# Characterisation of extracellular products produced by *Mycobacterium* spp. and their effects on the fish immune system

**Thesis submitted for the degree of**

**Doctor of Philosophy**

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**by**

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to my parent

**DECLARATION**

I hereby declare that this thesis has been composed entirely by myself and has not been submitted in any previous application for a degree.

The work of which it is a record has been carried out myself. The nature and extent of any work carried out by, or in conjunction with, others has been specifically acknowledged by reference.

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## ABBREVIATIONS

| | |
|---|---|
| AAHRI | Aquatic Animal Health Research Institute, Bangkok, Thailand |
| APS | Ammonium persulphate |
| ATCC | America Tissue Culture Center |
| BAN | Mycobacteria isolate from Bandon Hatchery, Oregon, USA |
| BCG | Bacilli Calmette-Guerin |
| BSA | Bovine serum albumin |
| CMI | Cell mediated immunity |
| Con A | Concanavalin A |
| CRP | C-reactive protein |
| CSF | Colony stimulating factors |
| DMSO | Dimethyl sulfoxide |
| EcF1 | A specific chromatographic fraction |
| ECP | Extracellular product |
| ELISA | Enzyme-linked immunosorbent |
| EMEM | Eagles Minimum essential medium |
| FCA | Freund's complete adjuvant |
| FCS | Foetal calf serum |
| fECP | Formalin toxoid extracellular product |
| FIA | Freund's incomplete adjuvant |
| HRP | Horse radish peroxidase |
| IFN-γ | Gamma Interferon |
| IL I | Interleukin I |
| IL II | Interleukin II |
| ILs | Interleukines |
| IM | Intramuscularly |
| IP | Intraperitoneally |
| IPNV | Infectious pancreas necrosis virus |

| | |
|---|---|
| IS | Intraswimbladder |
| kDa | Kilodalton |
| L-15 | Leibovitz-15 medium |
| LPS | Lipopolysaccharide |
| MAbs | Monoclonal antibodies |
| MAF | Macrophage activation factor |
| MC | *Mycobacterium chelonae* |
| MCA | *Mycobacterium chelonae* subspecies *abscessus* |
| MCC | *Mycobacterium chelonae* subspecies *chelonae* |
| MF | *Mycobacterium fortuitum* |
| MIF | Macrophage inhibition factor |
| mL | Mili liter |
| MM | *Mycobacterium marinum* |
| mM | Mili molar |
| NCIMB | National Collections of Industrial and Marine Bacteria, United Kingdom |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| Pen/Strep | Potassium bebzyl-penicillin/streptomycin sulphate |
| PMA | Phorbol myristate acetate |
| SC | Subcutaneous |
| SDS | Sodium dodecyl sulfate |
| SOD | Superoxide dismutase |
| TBS | Tris-buffered saline |
| TEMED | N,N,N,N,-tetramethyl-ethylenediamine |
| VHS | Viral hemorrhagic septicemia |

**ABSTRACT**

*Mycobacterium* spp. isolated from food and ornamental fish in Thailand (strains TB1, TB40, TB267, TB268), and the type strains *Mycobacterium marinum* (NCIMB 1298), *Mycobacterium fortuitum* (NCIMB 1294), and *Mycobacterium chelonae* (NCIMB 1474) were cultured in Long's medium, Eagle's minimum essential medium, Sauton's medium and modified Sauton's medium. The latter enabled excellent growth and production of extracellular products (ECP) from TB40, TB267, TB268 and *M. marinum* in particular, whereas growth and production of ECP for all strains was limited in Long's medium. SDS-PAGE protein profiles of ECPs from 14 day culture supernatants showed major bands at 65, and <14 kDa. After 2 days culture at the higher temperature of 37°C (heat shock), the production of ECP from all mycobacteria strains except *M. marinum* averaged approximately 4 to 10 fold higher than from strains cultured for 14 days at 28°C.

The major fibronectin binding proteins from ECP of *Mycobacterium* spp. isolated from infected fish were identified at 21-25 kDa. Cross reactivity was detected between ECP from *Mycobacterium* spp. and MAb anti-heat shock protein (60 kDa) and MAb anti-*M. Tuberculosis*. The 65 kDa antigen of TB267 is a strongly immunogenic protein eliciting antibodies in fish, rabbits and mice. Cross-reactivity was found between rabbit anti-65 kDa antibody and sonicated proteins from many other bacterial species. Therefore, the 65 kDa protein from *Mycobacterium* sp. isolated from snakehead fish may be a common protein in fish bacterial pathogens.

Eighteen MAbs to TB 267 and *M. chelonae* were produced. The epitopes to which the MAbs are against located on molecules susceptible to protease treatment. All MAbs recognized the 65

kDa protein. It is one of major proteins in the ECP, whole cell sonicates and lysates from *Mycobacterium* spp. and is located in the periplasmic space or cell wall, and is secreted in the medium during culture.

A primary intraperitoneal (IP) immunisation of extracellular products (ECP) from *Mycobacterium* spp., (strains TB40, TB267 or *M. marinum*) mixed with Freund's incomplete adjuvant (FIA), followed by a secondary IP injection at 8 wks, resulted in the elevation of both the non-specific immune response (by measuring nitroblue tetrazolium, lysozyme and phagocytosis activity) and the specific immune responses of rainbow trout, *Oncorhynchus mykiss* (by measuring specific antibody levels). Nile tilapia were immunised by injecting extracellular products (ECP) of *Mycobacterium* spp. (strain TB40, TB267 or the type strain *M. marinum*) into their swimbladders and this resulted in the elevation of the non specific immune response.

The cytological response of rainbow trout head kidney macrophages to ingested *Mycobacterium* spp was examined *in vitro*. The bacteria had previously been opsonised with either fresh rainbow trout serum (FS), or serum which had been heat-inactivated (HIS), or rainbow trout antiserum against the extracellular products (ECP) of *Mycobacterium* strains TB267 or *M. marinum*. MAbs against the ECP were also used as opsonins. Opsonisation of the mycobacteria was found to greatly enhance the phagocytic and killing activity of the rainbow trout macrophage.

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## General Introduction

Acid-fast organisms isolated from fish have been identified as *Mycobacterium* and *Nocardia* species. The family Mycobacteriaceae, comprising the single genus *Mycobacterium*, consists at present of some 54 recognised species of aerobic, non-motile, non-spore forming, polymorphic rods, with filamentous and coccoid forms also occuring which are characteristically acid-alcohol-fast at some stage of growth (Wayne and Kubica, 1986; Frerichs, 1993).

The first report of a mycobacterial infection in fish was attributed to Bataillon *et al.*, (1897) who isolated acid-fast bacilli from a tuberculous lesion in common carp (*Cyprinus carpio*). The carp isolate was subsequently identified as a distinct species and named *Mycobacterium piscium* (Bataillon *et al.*, 1902). The type culture of this organism (ATCC 9819) is however, no longer available and *M. piscium* is not recognised as a valid species in Bergey's Manual of systematic Bacteriology. Aronson, (1926) reported the first now well-established fish pathogen, *Mycobacterium marinum*, following isolation of the organism from tropical marine fish. *Mycobacterium platypoecilus*, isolated from a Mexican platyfish (*Platypoecilus maculatus*) by Baker and Hagan (1942) and *Mycobacterium anabanti* isolated from Siamese fighting fish (Besse, 1949) are regarded as synonymous with *M. marinum*.

*Mycobacterium marinum* and *M. fortuitum* are the as acid fast organisms most commonly isolated from fish and other cold blooded animals. Other mycobacteria have also been isolated. For example *M. avium* complex, serotype 8, was isolated from a sea turtle (Brock *et al.*, 1976); *M. chelonae* has been isolated from tuberculous-like lesions in fish, snake and turtle (Bernstad, 1974; Rhodin and Anver, 1977; Thoen, 1979; Wolke *et al.*, 1978) snakehead (Chinabut, *et al*

1990), Siamese fighting fish (Somsiri, 1993) and Swordtail (Gomez *et al.*, 1996). Mycobacteriosis or fish tuberculosis infects a wide range of hosts throughout the world. It not only represents a major problem in the farming of food fish through the world, but also within the aquaria trade where the worldwide export of ornamental fish is increasing. High mortalities due to mycobacteriosis in farmed fish have been reported in Thailand and Israel (Chinabut *et al.*, 1990; Colorni, 1992)

*M. marinum* is the best known opportunistic *Mycobacterium* that gives rise to cutaneous lesions in man (Jenkins, 1991). *M. marinum* usually causes a localised cutaneous lesion that many be nodular, or ulcerative, rarely involving deeper structure (Brown and Sanders, 1987). New nodules or ulcers may arise along lymphatic vessels draining the arm (Kator and Rhodes, 1991). Mycobacteria are difficult to isolate from infected tissue despite the presence of large numbers of acid-fast bacilli. Diagnosis is usually made by histology rather bacteriological examination of granulomatous lesions, for example using Ziehl-Neelsen (Frerichs, 1993). Histological methods of diagnosis, are both difficult and time-comsuming.

Resistance to infection with *M. tubeculosis* is efficiently evolked only by living mycobacteria (Collins, 1984). This suggests that certain antigens are secreted by living mycobacteria, but not by dead bacilli. These secreted antigens may become available for immune recognition at an early stage of mycobacterial infection. One of first antigens from the secreted proteins used as reagent in serological tests was the 38 kDa antigen (US-Japan antigen 5) (Daniel *et al.*, 1979). The assay using this antigen had a sensitivity of 70 % and specificity of 89% when the test was performed with sera from 27 patients with pulmonary tuberculosis. The 21 kDa and 30-31

kDa antigen have also been also used in serological testing for tuberculosis ( Jackett *et al.*, 1988; Sada *et al.*, 1990).

There is, however, no literature is available describing ECPs from *Mycobacterium* spp. isolated from fish. This thesis characterisation of ECPs produced by aquatic *Mycobacterium* spp. and investigates their effects on the fish immune system. The following areas were investigated.

1 An optimal medium capable of providing high concentrations of ECP from fish *Mycobacterium* spp. was selected and protein profiles, enzymatic activities and toxicity of the ECPs were also determined

2 The antigenicity of ECPs and sonicated antigens from *Mycobacterium* spp. were investigated by Western blot and ELISA

3 MAbs were produced to the ECP antigens and sonicated antigens from *Mycobacterium* spp. and these were characterised by Western blot and ELISA

4 The immune response of rainbow trout, *Oncorhynchus mykiss* and tilapia, *Oreochromuis nilotica* to the ECP from *Mycobacterium* spp was investigated.

5 Phagocytosis of *Mycobacterium* spp by fish macrophages was investigated by elecron microscopy.

## Chapter 1 Literature review

### 1.1 Historical background of Mycobacteriosis (fish tuberculosis)

The acid-fast organisms known to infect fish have been reviewed on a number of occasions and these clearly indicate that two families of acid fast bacteria, *Mycobacterium* spp. and *Nocardia* spp., are responsible for these infections (Conroy, 1966; Kusuda and Nakagawa, 1978; Kusuda, Kawakawi and Kawai, 1987; Chen, 1992). *Mycobacterium* spp. have been isolated from numerous warm and cold-blooded vertebrates and are the cause of some of the oldest and most serious infectious diseases known in both man and animals. *Mycobacterium tuberculosis* is the aetiologic agent of tuberculosis in man and other primates, while *M. bovis* and *M. avium* have resulted in significant economic losses to the cattle and poultry farming industry, respectively.

Mycobacterial diseases in fish were first reported at the end of the last century (Bataillon, Dubard and Terre, 1897) and are generally referred to as mycobacteriosis or fish tuberculosis. Mycobacteriosis has been diagnosed both in freshwater (Conroy, 1966; Majeed, Gopinath and Jolly, 1981; Bragg, Huchzermeyer and Hanisch, 1990; Chinabut, Limsuwan and Chanratchakool, 1990) and marine fish (Kusuda, Kawakami and Kawai, 1987; Mackenzie, 1988). The occurrence of the disease has frequently resulted in heavy losses to the fish farming industry (Hedrick, McDowell and Groff, 1987; Lawhaivinit, Hatai, Kubota and Suzuki, 1988; Bragg, *et al.*, 1990). However, high percentages of mycobacterial infections have also been found among populations of fish in their natural habitats (Hastings, MacKenzie and Ellis, 1982). The mycobacteria species most frequently isolated from fish are *M. marinum*, *M. fortuitum*, and *M. chelonae* (Nigrelli and Vogel, 1963; Ashburner, 1977; Hedrick *et al.*, 1987; Humphery *et al.*, 1987; Daoust, Larson and Johnson, 1989; Bragg *et al.*, 1990; Shamsudin *et al.*, 1990; Bozzetta *et al.*, 1995) (Table 1.1).

Table 1.1 The occurrence of *Mycobacterium* spp. in different fish species from 1977-1996.

| 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*) | UK |
| | | Goldfish (*C. auratus*) | |
| | | Kissing gourami (*Colisa labiosa*) | |
| | | Tetra (*Hyphessobrycon* sp.) | |
| | | Angel fish (*Pterohyllum* sp.) | |
| 1982 | Hasting *et al* | Mackerel (*S. 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 (*S. scombrus* L.) | Portugal |
| 1990 | Chinabut *et al* | Snakehead (*Channa steriatus* fowler) | Thailand |
| 1990 | Shamsudin *et al* | Gold fish (*C. auratus*) | Malaysia |
| | | Red eyeed tetra (*Moenkhansia sanctaefilomenae*) | |
| 1992 | Colorni | European sea bass (*Dicentrarchus labrax*) | Israel |
| 1993 | Gomez *et al* | Paradise fish (*Macropodus opercularis* L.) | Spain |
| | | Siamese fighting fish (*Betta splendens* Regan) | |
| | | Guppies (*Poecilia reticulata* Peters) | |
| | | Jewel tetra (*Hyphessobrycon callistus* Boulenger) | |
| | | Chanchito (*Cichlasoma facetum* Jenyns) | |
| | | Molly (*Poecilia velifera* Regan) | |
| 1996 | Gomez *et al* | Swordtail (*Xiphophorus helleri*) | Spain |

The mycobacterial fish pathogens reported here have also been implicated in external skin ulcers of humans. The *M. fortuitum* complex has been reported to cause diffuse pulmonary diseases in humans, although the incidence of these infection are rare (Nigrelli and Vogel, 1963; Howard *et al* ., 1987).

### 1.1.1 Aetiology

*Mycobacterium* spp. are Gram positive, acid fast, non-motile, pleomorphic rods, approximately 1.5-2.0 x 0.25-0.35 μm in size (Dulin, 1979). They produce pale cream to yellow/organge colonies on solid media and the optimum temperature for growth is 25°C, although some isolates will grow at 37°C. *M. anabanti* and *M. platypoecilus*, are regarded as synonyms of *M. marinum* (Van Duijn, 1981), and the slow-growing *M. salmoniphilum* (Ross, 1970) is synonymous with *M. fortuitum* (Gordon and Mihm, 1959)(Table 1.2). The relative pathogenicity of the three mycobacterial fish pathogens from most pathogenic are *M. marinum*, *M. fortuitum* and *M. chelonae*. *M. marinum* forms the largest proportion of all mycobacteria isolated from fish. Both tropical freshwater and marine fish are susceptible to *M. marinum* infections, but no authenticated case of naturally occuring *M. marinum* infection in a temperate water fish has been reported. The isolation of *M. fortuitum* from fish has been less frequently documented than *M. marinum*, but the prevalence of infection is probably more widespread than generally suspected (Frerichs, 1993). Fish from both tropical and temperate waters are susceptible to *M. fortuitum*, but this pathogen is most frequently isolated from freshwater fish. Infection by *M. chelonae* has so far only been identified in cold water salmonid species, occuring in the freshwater hatchery environment. However, one author has reported that *M. chelonae* infections can persist through the fresh and salt water phases of the fishes life cycle (Frerichs, 1993).

Table 1.2 *Mycobacterium* spp. isolated from fish ( Most of this data is from Dalsgarrd *et al.*, 1992)

| Described *Mycobacterium* | Synonym | Accepted name |
|---|---|---|
| *M. piscium* (Bataillon *et al.*, 1902) | - | - |
| *M. marinum* (Aronson, 1926) | *M. marinum* | *M. marinum* |
| *M. fortuitum* (Nigrelli, 1953) | *M. fortuitum* | *M. fortuitum* |
| *M. platypoecilus* (Baker and Hagan, 1942) | *M. marinum* | - |
| *M. anabanti* (Besse, 1949) | *M. marinum* | - |
| *M. salmoniphilum* (Ross, 1960) | *M. fortuitum* | - |
| *M. Poikilothermorum* (Amlacher, 1968) | - | - |
| *M. borstelense* (Bernstad, 1974) | *M. chelonae* | *M. chelonae* |
| *M. chelonae* (Arakawa and Fryer, 1984) *subsp. piscarium* | *M. chelonae* | - |
| *M. gordonae* (Bozzeta *et al.*, 1995) | - | - |

### 1.1.2 Clinical signs and gross pathology

Piscine mycobacteriosis is a systemic, chronic progressive disease with various clinical signs, which depend both on the *Mycobacterium* species and ecological conditions. The major clinical signs of the disease in tropical freshwater and marine aquarium fish include listlessness, anorexia, emaciation, exophthalmia, skin discoloration and external lesions ranging from scale loss to ulcers, nodules and fin necrosis (Wolke and Stroud, 1978; Dulin, 1979; Van Duijn, 1981; Chinabut *et al.*, 1990). In cold water salmonids there may be no external signs of disease other than mortality, or there may be variable degrees of skin discoloration, stunted growth and retarded sexual development (Ashburner, 1977; Abernethy and Lund, 1978). The nature and distribution of lesions which develop internally may be similar in both tropical and cold water fish species. Gross greyish-white miliary granuloma can be found scattered or grouped in

virtually any parenchymatous organ, especially the spleen, kidney and liver. Enlarged organs, peritonitis and oedema may all be apparent. The range of fish susceptible to mycobacteria is vast (Table 1.1). For example, mycobacteriosis was found to affect numerous stocks of European sea bass, *Dicentrarchus labrax* grown both in the Mediterranean and the Red sea coasts (Colorni, 1992). In broodstock sea bass, mortality was almost invariability correlated with extensive granulomatous lesions and enlargement of the abdominal organs, in particular the kidney and spleen. Diseased snakehead fish from Thailand, on the other hand, showed general immobility by floating on the surface of the water (Chinabut *et al.*, 1990). They were emaciated, with scale loss and had whitish patches along the length of their head. Small white foci could be observed on their gills. In many cases, there was severe exophthalmus with keratitis and cataracts. Lesions could be observed in fish as young as three months of age (Chinabut *et al.*, 1990).

### 1.1.3 Histopathology

The predominant pathological hallmark of mycobacterial infections in fish is the infiltration of lymphoid cells and macrophages with granuloma formation. Both the granulomatous tissue and surrounding areas produce a positive reaction with periodic acid-Schiff method (PAS) staining.

The typical lesions which occur in fish during mycobacteriosis consist of greyish nodules located on affected organs. The nodules are granulomas of varying sizes and degrees of development and are visible in infected tissue sections (Hematoxylin and Eosin, H&E). The nodules consist of clumps of epitheloid cells surrounded by a connective capsule of varying thickness, and areas of necrosis are often seen in the centre. In mycobacteriosis, the most severe granulomata are predominately located in the liver and the spleen of the fish.

Chronic inflammation occurs in teleost fish if persistent antigens are present, as in the case of mycobacteriosis. Antigens cause the inflammatory cells to become activated and highly phagocytic. These cells then differentiate into epitheloid cells which surround and isolate the antigenic agent. Epitheloid cells, once considered to be macrophages, are now thought to be differentiated mesothelial cells with characteristic desmosomes containing tonofilaments (Noga, Dykstra and Wright, 1989). Chronic inflammatory cells, characterized by desmosomes with tonofilaments, are found in conjunction with epitheloid cells. According to Conroy (1970) and Wolke (1975) this description is presumptive evidence of piscine tuberculosis. Timur, Roberts and McQueen (1977) described the formation of Langhans and foreign-body type giant cells in granulomas in experimentally infected plaice, *Pleuronectes platessa* L. They found that piscine *Mycobacterium* sp. invoked the formation of giant cells in these fish, but after 24-28 days the number of giant cells rapidly decreased. Mycobacterial lesions in mountain white fish, *Prosopium williamsoni* (Girard) are non-encapsulated, this suggests a lack of cellular response (Lund and Abernethy, 1978). The lesions in the mountain white fish suggests a chronic inflammatory response, and therefore, a more long-standing infection. These examples show that reactions to mycobacterial infections vary depending on the fish species involved. Parisot and Wood (1960) reported the pancreas and adjacent tissue were the primary targets organ for mycobacteriosis in juvenile salmonids. However, there observation may have been due to a massive invasion of *Mycobacterium* sp. into adjacent viscera causing widespread necrosis. Infiltration of bacteria from adjacent viscera is not naturally observed with most mycobacterial infections (Wolke and Stroud, 1978).

In fish, three cells types contain pigment. These are melanocytes, melanophores and melanomacrophage (Agius, 1985). All three cell types are associated with inflammatory lesions

in fish. (McQueen *et al.*, 1973; Roberts, 1975). Melanocyte cells and melanophores can be distinguished from the highly pigmented, melanomacrophage cells, which are normally present in chronic bacterial infections of the dermis (Roberts, 1975). The melanomacrophages are typically ovoid in shape and have melanosomes enclosed within membrane-bound vacuoles (Roberts, 1975). Melanocytes and melanophores, on the other hand, are usually dendritic to asteroide in appearance, with melanosomes free within the cytoplasm. The latter cells are not phagocytic.

### 1.1.4 Isolation and identification of mycobacteria

Attempts to isolate the aetiological agent of mycobacteriosis often fail. Some success has been achieved by inoculating pieces of infected tissue on standard culture media for mycobacteria, such as Petragnani, Lowenstein-Jensen, Middlebrook 7H10 and Dorset egg media. Growth of the bacteria on these media occurs after 2-28 days incubation at temperatures between 15 to 22°C (Dulin, 1979). Dubos agar, Sauton's agar and Ogawa egg media, all with similar formulations, have also been used to successfully isolate mycobacteria from fish (Ashburner 1977; Arakawa and Fryer, 1984). It is curious that Arakawa and Fryer (1984) reported success with standard bacteriological media such as Tryptone soya agar and brain heart infusion agar, while most authors have had difficulty in recovering mycobacteria from fish (Frerichs, 1993 ). Thoen and Schliesser (1984) found that fish mycobacteria could be cultured at temperatures between 20 to 30°C, and suggested that the cultures should be observed weekly for at least 12 weeks for the appearance of bacterial growth. If cultures are still negative after this time it may be necessary to increase the incubation period to 20 weeks. When growth is detected, smears of colonies should be stained with Ziehl-Neelsen (ZN) stain to confirm their acid-fastness. Colony morphology, pigmentation, and the time and temperature required for growth are all

important characteristics of individual mycobacteria species and should therefore be noted. The biochemical characteristics for some of the *Mycobacterium* sp. infecting fish are reported in Table 1.3 (Lansdell *et al.*, 1993).

Table1. 3 Biochemical test for identification of *Mycobacterium* species (Lansdell *et al.*, 1993)

| *Mycobacterium* spp and biochemical tests[a] | Reaction | Host |
|---|---|---|
| | Photochromogens | |
| *M. simiae* isolate 1 (0.94) | | Black acara |
| Niacin | + | |
| 68°C catalase | + | |
| Growth at 25°C | + | |
| *M. simiae* isolate 2 (0.72) | | Black acara |
| Niacin | - | |
| Nitrate reduction | - | |
| Tween-80 hydrolysis | - | |
| *M. marinum* (0.93) | | Striped bass |
| Nitrate reduction | + | |
| Tween-80 hydrolysis | + | |
| 14-d arylsulfatase | + | |
| | Scotochromgens | |
| *M. scrofulaceum* (0.84) | | Pacific staghorns sculpin |
| Tween-80 Hydrolysis | - | |
| 3-d arylsulfatase | - | |
| X-colony | - | |
| Catalase (SQ) | + | |
| *M. chelonae abscessus* (0.89) | | |
| Nitrate reduction | Rapid growers | Goldfish, fire-mouth |
| 3-D arylsulfatase | - | cichild, black acara |
| Catalase (SQ) | + | |
| Urease | + | |
| Tellurite reduction | + | |
| MacConkey spiral | + | |
| 5% NaCl L-J | + | |
| *M. fortuitum* (0.91) | + | Black acara |
| Nitrate reduction | | |
| 3-d arylsulfatase | + | |
| Catalase(SQ) | + | |
| Urease | + | |
| Tellurite reduction | + | |
| MacConkey | + | |
| 5% NaCl L-J | + | |
| | + | |

[a] number in parentheses denotes probability of correct identification, obtained from tables generated by Kent and Kubica (1985) SQ: semiquantiatative; L-J: Lowenstein-Jensen medium .

The biochemical characteristics of some mycobacteria strains (*M. marinum*, *M. piscicida*, *M. fortuitum*, *M. chelonae*, *M. parafortuitum* and *M. vaccae*), were reported by Somsiri, 1993, (Table 1.4). Most biochemical characteristics of isolates from Siameses fighting fish are similar to *M. marinum*.

Table 1.4 Biochemical characteristics of *Mycobacterium* spp. from Siameses fighting fish and reference strains (Somsiri, 1993)

| Characteristic | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| 1. | Growth rate | R | R | R | R | R | R | R |
| 2. | Growth at 22°C | + | + | + | + | + | + | + |
| 3. | Growth at 25°C | + | + | + | + | + | + | + |
| 4. | Growth at 28°C | + | + | + | + | + | + | + |
| 5. | Growth at 37°C | + | + | + | + | + | + | + |
| 6. | Growth at 42°C | - | - | - | - | - | - | - |
| 7. | Growth at 45 °C | - | - | - | - | - | - | - |
| 8. | Growth at 52 °C | - | - | - | - | - | - | - |
| 9. | Growth on Ogawa egg medium | + | + | + | + | + | + | + |
| 10. | Growth on L-J medium | + | + | + | + | + | + | + |
| 11. | Growth on modified Sauton's agar | + | + | + | + | + | + | + |
| 12. | Growth on Dorset's egg | + | + | + | + | + | + | + |
| 13 | Growth on CGY | + | + | + | + | + | + | + |
| 14. | Growth on BHI agar | - | + | + | + | + | + | + |
| 15. | Growth on TSA | - | - | + | + | - | - | - |
| 16. | Growth on NA | - | - | + | + | - | - | - |
| 17. | Growth on middlebrook 7H10 | + | + | + | + | + | + | + |
| 18. | Growth on PAS medium | + | + | + | + | + | + | + |
| 19. | Growth on MacConkey Agar without C. V. | - | - | - | - | + | + | - |
| 20. | Growth on $NH_2$. OH HCl (125µg/ml) | + | + | - | - | + | + | + |
| 21. | Growth on $NH_2$.OH.HCl (250µg/ml) | + | + | - | - | + | + | + |
| 22. | Growth on $NH_2$.OH .HCl (500µg/ml) | - | + | - | - | + | + | + |
| 23. | Growth on 0.1% Salicylate | + | + | + | + | + | + | - |
| 24. | Growth on 5 % NaCl | - | + | + | + | - | + | - |
| 25. | Growth on 1 % Tween | + | + | + | + | + | + | + |
| 26. | Growth at pH 6.0 | + | + | + | + | + | + | + |
| 27. | Growth at pH 8.0 | + | + | + | + | + | + | + |
| 28. | Acid fast stain | + | + | + | + | + | + | + |
| 29. | Fluorescent stain | + | + | + | + | + | + | + |
| 30. | Gram stain | + | + | + | + | + | + | + |
| 31. | Photochromogenic | + | + | + | + | - | - | + |
| 32. | Scotochromogenic | - | - | - | - | - | - | - |
| 33. | Nonphotochromogenic | - | - | - | - | + | + | - |
| 34. | Resistance to 0.1% $NaNO_2$ | - | - | + | + | + | + | - |
| 35. | Resistance to 0.2% $NaNO_2$ | - | - | + | + | + | + | - |
| 36. | Resistance to 0.1 picric acid | - | - | + | + | - | + | - |
| 37. | Resistance to 0.2 picric acid | - | - | + | + | - | + | - |
| 38. | Resistance to thiophene-2-carboxlic acid hydrazide (1µg/ml) | + | + | + | + | + | + | + |
| 39. | Resistance to 5 fluorouracil (20µg/ml) | + | + | + | + | + | + | - |
| 40. | Resistance to mitocine (5µg/ml) | + | + | - | - | + | + | + |
| 41. | Resistance to ethambutol (5µg/ml) | + | + | + | + | + | + | + |
| 42. | Resistance to Rifampicin(25µg/ml) | + | + | + | + | + | + | + |

Table1.4 (continued 1)

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 43. Resistance to p-nitrobenzoic acid | + | + | + | + | + | + | + |
| 44. Degradation of PAS | - | - | - | - | + | + | - |
| 45. Degradation on Salicylate | - | - | - | - | + | + | - |
| 46. Niacin production | - | - | - | - | - | - | - |
| 47. Tween hydrolysis(7 days ) | + | + | + | + | + | + | + |
| 48. Tween hydrolysis(14 days | + | + | + | + | - | - | - |
| 49. A-Esterase | - | - | - | - | + | + | - |
| 50. Acid phosphatase(3 hrs) | + | + | - | - | + | + | + |
| 51. Catalase, foam height. >45mm | + | + | + | - | + | - | - |
| 52. Catalase after 68°C | - | - | + | - | - | - | - |
| 53. B-Galactocidase | - | - | - | - | + | - | - |
| 54. Nitrate reduction (6 hrs ) | - | - | - | + | - | - | - |
| 55. Nitrate reduction (24 hrs) | - | - | - | + | - | + | - |
| 56. Tellurite reduction (3 days) | - | - | + | + | + | - | - |
| 57. Tellurite reduction (9 days) | + | + | + | + | + | + | - |
| 58. Arylsulfatase (3 days) | + | - | - | - | + | + | - |
| 59 Arylsulfatase (14 days) | + | + | + | + | + | + | + |
| 60. Acetamidase | - | - | + | + | - | + | - |
| 61. Benzamidase | - | - | + | - | - | - | - |
| 62. Urease | + | + | + | + | + | + | + |
| 63. Isonicotinamidase | - | - | + | - | - | - | - |
| 64. Nicotinamidase | - | - | + | - | - | + | - |
| 65. Pyrazinamidase | - | - | + | + | - | + | - |
| 66. Salincylamidase | - | - | - | + | - | - | - |
| 67. Allantoinase | - | - | + | - | - | + | + |
| 68. Succinamidase | - | - | - | - | - | - | - |
| 69. Iron uptake | - | - | + | - | - | + | - |
| 70. Glucose as C source(Glutamate-N) | + | + | + | + | + | + | + |
| 71. Acetate as source (Glutamate -N) | - | - | + | + | + | + | + |
| 72. Succinase as C source (Glutamate-N) | - | - | + | + | + | + | + |
| 73. Pyruvate as C source (glutamate-N ) | - | - | + | + | + | + | + |
| 74. Acetate as C source | + | + | + | + | + | + | + |
| 75. Citrate as C source | + | - | - | - | - | + | - |
| 76. Succinase as C source | + | - | + | + | - | + | - |
| 77. Malatase as C source | - | - | + | + | - | + | - |
| 78. Pyruvate as C source | + | + | + | + | + | + | + |
| 79. Benzoate as C source | - | - | - | - | - | - | - |
| 80. Malonate as C source | - | - | - | - | - | - | - |
| 81. Fumarate as C source | - | - | + | + | + | + | - |
| 82. Glucose as C source | + | + | + | + | + | + | + |
| 83. Fructose as C source | + | + | + | + | + | + | - |
| 84. Sucrose as C source | - | - | + | - | + | - | - |

Table 1.4 (Continued 2)

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 85. Ethanol as C source | + | + | + | + | + | + | + |
| 86. N-Propanal as C source | + | + | + | + | + | + | - |
| 87. Propylene glycol as C source | + | + | + | + | + | - | - |
| 88. 1, 3-Butylene glycol as C source | + | + | - | - | + | + | - |
| 89. 2,3-Butylene glycol as C source | + | + | - | - | + | + | - |
| 90. N-Butanol as C source | + | + | - | + | + | + | - |
| 91. Iso-butanol as C source | + | + | - | + | - | + | - |
| 92. Acid from glucose | + | + | + | + | + | + | - |
| 93. Acid from D-manose | - | - | - | + | + | + | - |
| 94. Acid from D-Galactose | - | - | - | - | - | - | - |
| 95. Acid from Arabinose | - | - | - | + | - | - | - |
| 96. Acid from D-xylose | - | - | - | + | - | - | - |
| 97. Acid from L-rhamnose | - | - | + | - | - | - | - |
| 98. Acid from trehalose | - | - | - | - | - | + | - |
| 99. Acid from Inositol | - | - | - | + | - | - | - |
| 100. Acid from mannitol | - | - | + | + | - | - | - |
| 101. Acid from sorbitol | - | - | - | - | - | - | + |
| 102. L-glutamate as N source | + | + | + | + | + | + | - |
| 103. L-serine as N source | + | + | + | + | + | + | - |
| 104. L-methionine as N source | + | + | + | + | + | + | - |
| 106. Benzamide as N source | - | + | + | + | - | - | + |
| 107. Urea as N source | + | + | - | + | + | + | + |
| 108. Pyrazinamide as N source | + | + | - | + | + | + | - |
| 109. Nicotinamide as N source | + | + | - | - | + | + | - |
| 110. Isonicotinamide as N source | + | + | - | + | - | + | + |
| 111. Succinamide as N source | + | + | - | + | + | + | + |
| 112. Nitrate as N source | + | + | - | + | + | + | - |
| 113. Nitrite as N suource | - | - | - | - | - | + | + |
| 114. Growth on 0.2% pyronin B | + | + | - | - | + | + | + |
| 115. Growth on 1% sodium dexycholate | - | - | - | - | - | + | + |
| 116. Growth 0.1% malachite green | + | + | + | + | + | + | + |
| 117. Growth on 0.01% methyl violet | + | + | - | - | + | + | + |

1: Isolated from Siamese fighting fish

| | |
|---|---|
| 2. *M. piscicida* | NJB 8601 |
| 3. *M. vaccae* | ATCC 15483 |
| 4. *M. parafortuitum* | ATCC 19686 |
| 5. *M. chelonae subsp. chelonae* | NCIMB 1474 |
| 6. *M. fortuitum* | NCIMB 1294 |
| 7. *M. marinum* | NCIMB 1298 |

R: Rapid growth

### 1.1.5 Pathogenicity

The pathogenicity of *M. chelonae subsp. piscarium* has been studied in some detail (Arakawa and Fryer, 1984 ). *M. chelonae* infections were experimentally induced in rainbow trout at a water temperature of 12°C by injecting (approximately $10^7$) bacteria intraperitoneally into fish. Cumulative mortalities ranged from 20 to 52 % with juvenile chinook salmon, with 98% of the mortalities being recorded within 10 days at a water temperature of 18°C (Arakawa *et al.*, 1984). The experimental Siamese fighting fish were intramuscularly injected with *Mycobacterium* sp. (1x $10^8$ ml). Infected fish did not show any external lesions. Various sizes of both hard and soft internal granulomata were observed in Siamese fighting fish (Pungkachonboon, Chinabut, and Shariff, 1994). The Pejerrey were inoculated with intramuscular injections of *Mycobacterium* sp., developed granulomatous lesions similar to the natural infection. The fish showed 100% mortality at 14 and 30 days after 10 and 1 mg/ml respectively of *Mycobacterium* sp suspension was injected, (Hatai *et al.*, 1988;1993).

