol	10ml
Distilled Water	90ml

Dissolve Safranine in Ethanol then add distilled water. Allow to stand for 24 hr then filter.

4) Alcohol/Acetone mixture

Ethanol	950ml
Acetone	50ml

2.1.7.2.2 Technique

1) Take a clean glass slide and, using a sterile loop, aseptically place a loopful of sterile saline onto the slide.

2) Remove a minute quantity of culture using a sterile loop. Emulsify the culture in the liquid on the slide and spread evenly.

3) Allow the slide to dry naturally.

4) Using foreseps hold the slide with the film upwards and slowly pass through a bunsen flame three times to fix the film.

5) Allow the slide to cool and place on a staining rack.

6) Cover the slide with crystal violet solution and leave for approximately one minute.

7) Wash off the residual stain with iodine soln. then cover the slide in iodine and leave for approximately one minute.

8) Wash with alcohol/acetone mixture until no further stain comes out.

9) Wash the slide thoroughly with water.

10) Counterstain with safranine solution for approx. two minutes.

11) Wash and dry the slide and carefully clean excess stain off the bottom of the slide.

12) Microscopically examine the stained slide under x40 objective then under oil immersion with X100 objective.

APPENDIX 3

3.1 Bacteriology

All the methods for bacteriology used during this study are those are routinely being used in the bacteriology laboratory of the Institute of Aquaculture, University of Stirling.

3.1.1 Motility test

This test demonstrates whether a bacteria is capable of independent movement, i.e. is motile.

Many species of bacteria are capable of motility by the movement of external appendages called flagella. This motility can be observed directly under the microscope using a suspension of living bacteria in a "hanging drop slide". A drop of bacterial suspension is hung from the underside of a coverslip and mounted, using soft paraffin, on a microscope slide.

Direct observation of the slide under the x40 objective lens of a microscope will then reveal whether the bacteria are motile.

It is important not to confuse true motility with vibration or Brownian movement. All bacteria in suspension exhibit movement which is quite random and non-directional whereas only some bacteria exhibit true motility which is non-random and directional.

3.1.1.1 Technique

1. Place Vaseline on the four corners of a coverslip.

2. Place the coverslip, Vaseline up, on the bench.

3. Using a sterile loop, pick up a loopful of sterile saline and place it on the coverslip. Re-sterilise the loop and pick up a minute amount of growth from an agar plate and gently emulsify the growth in the saline. Remember to sterilise the loop after use.

4. Gently lower a microscope slide onto the Vaseline mounds without allowing the slide to touch the drop of culture.

5. Quickly, but gently, invert the slide so that the drop is hanging from the coverslip.

6. Carefully place the slide onto the microscope and rack down the X40 objective lens until it is just touching the coverslip.

7. Rack up the objective lens slowly until the image is formed.

8. Examine for evidence of bacterial motility.

3.1.2. Oxidase test

This test demonstrates whether a bacteria possesses certain oxidase enzymes that are involved in electron transfer from electron donors. If the redox dye tetramethyl-p-phenylenediamine is used as the electron acceptor, this will be reduced and the reduced dye has a deep blue colour.

Growth from an agar culture of the organism under test is smeared onto a pre-prepared freeze-dried reagent strip (Oxidase strip) and the colour of the smear is noted after 30 seconds.

Cultures must be smeared using a platinum wire as other bacteriological wires may contain traces of iron which could catalyse the reaction and give a false positive result.

3.1.2.1 Technique

1. Place an "oxidase strip" into a clean petri dish.

2. Sterilise a platinum wire and allow to cool.

3. Pick up a heavy inoculum of pure growth from a culture plate using the tip of the wire.

4. Smear the inoculum over the area of filter paper containing the "oxidase reagent".

5. Re-sterilise the wire.

6. Observe the smear for up to 30 seconds and note any colour change.

Results

A deep blue or purple colour developing within 30 seconds indicates oxidation of the reagent and a positive result.

3.1.3 O-F test (Hugh and Liefson)

This test demonstrates whether bacteria can break down glucose aerobically (by oxidation) or anaerobically (by fermentation).

A culture of the organism is inoculated into freshly prepared tubes of O-F medium by a single stab with a straight wire. One tube is incubated in the presence of air (open, aerobic tube), the other is covered with a thick layer of liquid paraffin to exclude air (closed, anaerobic tube). The medium contains bromothymol blue pH indicator to indicate the formation of acid from the breakdown of glucose.