### 1.6 Diagnostic methods

Diagnosis of mycobacteriosis is usually achieved by observing the clinical signs of the disease and the presence of acid fast bacteria in histological tissue sections. However, attempts to culture the pathogen from tissue samples are also recommended. Mycobacteria are isolated from specimens by aseptically sampling lesions in the spleen. The sample is first treated with 4 % (w/v) NaOH for 10 minsto kill other bacteria, the pH neutralised with a few drops of 1N HCl then cultured on either TB (Thomas and Broom) broth, Dubos broth, Dubos blood agar, Petragnani medium, Middle brook 7H10 agar at both 25°C and 37°C (Thoe and Schliesser, 1984; Frerichs, 1993). Confirmation of the presence of acid-fast mycobacteria bacilli in tissue

samples is made by preparing smears from the cultured bacteria and staining with ZN stain. To identify and differentiate between mycobacteria isolates, the growth rate, colony morphology and pigment production are first observed, then the numerous biochemical tests are performed (Runyon *et al.*, 1974). These include growth in relation to temperature, nitrate reduction, catalase drop method, catalase test after heating bacteria to 68°C, urea hydrolysis and growth on MacConkey agar. Gomez *et al.*, (1993; 1996) used polyclonal antisera to identify the presence of mycobacterial antigens in the epitheloid cells of granulomas and macrophages from the intestine. The specific reaction by immunohistocytochemistry (IHC) was visible as a golden-brown colour within the cytoplasm of phagocytic cells. In general, it is diffficult to identify single bacteria because the reaction produces diffuse staining of the phagocyte cytoplasm. The IHC method proved to be more sensitive than ZN staining since positive regions were observed in the liver of infected fish by IHC which could not be seen or were only faintly observed stained with ZN stain. Monoclonal antibodies (MAbs), have also recently been used to identity mycobacteria within epitheloid cells of kidney granulomas (Gomez *et al.*, 1996). Fridman (1995) reported that a comparative study of detection methods for mycobacteriosis was carried out on formalin fixed tissue obtained from field and experimentally infected fish (*Chanmos striatus*, *Betta Splendens*, *Dicentrachus labrax*, *Siganus riunlatus* and *Chaetodon fasciatus*) from Thailand and Israel. Immunohistochemistry was found to be effective in the detection of mycobacterial antigens in tissue at the early stages of disease where conventional histological staining methods failed. Adams *et al.*, (1996) reported that *Mycobacterium* spp. can be detected in the spleen and kidney of fish by using a sandwich ELISA. It is intended that the MAbs in sandwich ELISA will provide a rapid alternative to the time-consuming methods of diagnosis currently in use. Immunohistochemical analysis proved less conclusive with only a very weak staining of the bacteria being observed. It may be

possible, however, to improve the sensitivity of the assay by processing the fish tissues in some way that would expose the reactive epitopes on the bacteria to the MAbs. An alternative approach to the identification of mycobacteria is the application of polymerase chain reaction (PCR) using 16S RNA primer. The amplified fragment of mycobacterial DNA yields a 220 bp fragment (Knibb *et al.*,1993). *Mycobacterium* spp have been identified to genus and species level using this method. Knibb *et al.*, 1993 and Puttinaowarat, 1995 recently reported on the use of the PCR for detection and identification of *M. marinum*. This is a rapid and and sensitive technique. Firstly, the samples were classified as *Mycobacterium* sp. then, PCR product (220 bp) was digested by a restriction enzyme (Hinf I ) to confirm *M. marinum* (180 bp fragment).

### 1.1.7 Transmission of infection

Mycobacterial infections in fish are thought to be transmitted naturally by the ingestion of contaminated food or aquatic detritus, or through bacterial invasion of damaged skin and gill tissue, especially since mycobacteria are common in soil and water, where they can remain viable for 2 years or more (Reichenbach-Klinke, 1972). Organisms may be released from lesions of diseased fish into the environment or from a wide range of amphibians and reptiles known to be susceptible to mycobacterial disease (Nigrelli and Vogel, 1963). The feeding of infected trash fish has been circumstantially shown to have disseminated diseased in cultured pacific salmon (Ross, Earp, and Wood, 1959) and snakehead fish (Chinabut *et al*, 1990). Experimental studies in mexican platyfish, *Platypoecilus maculatus* have also provided evidence for the transmission of infectious organisms by contaminated feed (Baker and Hagan, 1942). Evidence from an Australian fish hatchery (chinook salmon) has shown that mycobacteria can be introduced to eggs and then transmitted to the F1 generation (Ashbruner,

1977). This may occur by ovarian transmission or by peritoneal fluid containing the mycobacteria, but infection via the ovarian route has, however, been discounted as a means of spreading the disease in salmon (Ross and Johnson, 1962).

### 1.1.8 Immune response to infection

Bartos and Sommer (1981) reported that rainbow trout (*Oncorhynchus mykiss*) produce a typical delayed type hypersensitivity reaction to immunisation with *M. tuberculosis*, thus supporting the existence of a T-cell-like mediated immune response in teleost fish. The sensitized fish also produced a response when they were subjected to a skin test with *M. salmoniuphilum* (synonym *M. fortuitum*) showing some degree of antigenic similarity between the two strains of mycobacteria. Skin window studies support the presence of cell mediated immunity by demostrating the presence of activated macrophages in the dermal layers of the fish (Bartos *et al.*, 1981).

### 1.1.9 Antigenic relationship

Results from a number of serological and dermal hypersensitivity studies have shown *M. marinum* to be antigenically distinct from all other mycobacterial species examined and that they form a very homogeneous group of organisms comprising only one or possibility two serotypes (Wayne and Kubica, 1986). On the other hand, no serological studies specifically relating to *M. fortuitum* have been reported, but this species of mycobacteria is also recognised as being antigenically distinct from all other *Mycobacterium* species (Frerichs, 1993). *M. chelonae* and subsp. *abscessus* are presently only identified on the basis of biochemical characteristics and their classification has not yet been supported by chemical and serological analysis of cell surface antigens (Tsang *et al.*, 1984).

In 1972, the International Working Group for Mycobacterial Taxonomy reported that the two taxa, now recognized as *M. chelonae* subspecies *chelonae* and *M. chelonae* subspecies *abscessus*, could be differentiated phenetically, but could not be distinguished from each other by lipid and immunological analyses. The committee suggested that the subspecies designation, currently recognized by Bergey's Manual, be adopted (Runyon, Wayne and Kubica, 1974). Tsang *et al* (1984) using chemical and serological techniques, showed that there was no significant difference in the composition of the outer surface antigens of the two subspecies of *M. chelonae*.

In another study, antisera were prepared against (Mycobacteria isolate from Bandon Hatchery, Oregon, USA, BAN), *M. chelonae* subspecies *abscessus* (MCA) and *M. chelonae* subspecies *chelonae* (MCC) (Tsang *et al.*, 1984). Cross agglutinations occurred with the antisera against MCA, MCC and BAN (Table 1.5). Antibody titers of the three antisera were reduced to 5 % or less after a second adsorption with either homogenous or heterologous antigens, thus showing that the three strains could not be serological distinguished. No antigenic relationship was found between the strains of *M. chelonae* and *M. fortuitum* (Tsang *et al.*, 1984). There was no reaction with any of the antisera to *M. fortuitum* and titers of the antisera did not significantly decrease after adsorption with this species (Table 1.5). Examination of DNA-DNA homology comparisons (Baess, 1982) and electrophoretic pattern of cytoplasmic proteins (Haas, Michael and Sacks, 1974) have also supported synonymy between the two subspecies.

Arakawa, Fryer and Sanders (1986) found that salmonid isolates of *Mycobacterium* could not be serological differentiated from each other or from the two subspecies of *M. chelonae* obtained from America Tissue Culture Center (ATCC), thus suggesting a homogeneous group

of bacteria belonging to the species *M. chelonae*. Although the salmonid isolates could be phenetically differentiated from both *M. chelonae* subspecies, the authors suggested that the name *M. chelonae* subsp. *piscarium*, proposed in 1984 by Arakawa and Fryer, be withdrawn, and the salmonid isolates be considered as a single strain of *M. chelonae*.

Table 1.5. Antigenic analysis of *Mycobacterium chelonae* subspecies *chelonae*, *Mycobacterium chelonae* subspecies *abscessus* and the Bandon (BAN) *Mycobacterium* isolate (Arakawa *et al.*, 1986).

| Antisera | Adsorbing antigen | Agglutination titre when tested mycobacterial with antigen | | |
|---|---|---|---|---|
| | | BAN | MCA* | MCC† |
| BAN | Unadsorbed | 320 | 1280 | 320 |
| | BAN | 0 | 20 | 20 |
| | MCA | 0 | 20 | 20 |
| | MCC | 0 | 20 | 0 |
| | MF‡ | 160 | 1280 | 160 |
| MCA | Unadsorbed | 640 | 1280 | 640 |
| | MCA | 0 | 20 | 0 |
| | BAN | 0 | 20 | 0 |
| | MCC | 0 | 20 | 0 |
| | MF | 160 | 320 | 160 |
| MCC | Unadsorbed | 320 | 640 | 320 |
| | MCC | 0 | 20 | 0 |
| | BAN | 0 | 20 | 0 |
| | MCA | 0 | 20 | 0 |
| | MF | 160 | 640 | 160 |

**Mycobacterium chelonae* subspecies *abscessus*.
†*Mycobacterium chelonae* subspecies *chelonae*.
‡*Mycobacterium fortuitum*.

### 1.1.10 Drug sensitivity, treatment and control

No vaccines are available against fish mycobacteria, but the fact that fish are able to develop a cell mediated immune response to mycobacterial antigens indicates a potential for the

development of BCG like vaccine antigens (Bartos and Sommer, 1981). Such products might find particular application in the immunisation of valuable, long-lived species of fish, but are unlikely to be commercially justifiable for farmed food fish (Frerichs, 1993).

Some workers advocate that clinically diseased fish should be destroyed by incineration or burying in quick lime, because of the prolonged use of chemothreapeutic agents poses potential hazard to human health (Dulin, 1979; Van Duijn, 1981). Other scientists have reported that the natural spread of infection within a closed environment may be controlled by the addition of chloramine B or T at 10 mg/liter for 24 h to the tank. However, this practice is unreliable and it is recommended that fish for food processes must be pasteurized before use since freezing does not the kill the bacterium (Ross *et al.*, 1959). In Argentina, it has been shown that *Mycobacterium anabanti* (*M. marinum*) and other strains of acid-fast bacteria isolated from freshwater fish, Platyfish, *Platypoecilus maculatus* and Siamese fighting fish, *Betta splendens* are more sensitive to kanamycin sulphate than other antibiotics *in vitro* (Conroy and Solarolo, 1965) (Table 1.6). Spontaneous acid-fast bacterial infections in Siamese fighting fish were successfully treated by the addition of kanamycin sulphate at a concentration of 100 ppm to the aquarium water as a permanent bath over a 5 day period (Conroy, 1966). Two severely affected gouramies were also treatmented by this regime, after which the clinical signs of the disease had completely disappeared. The remainder of the gouramie population was similarity treated, thus leading to a full clinical recovery in all fish. No further losses were detected in the 3-month period which followed (Conroy, 1966).

Table 1.6 Mininum inhibitory concentration (μg/ml) antibiotics for *Mycobacterium* spp. (Conroy and Solarolo, 1965).

| Antibiotic | Organism | | | | |
|---|---|---|---|---|---|
| | *Mycobacterium anabanti* | M/A-4 | M/A-5 | M/A-6 | M/A-7 |
| INAH | 800 | 50 | 800 | 3.1 | 50.0 |
| PAS | 800 | 6.3 | 800 | 25.0 | 50.0 |
| Viomycin sulphate | 400 | 12.5 | 800 | 1.6 | 50.0 |
| Rifamycin SV | 25.0 | 1.6 | 200 | 3.1 | 12.5 |
| Streptomycin sulphate | 200 | 12.5 | 800 | 6.3 | 50 |
| Kanamycin sulphate | 3.1 | 1.6 | 100 | 1.6 | 6.3 |

M/A-4: Isolated from *Platypoecilus maculatus*, Argentina
M/A-5: Isolated from *Betta splendness*, Argentina
M/A-6: Isolated from *Cynolebias adloffi*, USA
M/A-7: Isolated from *C. wolterstorfi*, USA

Kawakami and Kusuda, (1989; 1990) reported that erythromycin, rifampicin and streptomycin appeared to be highly effective against some mycobacterial bacterial isolates. Howard (1994) found that ethambutol plus rifampin appears more useful than minocycline in treating cutaneous *M. marinum* infections. Sarafloxacin and amikacin appear to be more effective than kanamycin, oxolinic acid with *Mycobacterium* spp isolated from snakehead fish and Siamese fighting fish (K. Thompson, personal communication).

### 1.1.11 Public health

*M. marinum, M. fortuitum* and *M. chelonae* are all capable of infecting warm-blooded vertebrates, including man. *M. marinum* is the most frequently encountered of the three mycobacterial fish pathogens. It can cause cutaneous granulomas in man, usually resulting from abrasions incurred in swimming pools and is therefore known as, swimming pool granuloma, or fish tank skin ulcer (Barrow and Hewitt, 1971; Miller and Toon, 1973). The resulting granulomas are usually found on either the elbow, knee or foot of the patient. *M.*

*fortuitum* has also been cultured from patients with pulmonary disease and local abscesses, while there are a few recent reports of *M. chelonae* being isolated from patients who have undergone hetergraph heart valve transplants. Lesions have been detected in synovial fluid and muscle. *M. fortuitum* is a relatively uncommon opportunist pathogen of man with infections usually involving the skin following superficial trauma, although the organism has also been isolated from lungs, lymph nodes and other internal organs. *M. chelonae* has been isolated from knees, and other joints *M. chelonae,* but the strains capable of growth at 37°C appear phenotypically distinct from *M. chelonae* strains isolated from fish. Non-tuberculosis mycobacterial infections are not commonly diagnosed in Thailand (Kullavanijaya, Sirimachan, and Bhuddhavudhikrai, 1993). For a country with a high incidence of fish mycobacteriosis, only 10 reports have been published in Thailand relating to the incidence of mycobacteria in humans. These revealed that in a total of 44 cases, 38 of the cases of cutaneous infection were due to *M. marinum*. A history of previous trauma occurred in 11 cases (61.1%), most of which were negligible wounds or minor abrasions, and twelve cases (66.7%) came in contact with organism through their occupations or hobbies associated with fish or water. Human infections by aquatic mycobacteria have been reported with increased frequency (Brown and Sanders, 1987; Kator and Rhodes, 1991; Wayne and Sramek, 1992). and granulomatous skin lesions caused by *Mycobacterium* spp. have been recorded on numerous occassion in Thailand in people working on fish farms and attending home aquaria (T. Somsiri, personal communication).

### 1.2 Extracellular products (ECP) of *Mycobacterium* spp

In a series of reports, several antigens have been identified from the culture supernatants of *M. bovis* Bacilli Calmette-Guerin (BCG), *M. tuberculosis* and *M. leprae* (Abou-zeid *et al.*,

1986; Closs *et al.*, 1980; Kolk *et al.*, 1984). The 30-32 kDa and 65 kDa antigens are major proteins secreted by *M. tuberculosis* and *M. leprae* (Godfrey *et al.*, 1990; Anderson *et al.*, 1988; Shinnick, Vodkin and Williams, 1988). The 65 kDa is a heat shock protein (HSP), and one of the major immunogenic proteins in mycobacteria ECP. Fibronectin (FN) binding protein at 30-32 kDa is another major ECP protein. Both proteins are known to have important roles in the pathogenesis of mycobacteria in mammals (Espitia *et al.*, 1992; Buchanan *et al.*, 1987; Young *et al.*, 1987).

### 1.2.1 Heat shock protein

The HSPs are produced by cells exposed to a variety of stress stimuli including increased temperature, nutrient deficiency, low oxygen pressure, pH alterations or reactive oxygen metabolite attack (Lindquist, 1986). They can be divided into four major families, with members have molecular weights of about 90 kDa, 70 kDa, 60 kDa, and 10-30 kDa (Table 1.7) (Lydyard and Eden, 1990; Kaufmann, 1990). The 65 kDa antigen of *M. tuberculosis* and *M. leprae* is a well-characterized, strongly immunogenic protein, eliciting antibody and T cell responses in infected patients. Recent studies have disclosed regions of cross-reactivity between the 65 kDa antigen and proteins in many other bacterial species (Young *et al.*, 1987). These include the product of the ams gene in *E. coli* which is involved in the processing of RNA. The significance of the 65 kDa antigen and its possible role in the pathogenesis of mycobacterial and other diseases has been reviewed (Young *et al.*, 1987). The 65 kDa antigen has been described as cell-wall associated, since much of the antigen is found in the insoluble fraction remaining after disruption of mycobacterial cells. Other authors have proposed a periplasmic

location for this antigen and have demonstrated release of the molecule into culture supernatant during growth of *M. bovis* under conditions of zinc defiency (Young *et al.*, 1987).

Table 1.7 The Heat shock protein superfamily; physiological role and possible relation to the immune response of major HSPs ( Kaufmann, 1990)

| Family | Major members | Important physiological function | Possible role in the immune response |
|---|---|---|---|
| HSP90 | HSP90, HSP83 | Prevention of steroid receptor binding to DNA; tyrosine kinase phosphorylation | Tumor resistance; autoimmunity |
| HSP70 | HSP70,BiP,hsc70, Grp78, dnak | Protein folding and unfolding; protein translocation; assembly of multimeric complexes | Immunoglobulin assembly; class II antigen processing; antigen of many pathogens; autoimmunity |
| HSP60 | HSP65, groEL | Protein folding and unfolding ; assembly of multimeric complexes | Antigen of many pathogens; autoimmunity |
| Ubiquitin | Ubiquitin | protein degradation | Class-1-antigen processing; lymphocyte homing; autoimmunity |

### 1.2.2 Fibronectin binding protein

Understanding the role that mycobacterial components play in the infectivity and virulence of the bacterium may be as important as a knowledge of their immunogenic properties. Fibronectin is a multi-functional molecule abundant in plasma and connective tissue, which can interact with a number of molecules including IgG, C1q (a component of the complement cascade), collagen, gelatin, and surface cell receptors. Fibronectin, a 420 kDa glycoprotein, consists of two similar subunits joined by a disulphide bond near the C-terminal end. It is present in soluble and matrix forms in various body fluids and tissue (Tables 1.8a,b,c,d) (Hynes and Yamada, 1982). FN binds to specific site for attachment on eukaryotic cells such as macrophages and polymorphonuclear leucocytes. These sites include collagen, fibrin, heparin,

heparin sulphate, dextran sulphate, DNA, actin and hyluronic acid. In addition, fibronectin also has been reported to bind to bacteria including *Staphylococcus aureus* group A, C, and G, *Streptococci, Treponema pallidum, Escherichia coli* and *Salmonella dublin* (Hynes *et al.*, 1982; Speziale *et al.*, 1984; Proctor, 1987).

Table 1.8a Site of fibronectin synthesis *in vitro*

| Cell type | Comments |
|---|---|
| Fibroblast | Often decreased after oncogenic transformation |
| Endothelial cells | High rate of synthesis, large proportion secreted *in vitro* |
| Chondrocytes | Amount correlates inversely with differentiation |
| Myeloblast and myotubes | Quantity appears to differ according to source of cells |
| Transformed cells | Have less; myotubes often have less than fibroblasts |
| Macrophages | All or most is secreted |
| Hepatocytes | All or most is secreated plasma fibronectin |
| Amniotic cells | Amniotic FN is more heavily glycosylated |
| Glial cell lines | Glial cells *in vivo* probably do not synthesize FN |
| Intestinal epithelial cell | Small amount only |
| Mammary epithelial cell | Decreased on metastatic |
| Tetraocarcinomas | Change with differentiation |
| Early embryonic tissue | Cell from all three germ layers have been reported to fibronectin |

Table 1.8b Properties of fibronectins

| | |
|---|---|
| Subunit | 220,000+20,000-dalton chains in disulfide-bonded dimer |
| pI | 5.5-6.3 |
| Carbohydrate | 5-9% asparagine linked complex oligosaccharides. |
| Sulfhydryl groups | One or two in the C-terminal 30% |
| Disulfide bonds | ~20 per subunit. Intersubunit bond (s) are very near C-terminal. N-terminal 25% is very rich in intrachain disulfides |
| Secondary structure | No α helix, probably some structure. Tertiary structure. A symmetric and elongated with globular domains |
| High order associations | Form disulfide-bonded complexes and fibrils |

Table 1.8c Interactions of fibronectins

| |
|---|
| Gelatin, denatured collagens, collagens I-V |
| Fibrin, fibrogen Factor XIII a transglutaminase |
| Heparin |
| Proteoglycans |
| Cellls |
| Bacteria |
| Actin |
| DNA |
| Gangliosides |
| Asymmertic acetylcholinesterase |
| CIq component of complement |
| Thrombospondin |

Table 1.8d Functions of fibronectins

| |
|---|
| Cellular adhesion |
| Cellular morphology and spreading |
| Cytoskeletal (microfilament) organization |
| Oncogenic transformation |
| Cell migration/chemotaxis or haptotaxis |
| Phagocytosis |
| Hemostasis/thrombosis |
| Embryonic differentiation |

## 1.3 Fish immune response

The immune system in fish comprises non-specific and specific immune responses. The latter includes cell mediated immunity and humoral immunity (antibody production).

### 1.3.1 Non-specific immune response

Non-specific immunity or innate immunity is defined as the non-inducible and non-specific first line of defence, against invading pathogens. It has no memory component, and being nonspecific, it acts against a variety of pathogens. It relies on both cells and humoral factors as well as the physical characteristic of the fish. In fish the first barrier to infection is the intact

skin. Many pathogens, especially fungal ones, can only infect the fish if this barrier is breached. Once the pathogen has invaded the fish, natural denfense factors such as mucus, CRP (C-reactive protein), transferrin, lysozyme, chitinase, interferon, complement, and phagocytosis help to protect the animal.

### 1.3.1.1 Lysozyme

Lysozyme, sometime referred to as muramidase or N-acetylmuramide glycarbohydrolase, is a mucolytic enzyme of leucocytic origin which exhibits antibiotic properties. The enzyme is widely distributed in nature and is found in a large variety of animal secretions such as mucus and saliva, and in many tissues including blood and in the cell vaculoes of plants (Jolles, 1969). The optimum activity of lysozyme is reached at a pH between 6-7. It has an isoelectric point between pH 10.5-11.0 and an approximate molecular weight of 14400 kDa (Osserman, Canfield and Beychok, 1974). In humans, lysozyme can either have a direct bacteriolytic effect on Gram negative bacteria or with the aid of complement and antibody enhances complement-mediated bacteriolysis. Lysozyme has also been detected in the serum, mucus and certain tissues of both freshwater and marine fish (Fletcher and White, 1973; Murray and Fletcher, 1976). It occurs mainly in fish netrophils and monocytes with small amounts detectable in macrophages, a finding which is in keeping with the mammalian literature (Hansen, 1974). High activities of lysozyme were found against *Micrococcus lysodeikticus* in the lymphomyeloid (haematopoietic) tissue of elasmobranchs and in lymph and plasma of teleosts. Fletcher and White (1973) demonstrated the presence of lysozyme in flatfish, which unlike cod (*Gadus morhua*), was consistently present in its sera throughout the year. In other fish species such as haddock, *Melanogrammus aeglefinus*, seasonal variation in lysozyme levels have been found. Seasonal and sexual variations in the levels of serum lysozyme have also been reported

for rainbow trout, (Vladimirov, 1968) plaice and lumpsuckers (Fletcher and White, 1976; Fletcher, White and Baldo, 1977). Fletcher and White (1976) found that the enzyme in plaice possessed properties similar to those of mammalian lysozyme with a molecular weight of 14000-15000 kDa, an optimum activity at pH 5.4, exhibited cathodic mobility and weak chitinase activity. A 2-3 fold increase in lysozyme activity was considered to be an effective natural protective mechanism in fish (Vladimirov, 1968).

Mock and Peters, (1990) reported that strong stressors such as transport lasting 2 h or acute water pollution, reduced the lysozyme level significantly, the decrease of the lysozyme activity returned to normal within 2 week following confrontation with the stressor. Brattgjerd *et al.*, (1994) found that treatment of Atlantic salmon with M-glucan also resulted in enhanced serum lysozyme activity in week 3 of the experimental period.

### 1.3.1.2 C-rective protein (CRP)

CRP is found in man and a limited number of mammals. It is so named because of its property of precipitation with group-specific C-polysaccharide (mucopeptide and N-acetyl galactosamine-6-phosphate) on the cell walls of *Pneumococcus* sp in the presence of the calcium ions. CRP is not an antibody, though it's amino acid composition shows some homology with that of immunoglobulins (Marchalonis and Weltman, 1971). In fish, CRP appears to be a normal serum component and may be especially important at times when the specific humoral defence system is inhibited by low temperature (Marchalonis *et al.*, 1971). CRP-like precipitins have been demonstrated in the sera of healthy plaice, dab (Limanda limanda), and cod against extracts from bacteria, some fungi and a nematode worms using gel diffusion (Baldo and Fletcher, 1973). CRP-like proteins have also been implicated in

immediate hypersensitivity responses of flat fish (Baldo and Fletcher, 1975a,b ). Nakanishi *et al.*, (1991) found that CRP has a role in host defense during acute-phase response through the activation of the complement system enhancement of phagocytosis and suppression of bacterial growth. In fish, C-reactve protein is naturally present at most times and level are some 500 times greater than the normal levels in mammals (White *et al.*, 1981). In tilapia, *Sarotherodon massambicus*, C-reactive protein could not be detected in healthy animals (Ramos and Smith, 1978), however, following physical injury it was found. In the eel, *Anguilla Japonica*, the monomer of CRP had a molecular weight approximately 24 kDa, and the native molecule was 120 kDa by gel filtration.

### 1.3.1.3 Transferrin

Transferrins are non-heme, globular, iron-binding glycoproteins which are found in the sera of most vertebrates, in egg white and in mammalian milk. Each molecule of transferrin can bind 2 ferric ions, or other transitional metals thus forming the recognisable salmon-pink coloured complex (Putnam, 1975). Transferrin exhibits antimicrobial properties by chelating free iron, making it unavailable to the pathogen and thereby limiting their growth. In coho salmon, the various genotypes of transferrin may be responsible for its relative resistance to bacterial kidney disease (Suzumoto, Schreck and McIntyre, 1977). Roed *et al.*, (1995) produced polyclonal and monoclonal antibodies against salmon transferrin. These were used in an enzyme immunosorbent assay (ELISA). The sensitivity of the assay was high with the lowest detectable transferrin concentration being 0.5-1 ng/ml.

### 1.3.1.4 Chitinase

Chitinase has been detected in lymphomyeloid tissue with the exception of the thymus (Fange, Lundblad and Lind, 1976). In comparison to lysozyme, high chitinase activity was found in the spleen, plasma and lymph of all the teleosts studied. Insectivorous fish possess large amounts of the enzyme in the serum, pancreas and gut mucosa, in keeping with the site of localisation in other vertebrates (Jeuniaux, 1961; Micha, Dandrifosse and Jeuniaux, 1973). Clark *et al.*, (1984) reported that chitinase activity was shown to be optimal at pH 9.5. Sabapathy and Toe, (1993) reported that chitinase activity was recorded in the gut of sea bass, primarily in the intestine and the pyloric caeca.

### 1.3.1.5 Interferon

The first report of interferon-like activity in marine fish was published by Gravell and Malsberger (1965), in infectious pancreatic necrosis virus IPNV-infected fish cell cultures. Fish produce more than one type of interferon, depending on the type of inducer and type of cell stimulated (Alexander and Ingram, 1992). It is produced mainly by sensitised lymphocytes and other leucocytes *in vitro*. Hedrick *et al.*, (1994) found that the mechanism for the viral mediated resistance induced by cutthroat trout virus is unknown but the virus was shown to be a potent inducer of interferon like activity in anterior kidney cells isolated from rainbow trout (*Oncorhynchus mykiss*). Rogel-Gaillard (1993) found that interferon like activity was induced *in vitro* from blood and kidney leucocytes from rainbow trout by infectious or inactivated Egtved virus, viral hemorrhagic septicemia virus (VHSV) serotypes.

### 1.3.1.6 Complement

Fish possess a complement system similar to that of mammals. It can be activated by both the classical and the alternate pathway (Sakai, 1992). Certain components of fish complement are compatible with those present in human and guinea pig serum (Sakai, 1992).

The alternative pathway is activated, by bacterial polysaccharides and microbial enzymes in the absence of antigen-antibody complexes, and hence provide immediate defence against infection before the development of immunity. Once activated, it can destroy bacteria by lysing the cell membranes, and can inactivate viruses, as well as causing increased vascular permeability, the chemotaxis of leucocytes and their retention at sites of tissue injury and the enhancement of phagocytosis (Sakai, 1992).

Cushing (1945) demonstrated the first four components of complement system in carp serum, with reactions displaying a marked similarity to those of guinea pig system. Other teleosts have also been shown to possess C1, C2, C3 and C4 (Sirotinin; 1960; Gewurz *et al.*, 1966; Legler *et al.*, 1967a,b; Chiller, Hodgins and Weiser, 1969; Gigli and Austin, 1971). However, carp and rainbow trout complement, unlike that of mammals, is not affected by $NH_4OH$ and complement from a heterologous source can replace the heat-labile components of either species (Cushing, 1945; Hodgins, Weiser and Ridgway, 1967). Elasmobranch complement has 6 components, of which the first and last two show analogies with mammalian C1, C8 and C9 respectively (Jesen, Sigel and Ross, 1968; Nelson and Gigli, 1968).

Teleost complement has not been as well studied as that of the elasmobranchs, but is known to be activated by the both the classical and alternative pathways, and it has a zymosan binding

protein identified as the C3 coupled together with a C5 component. Woo and Thomas (1991) reported activation of the complement cascade *in vitro* by mammalian antibodies. In fish, the complement system is much less well-characterised than in mammals, but the rainbow trout (*O. mykiss*) appears to possess a complement system with structural and functional similarities to the mammalian system (Nonaka *et al.*, 1981). Rainbow trout C3 has been described as the central component of this system (Nonaka *et al.*, 1984). Evidence for opsonization of bacteria by fish complement for the phagocytic cells of salmonids, was reported by Sakai, (1984).

Others substances involved in the natural resistances of fish infection includes natural agglutinins (Sindermann and Honey, 1964), natural lysins (Legler *et al*, 1967a), precipitins (Janssen and Meyers, 1968), non-immunoglobulin antibody-like molecules (Marchalonis and Weltman, 1971), and naturally-occuring immunoglobulins (Leslie and Clem, 1970). Bandin *et al.*, (1995) reported that immune sera favoured survival to a greater extent than non-immune sera. Although heat-inactivated immune sera reduced the extent of bacterial killing, bacterial persistence was enhanced to the such an extent when both complement and antibody were present, that opsonized bacteria grew faster within macrophages than extracellularly.

### 1.3.1.7 Fish cytokines

Several approaches have been used to look for cytokines in fish (Secombes *et al.*, 1996 Table 1.9). The majority of studies have searched for biological activity in homologous assay systems, as reviewed previously by Coken and Haynes (1991) and Secombes (1994). Whilst activities akin to interferons (IFNs), interleukins (ILs), chemokines, macrophage migration inhibition factors (MIF) and macrophages activating factors (MAF) are well know, more recently colony stimulating factors (CSF) activity has been described in rainbow trout serum

following injection with lipopolysaccharide (LPS) (Kodama *et al.*, 1994). Factors released from stimulated (trout) macrophages have also been shown to have a positive autocrine effect on effect functions such as generation of bacterial reactive oxygen species (Jang *et al* 1995).

Table 1.9 Approaches taken to investigate cytokine activity on fish (Secombes *et al.*, 1996)

| | |
|---|---|
| (1) | Biological activity in homologous assay system<br>(e.g. Kodama *et al.*, 1994; Verburg van Kemenade *et al.*, 1995; Jang *et al.*, 1995) |
| (2) | Biological cross-reacivity<br>(a) fish leucocyte-derived supernatants on mammalian cell assays (e.g. Ellsaesser & Clem, 1994; Verburg van Kemenade *et al.*, 1995).<br>(b) Mammalian cytokines on fish cell assay (e.g. Ellsaesser & Clem, 1994) |
| (3) | Antigen cross-reactivity<br>(a) to cytokine (e.g. Ellsaesser & Clem, 1994; Jang *et al.*, 1995)<br>(b) to cytokine receptors (e.g. Jang *et al.*, 1995) |
| (4) | Genetin/sequence data<br>(a) hybridisation with mammalian cytokine probes (eg. Tengelsen *et al.*, 1991)<br>(b) gene/amino acid sequence (e.g. Tamai *et al.*, 1992) |

### 1.3.2 Specific immune response

Mycobacteriosis is one of the most common chronic infections of fish. It is a systemic disease that can affect virtually any tissue, most commonly the spleen and kidney (Wolke and Stroud, 1978).The classical reaction of mycobacterial infections is considered to be the induction of epitheloid granulomas. Rainbow trout have been shown to elicit a cell mediated immune response on immunisation with *M. tuberculosis* and *M. fortuitum* (Bartos and Summer,1981). The immune system of higher vertebrates is composed of lymphocytes, macrophages, monocytes, basophils, mast cells, eosinophils and neutrophils (Rowley and Ratcliffe, 1988). They posses both cellular and humoral immune response capabilities, which are mediated by

the interactions between the different sub-populations of cells. The most important dichotomy of lymphocyte types in mammals is the major division into T and B-lymphocytes, and accessory cells. Mammalian B cells are antigen specific lymphocytes which are most readily identified by the presence of surface immunoglobulin (sIg) and their ability to undergo clonal expansion and differentiation into antibody secreting cells in response to appropriate stimuli. T-cells are also antigen specific, clonally expandable lymphocytes, but in contrast to B cells, they do not exhibit Ig and develop in the thymus during ontogeny to differentiate into specialised sub-populations, capable of subsequently exhibiting either suppressor, cytotoxic or helper activities. In higher vertebrate species, each of these sub-populations of T cells exhibit distinctive cell surface markers (Bierer *et al.*, 1989). Accessory cells such as macrophages and monocytes are generally considered to be antigen-nonspecific and their primary functions are to secrete interleukin (IL-1), a cytokine involved in T-cell activation, and participating in antigen processing and presentation of antigen to B and T-cells (Unanue, 1984).

Immune responses in higher vertebrates can be divided into two general categories based on the type of lymphocytes involved: Thymus-dependent (TD) responses which require T-cell help and thymus independent (TI) responses which do not. It is important to note that in higher vertebrates, the T and B-lymphocytes do not only show functional differences, but they also show distinct developmental differences, with the T cells developing in the thymus, whilst the B cells remain in the bone marrow during development. Fish contain three peripheral cell populations: erythrocytes, thrombocytes and the leucocytes, comprising granulocytes, monocytes, and lymphocytes. Thrombocytes resemble mammalian platelets in being responsible for clot formation, and several authors suggest

that they are also phagocytic (Rowley *et al.*, 1988). Granulocytes in fish are composed of neutrophils, eosinophils, mast cells and basophils, but the relative proportion of the granulocyte populations is species dependent ( Rowley *et al.*, 1988).

Granulocytes are generally the first cells to migrate to sites of inflammation, where they nonspecifically destroy invading organisms by phagocytosis or cytotoxic killing (Finn and Nielson, 1971; MacArthur *et al.*, 1984). Monocytes from teleosts are similar to their mammalian counterparts, morphologically and functionally, making up some 1-4% of total leucocytes. They act as phagocytic cells moving to sites of acute and chronic infection (Rowley *et al.*, 1988). The role of macrophages in fish is more than simply one of phagocytosis. Macrophages, found in the lymphoid tissues, are thought to trap, process and present antigens to lymphocytes. Evidence suggests that macrophages interact with, and stimulate lymphocytes, and for this reason are accessory cells. The spleen of fish is composed of highly phagocytic macrophages, with in a reticulin fibre network, called an ellipsoid, in which immune complexes are trapped (Ellis, 1982). IL-1 is secreted by activated fish macrophages (Ellsaesser, 1989), which in turn may stimulate T lymphocytes to secrete interleukin-2 (IL-2). It is thought that macrophages are activated in two step process (Ruco and Meltzer, 1978). Firstly, inactivated macrophages are primed by the stimulus of low-dose lipopolysaccharide (LPS), thioglycolate, complement, lymphokine or interferon-$\gamma$ (IFN-$\gamma$). In the primed state macrophages have increased spreading, alteration in cell surface receptors and an increase in metabolic activity. They also become more susceptible to stimulation by the above and other cytokines, which triggers full activation. Unstimulated macrophages can be fully activated *in vitro* with either phorbol myristate (PMA), mitogens including concanavalian A (Con A) and LPS or by calcium ionophore

A23187 (West, 1990). In the activated state, macrophages have decreased chemotaxis and enhanced spreading and adherence. They show increased phagocytosis, elevated metabolic activity with a heightened respiratory burst activity, eicosanoid and cytokine production and the release of lysosomal enzymes. The result is an increase in bactericidal activity, and tumor and parasite cytotoxicity (West, 1990).

When normal unactivated macrophages are cultured with antigen-sensitized lymphocytes for 2-3 days, the macrophages undergo the physiological changes mentioned above which results in an enhanced ability to kill ingested bacteria. This observed activation of the macrophages is not dependent on the physical presence of sensitized lymphocytes, but rather a factor produced by the sensitized lymphocytes. If supernatants from cultures of activated lymphocytes are added to normal macrophages in the absence of the lymphocytes, macrophage activation will still occur (Secombes, 1987). The factor responsible for activation is called macrophage activation factor (MAF) and attempts to characterize it suggest that it may be a gamma interferon (IFN-$\gamma$) (Graham and Secombes, 1990).

## Chapter 2

## Extracellular products from fish *Mycobacterium* spp.

### 2.1 Introduction

It has been reported that live *Mycobacterium bovis* Bacilli Calmette-Guerin (BCG) vaccine induces higher levels of resistance to tuberculosis than dead bacilli, or their components, in experimental animals (Rook, 1980; Collins, 1984; Lovik and Closs, 1984; Orme, 1988). This may be explained by the secretion of immunostimulating antigens by the live *M. bovis* BCG in the host tissue. Secreted antigens are likely to provide the first stimulus *in vivo* for humoral and cellular response to mycobacteria and thus, may be more valuable in a serological test than *M. tuberculosis* antigens derived from dead bacilli. Secreted antigens may be also be produced by mycobacteria cultured *in vitro*, and several antigens have been identified from the culture supernatant of *M. bovis* BCG and *M. tuberculosis* (Closs, Harboe, Axelsen, Bunch-Christensen and Magnussen, 1980; De Bruyn, Weckx and Beumer-Jochmans, 1981; Abou-Zeid, Smith, Grance, Steele and Rook, 1986, Abou-Zeid, Smith, Grance, Ratliff, Steele and Rook, 1988a).

No literature is currently available, however, to describe the extracellular products (ECP) from *Mycobacterium* spp. isolated from fish. This chapter describes the selection of an optimal culture medium, capable of providing high concentrations of ECP from fish *Mycobacterium* spp. The protein profiles, enzymatic activities and toxicity of ECPs are also described.

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## 2.2 Materials and methods

### 2.2.1 Media preparation and culture of *Mycobacterium* spp.

Sauton's medium, modified Sauton's medium, Long's medium and Eagle's minimum essential medium (Sigma), supplemented with 0.4% (w/v) asparagine and 0.005% (w/v) ferric ammonium citrate were selected for the production of ECP from *Mycobacterium* spp. The composition of each medium is shown in Table 2.1. TB1, TB40, TB267, TB268 and NCIMB strains of mycobacteria (Table 2.2) were inoculated into the four different media and incubated for 14 days at 28°C . The bacteria were transferred at least 3 times under the same conditions to ensure nutrient adaptation prior to testing secreted responses. For preparation of ECP, 5 ml (1x $10^7$ CFU) of precultured bacteria were inoculated into 500 ml of each culture medium. These were cultured for 10, 11, 12, and 14 days at 28° C, then shifted from 28° C to 37°C for a further 4, 3, 2 and 0 days, respectively. Bacteria were killed by heating the suspension in a water bath at 75° C for 15 min. The suspensions were centrifuged at 7,000 x *g* for 1 h at 4°C and bacteria were then removed by sequential filtration through a Whatman No. 1 paper and a Millipore filter (0.2 μm). The supernatants were concentrated using polyethylene glycol (8 kDa, Sigma) and the concentrated samples were then dialysed against phosphate buffered saline ( 0.15M, pH 7.2 ) for 24 hrs at 4°C. The samples were centrifuged at 12,000 x *g* for 1 h at 4°C, and harvested supernatants were stored at -70°C.

### 2.2.2 Whole cells sonicated (WCS) antigen

Different strains of *Mycobacterium* spp., cultured at 28°C for 14 days in modified Sauton's medium, were centrifuged at 4000 x *g* for 1 h. The resulting pellet was washed three times with PBS and then were suspended in PBS (3 grams 10 $ml^{-1}$). The suspensions were sonicated using a 150 W ultrasonic distegrator (MSE, London, England) for four 5 mins burst period four

time with a 1 min interval. The suspension was centrifuged for 1 h at 12,000 x *g*. The supernatant was used as an antigen.

Table 2.1. Composition (%w/w)* of the four media for culture of *Mycobacterium* spp.

| | | MSM | SM | LM | MEM supplemented |
|---|---|---|---|---|---|
| 1. Asparagine | | | 0.4 | 0.5 | 0.4 |
| 2. Ammonium citrate | | | | 0.5 | |
| 3. Potassium acid phosphate | ($KH_2PO_4$) | 0.05 | 0.05 | 0.3 | |
| 4. Sodium carbonate | | | | 0.2 | |
| 5. Sodium chloride | | | | 0.1 | |
| 6. Magnesium sulphate | ($MgSO_4.7H_2O$) | 0.05 | 0.05 | | |
| 7. Ferric ammonium citrate | | 0.005 | 0.005 | 0.005 | 0.005 |
| 8. Citric acid | | 0.2 | 0.2 | | |
| 9. Sodium glutamate | | 0.4 | | | |
| 10. Glycerol | | 3.0 | 6.0 | 5.0 | |

* : %w/w, except for glycerol (% v/v)

MSM: Modified Sauton's medium.

SM : Sauton's medium.

LM: Long's medium

MEM: Eagle's minimum essential medium.

Table 2.2 Mycobacterium strains

| Strain | Source |
|---|---|
| TB1 | AAHRI*, Siamese fighting fish (*Betta spendens*) |
| TB40 | AAHRI, Siamese fighting fish (*Betta spendens*) |
| TB267 | AAHRI, Snakehead fish (*Channa striatus* ) |
| TB268 | AAHRI, Snakehead fish (*Channa striatus*) |
| *Mycobacterium chelonae* | NCIMB** 1474, Tortoise |
| *Mycobacterium fortuitum* | NCIMB 1294, A. J. Ross/Kidney of juvenile chinook salmon (*Oncorhynchus tschawytscha*) |
| *Mycobacterium marinum* | NCIMB 1298, Neon tetra (*Hyphessobrycon innesi*). |

* AAHRI: Aquatic Animal Health Research Institute, Bangkok, Thailand.

**NCIMB : National Collections of Industrial and Marine Bacteria, United Kingdom.

### 2.2.3 Dry weight determination

Bacterial pellets obtained by centrifugation were washed three times with nanopure water. The cells were then dried to a constant weight at 70°C and the dry weight (grams) determined.

### 2.2.4 Protein determination

Protein content was determined using a Bio-Rad protein assay kit, based on the Bradford dye (Coomassie brilliant blue G) binding procedure with bovine serum albumin (BSA) as a standard ( Bradford, 1976).

### 2.2.5 Protein analysis by polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) using 15% (w/v) acrylamide separating gels and 4% (w/v) acrylamide stacking gels. The Bio-Rad electrophoresis calibration kit used, contained the following molecular weight markers: rabbit phosphorylase B 97.4 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa, lysozyme 14.4 kDa. The ECP samples were diluted in sample loading buffer (0.064 M Tris HCl pH 6.8, 2 % (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 5% (v/v) mercaptoethanol) and were heated to 100°C for 5 min immediately prior to loading the gels. Gels were stained with Coomassie blue.

### 2.2.6 Gel processing

The gels were placed overnight in 0.1% Coomassie brilliant blue solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. The gel was then destained by shaking with a solution of 10% (v/v) methanol and 10% (v/v) acetic acid. Gels were stored in 12.5% acetic acid

### 2.2.7 Enzyme activity

The following enzyme activities were determined by the method of Kannan, Katoch, Sharma, and Bharadwas, (1987).

#### 2.2.7.1 Mucinase

Mucinase activity was detected by incubating 20 µl ECP in wells of 1 % agarose plates supplemented with 0.28% (w/v) porcine stomach mucin (Sigma). After incubation for 24h, the plates were flooded with 1% (w/v) calcium chloride. Mucinase activity was revealed by a zone of clearing.

#### 2.2.7.2 Protease

Protease activity was assayed by incubating 20 µl ECP overnight at 37°C in wells of 1 % agarose plates containing gelatin or casein (both at 1% w/v Difco, U.S.A.) in Tris HCl buffer (25 mM, pH 8.5) and subsequently flooding the plates with saturated ammonium sulphate solution. Protease activity was revealed by a zone of clearing.

#### 2.2.7.3 Lipase

Lipase activity was detected by incubating 20 µl ECP overnight at 37°C in wells of a 1 % (w/v) agarose plates supplemented with 1% (v/v) Tween-80 and 0.01% (w/v) $CaCl_2$. $H_2O$. A positive test was indicated by an opaque halo around the wells.

#### 2.2.7.4 DNase and RNase

DNA or RNA (Sigma; 0.2% w/v in deionised water) added to 1 % (w/v) agarose was used for the detection of DNase and RNase, respectively. ECP (20 µl) was added to the wells and

incubated overnight at 37°C. A positive reaction were indicated by a clear zone around the well when flooded with 1 N HCl.

### 2.2.8 Toxicity of extracellular products

#### 2.2.8.1 Toxicity of ECP to rainbow trout

A total of 170 healthy rainbow trout (10-15 g body weight) were used for the experimental study. The fish were maintained at 15±2 °C. They were divided equally into 17 treatment groups (10 in each group) in separate tanks and fed a commercial diet daily. Fish were inoculated with 0.2 ml of ECP containing various protein doses (50, 100, 200 and 400 μg/0.2ml, four tanks of each) from TB40, TB267, and *M. marinum* by intraperitoneal injection, respectively, and 400 μg only by intramuscular injection (thirteenth to fifteenth group). The sixteenth to seventeenth group of fish used as the control group and were intraperitoneal and intramuscular injected with PBS only.

#### 2.2.8.2 Toxicity of ECP to Nile tilapia

A total of 85 healthy Nile tilapia ( 30-40g body weight) were used for the experimental study. The fish were maintained at 25±2 °C. They were divided equally into 17 treatment groups (5 in each group) in separate tanks and fed a daily commercial diet. The toxicity experiment described in section 2.2.8.1 was also tested for tilapia.

### 2.2.9 Histopathology

#### 2.2.9.1 Fixation

Tissues (gills, kidney, heart, liver, spleen, stomach and intestine) from experimental and control fish were taken for histological studies. Samples were placed in fixative (10% neutral

buffered formalin saline) for at least 24 hours prior to cassetting. Individual tissues were of a suitable size to allow penetration of the fixative.

### 2.2.9.2 Tissue processing

The time schedule for the processor is shown in Appendix 1.

## 2.3 Results

Mycobacteria were grown on the surface of medium as pellicles for 14 days at 28°C. Strains TB1, TB40, TB267, TB268 and *M. marinum* (NCIBM 1298) could not be cultured on Long's medium (Table 2.3) and in general the growth and production of ECPs for all other strains of mycobacteria examined was limited in Long's medium. Successful growth, however, was achieved on modified Sauton's medium, Sauton's medium and MEM medium, especially in the former two media. Modified Sauton's medium enabled excellent growth and production of ECPs for TB40, TB267, TB268 and *M. marinum*. The dry weights of TB267, *M. marinum* and *M. chelonae* were higher than those of other strains in modified Sauton's and Sauton's medium (Table 2.3).

Mycobacteria produced approximately 1.5-2.5 mg ECP per litre of culture filtrate. SDS-PAGE protein patterns of ECPs from 14 day culture supernatants (12 days at 28°C then shifted to 37°C for 2 days) showed major bands at 65, and <14 kDa and faint bands in the 70, 45, 40, 38 and 25 kDa regions (Figure. 2.1a). The protein profile of TB1 most closely resembled the *M. marinum* profile, rather than the other type strains, showing major bands at 65, 16, and <14 kDa. The protein profiles of WCS (Figure 2.1b) and ECP from TB1, TB40, TB267, TB268, *M. chelonae*, *M. fortuitum* and *M marinum* were different, although the 65 kDa protein was

observed in both WCS and ECP gels. The SDS-PAGE protein profile of WCS from 14 day cultures showed major band at 68, 65, 55, 50, 31, 27, 16 and a faint band >97 kDa. The 65 kDa band was thicker in ECP than WCS gels. The low molecular weight (<14 kDa) protein (5-7 bands) was only detected in the ECP group and not in WCS gels.

Table 2.3. Production of ECP and growth from *Mycobacterium* spp cultured in four media for 14 day at 28°C

| Strain | ECP (mg protein/l ) producing following media | | | | Dry weight (g/l) of bacteria growth in following media | | | |
|---|---|---|---|---|---|---|---|---|
| | MSM | SM | LM | MEM | MSM | SM | LM | MEM |
| TB1 | 1.707 | 0.468 | NG | 0.085 | 0.87 | 1.43 | NG | 0.43 |
| TB40 | 2.660 | 0.840 | NG | 0.649 | 1.07 | 0.35 | NG | 0.31 |
| TB267 | 1.990 | 0.720 | NG | 0.118 | 2.71 | 0.84 | NG | 0.45 |
| TB268 | 2.503 | 0.052 | NG | 0.062 | 1.32 | 0.82 | NG | 0.42 |
| *M. chelonae* | 1.623 | 0.330 | 0.403 | 0.064 | 1.83 | 0.49 | 0.81 | 0.32 |
| *M. fortuitum* | 1.623 | 0.384 | 0.351 | 0.111 | 0.83 | 1.27 | 1.03 | 0.71 |
| *M. marinum* | 2.318 | 2.142 | NG | 0.084 | 2.33 | 1.59 | NG | 0.74 |

NG: No growth
MSM: Modified Sauton's medium.
SM : Sauton's medium.
LM: Long's medium
MEM: Eagle's minimum essential medium.

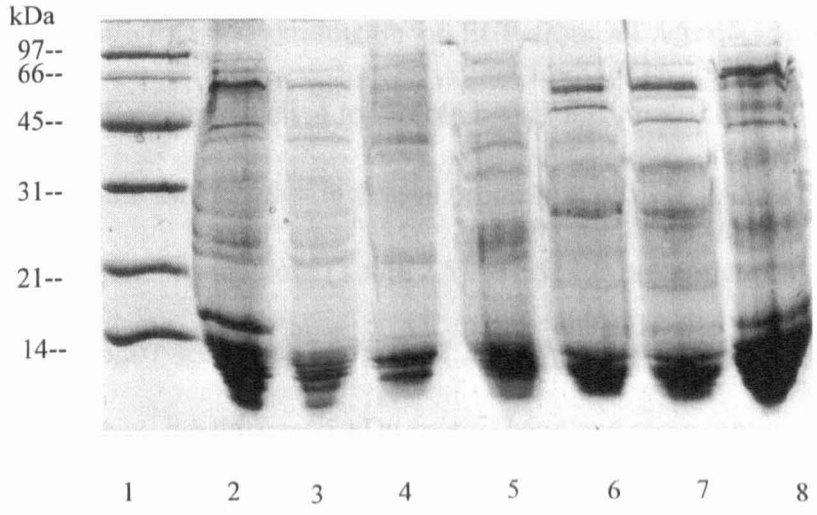



Figure 2.1a Coomassie blue stained 15% SDS-gel of 14 day extracellular products from *Mycobacterium* spp. Lanes: (1) Molecular markers, (2) TB1, (3) TB267, (4) TB40, (5) TB268, (6) *M. chelonae,* (7) *M. fortuitum*, (8) *M. marinum.*

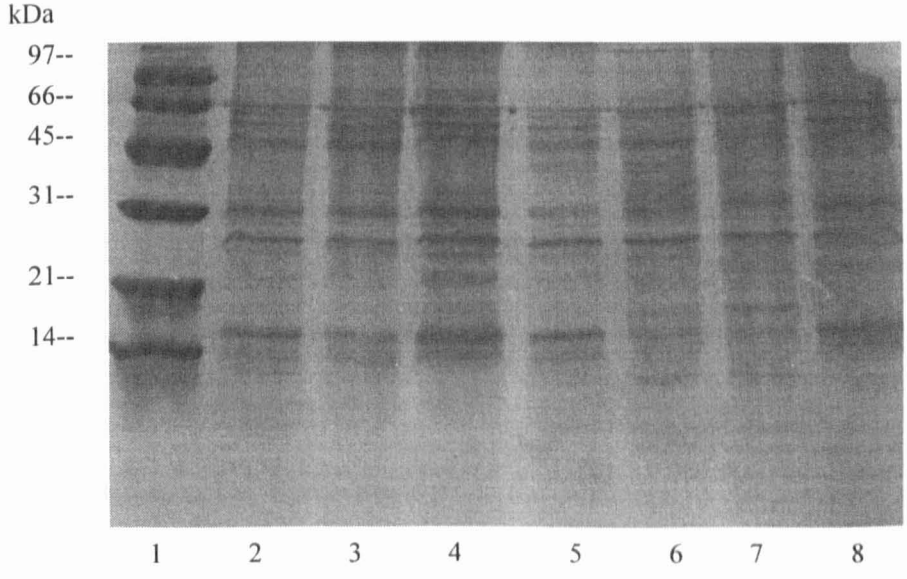



Figure 2.1b Coomassie blue stained 15% SDS-gel of 14 day whole cell sonicate from *Mycobacterium* spp. Lanes: (1) Molecular markers, (2) TB1, (3) TB40, (4) TB267, (5) TB268, (6) *M. chelonae,* (7) *M. fortuitum*, (8) *M. marinum.*

After a 2-day heat shock at 37°C, the production of ECP from all *Mycobacterium* strains, except *M. marinum* averaged approximately 4 to 10 fold higher than those cultured for 14 days at 28°C (Table 2.4). TB267 and *M. marinum* increased production of ECP at 2 and especially 3 day heat shock in modified Sauton's medium (Table 2.5). The ECP protein profiles before and after heat shock for strain TB267 are shown in Figure 2.2. The profiles were similar 2, 3 and 4 days after heat shock. Moreover, the thicker 65 kDa protein band and more protein between 60-16 kDa were only found under heat shock.

The activities of the various extracellular enzymes are shown (Table 2.6). No protease, mucinase, lipase, RNase and DNase activities were detected for TB1, TB40, TB267 and TB 268. Mucinase activity however was detected in *M. marinum* and lipase and RNase activities were positive for *M. chelonae* and *M. fortuitum*. Protease and DNase activities could not be detected in any of the species tested.

During the toxicity test only three rainbow trout and two Nile tilapia died during the entire trial. These fish had been injected im with 400 μg of ECP. Thus the LD50 of ECP to rainbow trout or Nile tilapia was greater than 400 μg. The mild haemorrhage was found in injected area and no significant lesions were detected by histopathology in the other organs examined.

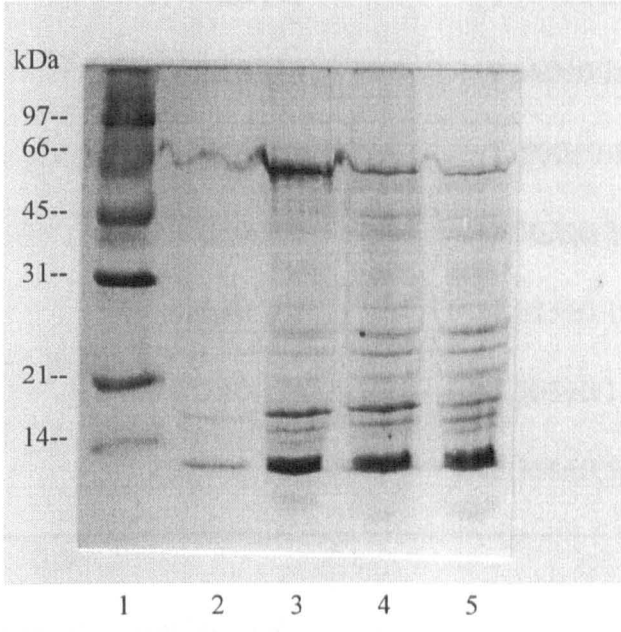



Figure 2.2 Coomassie blue stained 15 % SDS-gel of 14 day extracellular products of *Mycobacterium* spp. Lanes
(1) Molecular markers
(2) TB267 H-0 (cultured at 28°C for 14 days),
(3) TB267 H-2 (cultured at 28°C for 12 days then shifted to 37°C for 2 days)
(4) TB267 H-3 (cultured at 28°C for 11 days then shifted to 37°C for 3 days)
(5) TB267 H-4 ( cultured at 28°C for 10 days then shifted to 37°C for 4 days)

Table 2.4. Comparison of secreted protein production (μg/l) from non-heat shock and 2 day heat-shock in modified Sauton's medium [Four experiments (mean ± standard error )]

| Strain | *Non-heat shock | **Heat-shock |
|---|---|---|
| TB1 | 0.456±0.111 | 1.707±0.220 |
| TB40 | 0.466±0.114 | 2.660±0.263 |
| TB267 | 0.354±0.104 | 1. 990±0.432 |
| TB268 | 0.372±0.102 | 2.503±0.355 |
| *M. chelonae* | 0.486±0.167 | 1.623±0.135 |
| *M. fortuitum* | 0.152±0.124 | 1.563±0.126 |
| *M. marinum* | 2.227±0.167 | 2.318±0.516 |

* Cultured in modified Sauton's medium 14 day at 28°C

**Cultured in modified Sauton's medium 12 days and shift to 37°C for 2 days

Table 2.5. Comparison of ECP production under various heat shock culture conditions in modified Sauton's medium. [Four experiments (mean ± standard error )]

| Day | TB267 (mg/l) | *M. marinum* (mg/l) |
|---|---|---|
| H-0 | 0.354±0.104 | 2.227±0.167 |
| H-2 | 1.990±0.432 | 2.318±0.516 |
| H-3 | 7.020±0.830 | 6.975±0.750 |
| H-4 | 2.308±0.461 | 2.715±0.391 |

H-0: cultured in modified Sauton's medium 14 day at 28°C

H-2: cultured in modified Sauton's medium 12 days and shift to 37°C for 2 days

H-3: cultured in modified Sauton's medium 11 day and shift to 37°C for 3 days

H-4: cultured in modified Sauton's medium 10 day and shift to 37°C for 4 days

Table 2.6. Extracellular enzyme activities of *Mycobaterium* strains

| | Protease | | Mucinase | Lipase | Ribonuclease | |
|---|---|---|---|---|---|---|
| | C | G | | | DNase | RNase |
| TB1 | - | - | - | - | - | - |
| TB40 | - | - | - | - | - | - |
| TB267 | - | - | - | - | - | - |
| TB268 | - | - | - | - | - | - |
| *M. chelonae* | - | - | - | + | - | + |
| *M. fortuitum* | - | - | - | + | - | + |
| *M. marinum* | - | - | + | - | - | - |

C:Casein

G:Gelatin

## 2.4 Discussion

Modified Sauton's medium appears to be the best of the media tested for the culture of fish mycobacteria. A one litre culture filtrate contained typically 1.5-2.5 mg of protein. This is, however, substantially lower than the production of ECPs from *Mycobacterium bovis* BCG 1173P2 in Sauton's medium, as De Bruyn *et al* (1987) reported that *M. bovis* can produce 125-150 mg of protein per litre after 14 day culture in Sauton's medium at 37.5°C.

The 14 day culture supernatants from all mycobacterial strains tested showed major bands on SDS-PAGE at 65 and <14 kDa. Their major bands were similar to those of *M. tuberculosis* although many minor bands indicated strain differences (Verbon, Kuijper, Jansen, Speelman and Kolk, 1990). Different protein profiles were observed between WCS and ECP for all the mycobacterial strains tested. The low molecular weight proteins (<14 kDa) were only found in the ECP. This indicates that *Mycobacterium* spp either secrete these into the culture medium during the 14 day culture period or that the low molecular weight bands represent break down products. As low molecular weight products were not observed at any time with WCS stored either frozen or at 4°C, it seems likely that these proteins are in fact secreted by mycobacteria. More protein appeared to be secreted under heat shock conditions and the 65 kDa protein appears to be one of the heat shock proteins.

Extracellular enzymes are thought to play an important role in the pathogenesis of various fish diseases (Allan and Stevenson, 1981; Ellis, Hastings and Munro, 1981; Inamura, Muroga, and Nakai 1984; Magarineos, Santos, Romalde, Rivas and Barja, 1992). Mucinase may play a part in the penetration of pathogens, as in the case of *Vibrio cholerae* in the intestine (Freter, Allweiss, O'Brien, Halstead and Macsai, 1981) and DNase and RNase may be involved in the

inactivation of phagocyte nucleic acid after phagocytosis of micro-organisms. Tissue damage in mycobacterial disease, particularly leprosy and tuberculosis, is usually associated with attempts by the immune system to destroy the mycobacteria residing in the tissues. Extracellular mucinase and RNase have been reported to be activities associated with pathogenicity in mammalian mycobacteria (Kannan, *et al* 1987). The results presented in here indicate only the presence of mucinase, lipase and RNase in the type strains, however, none of these activities were detected in the *Mycobacterium* strains isolated from fish in Thailand.

The ECP from fish pathogens has been reported to be toxic in some cases. *Vibrio anguillarum* ECP exhibits high toxicity with a lethal dose of approximately 80 μg/fish (goldfish) (Inamura, *et al* 1984), while the LD50 of ECP from *Aeromonas hydrophila* was reported to be 19 μg/g fish (channnel catfish) (Thune, Graham, Riddle and Amborski, 1982). Mycobacteria ECP, however, appears to be of low toxicity in trout and tilapia as doses of 400 μg/ fish resulted in low mortalities

## Chapter 3

## A comparison of the antigenicity of the extracellular products (ECP) and whole cell sonicates (WCS) from *Mycobacterium* spp. in rabbits, mice and fish by Western blotting and enzyme-linked immunosorbent assay (ELISA)

### 3.1 Introduction

Previous studies have shown that extracellular products (ECP) are produced by mycobacteria cultured *in vitro* ( Chapter 2; Chen. *et al.*, 1996a), however, there is no literature available describing the antigenicity of these ECPs. The aim of the present study was to examine the antigenicity of ECP derived from *Mycobacterium* spp. isolated from snakehead fish (*Channa striatus*) and Siamese fighting fish (*Betta spendens*). The reactivity of sera from rabbits, mice and fish immunised with ECP from mycobacteria isolated from Siamese fighting fish and snakehead fish, was examined by immunoblotting and enzyme-linked immunosorbant assay (ELISA). Cross-reactivity of anti-*M. tuberculosis* and anti-human heat shock protein monoclonal antibodies (MAbs) with the ECP from these mycobacteria strains, and detection of the fibronectin binding protein was also examined.

### 3.2 Materials and methods

#### 3.2.1 Preparation of ECP

*Mycobacterium* spp., strains TB40, TB267 and *M. marinum* (Table 3.1), were cultured in modified Sauton's medium (MSM). Five ml of pre-culture was inoculated into 500 ml of MSM and cultured for 12 days at 28°C, followed by increasing the temperature to 37°C for a further 48 h. (as described in section 2.2.1).