3.1.3.1 Technique

1. Sterilise a straight wire and allow to cool.

2. Pick up an inoculum of pure growth from a culture plate using the tip of the straight wire.

3. Make a single stab into the agar of one tube using the inoculated straight wire.

4. Inoculate the second O-F tube in an identical manner.

5. Aseptically cover the agar in one tube with a 5-10 mm layer of liquid paraffin.

6. Incubate both tubes.

After suitable incubation the results are interpreted as follows;

Open tube	Closed tube	Result
Green	Green	No reaction on glucose
Blue at top	Green	Alkaline reaction
Yellow	Green	Oxidative
Yellow	Yellow	Fermentative

3.1.4 0/129 Sensitivity

0/129 (2,4-Diamino- 6,7-di-iso-propyl pteridine phosphate) is a bacteriostatic agent effective against *Vibrio* species. Knowing whether an organism is sensitive to 0/129 is of great value in differentiating *Vibrio* species from other Gram-negative rods and particularly from *Aeromonas* species which are characteristically resistant to 0/129.

Sensitivity is determined by placing filter paper discs impregnated with 0/129 (10 mg and 150 mg) on an agar plate which has been spread with a suspension of the organism under test. After incubation, sensitivity to 0/129 is indicated by a clear area of no growth surrounding the discs. An organism may be sensitive, partially sensitive or resistant to 0/129.

Media used for determining sensitivity must contain an adequate sodium chloride concentration for the growth of *Vibrio* species. Sensitivity agar supplemented with 1.5% NaCl may therefore be required. The use of marine agar is not recommended.

3.1.4.1 Technique

1. Remove several colonies of growth using a loop and inoculate a small quantity of sterile saline with the culture.

2. With the bottle cap replaced tightly, suspend the culture in the saline by gentle shaking.

3. Using a sterile pipette, remove a small volume of the suspension and carefully pipette 5 drops of suspension onto the surface of the agar.

4. Sterilise a glass spreader by immersing it in 70% ethanol, then pass the spreader through a Bunsen flame to burn off the excess ethanol.

5. Gently spread the suspension over the whole surface of the agar plate.

6. Replace the lid and allow the plate to dry for approximately one minute.

7. Place one 10 µg disc and one 150 µg disc of 0/129 onto the plate.

8. Invert the plate and incubate at a suitable temperature.

3.1.5 API 20E Microbial Identification Kit

The API 20E™ system is a standardised miniaturised version of conventional procedures for the identification of certain Gram-negative bacteria. It is a microtube system enabling 23 standard biochemical tests to be carried out on a bacterial culture.

Although designed primarily for identification of Enterobacteriacea and some other Gram-negative bacteria from human clinical specimens this system has proved of value in the identification of some fish pathogens.

A suspension of the culture under test is prepared and inoculated into small plastic cups containing dehydrated media. After suitable incubation, results are obtained by direct reading or after addition of reagents.

Identification of the organism under test may then be possible by comparing the results obtained with known results for certain fish pathogens.

3.1.5.1 Preparations

3.1.5.1.1 Preparation of strip

1. Dispense a small volume of tap water into the incubation tray using a plastic squeeze bottle. This provides a humid atmosphere during incubation.

2. Remove API strip from the sealed envelope and place in the tray.

3.1.5.1.2 Preparation of bacterial suspension

1. Sterilise a bacteriological loop and allow to cool.

2. Pick up an agar plate containing a pure culture of the organism under test and using the sterile loop remove several colonies of growth.

3. Inoculate 5ml of sterile distilled water or sterile saline (if organism requires salt for growth) by tilting the bottle and gently rubbing the culture from the loop on the inner surface of the bottle. Avoid unnecessary vibration of the loop.

4. Re-sterilise the loop.

5. With the bottle cap replaced tightly, suspend the culture in the water/saline by gentle shaking.

3.1.5.2 Technique

3.1.5.2.1 Inoculation of strip

1. Remove a sterile pipette from its container and pipette up a quantity of the culture suspension.

2. Tilt the API incubation tray and carefully fill the tube section of the microtubes by placing the pipette tip against the side of the cupule

3) Fill both the tube and the cupule section of the ⌊CIT⌋, ⌊VP⌋ and ⌊GEL⌋ tubes.