### 3.2.2 Preparation of whole cell sonicated (WCS) antigen

Strains of mycobacteria, outlined in Table 3.1, were cultured for 14 days at 28°C. Bacteria were killed by incubation in a water bath at 75°C for 15 mins and then sonicated as described in section 2.2.2. WCS from a variety of non-mycobacteria (Table 3.1) were also prepared to examine the cross-reactivity of the rabbit anti-65 kDa serum prepared below. *Nocardia asteriodes* was grown on MSM for 14 days, *Renibacterium salmoninarum* was cultured in selective kidney disease medium with charcoal for 24 days and *Pasteurella piscicida, Vibrio anguillarum* and *Vibrio ardalli* were cultured in tryptic soy broth (TSB) with 2% NaCl for 24h. All the other remaining bacteria (Table 3.1) were cultured in TSB for 24 h. Non-mycobacteria were sonicated for two 3 min periods with a 1 min interval between. Sonicates were centrifuged for 60 min at 12,000 x *g* and the resulting supernatants were used as an antigen.

### 3.2.3 Electro-elution of 65 kDa protein

The 65 kDa protein present in the ECP of strain TB267 (Chen *et al.*, 1996a) was purified by electro-elution according to Neelam *et al.*, 1995. The protein was isolated on a 12 % (w/v) polyacrylamide slab gel by SDS-PAGE. The sample for each gel (150 µl of ECP containing 100 µg protein mixed 1:1 with sample buffer) was prepared by heating for 5 mins at 100°C, then centrifuging for 5 min at 13000 x *g*. The 65 kDa protein band was located by lightly staining the gel with Coomassie blue, then excised from the gel with a scalpel. It was electro-eluted overnight at 100 V using a Biotrap BT 1000 apparatus (Schlecicher and Schuell, Dassel, Germany). The elution buffer was 25 mM Tris-HCl, 192 mM glycine, 0.025 % (w/v) SDS. At the end of the run the polarity was reversed for 20 Sec. at 200 V before collecting the sample.

Table 3.1 Bacteria used to test cross reactivity of rabbit antisera

| Species | Source | Medium | Temperature (°C) |
|---|---|---|---|
| *Aeromonas hydrophila hydrophila* | NCIMB 87 | TS | 22 |
| *Aeromonas salmonicida acromogenes* | NCIMB 1110 | TS | 22 |
| *Aeromonas salmonicida masoucida* | NCIMB 2020 | TS | 22 |
| *Aeromonas salmonicida salmonicida* | IOA | TS | 22 |
| *Corynebacterium aquticum* | IOA | TS | 22 |
| *Micrococcus luteus* | NCIMB 570 | TS | 22 |
| *Nocardia asteroides* | NCIMB 1290 | MSM | 22 |
| *Streptococcus faecalis* | IOA | TS+N | 22 |
| *Pesudomonas aeruginosa* | NCIMB 8295 | TS | 22 |
| *Pseudomonas fluorescences* | NCIMB 1283 | TS | 22 |
| *Vibrio anguillarum* | NCIMB 571 | TS+N | 22 |
| *Vibrio ordalli* | NCIMB 2167 | TS+N | 22 |
| *Yersinia ruckeri* | IOA | TS | 22 |
| *Arthrobacter aurescens* | NCIMB 8912 | TS | 22 |
| *Pasteurella piscicida* | Japan | TS+N | 22 |
| *Pasteurella piscicida* | Greece | TS+N | 22 |
| *Escherichia coli* | IOA | TS | 22 |
| *Edwardsiella tarda* | NCIMB 2034 | TS | 22 |
| *Edwardsiella ictaruli* | IOA | TS | 22 |
| *Renibacterium salmoninarum* | IOA | BKDM-C | 22 |
| *Bacillus subtilis* | IOA | TS | 22 |
| *Bacillus cereus* | IOA | TS | 22 |

NCIMB : National Collection of Industrial and Marine Bacteria, Scotland
IOA: Institute of Aquaculture, University of stirling.
MSM: Modified Sauton's medium.
TS: Tryptic soy broth
TS+N: Tryptic soy broth+NaCl
BKDM-C: Bacterial Kidney Disease Medium-Charcoal.

### 3.2.4 Preparation of antisera

#### 3.2.4.1 Rabbit anti-ECP serum

Two New Zealand White rabbits, obtained from the animal house, University of Stirling, were immunised with ECP from either strain TB40 or strain TB267. The ECP (200 μg) was mixed 1:1 with Freund's incomplete adjuvant (FIA) and 1 ml was delivered subcutaneously (SC) into several sites. The rabbits received a secondary boost of ECP in Freund's incomplete (FIA) 30 days later. A final boost of ECP in sterile saline was given intravenously (IV) 4 weeks later. The rabbits were then bled out by cardiac puncture after 10 days.

#### 3.2.4.2 Rabbit anti-65 kDa protein serum

Two New Zealand White rabbits were immunised with the 50 μg of the electro-eluted 65 kDa protein following the protocol described above. The rabbit, however, received an additional booster vaccination, SC, on day 60 of the vaccination regime.

#### 3.2.4.3 Mouse anti-ECP serum

Six Balb/c mice, maintained in isolation, were provided with sterile bedding and filtered water (Adams *et al.*, 1996). Three mice were immunised with ECP from strain TB40 and another three with ECP from strain TB267. The ECP had been mixed 1:1 with Titermax adjuvant (Vaxcel$^{tm}$, InC. ) and each mouse received 25 μg of protein as a primary and secondary intraperitoneal (IP) injection on days 0 and 30, respectively. Levels of serum antibodies were tested by ELISA and Western blotting 10 days after the second injection (Adams *et al.*, 1996).

### 3.2.4.4 Fish anti-ECP serum

Rainbow trout (*Oncorhynchus mykiss*) (800-900 g) were obtained from a local fish farm, Perth, Scotland. They were maintained on a commercial diet (Ewos, Westfield, Scotland) in 150 l tanks supplied with aerated, flow-through water. The water temperature was 15±2°C throughout the study. A total of 24 fish, randomly divided into 3 groups of eight fish were placed into separate tanks. The fish were immunised IP with 0.3 ml of ECP mixed 1:1 with FCA. Groups 1, 2 and 3 were injected with ECP from strains TB40, TB267 or the type strain *M. marinum,* respectively. Each fish received 150 μg of protein as a primary vaccination and again 8 weeks later as a secondary booster vaccination. Fish were bled at 12 weeks post primary immunisation (Chen *et al.,* 1996b ).

**3.2.5 SDS-PAGE** (SDS PAGE was performed using the method described in section 2.2.5).

### 3.2.6 Western Blot analysis

#### 3.2.6.1 Reaction of anti-mycobacteria sera with ECP

Antigens were transferred to nitrocellulose paper using a wet system (Hoefer Scientific instruments 654, Minnesotastreet, PO box 77383, Sanfrancisco. California 94107-0387) by applying 40 V for 1 hr. The buffer used for the transfer was 25 mM Tris-hydrochloride, 192 mM glycine, 20% (v/v) methanol, pH 8.3. The nitrocellulose membrane was incubated in 1% (w/v) bovine serum albumin (BSA) in PBS for 2 hrs, then washed with PBST (PBS containing 0.001 % (v/v) Tween-20). The membranes were incubated for 2 hrs with sera from either immunised rabbits, mice or fish, diluted 1/2500, 1/2000, and 1/50 respectively, in PBS containing 1% (w/v) BSA, respectively. They were then washed the three times with PBST. Membranes incubated with only fish serum were then incubated with tissue culture supernatant

containing an anti-rainbow trout IgM immunoglobulin MAb (C410, Courtesy of Dr. Ann Thuvander, Department of Pathology. Swedish University of Agricultural Science, S-75007 Uppsala, Sweden) for 60 mins at 20°C, followed by a further 3 washes with PBST. All membranes were incubated with either goat anti-mouse or anti-rabbit IgG-horse radish peroxidase (HRP, Scottish Antibody Production unit, Lanark, Scotland) (1/100 in PBS) for 60 min at 20°C then again washed with PBST. Reaction of the antisera with the immobilised antigens was visualised by incubating the membranes in substrate buffer (20 mM Tris-hydrochloride, 500 mM NaCl, pH 7.5) containing 6 mg 3,3-diaminobenzidine tetrahydrochloride dissolved in 10 ml TBS with 30 µl $H_2O_2$. The reaction was stopped after 10 mins with distilled $H_2O$.

### 3..2.6.2 Detection of fibronectin binding protein in mycobacterial ECP

After blocking the nitrocellulose membrane with BSA as described above, the membrane was incubated with fibronectin (Sigma) (100 µg/ml in PBS) for 2 hrs at 20°C. Bound fibronectin was detected using a rabbit anti-fibronectin Ig-HRP conjugate (Dako, High Wycombe, Bucks, UK ) diluted 1/50 in PBS containing 1% BSA. The reaction was developed as outlined in section 3.2.6.1.

### 3.2.6.3 Reaction of MAbs directed against human 60kDa heat shock protein with ECP from mycobacteria

After blocking with BSA as described above, the nitrocellulose membrane was incubated with MAbs directed against human 60kDa heat shock protein (Sigma) for 2 hrs at 20°C. After washing, membranes were incubated with goat anti-mouse IgG-HRP, diluted 1/500 in PBS

containing 1% (w/v) BSA for a further 2 hrs at 20°C. The reaction was developed as outlined in 3.2.6.1.

### 3.2.6.4 Cross reactivity of anti-*M. tuberculosis* MAbs with ECP

After blocking the nitrocellulose membrane with BSA as described in (section 3.2.6.1), the membrane was incubated with anti-*M. tuberculosis* MAbs F29-47 or F67-19 MAbs, (Dr. Kolk, Royal Tropical Institute (KIT) N. H. Swellengrebel Laboratory of Tropical Hygiene, The Netherlands) for 2 hrs at 20°C. The membranes were incubated with goat anti-mouse IgG-HRP, diluted 1/500 in PBS containing 1% BSA for a further 2 hrs at 20°C. The reaction was developed as described above.

### 3.2.6.5 Cross reactivity of rabbit anti-65 kDa protein serum with non-mycobacteria WCS

The protocol described in section 3.2.6.1 was followed to examine the cross-reactivity of rabbit anti-65 kDa protein serum with non-mycobacteria WCS. SDS-PAGE was performed by applying 12μg of non-mycobacteria WCS from each of the bacteria described in Table 3.1

### 3.2.7 Enzyme linked immunosorbent assay (ELISA)

Cross reactivity of rabbit anti-65 kDa protein serum with non-mycobacteria WCS was also examined by ELISA. The ELISA used here was a modification of the indirect ELISA described by Adams *et al.*, (1996). Briefly, ELISA plates were coated with 1 μg/ml of sonicated antigen diluted in coating buffer (0.125 M carbonate/bicarbonate buffer, pH 9.6) pH 9.6 (100 μl/well). Plates were incubated overnight at 4°C and washed three times with low salt wash buffer (LSW) (0.02M Trisma base, 0.38M NaCl, and 0.05% Tween-20 pH 7.4). Nonspecific binding sites were blocked with 250 μl/well of 3% (w/v) skimmed milk solution

(Marvel, Cadburys, U.K ) for 60 min at 20°C. The plates were again washed three times LSW buffer. The rabbit anti-65 kDa protein serum diluted 1/2500 in PBS containing 1% BSA was added to the wells (100 µlwell$^{-1}$). A negative control of normal rabbit serum was also included. The plates were incubated for 60 minutes at 20°C, washed five times with high salt wash buffer (HSW) (0.02M Trisma base, 0.5M NaCl and 0.01% (v/v) Tween-20, pH 7.8), then incubated for 60 mins with goat anti-rabbit IgG-HRP (1/1000 in PBS) at 20°C. After washing the plates five times with HSW buffer, 100µl chromogen/substrate was added to the wells and incubated for 5 min at 20°C. The reaction was stopped with 50 µl/well 2M $H_2SO_4$ and read spectrophotometrically at 450 nm, using chromogen, substrate buffer and 2M $H_2SO_4$ as a zero reference. Wells showing an absorbance three times that of the background absorbance were considered positive.

### 3.3 Results

#### 3.3.1 Reactivity of rabbit anti-*Mycobacterium* ECP

The response of rabbit anti TB40 ECP and TB267 ECP sera was examined by Western blot analysis; firstly against the ECP from mycobacterial strains *M. marinum, M. chelonae, M. fortiutum*, TB1, TB40, TB267 and TB268, and secondly against WCS preparations from these mycobacteria. Various bands in the ECP antigenic profiles were identified by the antisera, and these are summarised in Table 3.2. The major bands recognised were found at 65, 40 and 21-<8 kDa (Figure 3.1a and 3.1b), while the major bands recognised in the WSC were found at 65, 43, 40, 36, 27, 25, and 21-<8kDa (Figure 3.2a and 3.2b). The Western blot profiles of ECP from TB1, TB40, TB267 and TB268 incubated with rabbit anti-TB40 ECP or TB267 sera were most similar with to that of the ECP from *M. marinum.* In contrast, <21 kDa protein was not detected in ECP profiles from *M. cheloane.*

Table 3.2 The reactivity of rabbit anti-*Mycobacterium* ECP antibody by Western Blot analysis

| | Bands recognized kDa | | | |
|---|---|---|---|---|
| | Rabbit anti-TB40 ECP | | Rabbit anti-TB267 ECP | |
| | ECP | WCS | ECP | WCS |
| TB1 | 65,40,21,16 | 65,43,40,36, 16,14 | 65,40,21-<8 | 65,36,25, 21,18,16,10 |
| TB40 | 65,43,40,21-<8 | 65,25,18, 16,14,12,8 | 65,40,21-<8 | 65,25,18,10 |
| TB267 | 65,40,21-<8 | 65,43,40,27,25,21-<8 | 65,40,21-<8 | 65,27, 25,21-<8 |
| TB268 | 65,40, 21-<8 | 65,43,40,27,25,21-<8 | 65,40,21-<8 | 65, 27,25,21-<8 |
| MC | 65,21,16 | 65,43,40,27,25,18 | 65 | 65,43,40 |
| MF | 65,36,21 | 65,S43,40,27 | 65,21,12 | 65,43,40 |
| MM | 65,40,21-<8 | 65,43,40,27,25,18-<8 | 65,40,21-<8 | 65,25,21,18-<8 |

ECP: Extracellular products

WCS: Whole cell sonicated antigen

MC: *Mycobacterium chelonae*

MF: *Mycobacterium fortuitum*

MM: *Mycobacterium marinum*

21-<8: A band of smearing between 21-<8 kDa.

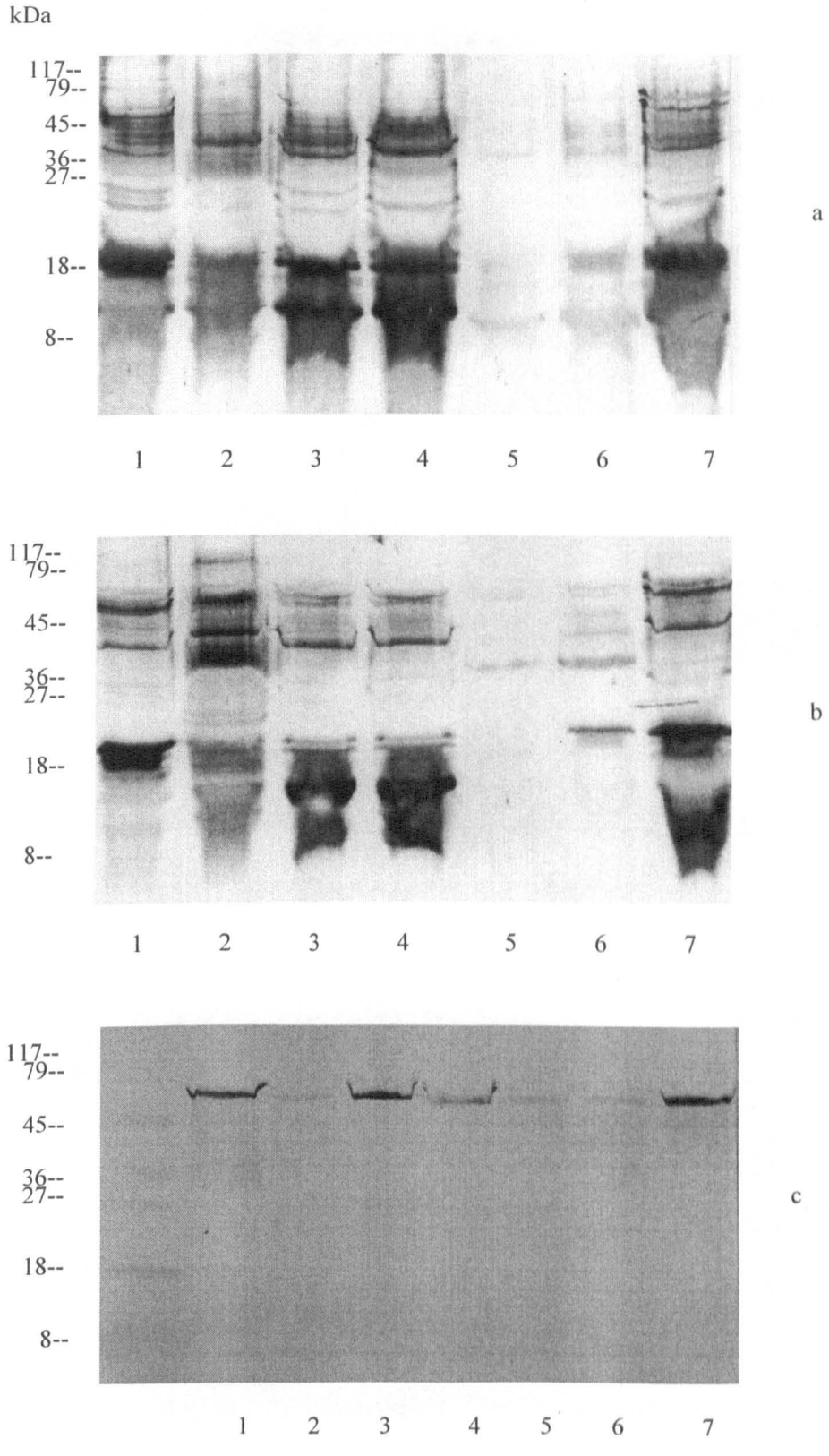



Figure 3.1 Western blot analysis of rabbit anti-*Mycobacterium* ECP serum against ECP from strains (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. fortuitum*, (7) *M. marinum*, (a) rabbit anti-TB40 serum, (b) rabbit anti-TB267 serum, (c) rabbit anti-TB267 65 kDa protein serum

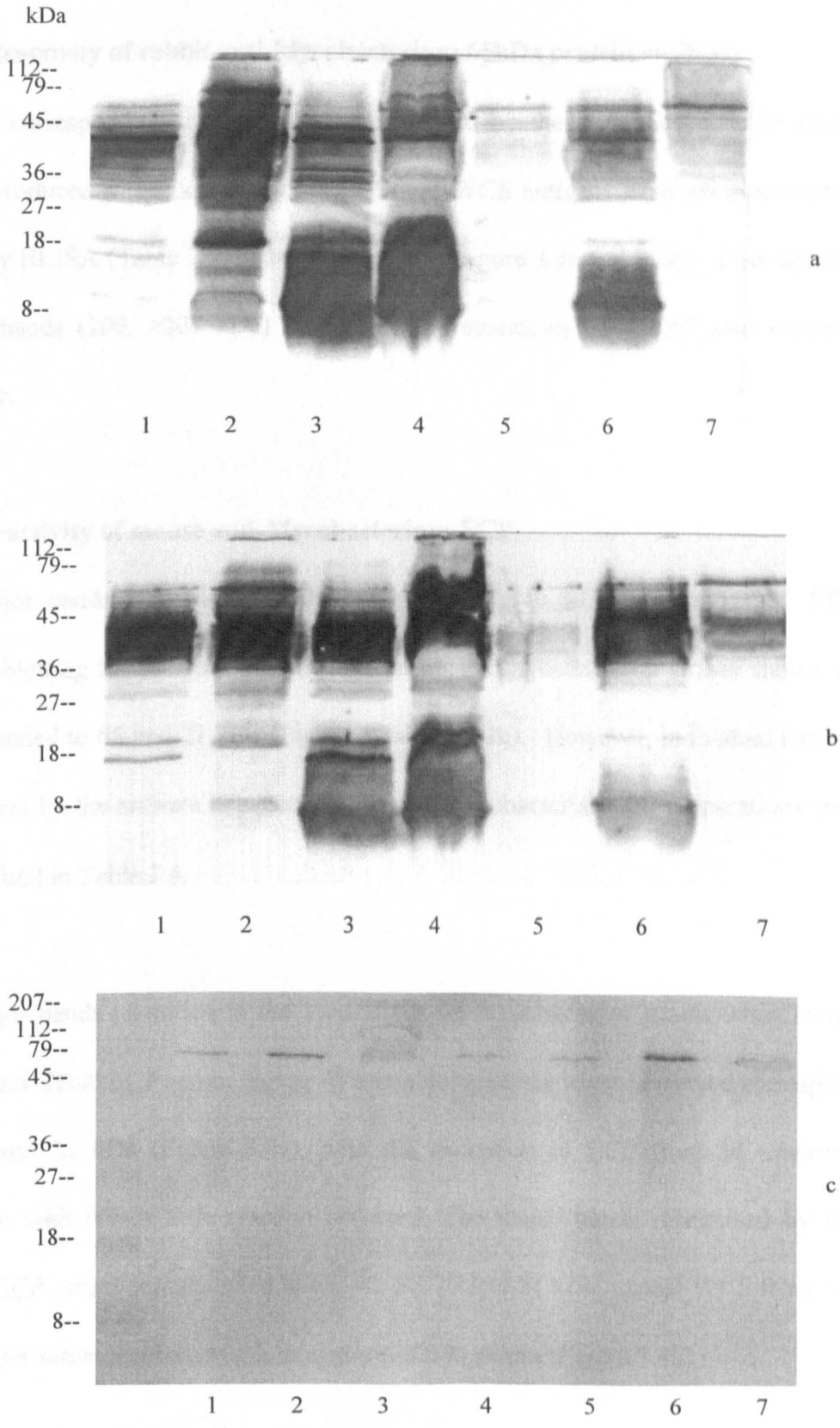



Figure 3.2 Western blot analysis of rabbit anti-*Mycobacterium* ECP serum against ECP from strains (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. marinum*, (7) *M. fortuitum*; (a) rabbit anti-TB40 serum, (b) rabbit anti-TB267 serum, (c) rabbit anti-TB267 65 kDa protein serum

### 3.3.2 Reactivity of rabbit anti-Mycobacterium 65kDa protein antibody

A band corresponding to 65 kDa was recognised by the rabbit anti-65 kDa protein serum, which produced a reaction with both ECP and WCS antigens from all mycobacterial strains tested by ELISA (Table 3.3) or by Westernblot (Figure 3.1c and 3.2c). Two higher molecular weight bands (100, >207 kDa) in the WCS preparations of TB267 also reacted with this antibody.

### 3.3.3 Reactivity of mouse anti-Mycobacterium ECP

The major bands recognised by the mouse anti-TB40 ECP or anti-TB267 ECP sera by Westernblotting the antisera with ECP from all the mycobacterial strains shown in Table 1, corresponded to 65 and 21 kDa (Figure 3.3a and 3.3b). However, individual bands were also recognised by the antisera between the various mycobacterial ECP preparations and these are summarised in Table 3.4.

The major bands identified in the WSC from all mycobacterial strains when incubated with mouse anti-TB40 ECP serum during Westernblot analysis when incubated corresponded to 65, 40, 25 and 21 kDa (Figure 3.4a), with the exception of ECP from *M. chelonae* and *M. fortuitum* with which little reaction occurred. The major bands recognised by mouse anti-TB276 ECP serum corresponded to 65, 40, 37, 25 and 21 kDa, except WCS from *M. chelonae* and *M. fortuitum* incubated with mouse anti-TB40 serum (Figure 3.4b).

Table 3.3 Cross reactivity of rabbit anti-65 kDa protein serum with mycobacteria and non-mycobacteria WCS by Westernblotting and ELISA.

| Species | Source | ELISA | Western blot |
|---|---|---|---|
| TB1 | AAHRI | ++ | ++ |
| TB40 | AAHRI | ++ | ++ |
| TB267 | AAHRI | ++ | ++ |
| TB268 | AAHRI | ++ | ++ |
| *Mycobacterium chelonae* | NCIMB 1474 | ++ | ++ |
| *Mycobacterium fortuitum* | NCIMB 1294 | ++ | ++ |
| *Mycobacterium marinum* | NCIMB 1298 | ++ | ++ |
| *Aeromonas hydrophila hydrophila* | NCIMB 87 | ++ | ++ |
| *Aeromonas salmonicida acromogenes* | NCIMB 1110 | - | ++ |
| *Aeromonas salmonicida masoucida* | NCIMB 2020 | - | ++ |
| *Aeromonas salmonicida salmonicida* | IOA | - | ++ |
| *Corynebacterium aquticum* | IOA | ++ | - |
| *Micrococcus luteus* | NCIMB 570 | - | - |
| *Nocardia asteroides* | NCIMB 1290 | ++ | ++ |
| *Streptococcus faecalis* | IOA | ++ | ++ |
| *Pesudomonas aeruginosa* | NCIMB 8295 | ++ | + |
| *Pseudomonas fluorescences* | NCIMB 1283 | - | ++ |
| *Vibrio anguillarum* | NCIMB 571 | - | ++ |
| *Vibrio ordalli* | NCIMB 2167 | ++ | ++ |
| *Yersinia ruckeri* | IOA | ++ | ++ |
| *Arthrobacter aurescens* | NCIMB 8912 | - | ++ |
| *Pasteurella piscicida* | Japan | - | ++ |
| *Pasteurella piscicida* | Greece | ++ | ++ |
| *Escherichia coli* | IOA | ++ | ++ |
| *Edwardsiella tarda* | NCIMB 2034 | ++ | ++ |
| *Edwardsiella ictaruli* | IOA | ++ | ++ |
| *Renibacterium salmoninarum* | IOA | - | ++ |
| *Bacillus subtilis* | IOA | - | - |
| *Bacillus cereus* | IOA | - | - |

AAHRI : Aquatic Animal Health Research Institute, Bangkok, Thailand.
NCIMB : National Collection of Industrial and Marine Bacteria, Scotland
IOA : Institute of Aquaculture, University of Stirling.

Table 3.4 Reactivity of mouse anti-*Mycobacterium* ECP antibody by Western blot analysis

| | Bands recognized kDa | | | |
|---|---|---|---|---|
| | Mouse anti-TB40 ECP | | Mouse anti-TB267 ECP | |
| | ECP | WCS | ECP | WCS |
| TB1 | >207, 65,40,21 | 65,40,21 | >207,65-25,21,14 | 65, 40,38,25,18,16,10 |
| TB40 | >207,72,65,43,40-27 | >207, 65,40 | >207,65,43,25,21, | 65,40,38,25,21,18 |
| TB267 | 65,40,21 | 40,25 | 65-27, 25,21,14 | >207-65,40,38 25-8 |
| TB268 | 65,40,,25-21 | 40,25 | 65,40, 25-14 | 65,40,37,25,18-8 |
| MC | 65 | - | 65,21 | 65,44,43 |
| MF | 65,36,21 | - | 65-27,21, | 72-40,36,25,18 |
| MM | 65-27, 21 | 65,40,27-16 | 67-25,21,14 | 100-40, 36, 25 ,18 |

ECP: Extracellular products

WCS: Whole cell sonicated antigen

MC: *Mycobacterium chelonae*

MF: *Mycobacterium fortuitum*

MM: *Mycobacterium marinum*

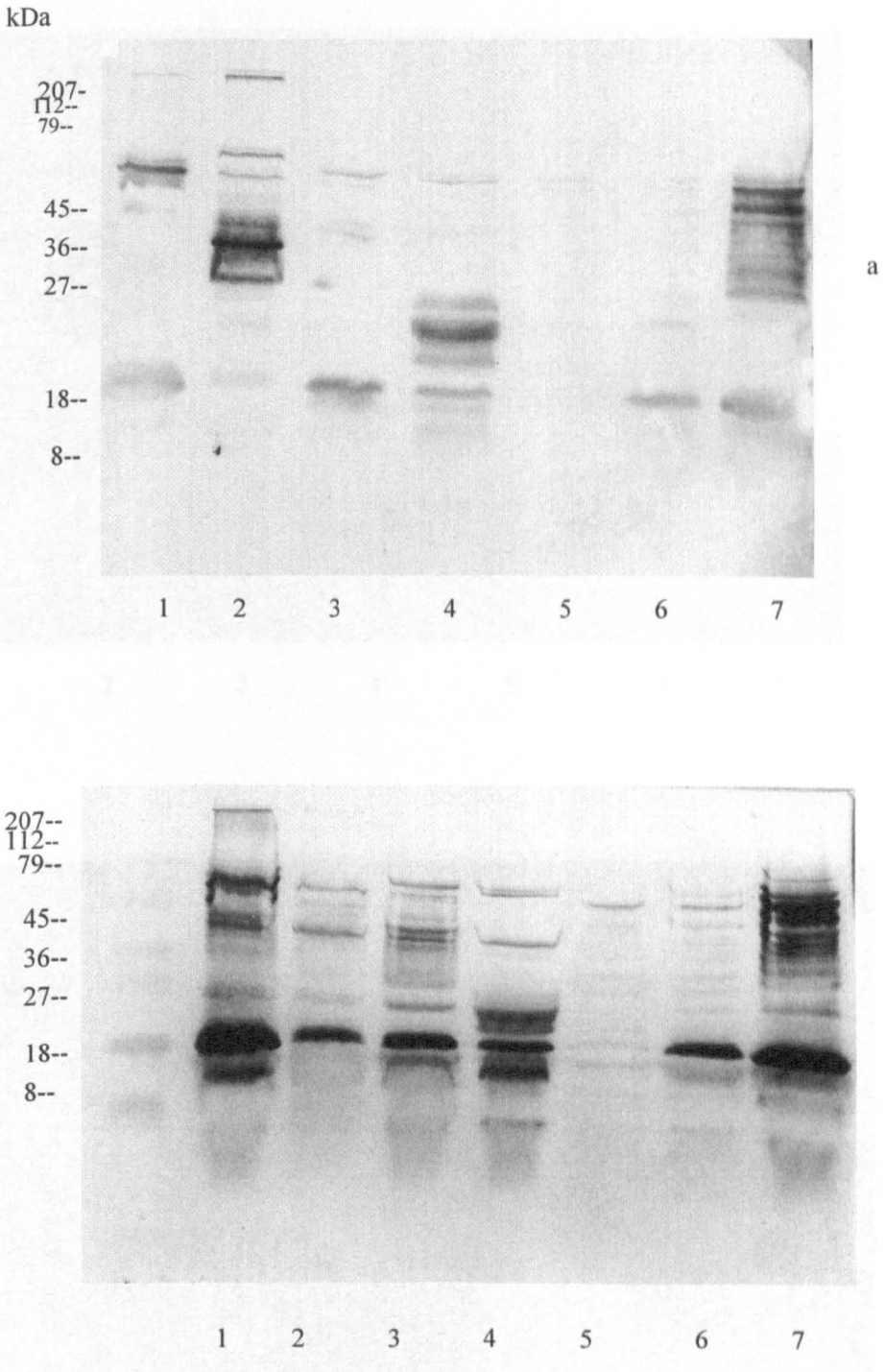



Figure 3.3 Western blot analysis of mouse anti-*Mycobacterium* ECP serum against ECP from strains (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. fortuitum*, (7) *M. marinum*, (a) mouse anti-TB40 serum, (b) mouse anti-TB267 serum

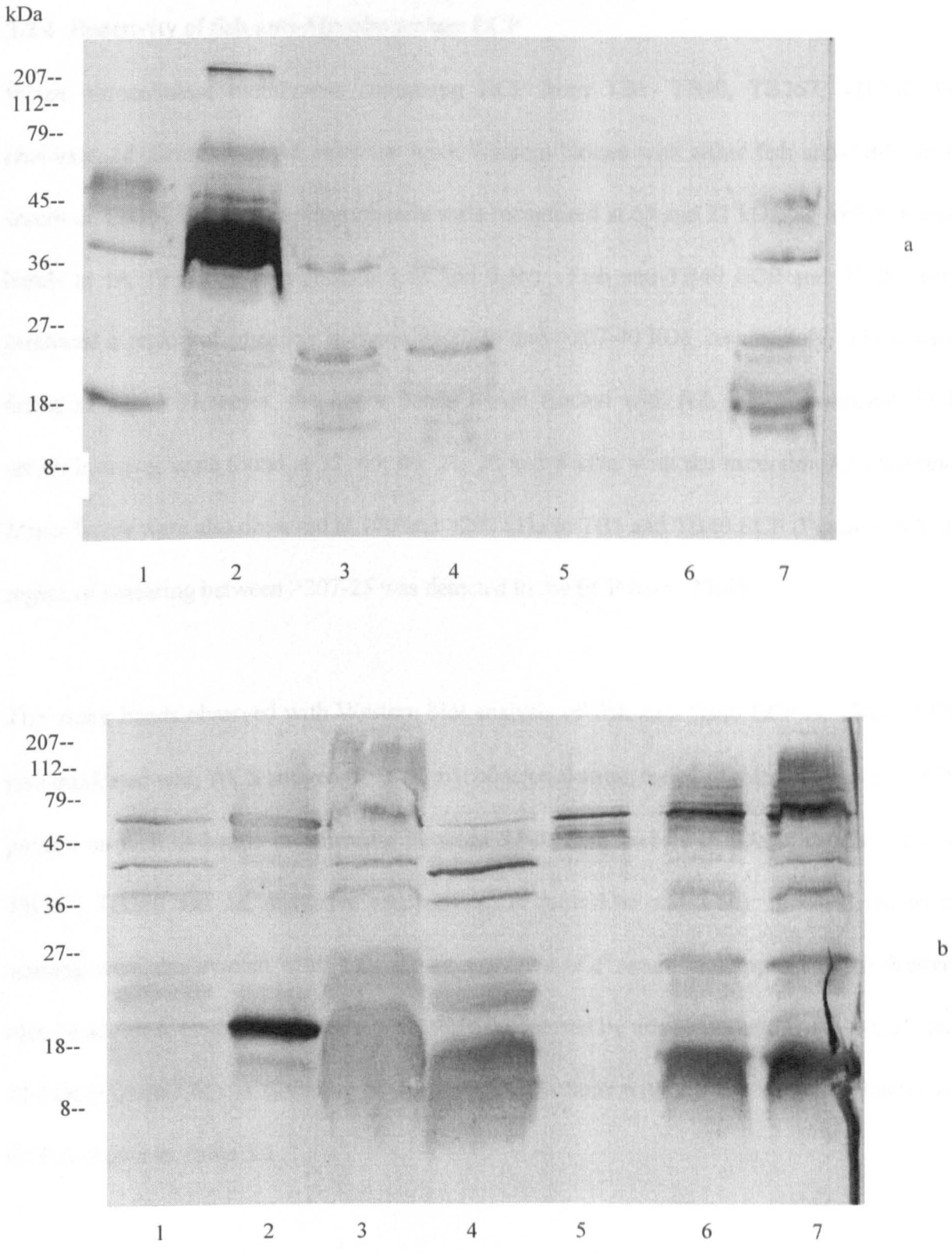



Figure 3.4 Western blot analysis of mouse anti-*Mycobacterium* ECP serum against WCS from strains (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. fortuitum*, (7) *M. marinum*, (a) mouse anti-TB40 serum, (b) mouse anti-TB267 serum

### 3.3.4 Reactivity of fish anti-*Mycobacterium* ECP

When nitrocellulose membranes containing ECP from TB1, TB40, TB267, TB268, *M. chelonae*, *M. fortuitum* or *M. marinum* were Western blotted with either fish anti-TB40 ECP serum or TB267 ECP serum, major bands were recognised at 65 and 21 kDa, as well as minor bands at 14, 12 and 8 kDa (Figure 3.5a and 3.5b). Fish anti-TB40 ECP and TB267 sera produced a region of smearing between >207-25 and >207-40 kDa, respectively, which were found in TB40. However, the major bands which reacted with fish anti-*M. marinum* ECP serum however were found at 72, 65, 40, 21, 12 and 8 kDa, with the exception *M. chelonae*. Minor bands were also observed at 100 and >207 kDa in TB1 and TB40 ECP (Figure 3.5c). A region of smearing between >207-25 was detected in the ECP from TB40.

The major bands observed with Western blot analysis of fish anti-TB40 ECP or TB267 ECP sera incubated with WCS antigen from all mycobacterial strain tested (Table 3.5) was a 65 kDa protein as well as bands of smearing between 21-8, 25-8 and 16-8 kDa with WCS from TB267, TB268 and *M. marinum*, respectively. (Figure 3.6a and 3.6b). Similar regions of staining were also evident when fish anti-*M. marinum* ECP serum was applied to the Western blot. In addition, major bands in the WCS were recognised by this serum at 65, 44, 40, 25, and 21 kDa (Figure 3.6c). A summary of the different reactions with the fish anti-Mycobacterium ECP is shown in Table 3.5.

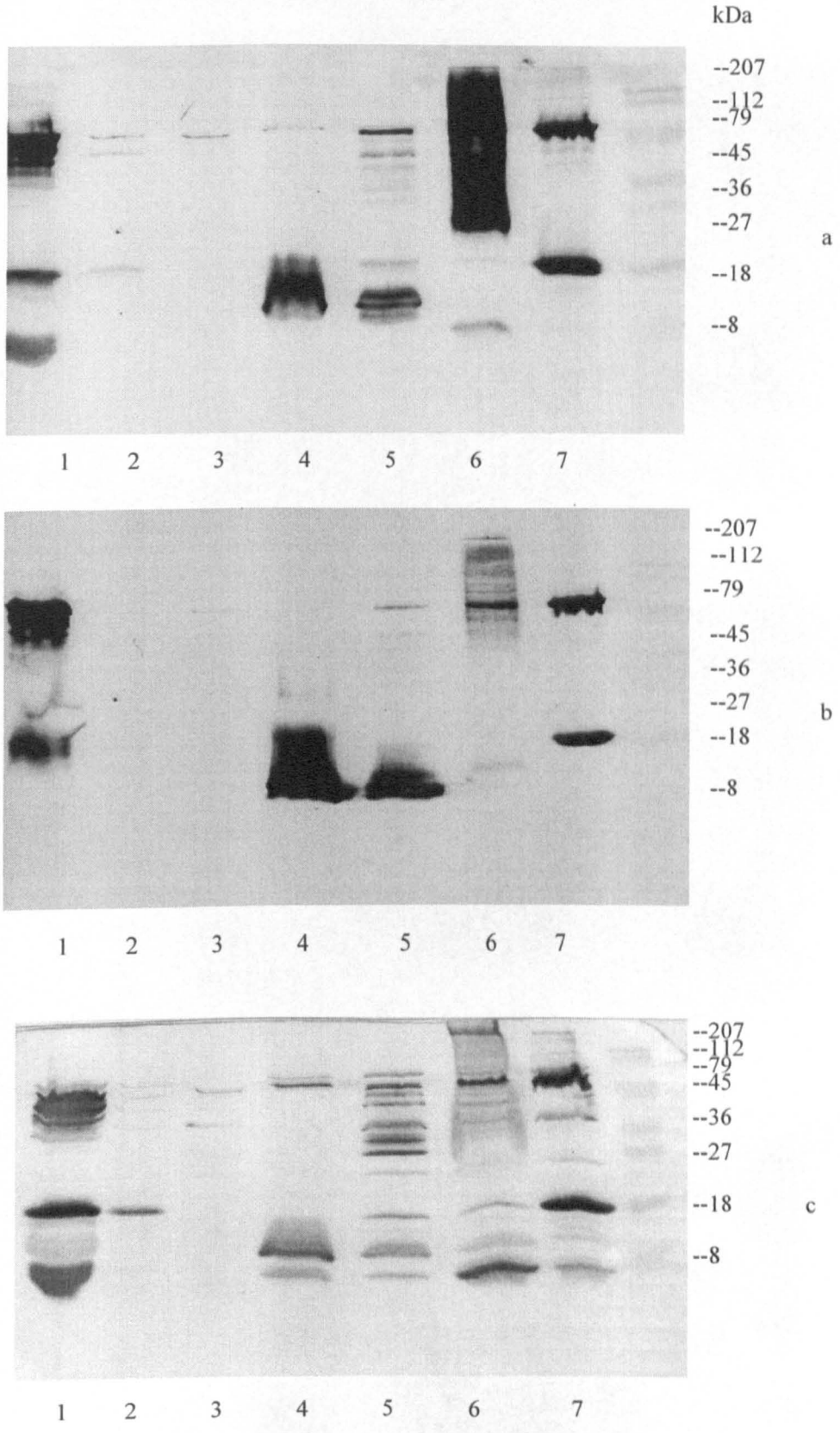



Figure 3.5 Western blot analysis of fish anti-*Mycobacterium* ECP serum against ECP from strains (lanes) (1) *M. marinum* (2) *M. fortuitum*, (3) *M. chelonae*, (4) TB268, (5) TB267, (6) TB40, (7) TB1, (a) fish anti-TB40 serum, (b) fish anti-TB267 serum, (c) fish anti-*M. marinum* serum

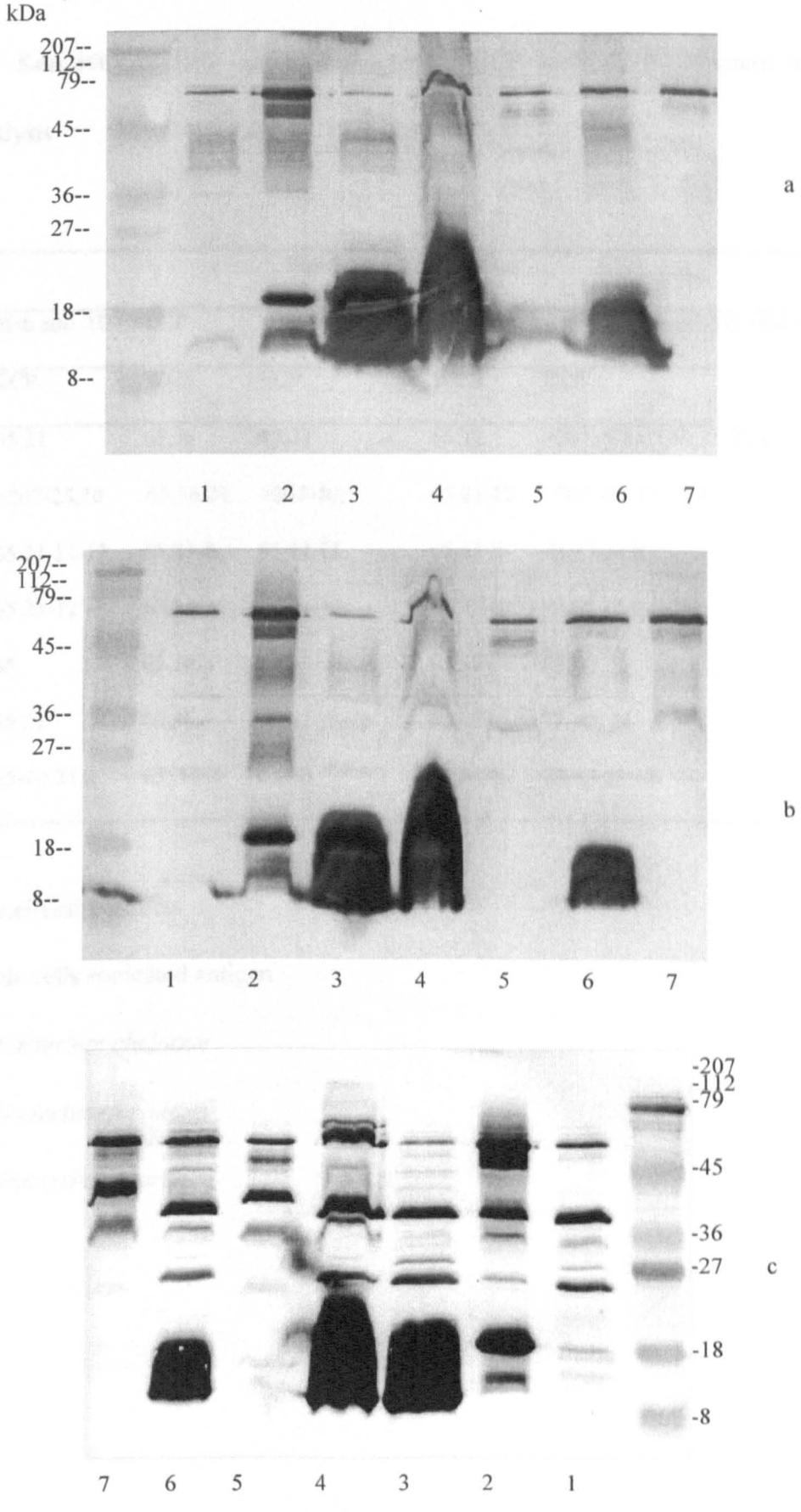



Figure 3.6 Western blot analysis of fish anti-*Mycobacterium* ECP serum against WCS from strains (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. marinum*, (7) *M. fortuitum*, (a) fish anti-TB40 serum, (b) fish anti-TB267 serum, (c) fish anti-*M. marinum* serum

Table 3.5 Reactivity of fish anti-*Mycobacterium* ECP antibody by Western blot analysis

| | Bands recognized (kDa) | | | | | |
|---|---|---|---|---|---|---|
| | fish anti-TB40 ECP | | fish anti-TB267 ECP | | fish anti-TB MM ECP | |
| | ECP | WCS | ECP | WCS | ECP | WCS |
| TB1 | 65,21 | 65,36 | 65,21 | 65,12 | >207,100,65,40,25,21,8 | 65,40,36,27,25 |
| TB40 | >207-25,10 | 65,36,21 | >207-40, | 65,21,12 | >207-27, 21,12,8 | 65,40,37,25,21 |
| TB267 | 65,21,14-12 | 65,21-8 | 65,14,12 | 65,21-8 | 72-27,21,8 | 65,40,27,25,21-8 |
| TB268 | 65,21-12 | 65,25-8 | 65,21-12 | 65,25-8 | 72,65,12,8 | 72,65,41,38,36,25-8 |
| MC | 65 | 65,36 | 65 | 65,12 | 65,40 | 65,43,36 |
| MF | 65,21 | 65,36 | - | 65 | 72-40, 21 | 65,44,38 |
| MM | 65-40 21,8 | 65, 16-8 | 65-40 | 65,21-8 | 72-40,21-<8 | 65,40,38,25,16-8 |

ECP: Extracellular products

WCS: Whole cells sonicated antigen

MC: *Mycobacterium chelonae*

MF: *Mycobacterium fortuitum*

MM: *Mycobacterium marinum*

### 3.3.5 The reactivity ECP from mycobacteria with MAbs directed against human 60 kDa heat shock protein, *M. tuberculosis* or with fibronectin, by Western blot analysis

Monoclonal antibody (MAb) against anti-human 60 kDa heat shock protein cross-reacted with some of the ECP from the *Mycobacterium* spp. examined. It reacted with the 65 kDa band in the profile from TB1 and *M. marinum* ECP, as well as with bands at 23 kDa from TB1, TB40, TB267 and TB268 ECP (Figure 3.7a).

MAb (F29-47) against *M. tuberculosis* displayed a strong reaction with all mycobacterial ECPs tested, except with that from *M. chelonae* (Figure 3.7b). The cross reactivity observed with MAb (F67-19), another MAb against *M. tuberculosis*, was much weaker than with MAb F29-47, but did, however, react with ECP from TB1, TB40, TB267, TB268 and *M. marinum* and it may be a non specific reaction (Figure 3.7c).

The major fibronectin binding proteins in the ECP from *Mycobacterium* spp. were detected at around 21-25 kDa. Differences were observed between the different isolates in the molecular weight of the bands corresponding to the fibronectin binding protein. The ECP from strains TB1 and TB40, isolated from Siamese fighting fish produced a strong reaction with a band found at >207 kDa, while ECP from *M. chelonae* and *M. fortuitum* gave a weak reaction with a band at 40 kDa (Figure 3.7d). The reactions described in this section are outlined in Table 3.6.

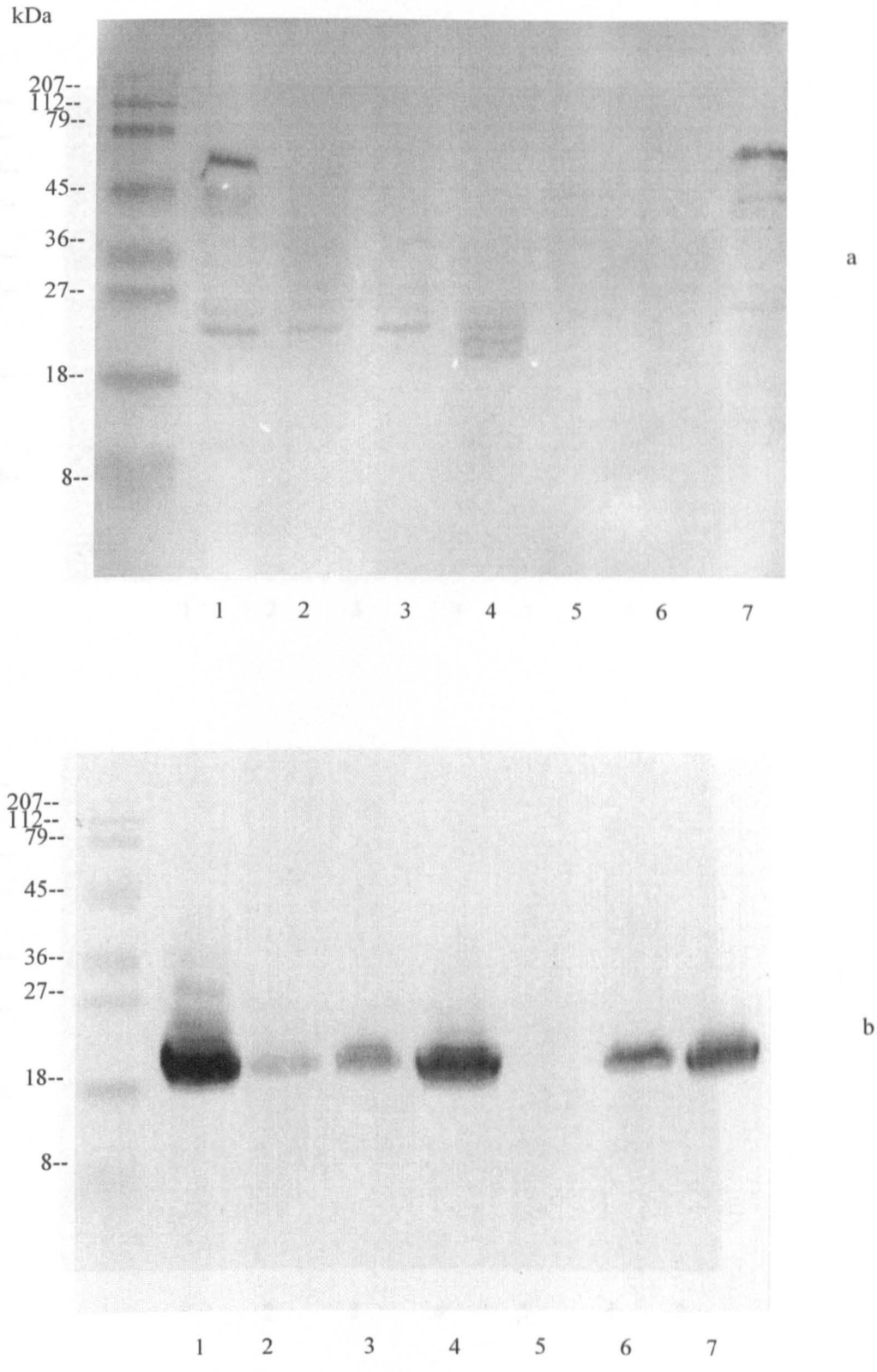



Figure 3.7 Reactivity of MAbs directed against human 60kDa heat shock protein, *M. tuberculosis* or the fibronectin binding protein to *Mycobacterium* ECP in Western blot analysis (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. fortuitum*, (7) *M. marinum*, (a) anti-human 60kDa heat shock protein MAbs, (b) *anti-M. tuberculosis* MAb (F29-47),

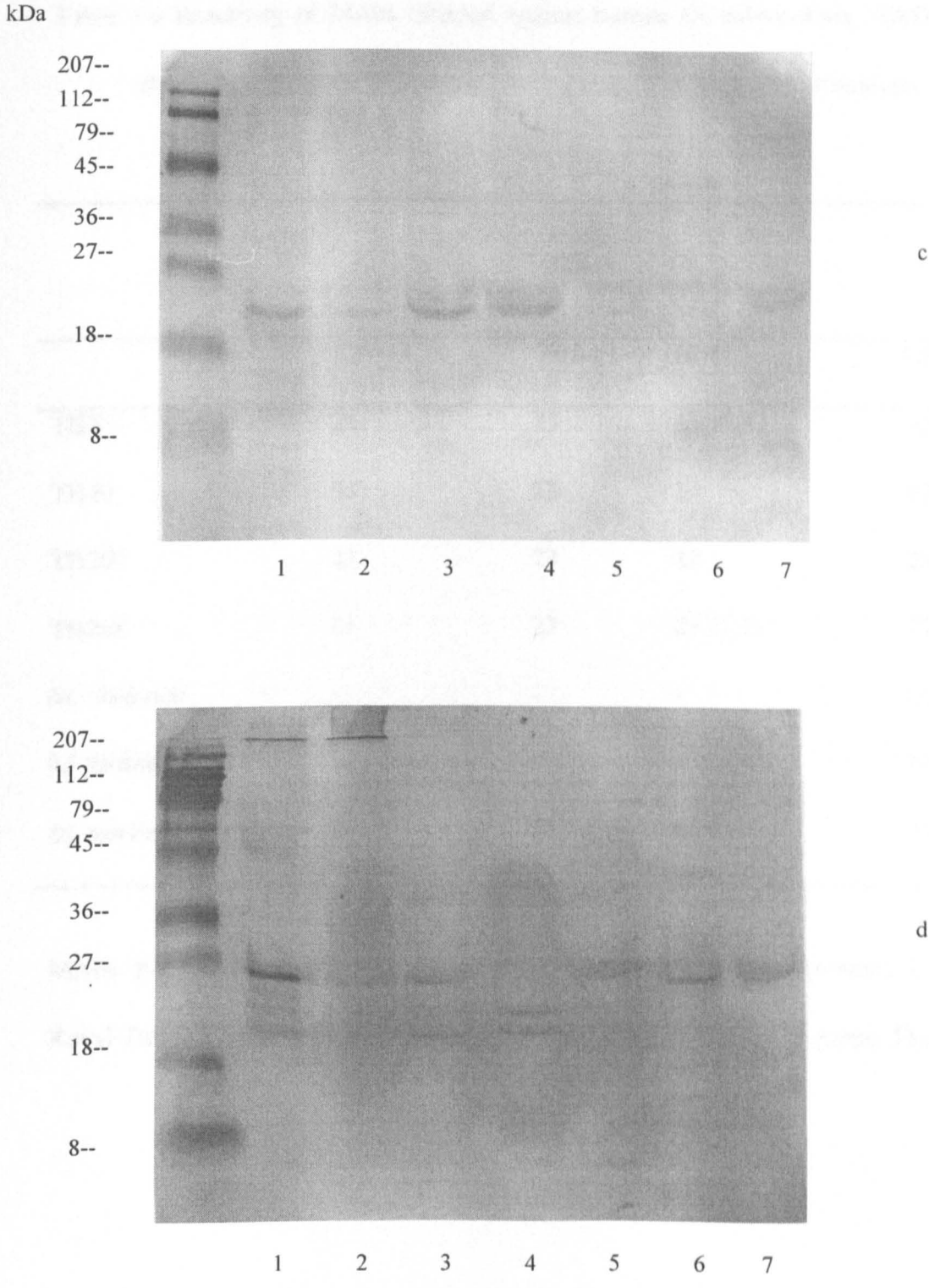



Figure 3.7 Reactivity of MAb directed against human 60kDa heat shock protein, *M. tuberculosis* or the fibronectin binding protein to *Mycobacterium* ECP in Western blot analysis (lanes) (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. fortuitum*, (7) *M. marinum*, (c) *anti-M. tuberculosis* MAb (F67-19), (d) anti-fibronectin binding protein MAbs

Table 3.6 Reactivity of MAbs directed against human *M. tuberculosis*, 60kDa heat shock protein, or the fibronectin binding protein in Western blot analysis

| | Bands recognized kDa | | | |
|---|---|---|---|---|
| | F29-47 | F67-19 | HSP | Fibronectin |
| TB1 | 21 | 23 | 65,43,23 | >207,25,24,23,21 |
| TB40 | 21 | 23 | 23 | >207,25,21 |
| TB267 | 21 | 23 | 23 | 25,23,21 |
| TB268 | 21 | 23 | 23,22,21 | 23,21 |
| *M. chelonae* | - | - | - | 25,23,21 |
| *M. fortuitum* | 21 | - | - | 24,16,12 |
| *M. marinum* | 21 | 23 | 65,43 | 24,16,12 |

MAbs F-29-47 (21 K secreted protein) and F-67-19 (38 K secreted protein): From Dr. Kolk, Royal Tropical Institute N. H. Swellengrebel Laboratory of tropical Hygiene, The Netherlands.

### 3.3.6 Cross reactivity of rabbit anti-65 kDa protein serum with non-mycobacteria WCS by immunoblotting and ELISA

Rabbit anti-65 kDa serum exhibited a strong response with both ECP and WCS antigens from all of the mycobacterial strains tested by ELISA and Western blotting (Table 3.3). The antisera also cross-reacted with 11 strains of the 22 non-mycobacteria examined by ELISA. However, when Western blot analysis was performed on these bacteria, 18 of the 22 strains tested cross-reacted with the rabbit anti-65 kDa antibody (Figure 3.8a, b and c).

## 3.4 Discussion

As yet it has not proven possible to classify the mycobacterial strains isolated from Siamese fighting fish and snakehead fish during outbreaks of mycobacteriosis in Thailand as particular type strains (Adams *et al.*, 1996). However, both the biochemical characteristics of these mycobacteria and the antigen profiles of their ECPs appear most similar to *M. marinum*. The PCR method is very useful to differentiate different strain. The TB267 and TB268 used in this study, had band at 180 bp position of the marker. So, it was confirmed that they were *M marinum* (Puttinaowarat, 1995).

Several distinct bands were identified in both ECP and WCS preparations of the different mycobacterial strains when tested by immunoblot analysis with rabbit anti-TB40 ECP or anti-TB267 ECP sera. The major bands recognised in the ECP and WCS preparations of the different mycobacterial strains by mouse anti-TB40 ECP and ant-TB267 ECP sera were similar to those identified by the rabbit sera, but fewer bands were identified by the mouse sera (bands at 65, 40, 25, and 21 kDa were identified with mouse sera compared with bands at 65, 43, 40, 41, 27, 21, 16 and 12 kDa with the rabbit sera).

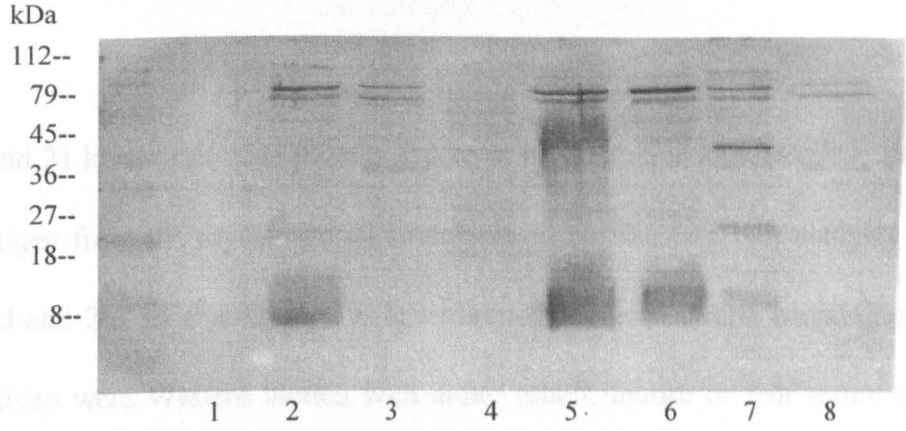



Figure 3.8a Cross reactivity of rabbit anti-65 kDa protein serum with non-mycobacteria WCS by immunoblotting: (lanes) (1) *Nocardia asteroides*, (2) *Edwardiella ictaruli*, (3) *Vibrio anguillarum* (4), *Streptococcus faecalis*, (5) *Edwardsiella tarda*, (6) *Pseudomonas aeruginosa*, (7) *Escherichia coli*, (8) *Vibrio ordalli*

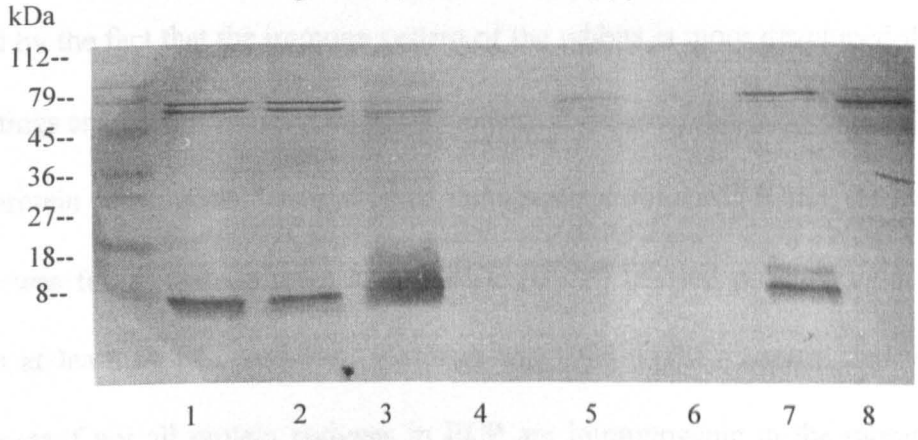



Figure 3.8b (lanes) (1) *pasteurella piscicida* (Japan), (2) *P. piscicida* (Greece), (3) *Aeromonas hydrophila*, (4) *Arthrobacter aurescens* , (5) *Aeromonas salmonicida achromogens*, (6) *Renibacterium salmoninarium*, (7) *Aeromonas salmonicida masoucida*, (8) *Aeromonas salmonicida salmonicida*

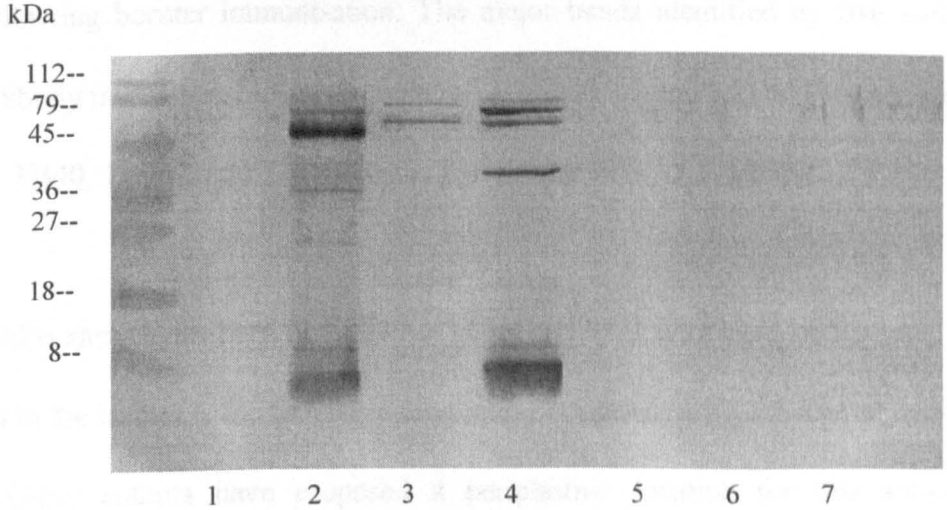



Figure 3.8c (lanes) (1) *Micrococcus luteus*, (2) *Corynebacterium aquaticum*, (3) *Pseudomonas fluorescens*, (4) *Yersinia ruckeria*, (5) *Bacillus subtilis*, (6) *Carbobacterium pisciola* (7) *Bacillus cereus*

The 65 and 21 kDa, or 65 kDa protein appear to be the major immunogenic bands in ECP or WSC antigen from the mycobacterial strains tested by Western blot analysis with fish anti-TB40 and anti-267 ECP antibody. When nitrocellulose membrane containing either ECP or WCS antigen were Western blotted with either rabbit, mouse or fish serum (anti-TB40 and anti-TB267), more bands were recognized by the rabbit and mouse sera than fish sera. This has been shown previously for other fish pathogens (e.g *A. salmonicida*) and has been explained by the fact that the immune system of the rabbits is more developed than that of the fish (Hastings and Ellis, 1988). Analysis by isoelectric focusing indicated that ECP contained at least 14 protein constituents. Using crossed immunoelectrophoresis (CIE), (Hastings and Ellis, 1988), it was found that formalin toxoid ECP (fECP) elicited production of antibodies in rabbits to at least 14 ECP antigens. Hastings and Ellis, (1988) suggest that when formalin treated, most if not all protein antigens in ECP are immunogenic in the rabbits. In contrast, antibodies to only four ECP antigens were detected in rainbow trout which were immunised with f-ECP, suggesting that many ECP components may be poorly immunogenic in the trout, even following booster immunisation. The major bands identified by fish anti-*M. marinum* ECP antibody in Western blot analysis were 72, 65, 40, 12 and 8 kDa. Western blotting profiles of TB1, TB40, TB267, and TB268 were most similar with *M. marinum*.

The 65 kDa antigen has been described as being cell-wall associated since much of the antigen is found in the insoluble fraction remaining after disruption of mycobacterial cells (Gillis *et al.*, 1985). Other authors have proposed a periplasmic location for this antigen and have demonstrated release of the molecule into culture supernatant during growth of *Mycobacterium bovis* under conditions of zinc deficiency (De Bruyn *et al.*, 1981). Recent studies by our group have suggested that the 65 kDa is a heat shock protein in *Mycobacterium* sp. isolated from fish

(Chen *at al*, 1996a), and the present study has confirmed as cross-reaction was observed with the anti-human heat shock 60 kDa MAb. The 65 kDa protein found on the SDS-PAGE profiles of *Mycobacterium* species isolated from snakehead fish (TB1 and TB40) was strongly immunogenic, eliciting an antibody response both in rabbits, mice and fish and appears to be a major protein in both ECP and WCS preparations of *Mycobacterium* spp. (Young *et al.*, 1987; Anderson *et al.*, 1988). The 65 kDa protein may indeed have a periplasmic location since the release of this molecule into the culture medium has been demonstrated during the growth of *Mycobacterium* spp. under heat shock conditions (Young *et al.*, 1987).