4) After inoculation completely fill the cupule section of the ADH, LDC, ODC H_2S and URE tubes with sterile liquid paraffin using a sterile pipette.

5) Replace the lid on the strip then incubate at a suitable temperature. For incubation at 20-22°C. up to 3 days may be required before reading results.

3.1.5.2.2 Reading the results

After examining the strip, record all reactions not requiring reagents as detailed in "Summary of Results".

(a) Add 1 drop of TDA reagent to the TDA tube.

(b) Add 1 drop of VP1 then 1 drop of VP2 reagent to the VP tube.

(c) Add 1 drop of IND reagent to the IND tube.

Record reactions as detailed in "Summary of Results".

3.1.5.2.3 Identification of organism

Once all results are recorded, these, along with the results of the primary tests, can be compared with known results for specific fish pathogens. In many cases this is sufficient for identification of the organism.

Care must be taken however, as discrepancies may occur between reactions obtained with conventional and API 20E tests. This should be borne in mind when interpreting characterisation tests.

API 20E "SUMMARY OF RESULTS"

Results

Test	Substrate	Reaction / enzyme	Negative	Positive
ONPG	Ortho-nitrophenyl galactosidase	beta-galactosidase	Colourless	Yellow
ADH	Arginine	arginine dihydrolase	Yellow	Red/Orange
LDC	Lysine	lysine decarboxylase	Yellow	Orange
ODC	Ornithine	ornithine decarboxylase	Yellow	Red
CIT	Sodium Citrate	citrate utilisation	Yellow	Blue/Green
H_2S	Sodium Thiosulphate	H_2S production	Colourless	Black Deposit
URE	Urea	urease	Yellow	Red/Orange
TDA	Tryptophane	tryptophane deaminase	Yellow	Dark Brown
IND	Tryptophane	indole production	Yellow Ring	Red Ring 2 minutes
VP	Sodium Pyruvate	acetoin production	Colourless	Pink/Red 10 minutes

GEL	Gelatine	gelatinase	No Black Diffusion	Black Diffusion
GLU	Glucose	fermentation/oxidation	Blue/Green	Yellow
MAN	Mannitol	fermentation/oxidation	Blue/Green	Yellow
INO	Inositol	fermentation/oxidation	Blue/Green	Yellow
SOR	Sorbitol	fermentation/oxidation	Blue/Green	Yellow
RHA	Rhamnose	fermentation/oxidation	Blue/Green	Yellow
SAC	Sucrose	fermentation/oxidation	Blue/Green	Yellow
MEL	Melibiose	fermentation/oxidation	Blue/Green	Yellow
AMY	Amygdalin	fermentation/oxidation	Blue/Green	Yellow
ARA	Arabinose	fermentation/oxidation	Blue/Green	Yellow

N.B.

Reagents to be added to TDA, IND, VP tests.

Once reagents are added the strip cannot be re-incubated.

H_2S and Citrate test is not very reliable for fish bacteria incubated at low temperatures.

3.1.6 Preparation of media for bacteriological tests

3.1.6.1 Tryptone Soya Agar (TSA)

It is a general purpose medium for the growth of a wide variety of organisms.

Formula	gr/litre
Tryptone	15.0
Soya peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.3 ± 0.2	

Suspend 40g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

3.1.6.2 Tryptone Soya Broth (TSB)

This is a highly nutritious general purpose medium for the growth of bacteria and fungi.

Formula	gr/litre
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5
pH 7.3 ± 0.2	

Suspend 30g in 1 litre of distilled water and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes.

APPENDIX 4

Control fish

Control fish were injected intramuscularly on the left myotome with normal saline at the same volume as used for the injection of experimental fish.

The control fish were sampled, processed and sectioned using the same procedures as experimental fish.

Histological changes observed in the control fish injected with sterile normal saline were minor compared with the experimental fish.

A mild haemorrhage and muscle degeneration with no inflammatory cell infiltration was detected in the area of injection at early stages post-injection (Fig. 1 & 2). The damage to the muscle area of the control fish was due only to the passage of the needle.

Fig. 1 At 1 hour after injection of sterile normal saline in control fish, the area of injection showed haemorrhage (arrow heads) and some muscle degeneration (arrow). (H & E, X 44).

Fig. 2 At 6 hours post-injection of normal saline, haemorrhage (arrow heads) and more muscle degeneration (arrows) were observed. (H & E, X 110).