Understanding immunogenicity of mycobacterial components that play a role in the infectivity and virulence of the bacterium is very important. It has been suggested that fibronectin is an adherence factor, important in the pathogenesis of infective endocarditis (Hamill, 1987). Recently, it has been shown that molecules with an affinity for fibronectin are present in *M. tuberculosis* or *M. bovis* BCG culture filtrates (Abou-zeid *et al.*, 1988b). It is believed that mycobacteria can use fibronectin to evade immune detection, or as a bridge to interact with molecules and cells of the host (Espitia *et al.*, 1992). Fibronectin-binding proteins are prominent components in supernatants after short-term culture of *M. tuberculosis* (Abou-Zeid *et al.*, 1988b). In 3 day-culture supernatants, a 30 kDa protein was identified as the major fibronectin-binding molecule, while in 21-day-culture supernatants, fibronectin bound to a double protein band at 30 and 31 kDa, as well as to a group of antigens at 57 to 60 kDa. Fibronectin-binding molecules of this size were also found in sonicates of the bacteria (Abou-Zeid *et al.*, 1988b).

A 210-kDa fibronectin binding protein was isolated by affinity chromatography followed by gel chromatography of a bacterial lysate (Froman *et al.*, 1987). Evidence suggests that the large receptor protein contains several binding sites for fibronectin and binding studies indicate that one receptor molecule can bind six to nine molecules of fibronectin. (Froman *et al*, 1987). In the present study, proteins of molecular weights from the >207, 40, and 21-25 kDa ECP of *Mycobacterium* spp. isolated from fish were observed to bind fibronectin. Major fibronectin binding proteins were observed at 21-25 kDa in all the mycobacterial ECPs tested. The >210 kDa fibronectin binding protein was, however, only detected in *Mycobacterium* spp. isolated from Siamese fighting fish.

Western blot analysis gives qualitative results and has an advantage over the ELISA method in that specific antigens can be detected, thus helping to evaluate weak or negative results obtained by ELISA analysis. This was observed in the present study when *Aeromonas hydrophila, A. salmonicida masoucida, A. salmonicida salmonicida, Pseudomonas fluorescens, Vibrio anguillarum, Arthrobacter aurescens, Pasteurella piscicida and Renibacterium salmoninarum* gave a negative response in the ELISA, but were clearly positive by Western blotting. This phenomenon may be explained by a demasking of specific epitopes on the antigen as result of SDS treatment prior to SDS-PAGE. The 65 kDa protein may have a possible role in the pathogenesis of mycobacterial infections and others diseases in human (Young *et al.*, 1987).

## Chapter 4

## Development of monoclonal antibodies (MAbs) to the extracellular products and lysates of *Mycobacterium* spp.

### 4.1 Introduction

Numerous approaches have been made to fractionate *M. tuberculosis* antigens and to isolate and define biologically active components. So far, it has not been possible by classical physical-chemical methods to isolate species specific antigens. Hybridoma technology has introduced the possibility of identifying single antigen determinants, which permits a powerful new approach to the characterisation of antigens. In the past 10 years, several monoclonal antibodies (MAbs) have been produced to *M. tuberculosis* (Andersen, *et al*, 1986; Gillis *et al*, 1985; Buchanan *et al*.,1987; Verborn *et al*.,1990; Mason, *et al* 1993).

Some antibodies were evaluated as possible tools for the serological diagnosis of patients with tuberculosis. Most investigators have searched for species-specific antigens and antibodies, but so far, only two different groups of MAbs exist. These are directed against a 38 and a 14 kDa antigen and are specific to the *M. tuberculosis* complex.

The purpose of this part of the study was to establish if a combination of antigens released into the culture medium by aquatic mycobacteria *in vitro* can be utilised in a diagnostic test which differentiate fish *Mycobacterium* spp. and other pathogenic bacteria. MAbs were produced against the ECP from *Mycobacterium* sp. (TB267) isolated from snakehead fish during an outbreak of the disease in Thailand, and the type strain *M. chelonae*. The MAbs were then

used to characterise antigens in the culture supernatant (ECP), WCS and bacterial lysate by ELISA and Western blotting.

## 4.2 Materials and methods

**4.2.1 Growth of bacteria** (Bacteria were cultured as described in section 3.2.2)

### 4.2.2 Preparation of antigens

#### 4.2.2.1 Preparation of ECP and WCS antigens

(Preparation of antigens [ECP and WCS] are described as in section 3.2.1 and 3.2.2).

#### 4.2.2.2 Preparation of cell lysates

Mycobacterial strains (Table 3.1) were cultured on MSM for 2 weeks at 28°C. Bacteria were gently scraped off the agar using a sterile inoculating loop and resuspended in sterile deionised water. The bacteria were washed three times with deionised water by centrifugation at 6000 x *g* for 30 min. Glass beads (150-212 μm, Sigma Chemical Co.,) were added to the pellet at a ratio of 1:1. Deionised water (5 ml) was added, the tube sealed with nescofilm, vortexed for 10 mins, and centrifuged at 7,000 x *g* for 30 min. The resulting supernatants were centrifuged further at 35,000 x *g* for 30 min, filtered through a 0.45 μm filter and stored in -70°C. and the absorbance measured at 280nm using an Ultrospec II spectrophotometer. Protein estimations were made using the relationship OD 1.4 = 1 mg/ml and also by using the Biorad protein test with bovine serum albumin (BSA) as the standard.

### 4.2.3 Immunisation of mice

#### 4.2.3.1 ECP immunisation schedule

Mice were immunised following the method in section 3.2.4.3. Mice were test bled 7-10 days after the secondary injection and an ELISA and Western blotting were performed to determined titre and specificity of the response. The spleen was used in a fusion 4 days later.

#### 4.2.3.2 Lysate immunisation schedule

TB1 lysates from snakehead fish were mixed (1:1) into an emulsion with Titermax adjuvant and injected intraperitoneally (i.p.) into four 6 wk old balb/c mice (0.5 ml/mouse, 50mg). Booster injections (0.5ml/mouse, 50 mg) in adjuvant were given after 4 wks. The antibody response was evaluated using the ELISA protocol described below. Four weeks later one mouse was boosted i.v. with 0.25 ml of TB1 lysate (25mg) in sterile phosphate buffered saline. The spleen was used in a fusion 4 days later.

### 4.2.4 Production of MAbs

MAbs against ECP and whole cell lysate of *Mycobacterium* spp. were developed using the methods described by Campbell (1984); Harlow and Lane (1988) and Adams *et al.*, (1992).

#### 4.2.4.1 Cell lines and culture conditions

Myeloma cell lines (sp2/0-Ag-14) was purchased from ICN Flow laboratories, UK. Myeloma and hybridoma cells were cultured, unless specified, in the Dulbecco's modification of Eagle's medium (DMEM) with 3.7 g $l^{-1}$ sodium bicarbonate and supplemented with L-glutamine (200 mM stock solution, 10 ml $l^{-1}$), penicillin/streptomycin (5000 IU of each, 10 $ml^{-1}$), heat

inactivated FCS (20 % v/v) and sodium pyruvate (100 mM x200 stock solution, 5 $ml^{-1}$). Cells were cultured at 37°C in a $CO_2$ atmosphere (5%) and maintained around 1-2 $x10^5$ cells $ml^{-1}$. They were cultured every 2-3 days by diluting the culture 1/10 or 1/20 in fresh medium.

#### 4.2.4.2 Preparation of myeloma cells

Myeloma cells were prepared seven days prior to the fusion. FCS (Sigma Chemical Company, 1 ml) was placed carefully in the bottom of a 10 ml conical tube containing nine ml of DMEM and allow to warm at 37°C for 15 minutes. A vial of frozen non-secreting myeloma cells containing $1x10^5$ cells $ml^{-1}$ was removed from the liquid nitrogen and thawed in a water bath at 37°C until little pieces of ice were left, and then transferred to a sterile hood where the vial was wiped with 70% alcohol. Cells were then transferred to the culture medium, previously made up, and centrifuged at 1000 rpm (150 x *g*) for 10 minutes in a Wifug centrifuge. The supernatant was carefully removed and the cell pellet transferred to a small tissue culture flask containing 10 ml of complete tissue culture media DMEM. The culture was incubated as described in section 4.2.3.1. until the fusion day.

#### 4.2.4.3 Fusion

On the fusion day, the meyloma cells grown at midlog phase were centrifuged at 1000 rpm for 10 minutes. The cell pellet was resuspended in 20 ml of prewarmed serum-free DMEM in a conical universal tube and left at 37°C. Meanwhile the percentage viability and the total number of viable cells per ml were determined. A small volume (50 µl) of the cell suspension was mixed with the same volume of Trypan blue dye in an Eppendorf tube. One drop (20µl) of this mixture was loaded on an improved Neubauer chamber and at least 200 cells were counted using a light microscope at 40x magnification. Dead cells appeared

blue in colour while viable cells remain clear. The following formula were used to determine the required parameters:

N° viable cells/ml = N° viable cells x 2 x $10^4$ / N° squares counted

% Viability = N° viable cells x 100/ total N° cells

The previously boosted mouse was killed using $CO_2$, bled out by cardiac puncture, and immersed in 70% ethanol. The spleen was removed using sterile instruments and placed in a small petri dish containing 5 ml of prewarmed DMEM medium without FCS at 37°C. The fatty tissue was carefully removed and the spleen cells teased from the capsule using two 21gauge needles. Cells were then taken up into 10 ml syringe and gently pushed through a 21G needle. The procedure was repeated once more with a 21gaude needle and twice with 25 gauge needle. Cells were observed under the microscope to check that they were dispersed. If there were many clumps, then the pipetting was repeated using 25G needle. The cell suspension was then placed in a conical universal tube and allowed to stand for two to three minutes to allow any remaining clumps to settle out. Spleen cells were then transferred to a universal tube and centrifuged at 1000 rpm for 10 minutes. The cell pellet was washed twice by resuspension in 10 ml of DMEM without FCS and further centrifugation. Spleen cells were then counted by mixing 50 µl of cell suspension with 300 µl of ammonium chloride which causes the cells to swell making then easier to observe under the microscope. Five ml of myeloma cells ($5x10^6$ cells $ml^{-1}$) were mixed with 5 ml of spleen cells ($5x10^7$ cells $ml^{-1}$) in a conical universal and centrifuged at 500 rpm for 10 minutes. The supernatant was removed to leave as dry a pellet as possible and 1 ml of 50% PEG (prewarmed at 37°C) added to the pellet over 30 seconds, flicking the tube all the

time, then pipetted up and down over 30 seconds, then allowed to stand for further 30 seconds. Two ml of DMEM without FCS was added dropwise over two minutes shaking the cells gently all the time. Finally, another 5 ml of DMEM without FCS was added and allowed to stand for three minutes before centrifugation at 800 rpm for ten minutes at room temperature. The supernatant was carefully removed and the cell pellet diluted in 50 ml of HAT medium containing 1.5 ml of red blood cells as feeder cells to a final dilution of $1x10^6$ cells $ml^{-1}$. Five 96 well plates were loaded with 100 µl $well^{-1}$ of this cell suspension. Control myeloma and non fused spleen cells were diluted at $1x10^6$ cells $ml^{-1}$ and plated out in a separate 96 wells plate labelled as control. The plates were incubated for seven days at 37°C with 5% $CO_2$ and checked every day using an inverted microscope.

#### 4.2.4.4 Screening of hybridomas

Seven days after the fusion, all the wells containing hybridoma clones were screened by ELISA using the methods described in sections 3.2.7. Positive clones with the highest optical density were rescreened using the same method. Selected clones were subcloned by the limiting dilution technique described by Campbell (1984) and rescreened by ELISA until the clones became monoclonal. MAbs were further characterized by Western blot (see section 3.2.6.1.).

### 4.2.5 Isotyping of monoclonal antibodies

Isotyping of the MAbs was performed using a Sigma immuochemicals ImmunoType Kit™. following the instructions provided by the manufacturers.

### 4.2.6 Protease treatment

ECP (100 μg) was digested with protease in PBS (Boehringer) for 45 min at 37°C. The reaction was stopped by boiling the samples in the presence of SDS and 2-mercaptoethanol. The digested material was analysed by Western blot.

### 4.2.7 SDS-PAGE and Western blot analysis

(SDS-PAGE and Western blot analysis were performed using the materials described in section 2.2.5 and 3.2.6.1).

### 4.2.8 Preparation of bacteria for immunogold staining

Mycobacteria were cultured in MSM at 28°C for 14 days and harvested by centrifugation (4,000 x *g*), washed three times with PBS, fixed with 5% gluteraldahyde/0.2M PIPES buffer (Sigma, Piperazine-N, N,-bis-[2-ethanesulfonic acid]) for 3 h, then rinsed three times with 0.2M PIPES and three times with PBS. The bacteria were dehydrate through a series of alcohol concentrations (50% - 100%), then infiltrated with 70% alcohol:LR White resin (London Resin Co.)(1:1), followed by infiltrated with 100% LR white resin. To do this, bacteria were incubated with 70 % alcohol: LR White resin changing the solution twice, every hour. They were then incubated overnight with 100% LR white resin changing the solution once the following morning. Bacteria infiltrated with resin were placed in gelatin capsules filled to the brim with resin. The capsules were polymerise at 50°C for 24 h. The bacteria in the resin were removed from the capsules, cut into ultra thin sections and mounted on Nickel grids.

### 4.2.9 Immunogold staining

The grids were floated, section side down, on a drop of wash buffer (0.2M TBS, 1% Tween 20, 1% BSA, 10 % FCS), placed on the nescofilm in petri dishes containing a wet filter paper. These were incubated overnight at 4°C. Grids were transferred to drops of MAb supernatant and again incubated overnight at 4°C. Sections were washed extensively by floating the grids on wash buffer in 24 well plates for 90 mins, with occasional agitation. The grids were transferred to fresh wash buffer and washed for another 90 mins. These were incubated overnight, section side down, on drops of 5 nm gold probe (Sigma), diluted 1/40 in wash buffer, after which they were washed as described above, followed by a rinse with distilled water to remove the wash buffer. The gold probe was enhanced by placing the grids on drops of silver enhancing solution (British Bio Cell Cardiff, UK Agar Scientific LTD, 66a Cambridge Road, Stansted, Essex CM24 8DA) for 2 minutes. The reaction was stopped with distilled water and the sections were counterstained using uranyl acetate and lead citrate. The bacteria were viewed under 80 kv.

## 4.3 Results

Thirteen MAbs were produced against the ECPs from *Mycobacterium* sp. (TB267) isolated from snakehead fish, and five MAbs were prepared against the reference strain *M. chelonae*. The immunising antigen, the isotype of the MAbs and the molecular weight of antigens recognised by the MAbs in the ECP, WCS and lysates *Mycobacterium* is presented in Table 4.1. All MAbs were of an IgG1 subclass, except MAbs 4H5, and 8B9 and 9C2 which are IgG3 and IgM antibodies, respectively.

Table 4.1. Characterization of MAbs

| Antibody | Antigen | Isotype | Mol. wt. (kDa) of reactive | | |
|---|---|---|---|---|---|
| | | | ECP | Sonicate | Lysate |
| 1D11 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 4B9 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 4E9 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 5E2 | TB267 ECP | IgG1 | 65,55 | 65,55,25 | 65,55,40 |
| 7B8 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 12C6 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 12D2 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 12H11 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 1D4 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 3F10 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 4H10 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 5H8 | TB 267 ECP | IgG1 | 65 | 65 | 65 |
| 11B11 | TB267 ECP | IgG1 | 65 | 65 | 65 |
| 4H5 | MC ECP | IgG3 | 65 | 65 | 65 |
| 7B7 | MC ECP | IgG1 | 65 | 65 | 65 |
| 8B9 | MC ECP | IgM | 65 | 65 | 65 |
| 9C2 | MC ECP | IgM | 65 | 65 | 65 |
| 10B7 | MC ECP | IgG1 | 65 | 65 | 65 |

MC: *Mycobacterium chelonae*
ECP: Extracellular products

Different mycobacterial antigen preparations were produced to examine the reactivity of the different MAbs. The protein components of ECP (Figure 2.1a) and WCS (Figure 2.1b) from mycobacterial strains TB1, TB40, TB267, TB268, *M. chelonae*, *M. fortuitum* and *M. marinum*, were resolved by SDS-PAGE and the gels were stained with coomassie brilliant blue. Variation in the protein profiles of different ECP preparations was greater between the various mycobacterial strains tested than was observed with the WCS preparations, where little variation occurred between the protein profiles (as described in chapter 2).

The ECP and WCS preparations of each mycobacterial strain were used as antigens in ELISA experiments to examine the reactivity of the MAbs (Table 4.2). The response of the MAbs to the ECP and WCS preparations was also examined by Western blot analysis (Table 4.3). For the sake of clarity, MAb responses were divided into three groups: ++, a strong response; +, a weak response; and -, no response.

Table 4.2 The response of monoclonal antibodies to extracellular products and whole cell sonicate antigen of mycobacteria determined by enzyme linked immunosorbent assay

| | TB1 | | TB40 | | TB267 | | TB268 | | TBMC | | TBMF | | TBMM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E | S | E | S | E | S | E | S | E | S | E | S | E | S |
| 1D11 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | ++ | ++ | ++ |
| 4B9 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | ++ | ++ | ++ |
| 4E9 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | ++ | ++ | ++ |
| 5E2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | - | ++ | ++ |
| 7B8 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - | - | - | ++ | ++ |
| 12C6 | ++ | + | ++ | ++ | + | ++ | + | ++ | - | - | - | - | ++ | ++ |
| 12D2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - | - | - | ++ | ++ |
| 12H11 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | ++ | ++ | ++ |
| 1D4 | ++ | + | ++ | ++ | - | ++ | - | ++ | - | ++ | - | ++ | ++ | ++ |
| 3F10 | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | - | ++ | - | ++ | ++ | ++ |
| 4H10 | ++ | ++ | ++ | ++ | + | ++ | - | ++ | - | ++ | - | ++ | ++ | ++ |
| 5H8 | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | ++ | - | ++ | ++ | + |
| 11B11 | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | - | ++ | - | ++ | ++ | + |
| 4H5 | ++ | ++ | ++ | - | - | ++ | - | - | ++ | - | + | + | - | + |
| 7B7 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ |
| 8B9 | - | ++ | ++ | - | - | ++ | - | ++ | ++ | - | + | ++ | ++ | ++ |
| 9C2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ |
| 10F7 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ |

Mean results from four experiments (++, optical densities exceeding three time the mean background absorbance; +, optical densities close to twice the mean background absorbance. E, Extracellular products; S, Whole cell sonicate antigen. MC: *Mycobacterium chelonae*; MF:*M. fortuitum*; MM: *M. marinum*.

Table 4.3. The response of monoclonal antibodies to extracellular products and whole cell sonicate antigen of mycobacteria determined by Western blotting

| | TB1 | | TB40 | | TB267 | | TB268 | | TBMC | | TBMF | | TBMM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E | S | E | S | E | S | E | S | E | S | E | S | E | S |
| 1D11 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - | ++ | + | ++ |
| 4B9 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | + | ++ | ++ | ++ |
| 4E9 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | + | ++ | ++ | ++ |
| 5E2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - | ++ | ++ | ++ | ++ |
| 7B8 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - | ++ | ++ | ++ | ++ |
| 12C6 | ++ | ++ | ++ | ++ | + | ++ | + | ++ | - | - | - | - | ++ | ++ |
| 12D2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | ++ |
| 12H11 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | ++ |
| 1D4 | ++ | - | ++ | ++ | - | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | ++ |
| 3F10 | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | ++ |
| 4H10 | ++ | + | ++ | ++ | + | ++ | ++ | ++ | - | ++ | ++ | ++ | + | ++ |
| 5H8 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | + |
| 11B11 | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 4H5 | - | ++ | ++ | - | - | ++ | - | - | ++ | - | - | - | - | ++ |
| 7B7 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 8B9 | - | ++ | ++ | - | - | ++ | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ |
| 9C2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 10F7 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

++: Strong reaction
+ : Weak reaction
- : No reaction
E : Extracellular products
S : Whole cell sonicate antigen
MC: *Mycobacterium chelonae*
MF: *Mycobacterium fortuitum*
MM: *Mycobacterium marinum*

All MAbs reacted with a 65 kDa protein found with each of the *Mycobacterium* spp examined. The thirteen MAbs produced against strain TB267 did not react with the ECP from *M. chelonae* and *M. fortuitum* by ELISA (Table 4.2), but eleven of them did react with ECP from *M. fortuitum* by Western blotting. Eighteen of the MAbs produced against either strain TB267 or *M. chelonae* reacted with the WCS antigen from *M. chelonae* and fourteen reacted with WCS from *M. fortuitum*. None of the MAbs from mice immunised with ECP from TB267 reacted with the 65 kDa protein in *M. chelonae* ECP by Western blotting but they did react with 65 kDa protein in WCS and lysate from *M. chelonae* by Western blot analysis.

All MAbs from the mouse immunised with ECP reacted with protease sensitive epitopes. MAbs produced against strain TB267 or *M. chelonae* reacted with the mycobacterial lysate from TB1, TB40, TB267, TB268, *M. chelonae, M. fortuitum and M. marinum* by Western blotting (Figure 4.1a, ,b, c). Figure 2 shows a sample representation of the MAb response against mycobacterial preparations using MAb 12D2. MAb 5E2, however, also reacted with proteins with a low molecular weight, except the 65 kDa protein (Figure 4.2a, b, c).

The cross-reactivity of the MAbs with WCS antigen prepared from a variety of mycobacterial strains isolated from snakehead fish or Siamese fighting fish were examined by ELISA, and the results are shown in Table 4.4. The percentage response of the MAb to the various mycobacterial stains, ranged from 22.2%-100%, using the absorbances of the type strain *M. marinum* as a reference.

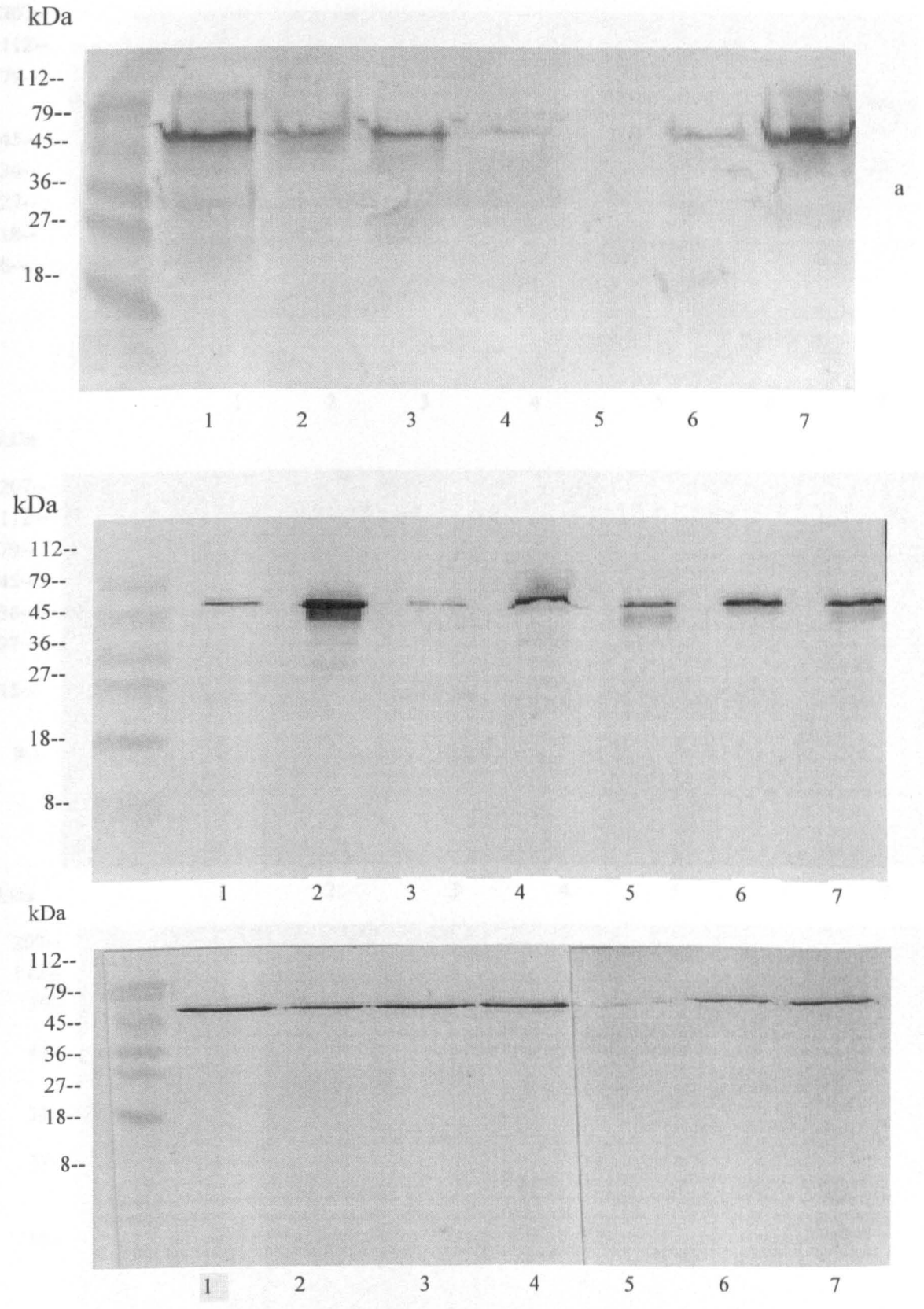



Figure 4.1 Western blot analysis; 12D2 incubated with ECP from (lanes); (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*. (6) *M. fortuitum*, (7) *M. marinum*. Molecular weight in thousands is indicated on the left. a (ECP), b (WCS), c (lysate).

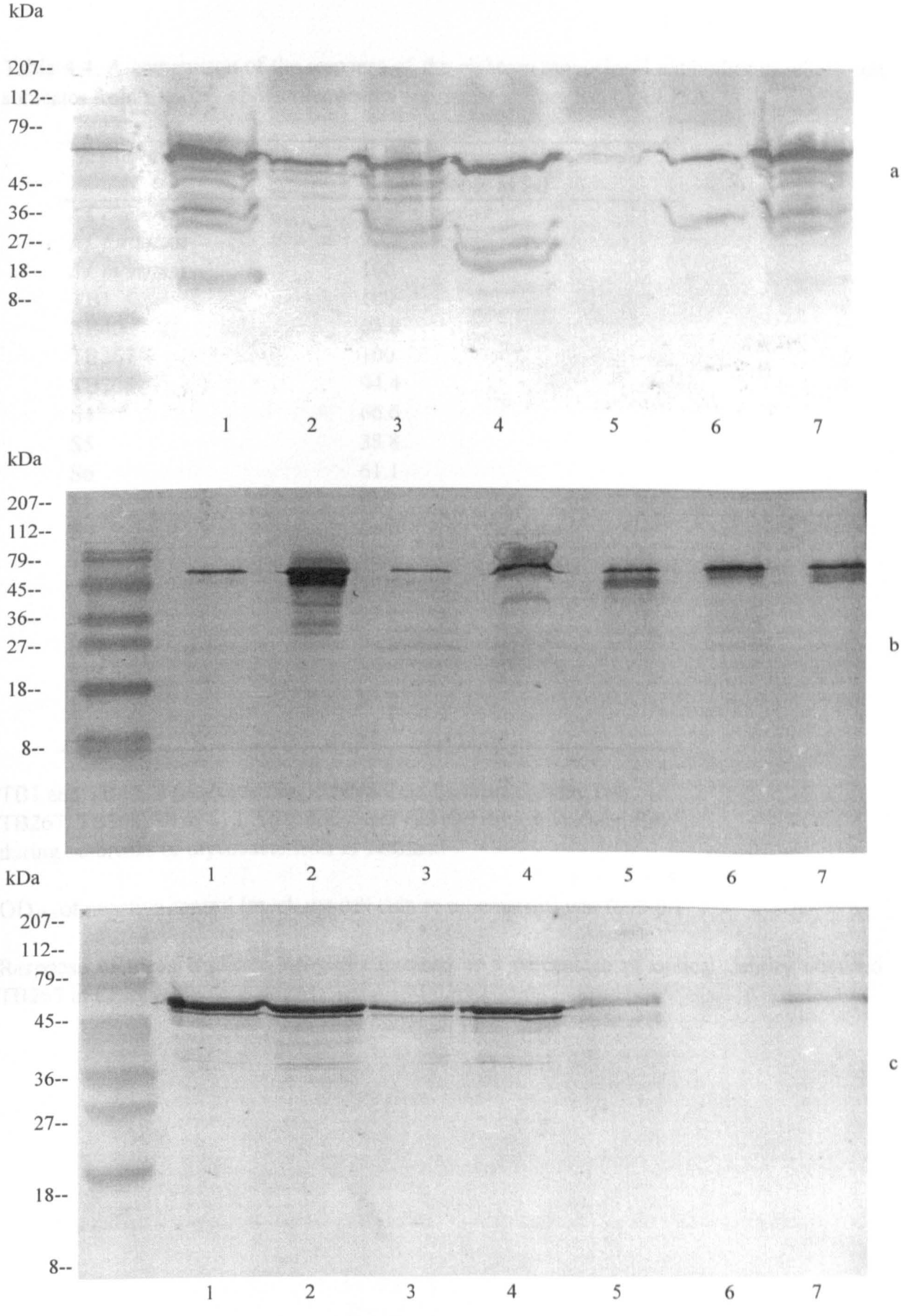



Figure 4.2 Western blot analysis; MAb 5E2 incubated with lysate antigen From (lanes); (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae*, (6) *M. fortuitum*, (7) *M. marinum*. Molecular weight in thousands is indicated on the left, a (ECP), b (WCS), c (lysate).

Table 4.4. A comparison of the response of the eighteen monoclonal antibodies to whole cell sonicates from a variety of *Mycobacterium* spp. isolated from fish by ELISA.

| Strains of mycobacteria | Whole cell sonicate antigen ( Response as %) |
|---|---|
| *M. chelonae* | 72.2 |
| *M. fortuitum* | 77.7 |
| *M. marinum* | 100 |
| TB1 | 100 |
| TB40 | 88.8 |
| TB267 | 100 |
| TB268 | 94.4 |
| S4 | 66.6 |
| S5 | 38.8 |
| S6 | 61.1 |
| S8 | 61.1 |
| S9 | 66.6 |
| S10 | 66.6 |
| S11 | 27.7 |
| S12 | 22.2 |
| S13 | 38.8 |
| S14 | 27.7 |
| S15 | 88.8 |
| S18 | 83.3 |

TB1 and TB40 : *Mycobacterium* isolated from Siamese fighting fish
TB267, TB268, S4 to S18: *Mycobacterium* isolated from snakehead fish during outbreaks of mycobacteriosis in Thailand

$OD_{450}$ of negative control (myeloma cell culture supernatant) was 0.06-0.1

Response of mean triplicate samples expressed as a percentage of optical density obtained TB267 in ELISA.

Twenty one different species of non-mycobacteria were tested by ELISA to determine if the MAbs cross-reacted with bacterial species other than mycobacteria. MAb 8B9 reacted with *Nocardia asteroides*; MAbs 1D11 and 3F10 reacted with *Yersinia ruckeri*; MAbs 1D11, 12D2 and 1D4 reacted with *Micrococcus luteus*; MAbs 1D11, 4B9, 4E9, 12H11 and 10F7 reacted with *Renibacterium salmoninarum*; and two MAbs, 1D11 and 3F10, reacted with *Carnobacteria piscicola*. The percentage cross-reactivity of MAbs with non-mycobacteria was also determined using *M. marinum* as a reference (Table 4.5) by ELISA (*M. luteus* 16.6%, *N. asteroides* 5.0%, *Yersinia ruckeri* 5.0%, *R. salmoninarum* 27.7%).

The MAbs against TB1, with the exception of MAb 14B7, recognised a doublet at 25 kDa and 33 kDa in the ECP from type strains *M. fortuitum* and *M. marinum* (Figure 4.3a). The same bands were recognised in the ECP of all the *Mycobacterium* spp. isolated from the snakehead fish and the Siamese fighting fish examined., however, they were not observed in *M. chelonae* ECP (Table 4.6), MAb 14B7, on the other hand, recognised a single product around 21 kDa in the ECP of type strains *M. marinum* and *M. fortuitum*. As with the other MAbs against TB1 lysate, MAb 14B7 did not recognise the 21 kDa band in the ECP from *M. chelonae* (Figure 4.3b). The MAbs against TB268 lysate recognised the heat shock protein at 65 kDa in the ECP of all mycobacteria examined (Table 4.6).

Some of the basic structural features of TB267 or *M. marinum* were characterised using ultra thin sections of the bacteria. As shown in Figures 4.4 and 4.5 an inner most fibrillar nuclear region of low electron density was surrounded by a higher electron dense cytoplasm, rich in ribosomes; this was externally bounded by the plasma membrane, outside of which was the cell wall complex, the outer region of which appeared to be continuous with a peculiar capsule-like

electro-transparent zone or clear substance. The latter material was found to be a constant feature, but did vary in thickness. The 65 kDa protein, recognised by MAbs 4B9 and 12D2 in the immunogold electron microscopy studies, was located in the periplasmic space or cell wall of the bacteria (Figures 4 and 5). This protein was secreted into the culture medium during growth. Most of the 65 kDa protein molecules were detected in the periplasmic space, but some were also found in the cell wall of the bacterium.

Table 4.5. The cross reactivity of eighteen MAbs produced against *Mycobaterium* spp with non-mycobacteria determined by ELISA

| Bacteria | Whole cell sonicate antigen (Response as %) |
|---|---|
| *Aeromonas hydrophila hydrophila* | 0 |
| *Aeromonas salmonicida achromogenes* | 0 |
| *Aeromonas salmonicida masoucida* | 0 |
| *Aeromonas salmonicida salmonicida* | 0 |
| *Carnobacterium piscicola* | 11 |
| *Corynebacterium aquaticum* | 0 |
| *Micrococcus luteus* | 16.6 |
| *Nocardia asteroides* | 5.0 |
| *Streptococcus faecalis* | 0 |
| *Pseudomonas aeruginosa* | 0 |
| *Pseudomonas fluorescens* | 0 |
| *Vibrio anguillarum* | 0 |
| *Vibrio ordalli* | 0 |
| *Yersinia ruckeri* | 5.0 |
| *Arthrobacter aurescens* | 0 |
| *Pasteurella piscicida* (Japan) | 0 |
| *Pasteurella piscicida* (Greece) | 0 |
| *Escherichia coli* | 0 |
| *Edwardsiella tarda* | 0 |
| *Edwardsiella ictaruli* | 0 |
| *Renibacterium salmoninarum* | 27.7 |
| *Bacillus subtilis* | 0 |
| *Bacillus cereus* | 0 |

$OD_{450}$ of negative control (myeloma cell culture supernatant) was 0.06-0.1

Response of mean triplicate samples expressed as a percentage of optical density obtained TB267 in ELISA.

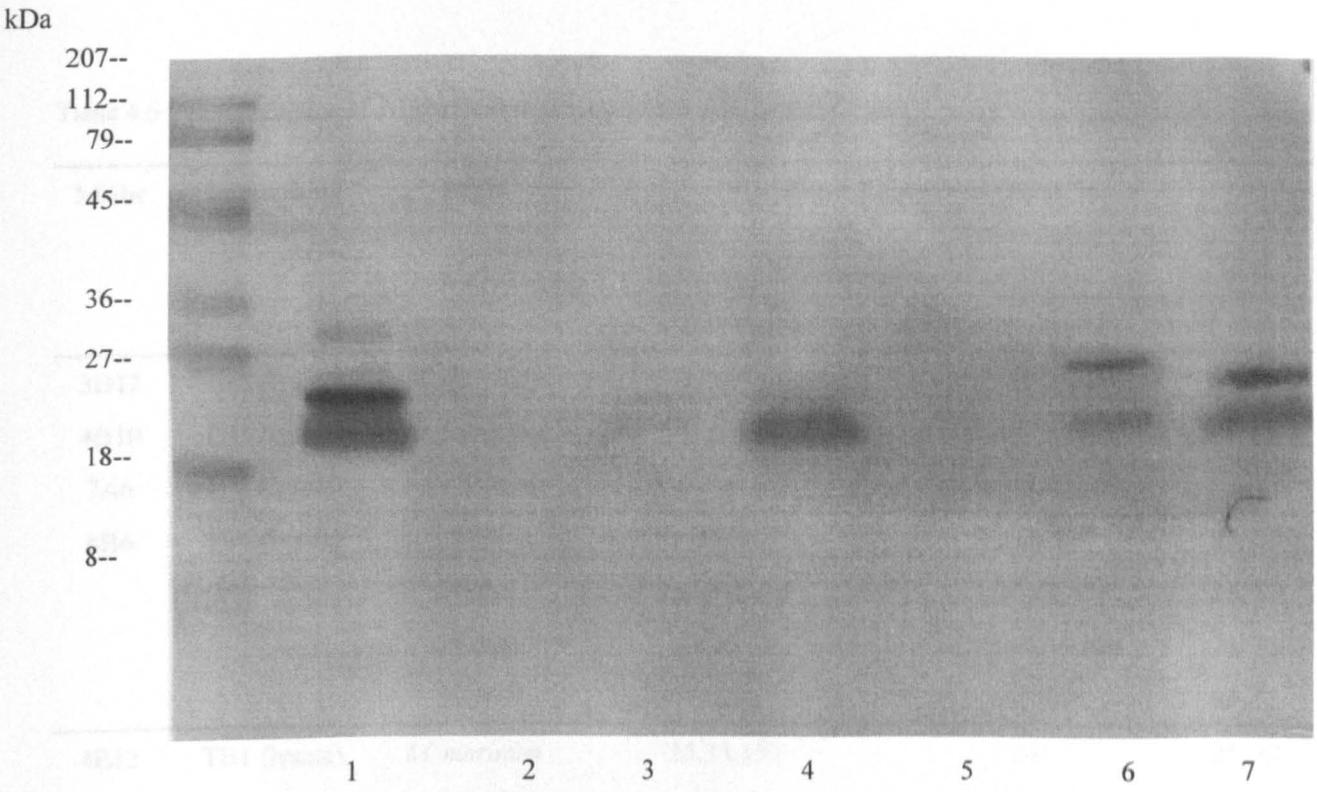



Figure 4.3 Western blot analysis MAbs 4E2 incubated with lysate antigen from (Lanes); (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae,* (6) *M. fortuitum*, (7) *M. marinum*. Molecular weight in thousand is indicated on the left.

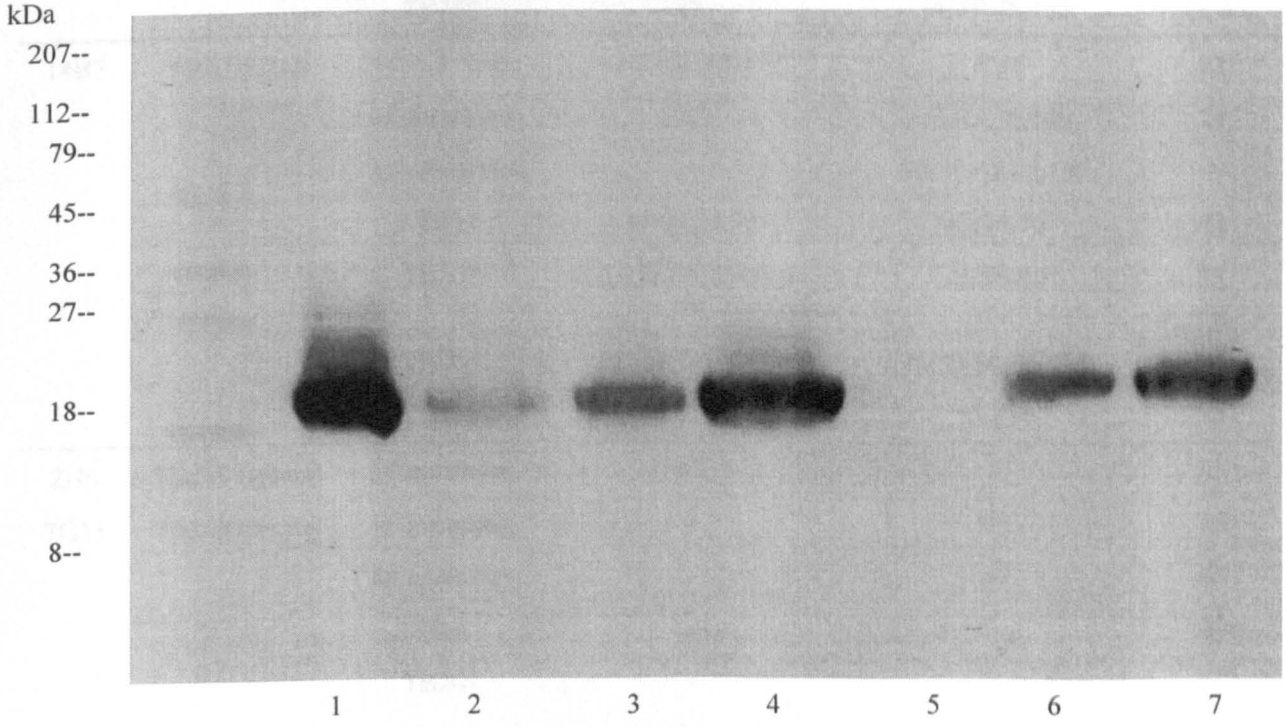
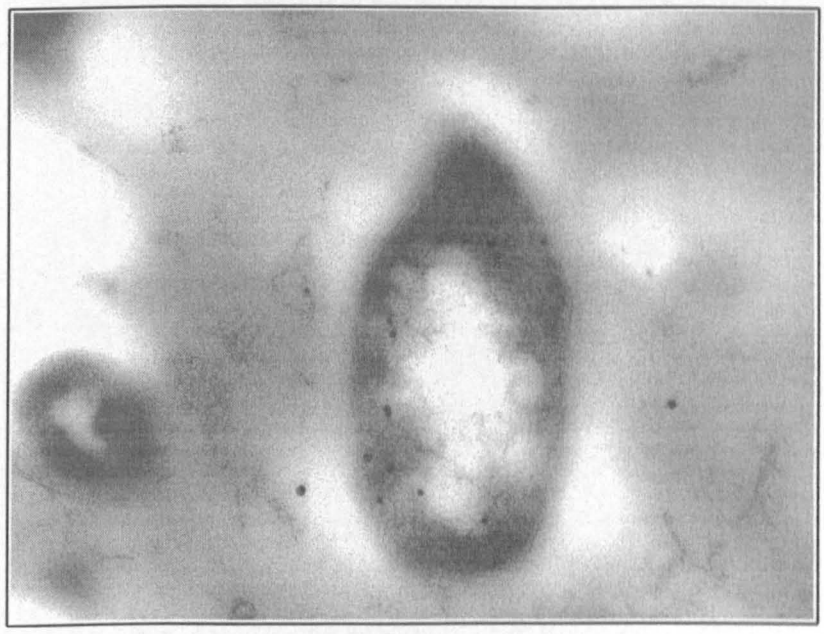



Figure 4.3 Western blot analysis MAb 14B7 incubated with lysate antigen from (Lanes); (1) TB1, (2) TB40, (3) TB267, (4) TB268, (5) *M. chelonae,* (6) *M. fortuitum*, (7) *M. marinum*. Molecular weight in thousand is indicated on the left.

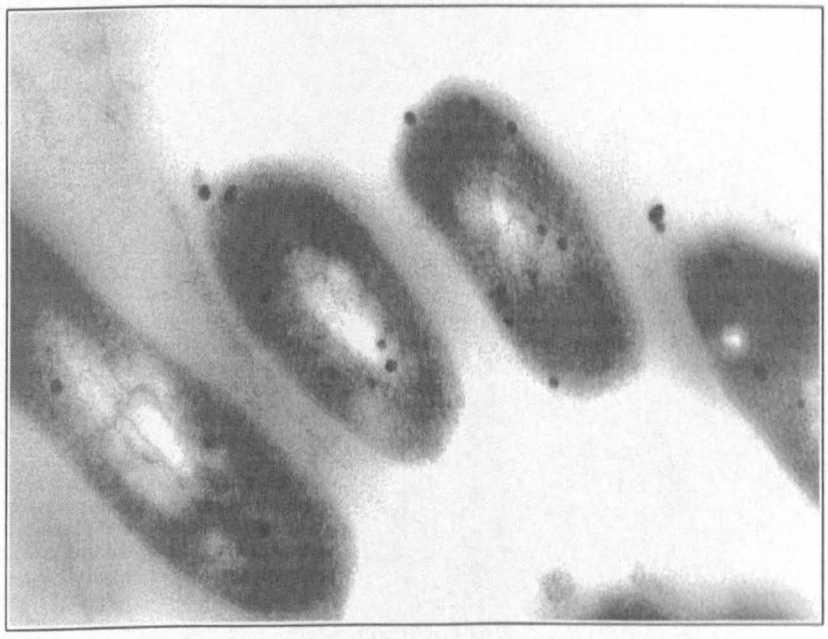
Table 4.6 Characterisation of MAbs from mice immunised with lysate antigen

| MAbs | Immunising antigen | Strain | Bands Recognised (kDa) | | |
|---|---|---|---|---|---|
| | | | sonicate | | ECP |
| | | | Major bands | Minor Bands | |
| 3D12 | TB1 (lysate) | *M. marinum* | 25,33,150 | 100 | 25, 33 |
| 4B10 | TB1 (lysate) | *M. fortuitum* | 25 | 33-45,70,100 | 25, 33 |
| 7A6 | TB1 (lysate) | *M. chelonae* | - | 25,33-45,70,100 | - |
| 8B6 | TB1 (lysate) | TB1 | 25,33 | 45,65-70 | 25, 33 |
| | | TB267 | 25,33-36,50 | 21,36-40,45 | 25, 33 |
| | | TB268 | 25 | 21,55,60,65,100 | 25, 33 |
| | | TB269 | 25 | 55,150 | 25, 33 |
| 4E12 | TB1 (lysate) | *M. marinum* | 25,33,150 | 100 | 25, 33 |
| | | *M. fortuitum* | 25 | 33-45,70,100 | 25, 33 |
| | | *M. chelonae* | - | 25,33-45,70,100 | - |
| | | TB1 | 25,33 | 45,65-70 | 25, 33 |
| | | TB267 | 25,33-36,50 | 14-21,36-40,45 | 25, 33 |
| | | TB268 | 25 | 21,14-19,55,60,65,100 | 25, 33 |
| | | TB269 | 25 | 14-19,55,150 | 25, 33 |
| 14B7 | TB1 (lysate) | *M. marinum* | 14,19,25,33,150 | 100 | 21 |
| | | *M. fortuitum* | 25 | 33-45,70,100 | 21 |
| | | *M. chelonae* | - | 25,33-45,70,100 | - |
| | | TB1 | 14-19,25,33 | 45,65-70 | 21 |
| | | TB267 | 14-21,33-36,45-55,60-70 | 25,36-40 | 21 |
| | | TB268 | 14-19,25 | 21,55,60,65,100 | 21 |
| | | TB269 | 14-19,25 | 55,150 | 21 |
| 2H6 | TB268 (lysate) | *M. marinum* | 65 | - | 65 |
| 7G11 | TB268 (lysate) | *M. fortuitum* | - | 65 | 65 |
| | | *M. chelonae* | - | 65 | 65 |
| | | TB1 | - | - | 65 |
| | | TB267 | - | - | 65 |
| | | TB268 | 65 | - | 65 |
| | | TB269 | 65 | - | 65 |

ECP-extracellular products, Minor bands- faint staining on Western blot

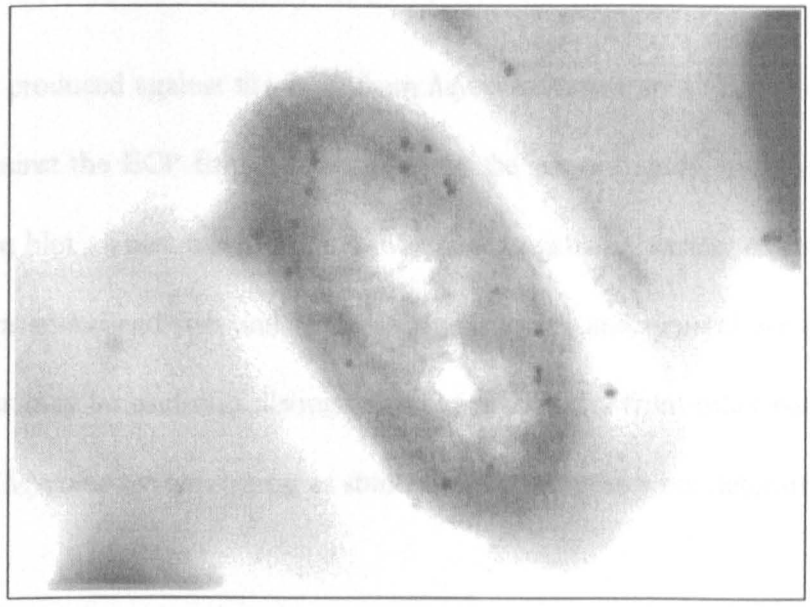


a

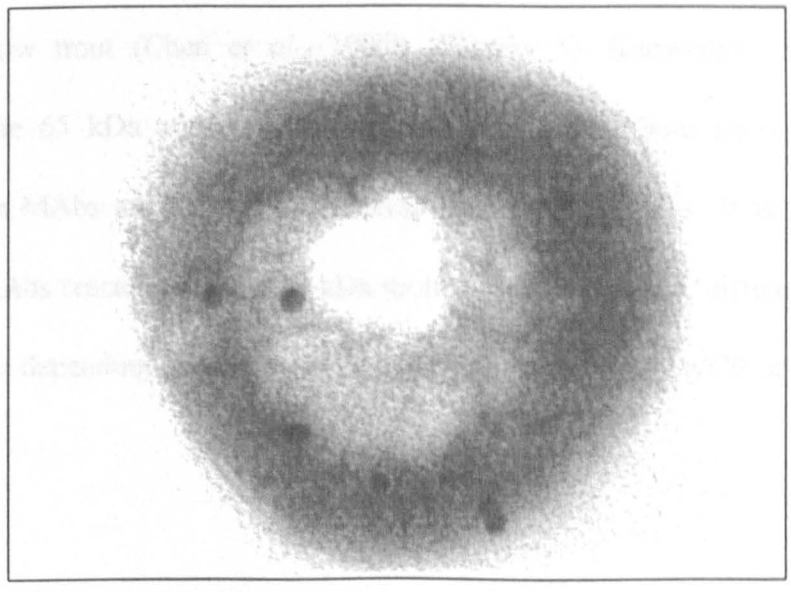


b

Figure 4.4. Immunogold electron microscopy. The antibody used was MAb 4B9 directed against cell wall and periplasmic space. a, TB267, X 36,000; b, *M. marinum*, X 45,000.



a



b

Figure 4.5 Immunogold electron microscopy. The antibody used was MAb 12D2 directed against cell wall and periplasmic space. a, TB267, X 36,000; b, *M. marinum*, X 45,000.

## 4.4 Discussion

The thirteen MAbs produced against the ECP from *Mycobacterium* sp. (TB267) and the five MAbs produced against the ECP from *M. chelonae* in the present study, were evaluated by ELISA and Western blot. Their reactivity also was tested against a variety of mycobacterial strains isolated from snakehead fish and Siamese fighting fish and general aquatic bacteria. These characteristics may be useful in distinguishing mycobacteria from other bacteria and in the identification of *Mycobacterium* strains, as strain specific antigens were determined.

All eighteen MAbs produced against mycobacteria ECPs reacted with the 65 kDa protein, suggesting that the 65 kDa antigen is very immunogenic. This particular antigen from *Mycobacterium* spp. infecting humans is in fact believed to be a super antigen (Born *et al.*, 1990), and the 65 kDa protein from mycobacteria infecting fish is also a major immunogenic protein for rainbow trout (Chen *et al.*, 1996b, Chapter 5). Knowedge of the chemical composition of the 65 kDa antigen is limited, although it has been shown that epitopes recognized by the MAbs are located on protease sensitive molecules. It is interesting that although all 18 MAbs reacted with the 65 kDa molecule, they did have different affinities for the 65 kDa protein depending or whether it originated from the ECP, WCS or lysates antigen preparations.

MAbs, 1D11, 4B9, 4E9 and 12D11 reacted strongly with *Renibacterium salmoninarum* in the ELISA, while MAb 8B9 reacted strongly with *Nocardia asteroides*. In a previously study, rabbit anti-65kDa protein polyclonal serum was shown to react with diverse bacterial genera. The 65 kDa antigen appears to be a common bacterial protein found in many pathogens

(Chapter 3). MAbs 5E2, 7B8, 12C6, 4H10, 11B11, 4H5, 7B7 and 9C2 only reacted with 65 kDa of ECP from *Mycobacterium* spp and did not react with other bacteria. These MAbs may prove useful in the identification of aquatic mycobacteria due to their specificity. In a previous study, in which eight MAbs were produced to lysate antigens from mycobacteria isolated from Siamese fighting fish (TB1) and snakehead fish (TB268), the MAbs were not strain specific and were unable to differentiate between the type strains and bacteria isolated from snakehead or Siamese fighting fish when used in diagnostic test (Adams *et al.*, 1996; Thompson *at al.*, 1996).

MAbs produced from strain TB267 gave a negative response in Western blot analysis with the 65 kDa from *M. chelonae* ECP, but reacted positively with this protein when WCS were tested. This could be explained by structural differences in the two molecules, perhaps due to cleavage of the antigenic epitope upon secretion. Different responses with MAbs 1D11 and 4B9 were also observed between ELISA and Western blot with ECP antigens. This observation may, however, be explained by demasking of epitopes during SDS treatment of the antigen for Western blot analysis (Andersen *et al.*, 1986). Another consideration may be that, in the ELISA, not all of the mycobacterial antigens are bound equally well to the polystyrene plate (Andersen *et al.*, 1986).

MAbs directed against the 65 kDa antigen frequently bind to bands with multiple molecular weights during Western blotting of extracts from *M. tuberculosis* and *M. leprae*, although this banding pattern seem to be less marked in the case of more rapidly growing mycobacteria (Gillis *et al.*, 1985). The most likely reason for the multiple banding is that the 65 kDa protein

undergoes progressive proteolytic degradation to multiple fragments, each containing a subset of the total antibody binding site from the intact molecule.

In this study, the 65 kDa protein was detected in ECP, lysate and WCS from *Mycobacterium* and immunogold staining showed that it did indeed have a periplasmic location within the cell wall.

Competitive binding assays have shown that MAbs can recognize at least 14 spatially separate epitopes on the 65 kDa protein (Buchanan *et al.*, 1987). The eighteen MAbs produced from a mouse immunised with the 65 kDa protein from TB267 and *M chelonae* in this study may also recognise different epitope. There is evidence that at least some of them do. The 65 kDa protein is a major mycobacterial antigen that induces specific antibody (Meeker, *et al* 1989). The human mycobacterial 65-kDa protein is a set of highly conserved proteins, synthesized in response to a sudden increase in temperature or to other environmental stresses (Lindquist 1986; Schlesinger, Ashburner, and Tissieres 1982; Shinnick, Vodkin and Williams, 1988). The 65 kDa protein is, therefore, known as a heat-shock protein (HSP). The band identified at 65 kDa by the MAbs is believed to be a HSP, and has been reported in mycobacteria infecting both mammals (Young *et al.*, 1987) and fish (Chen *et al.*, 1996a and Chapter 2).

## Chapter 5

**Immune response of rainbow trout, *Oncorhynchus mykiss* and tilapia, *Oreochromis nilotica* to the extracellular products from *Mycobacterium* spp.**

### 5.1 Introduction

Fish protect themselves against invasive pathogens by both non-specific and specific defense mechanisms. The non-specific defenses include physical barriers, such as an epithelial shield of scales, skin and also mucus. However, if a pathogen manages to breach these initial barriers, then chemical defenses such as serum lysozyme, lectins and complement components may coat the pathogen, opsonizing them for further destruction (Fletcher, 1981).

Other components of the non-specific immune system can also be activated by invading stimuli (Ellis, 1981). Phagocytic cells, for example, play an important role in the defense mechanisms of the host, by adhering to and engulfing invading particles. Such cells include tissue macrophages, circulatory monocytes and neutrophils. There are numerous reports of microbial products having immunostimulatory qualities that stimulate phagocytic activity. Such immunostimulants include BCG (Sher *et al.*, 1975), *Corynebacterium parvum* (Sher *et al.*, 1975; Berd, 1978), endotoxin (Dubos and Schaedler, 1956), glucan (Reynolds *et al.*, 1980), *Nocardia rubra* cell wall skeleton (Masuno *et al.*, 1979) and synthetic compounds such as muramyl, and its analogs (Parant *et al.*, 1980; Fraser-Smith *et al.*, 1983; Sakai *et al.*, 1983). Beta-1,3 and -1,6 linked glucans (ß-glucan and M-glucan) are major structural polysaccharides in the cell wall of most fungi and these too have been shown to enhance the non-specific defenses of fish and mammals (Di Luzio, 1985; Yano, Mangindaan and Matsuyama,

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1989; Robertsen *et al.*, 1990; Jorgensen, Lunde and Bobertsen, 1993; Chen and Ainsworth, 1992). Cipriano and Pyle (1985) showed increased protection in eastern brook trout (*Salvelinus fontinalis*) injected with FIA and EcF1, a chromatographic fraction of *A. salmonicida*. Similarly, modified FCA in combination with *Aeromonas salmonicida* formalin-killed bacterin increased protection in coho salmon (*Oncorhynchus kisutch*). Olivier, Evelyn and Lallier (1985) demonstrated that adjuvant alone protected coho salmon against *A. salmonicida* infection. Activation of macrophages by the adjuvant was believed to be responsible for this increase in disease resistance. The ECP of *A. salmonicida* has also been reported to increase rainbow trout macrophage activity *in vitro* (Francis and Ellis, 1994).

As explained in chapter 1, the specific immune responses include T-lymphocyte function and the production and release of antibodies from B-lymphocytes into the circulation. Kitao and Yoshida (1986) studied the effect of a *Streptmyces olivaceogriseus* sp. nov derivative, FK-565, on the immune response in rainbow trout, *Oncorhynchus mykiss*, and found that this immunopotentiator elevated the numbers of plaque forming cells (PFC) and circulatory antibody titers. Compared to non-specific responses, specific responses in fish, as with mammals, require a longer period of time to develop after the animal has been exposed to invasive materials, or after it has been subjected to active immunization (Anderson and Siwicki, 1989).

This chapter reports the dynamics of nitroblue tetrazolium (NBT) reduction, phagocytosis, lysozyme and antibody production in rainbow trout following IP injection of ECP from *Mycobacterium* spp. (ie. the type strain *M. marinum*; strains isolated from Siamese fighting fish (*Betta spendens*) and snakehead fish (*Channa striatus*) (see Table 2.2).

and an examination of the immunostimulatory properties of the ECP of *Mycobacterium* spp. on some of the non-specific immune responses of Nile tilapia *Oreochromuis nilotica, in vivo* is also presented. Moreover, a comparison of the non-specific immune response, in tilapia between fish injected with a variety of adjuvants is reported. The tilapia were immunised by injecting ECP into their swimbladders, a method previously reported as serving as an ideal depot for the delivery of antigen (Endo *et al.*, 1995). Stimulation of head kidney macrophages from tilapia by *Mycobacterium* ECP *in vitro* is also described.

## 5.2 Materials and methods

### 5.2.1 Preparation of ECPs

*Mycobacterium* spp., TB1, TB40, TB267, TB268, *Mycobacterium chelonae*, *M. fortuitum* and *M. marinum* (Table 2.1 and 2.2) were cultured in MSM (as described in section 3.2.1).

### 5.2.2 Fish

#### 5.2.2.1 Rainbow trout

Rainbow trout with weights ranging from 800-900 gm were obtained from a local farm and kept in 150 l tanks. The fish were fed with commercial pellets and supplied with aerated fresh water. The temperature was 15±2°C throughout the experiment. A total of 32 healthy rainbow trout were used for the experimental study. They were allotted equally into four treatment groups (8 in each group) in separate tanks. Groups 1, 2 and 3 were immunized 0.3 ml ECP from TB40, TB267 and *M. marinum*, respectively. The ECP was mixed into an emulsion with Freund's incomplete adjuvant (1:1). Each fish received 150 µg of protein (0.3 ml) during primary and secondary injections at 0 and 8 weeks, respectively. The fourth group was immunised with only FIA as controls.

#### 5.2.2.2 Nile tilapia

Nile tilapia, *Oreochromis nilotica,* (40-50 gm) were obtained from the Institute of Aquaculture, University of Stirling. Seven groups of fish (8 fish per group) were placed in separate tanks. The tanks held in 50 l of tanks, with aerated water at a temperature of 25±2°C and the fish were maintained on a commercial diet. Groups 1-6 were immunised with either ECP from TB40, TB267 or *M. marinum* (50 μg $fish^{-1}$), or with FCA, FIA, or Titre Max, respectively. The seventh group of fish, injected with PBS, was used as a control group. The fish were immunised by injecting 0.5 ml of the appropriate substance into their swimbladder (Endo *et al.*, 1995).

### 5.2.3 Adherence/Nitroblue tetrazolium (NBT) assays

#### 5.2.3.1 Rainbow trout

Blood samples were taken at 2, 4, 6, 8, 10, and 12 weeks post primary immunization from the caudal vessels of experimental fish which had been anaesthetized with benzocaine. Individual drops of blood were mixed with a drop of NBT (0.2% w/v in saline) on a glass coverslip. The coverslips were incubated in a humidified chamber (a petri plate containing a piece of moist filter paper) at 20°C for 30 min, before gently washing them with PBS. Excess liquid was drained from the coverslip by placing one edge against a paper towel. The coverslips were then placed upside down on a microscope slide, onto a drop of NBT solution and examined using a compound microscope (400x). Numbers of blue stained, adherent cells were counted in 10 fields and the number of positively stained cells were compared with that of control fish.

### 5.2.3.2 Nile tilapia

Blood samples were taken from the caudal vein of fish which had been anaesthetised with benzocaine, 4 and 8 days after immunisation. At the same time, cells were harvested from the swimbladder of the fish by firstly disinfecting the ventral surface of the fish with 70% (v/v) ethanol, then injecting 2 ml of cold Dulbecco's modified essential medium (DMEM)(Sigma) containing 10 i.u. heparin, 100 i.u. penicillin (pen) and 100 μg streptomycin (strep.) into the swimbladder of the fish. To stain the cells, a drop of blood or cell suspension was firstly mixed (as described in section 5.2.3.1).

### 5.2.4 Phagocytic assays for rainbow trout

Blood samples were taken as above at weeks 2, 4, 6, 8, 10, and 12 post primary immunisation. Individual drops of blood were mixed with one drop of formalin-killed *Aeromonas salmonicida* ($1 \times 10^7$ cells$ml^{-1}$) in PBS (pH 7.2) on a glass coverslip, and placed in a humidified chamber for 30 min at 20°C. The coverslips were washed with PBS as described above, and the cells fixed with methanol for 5 min. They were stained with Giemsa for 30 min and washed three times with distilled water. The coverslip was turned upside down onto a drop of mounting fluid on a glass microscope slide and the results were read using oil immersion light microscopy (x1000). Phagocytic cells (engulfing more than three bacterium) were counted over the whole slide and the number compared to control fish.

### 5.2.5 Lysozyme assay for rainbow trout and Nile tilapia

Lysozyme activity was measured with the tubidimetric method described by Parry *et al* (1965) using 0.2 mg $ml^{-1}$ lyophilised *Micrococcus lysodekticus* as the substrate in 0.04 M sodium phosphate buffer at pH 5.75. Serum (40 μl) was added to 3 ml of the suspension and the

reduction in absorbance at 540 nm was measured after 0.5 min and 4.5 min at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 $min^{-1}$.

### 5.2.6 Isolation of head kidney macrophages from Nile tilapia

Head kidney macrophages from non-vaccinated nile tilapia were incubated *in vitro* for 2 days with ECP from *Mycobacterium* spp. before assaying their ability to reduce NBT by the method described by Graham and Secombes (1988;1990). Macrophage cell suspensions were prepared from head kidney tissue according to the method of Secombes (1993), by teasing the tissue through a 100 μm nylon gauge into Leibovitz-15 medium (L-15)(Sigma) containing 10 i.u. heparin/ml, 1% penicillin /streptomycin (P/S) and 2% foetal calf serum (FCS). The resulting cell suspension was layered onto a 34%/51% (v/v) Percoll gradient and centrifuged for at 400 x *g* 30 min at 4°C. The leucocytes at the interface were collected and washed with L-15 medium by centrifuging at 400 x *g* for 10 min. The cell pellet was resuspended in L-15 containing 1% P/S and the cell concentration was adjusted to 1.0 x $10^7$ cells $ml^{-1}$. In order to obtain sufficient numbers of macrophages to carry out the test in a single assay, cells from six control fish were pooled. Macrophage monolayers were incubated at 22°C for 2 h before washing the plate three times in L-15 medium to remove non-adherent cells. L-15 medium containing 1% (v/v) pen/strep and 5% (v/v) FCS was added to the wells (100 μl $well^{-1}$). Triplicate wells were set up for each sample and the plates were incubated at 22°C overnight. The following day, the culture medium was removed from the macrophage monolayers and was replaced with culture medium containing 1, 10 or 100 $\mu g ml^{-1}$ ECP (100 μl $well^{-1}$). Triplicate wells were set up for each of the ECPs from the mycobacteria shown in Table 2.2. After culturing the macrophages with the various ECPs for 48h at 22°C, the respiratory burst by the macrophages was assesssed by NBT reduction.

### 5.2.7 NBT assay

Supernatant samples were removed from macrophage monolayers, cultured as described in section 5.2.6. The macrophage monolayers were then incubated with phorbol myristate acetate (1 mg $ml^{-1}$) (PMA) in PBS (Sigma) for 30 min. The reaction was stopped by fixing the cells in methanol. After washing with 70% (v/v) methanol, the wells were air dried and the insoluble formazan was dissolved by adding 120 µl 2M KOH and 140 µl dimethyl sulphoxide (DMSO, Sigma). The contents of each well was carefully mixed and air bubbles were removed with a needle. The absorbance was determined at 610 nm using a plate reader (Dynatech). The number of macrophages $well^{-1}$ was determined by adding of lysis buffer (0.1M citric acid, 1% Tween 20, 0.05% crystal violet) to control wells for 2 min before counting. The number of released nuclei were counted using a haemocytometer. The results were adjusted to give the the absorbance at 610 nm per $10^5$ cells. Samples were compared using analysis of variance.

### 5.2.8 Determination of rainbow trout anti ECP antibody by enzyme-linked immunosorbent assay (ELISA)

The plates were coated with 1 µg $ml^{-1}$ of ECP from TB267 or *M. marinum* (100 µl $well^{-1}$), which had been diluted in coating buffer. Plates were incubated overnight at 4°C, washed three times with low salt wash buffer (LSW) and non-specific binding sites then blocked by adding 250 µl of 3% w/v casein solution (powdered skimmed milk) in distilled water to all the wells. Plates were washed three times with LSW buffer, before adding sera from immunised rainbow trout [100 µl $well^{-1}$ of a 2 fold serial dilution of serum diluted in PBS containing 1% w/v bovine serum albumin (BSA)] to the wells. The ELISA was the continued using the method described in section 3.2.7. Results (OD values) which were three times greater than the background value (i.e. normal fish serum) were considered positive.

**5.2.9 Western blot** Western blot was performed using the method described in section 3.2.6.1.

### 5.2.10. Statistics

The results are presented as means +(S.E.). One way ANOVA and Student's t test were used to calculate P. $P < 0.05$ were considered statistically significant (95% confidence interval).

## 5.3 Results

### Rainbow trout

Non-specific immune responses were determined by measuring NBT, phagocytosis, and lysozyme activity in the blood of rainbow trout. The time course of the appearance of NBT activity in response to ECP antigens of *Mycobacterium* spp. was determind as shown in Figure 5.1. NBT-positive cells peaked 2 weeks post primary immunization. The differences between the immunized fish (TB40 group and TB267 group) and the control fish were significant. Although the level of NBT activity dropped four weeks post-primary immunization, the difference between the TB267 group and the control group was still significant. The level of NBT peaked again at 6 and 10 weeks post-primary immunization.

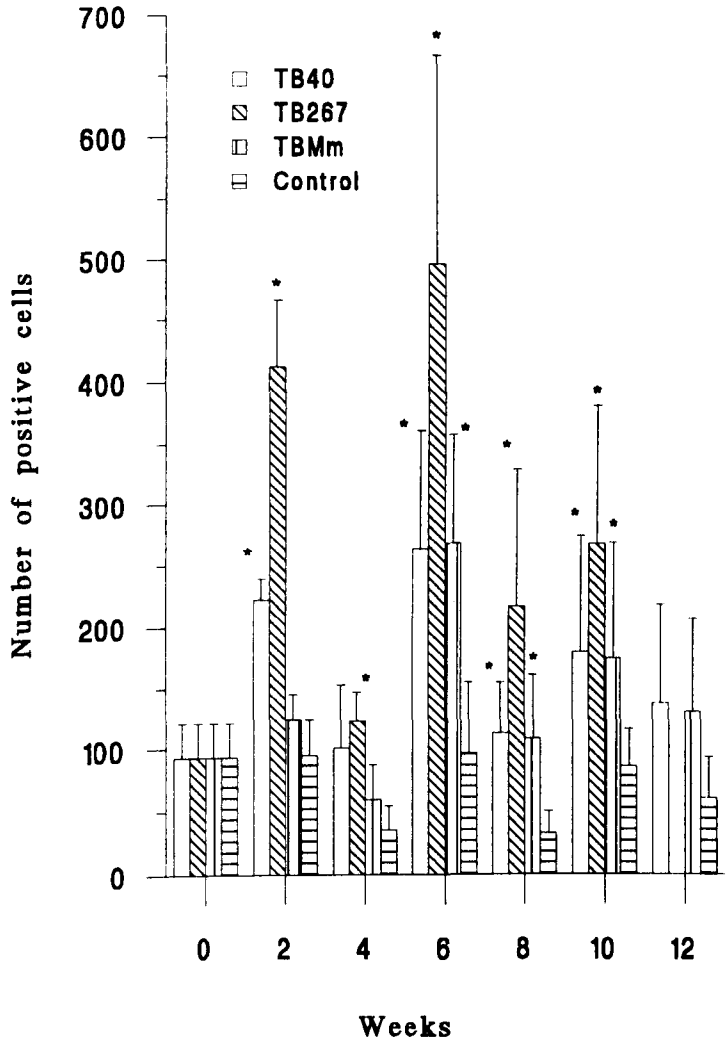


Figure 5.1 Nitroblue tetrazolium positive cells in the serum of rainbow trout following immunization with extracellular products from *Mycobacterium* spp. Results are expressed as mean +SE for 8 fish. (* $p < 0.05$). The data for TB267 at week 12 was not collected

The trend in phagocytic activity was remarkably similar to that of NBT activity (Figure 5.2), while lysozyme followed a slightly different pattern of activity. Lysozyme activity (Figure 5.3) peaked 2 weeks post-primary vaccination, with fish in the the *M. marinum* groups showing significantly higher lysozyme levels than the control group. Activity dropped at 6 weeks, but then increased again at 8 weeks. At 8 weeks, the vaccinated group again showed higher lysozyme activity than control group. A third peak in lysozyme activity was observed at week 10 post-primary immunization. ($p < 0.05$ for the TB267 group and the *M. marinum* group).

The specific immune response was measured by determining antibody titres of vaccinated fish using the indirect antibody capture ELISA. Vaccinated fish displayed a primary immune response which peaked at week 4 (Figure 5.4). The level of the antibody titres in serum from the TB267 group remained constant from week 4 to week 8, while the serum antibody titre of the other two groups gradually increased. The antibody titre of all three groups progressively increased after the booster vaccination at week 8. Western blot analysis showed that rainbow trout serum antibodies from immunised fish, reacted strongly with proteins at 65 and 16 kDa and also with minor antigens at 48, 46,40, and 36 kDa in the ECP of TB267 (Figure 5.5). No protein bands were recognized by control fish serum.

**Nile tilapia**

The non-specific immune response of vaccinated Nile tilapia was assessed by the measuring the activation of neutrophils from the blood and from the swimbladder of fish by the reduction of NBT. Serum lysozyme activity was also examined. The stimulation of blood and

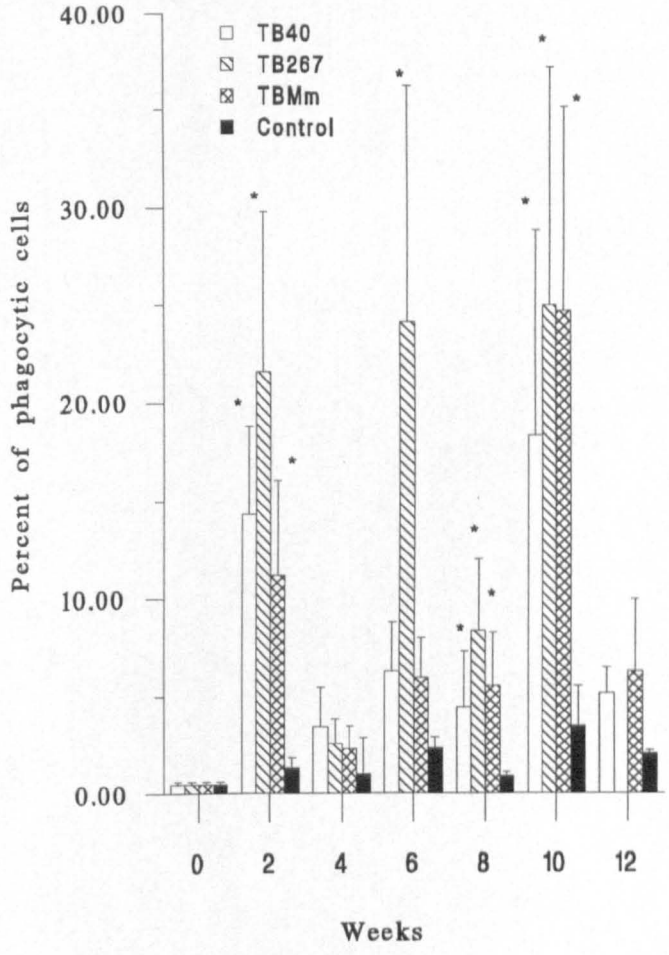


Figure 5.2 Phagocytic cells in the serum of rainbow trout following-immunization with extracellular products from *Mycobacterium* spp. Results are expressed as mean percentage of phagocytic cells which have engulfed three or more bacteria +SE for 8 fish. (* $p < 0.05$). The data for TB267 at week 12 was not collected.

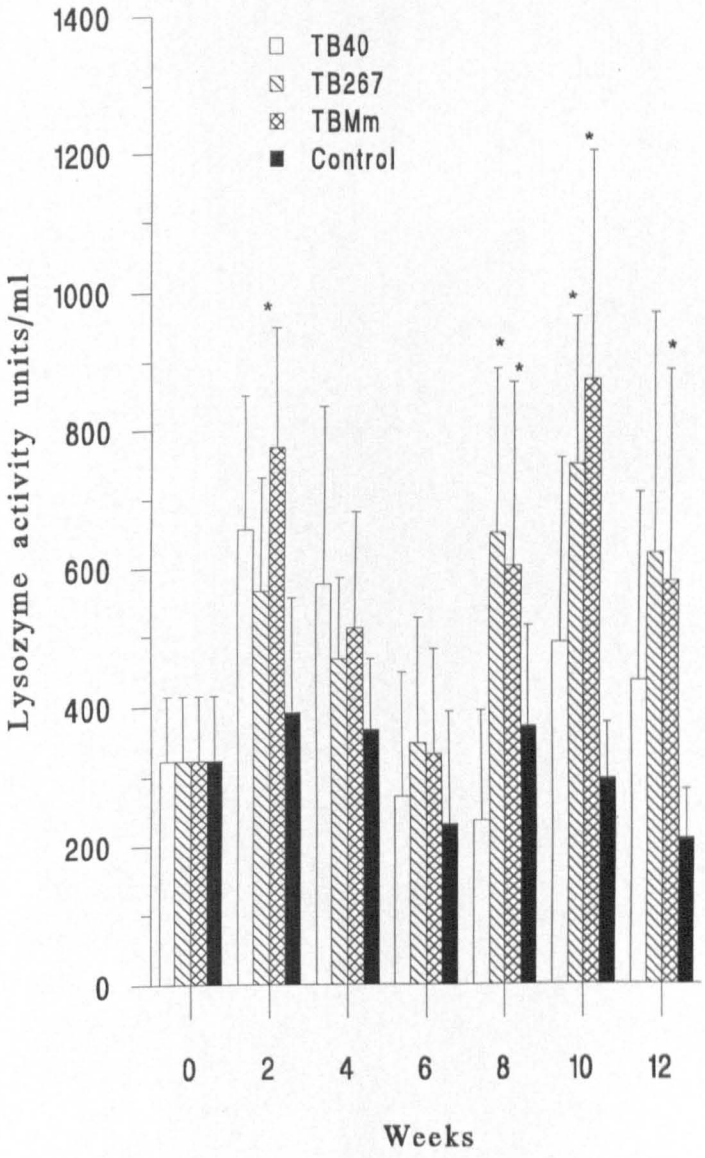


Figure 5.3 Lysozyme activity in the serum of rainbow trout following immunization with extracellular products from *Mycobacterium* spp. Results are expressed as mean lysozyme units/ml +SE for 8 fish. (* $p < 0.05$).

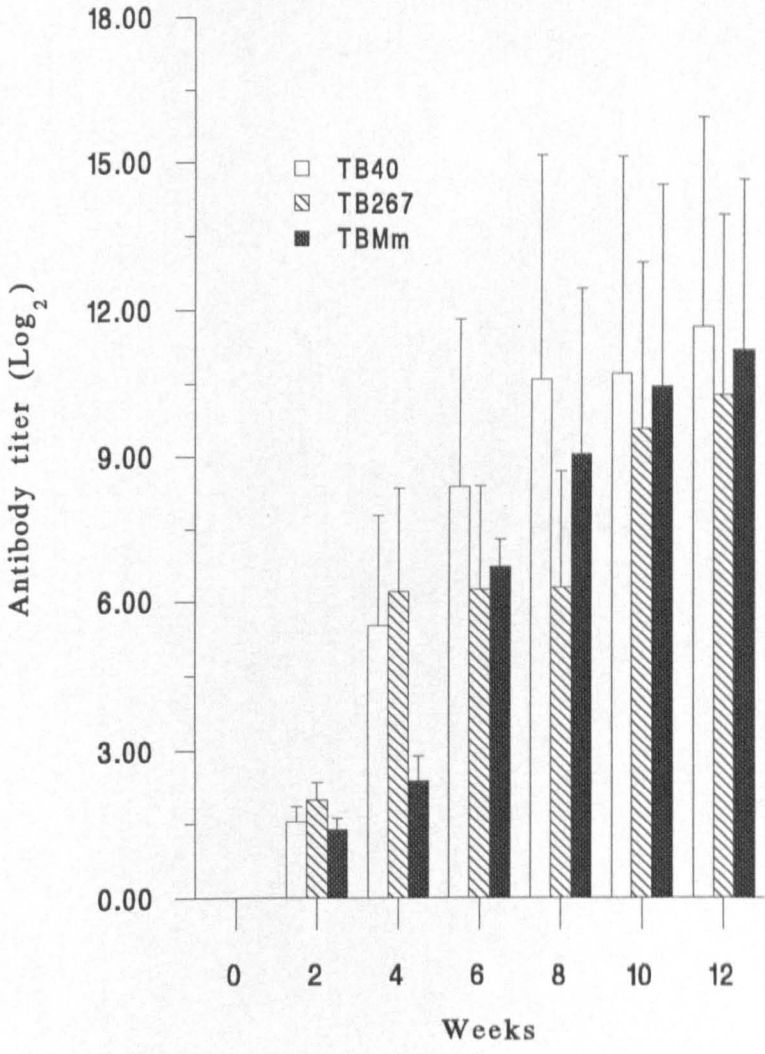


Figure 5.4 Anti-ECP antibody titers for rainbow trout serum following immunization with extracellular products from *Mycobacterium* spp. Results are expressed as mean $Log_2$+SE for 8 fish and have been adjusted to exclude nonspecific antibodes (from control fish) which have been subtracted from all values. Antibody titers from the serum of rainbow trout are higher significant than those of control fish serum.

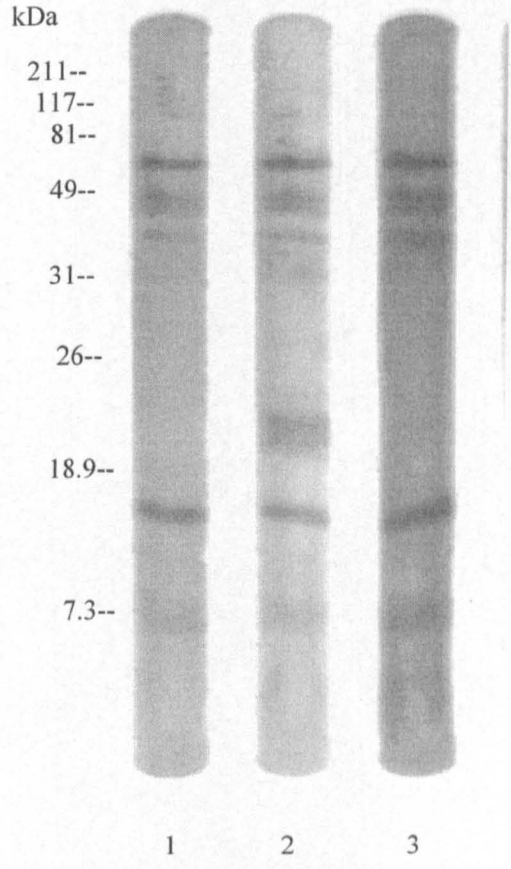


Figure 5.5 Western-blot analysis of serum from rainbow trout immunized with ECP of *Mycobacterium* spp. lane 1. Trout anti-TB40 ECP antibody; lane 2. Trout anti-TB267 ECP antibody; lane 3. Trout anti-*M. marinum* ECP antibody. TB267 ECP antigens were subjected to SDS-PAGE, electrotransferred onto nitrocellulose paper, and incubated with rainbow trout serum, anti-trout Immunoglobulin monoclonal antibody and finally goat anti-mouse IgG-HRP

swimbladder neutrophils by the ECP of the various *Mycobacterium* spp. or by the adjuvants, determined by NBT-staining of the cells is shown in Figure 5.6 and Figure 5.7 respectively. NBT-positive cells increased both in the blood and in the swimbladder of the fish four days after administering the various ECP and adjuvants, compared to the PBS injected control fish. This was with the exception of neutrophils from the blood of fish injected with FCA and FIA. By the eighth day, fewer NBT-positive cells were found in the blood and the swimbladder of fish immunised with ECP from mycobacteria strains TB40 and TB267, than in the other five groups of fish.

The level of lysozyme activity four days after immunisation of the fish, was found to be significantly higher in the serum of fish immunised with ECP from the different mycobacteria than was found in the serum of control fish (205 vs 135 unit $ml^{-1}$) (Figure 5.8). At day 8, the lysozyme activity of fish immunised with TB40 or FIA was still greater than levels observed in the control fish.

The ability of ECP, from a variety of mycobacteria ( see Table 2.2), to stimulate head kidney macrophages *in vitro* is shown in Figure 5.9. The reduction of NBT by the head kidney macrophages was used as a measure of cell activation. Supplementing ECP into the culture medium at 1 $\mu gml^{-1}$ resulted in a significant increase in NBT reduction by the macrophages. This was found to be the case with all of the mycobacteria strains examined. However, increasing the ECP concentration to 10 or 100 $\mu gml^{-1}$ caused a suppression of this activity.

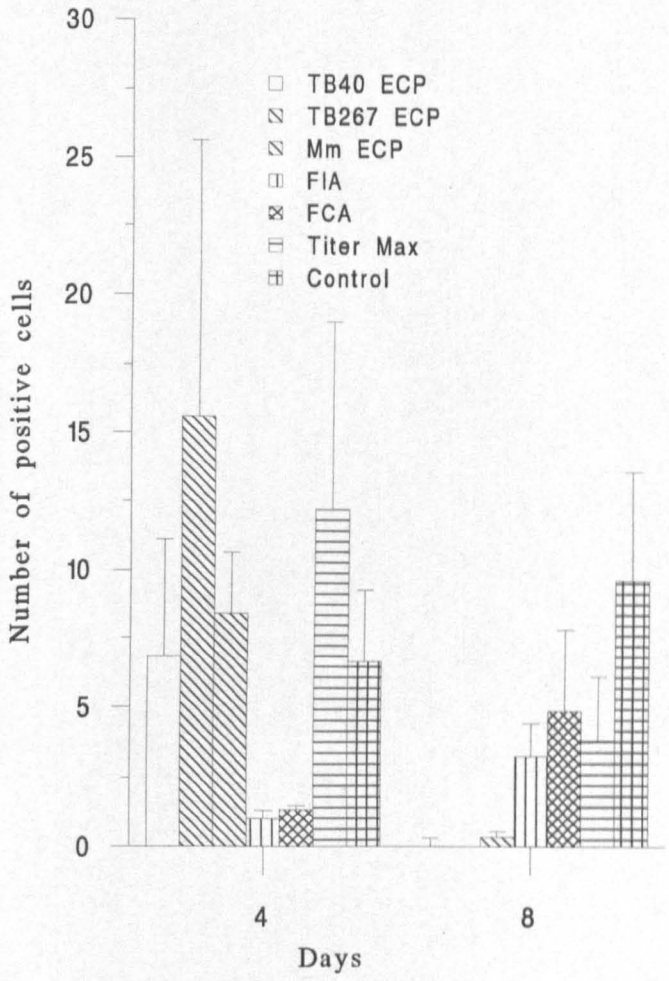


Figure 5.6 The number of NBT-positive cells in the peripheral blood of nile tilapia following injection with ECP from mycobacteria or adjuvants into their swimbladders. Results are expressed as the mean number +SE for four fish. Control fish were injected with phosphate buffered saline

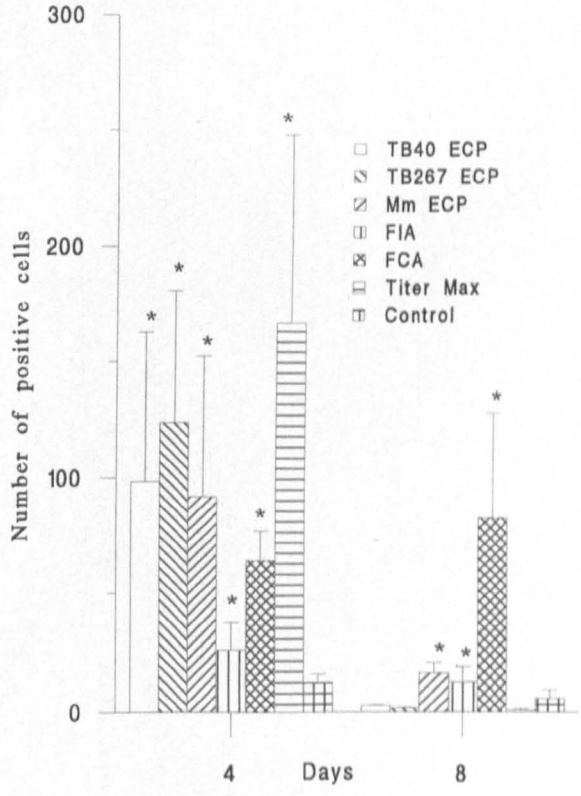


Figure 5.7 The number of NBT-positive cells in the swimbladder of nile tilapia. following injection of ECP from mycobacteria or adjuvants into their swimbladders. Results are expressed as the mean number +SE for four fish. Control fish were injected with phosphate buffered saline. (*P<0.05).

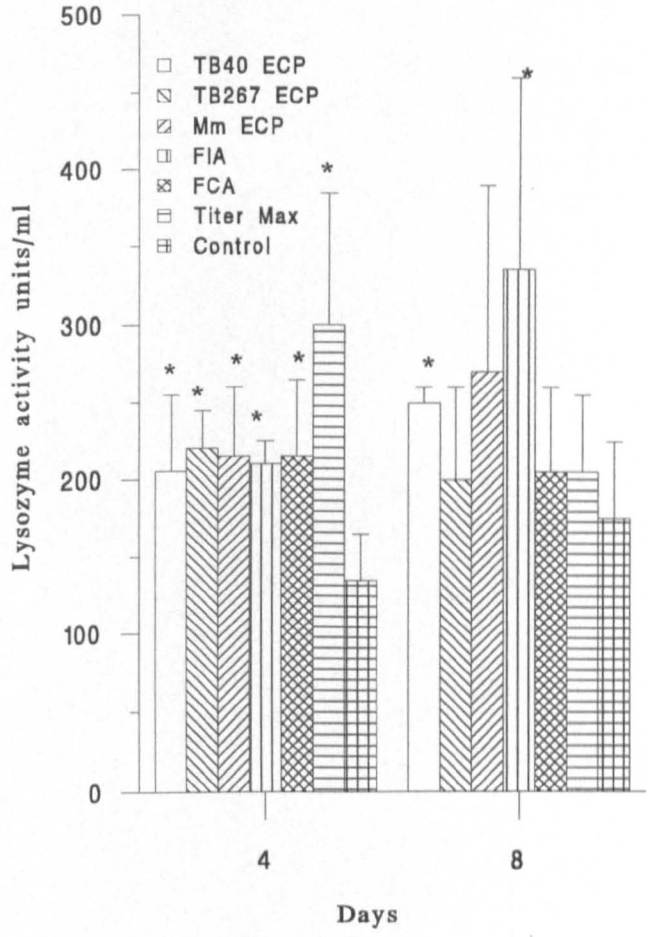


Figure 5.8 The serum lysozyme activity of nile tilapia following injection with ECP from mycobacteria or adjuvants injected into their swimbladders. Results are expressed as the mean units $ml^{-1}$ +SE for four fish. Control fish were injected with phosphate buffered saline. (*$P<0.05$).

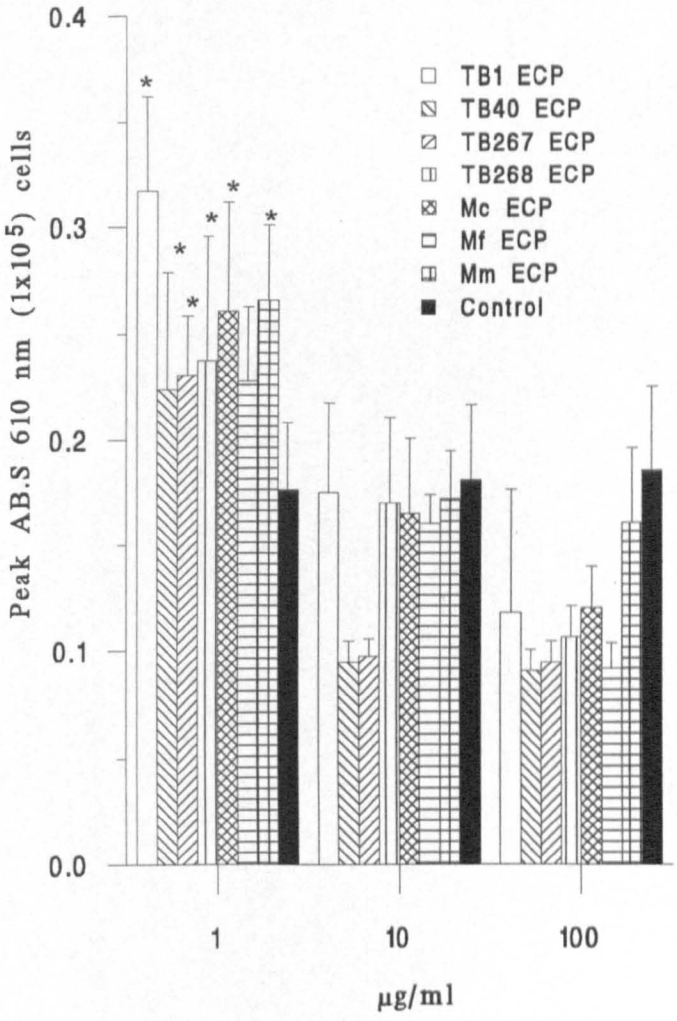


Figure 5.9 NBT reduction by head kidney macrophages from nile tilapia. The cells were cultured *in vitro* with ECP from an array of *Mycobacterium* spp. Results are expressed as the mean absorbance at 610nm for triplicate samples+SE for four fish. (*P<0.05)

## 5.4 Discussion

Documented reports of non-specific assays have demonstrated elevated levels of macrophage and monocyte function, such as phagocytosis and chemotaxis (Weeks and Warinner, 1986). Another important non-specific defense mechanism referred to involves peripheral blood neutrophils. It has been shown that these cells become more adherent to tissue cell surfaces by the production of adhesion proteins, facilitating their migration from the capillaries to the sites of injury (Kishimoto *et al.*, 1989; Magnuson *et al.*, 1989). In addition, they exhibit heightened production of oxygen radicals ($O^{-}_{2}$, $OH^{-}$) released during the oxidative burst process. These reactive species are then capable of destroying the invading pathogens (Hassett and Cohen, 1989).

It has been reported that neutrophil activity in juvenile rainbow trout increases on stimulation with the *Yersinia ruckeri* O-antigen bacterin (Anderson, Moritamo and Grooth, 1992). Levels of reactive oxygen species in the cytoplasm of cells can be determined using the differential stain, NBT. In the presence of the oxygen radicals, the soluble dye changes from yellow to the insoluble dark blues of formazan (Anderson *et al.*, 1992). The attachment of neutrophils to glass and their staining with NBT were used to determine the effectiveness of the immunization regime. The results reported in this chapter show that NBT positive cells appear to peak every second week both in experimental and control rainbow trout. This may be a physiological phenomena in rainbow trout, with NBT cells having a 4 week cycle. Nevertheless, a significant difference between immunized and control group was observed. The phagocytic activity of neutrophils in rainbow trout appeared to follow the same cyclic trend as NBT activity, with peaks at 2, 6, and 10 wks post-primary immunization.

Lysozyme activity was also determined in the present study, and the results showed that immunizing rainbow trout with ECP from *Mycobacterium* spp. stimulated the release of lysozyme into the serum of the fish. This elevation is not likely to be caused by the resting levels of lysosomal enzymes, but is more likely to be due to firstly, increased amounts of neutrophils or macrophages and secondly, increased synthesis and release of lysozyme, as observed with mice treated with glucans (Di Luzio *et al.*, 1979). Engstad, Robertsen and Frivold, (1992) suggested that observed increases in serum lysozyme levels in fish may be induced by glucans associated with the infecting bacteria.

Lysozyme plays a role in the non-specific defense against pathogens. It splits β (1-4) linked N acetyl-D- glucosaime, N-acetyl-muramic acid peptidoglycan in bacterial cell walls, especially in Gram positive bacteria. This usually results in rupture of the bacterial plasma membrane due to osmotic stress and is followed by cell death. Lysozyme has been detected in the blood, mucus and organs of various fish and it is known that increases in lysozyme concentration in fish can be caused by infection or exposure of foreign materials (Fletcher and White, 1973, Studnicka, Siwicki and Ryka, 1986). Non-specific antimicrobial agents such as lysozyme, may be more important in fish than in the mammals, since the former apparently has a less developed specific immune system (Ingram, 1980).

The ECP from *Mycobacterium* spp. induced high antibody titers (TB40, TB267, and *M. marinum*) in the vaccinated fish, especially in the TB40 and *M. marinum* immunized fish. It is interesting to note that the antibody titre of the TB267 group was lower than the level in fish from the TB40 or *M. marinum* groups, but gave the highest number of NBT positive cells and the highest phagocytic activity. This possibly reflects the ability of fish to coordinate humoral

and cell-mediated immune responses to combat pathogen infections and immunization. The reaction pattern of the immunized rainbow trout sera in the Western blot were similar to those obtained with sera from a rabbit and a mouse immunized with ECP from *Mycobacterium* spp.

**Nile tilapia**

It was reported in the present chapter that NBT-positive cells significantly increased in number in the blood and in the swimbladder of fish four days after they were injected with ECP from mycobacteria. It is interesting to note that the number of NBT positive cells in the blood in the immunised group were less than those of the control group at day 8. It may be that the neutrophils migrate from the blood into the swimbladder of the immunised fish. Serum lysozyme activity detected four and eight days after immunisation had also significantly increased compared to control fish.

The ECP from *Mycobacterium* spp and adjuvants, all appear to be good immunogens for nile tilapia, inducing an increased NBT and lysozyme response.

Culturing tilapia macrophages *in vitro* with media supplemented with low levels of *Mycobacterium* ECP (1 $\mu gml^{-1}$) enhanced the macrophages ability to reduce NBT. Rainbow trout head kidney macrophages similarly exhibited an enhanced NBT reduction when cultured *in vitro* with media supplemented with 1 $\mu gml^{-1}$ *Mycobacterium* ECP (data not shown). Increasing these levels, however, had an immunosuppressive effect on the macrophages. The outer membrane protein of *Aeromonas salmonicida* (10 or 100$\mu g\ ml^{-1}$) has also been reported to produce a stimulatory affect on the respiratory burst of head kidney macrophages from Atlantic salmon, as measured by NBT reduction (Francis and Ellis, 1994).

## Chapter 6

## Electron microscopy studies to investigate the phagocytosis of *Mycobacterium* spp.

### 6.1 Introduction

Mycobacterial infections induce systemic granulomas, especially in the kidney and spleen of infected fish (Chinabut *et al.*, 1990; Noga *et al.*, 1989, 1990). The phagocytic and bactericidal activity of the macrophage have been extensively studied in mammals. Phagocytosis, although a non-specific defense mechanism, is regarded as an important initial step in the triggering of the specific immune response in fish (Clem *et al.*, 1985). It has been shown that opsonisation, or coating of bacteria by specific antibodies, makes the bacteria more susceptible to engulfment (Griffin, 1983; Sakai, 1984). Little information is available, however, on the effect that immunization has on phagocytosis, and on the intracellular killing of bacteria by fish macrophages, or on the role of antibody and complement in the opsonisation of bacteria.

One of the major organs involved in the immune defense of fish is the head kidney, which is particularly rich in macrophages (Secombes, 1993). Phagocytosis of most organisms is normally followed by the rapid fusion of lysosomes and phagosomes within the macrophage to form phago-lysosomes. No literature is currently available to describe the interaction between *Mycobacterium* sp. and fish macrophages. In view of this, macrophages from the head kidney of rainbow trout were used as a model to study the pathogenesis of mycobacteria at a cellular level. Prelabelling dense granules, or secondary

lysosome, with ferritin allows the identification, occurrence and the frequency of phagosome-lysosome fusion to be investigated (Armstrong and Hart, 1971).

## 6.2 Materials and Methods

### 6.2.1 Fish

Rainbow trout, ranging from 300 to 900 g, were obtained from a local fish farm. They were maintained in 150 l tanks, which were supplied with aerated flow-through dechlorinated water. The water temperature during the experimental period was 15±2°C. and the fish were fed with a commercial feed. Rainbow trout weighing 300 to 400 g were used for macrophage isolations, while trout weighing 800 to 900 g were used to produce antisera against *Mycobacterium* (as described in section 3.2.4.4).

### 6.2.2 Rainbow trout serum preparation and macrophage isolation

Fish were anaesthetised with MS 222 and bled by syringe from the caudal vein. Blood was allowed to clot at room temperature for 30 mins, centrifuged at 800 *x* g for 10 mins and serum collected as rainbow trout fresh serum (FS). To inactivate the complement, FS was incubated at 48°C for 20 min to produce rainbow trout heat inactivated serum (HIS). Both sera were used immediately. Briefly, macrophages were isolated on 51%/34% Percoll gradients and macrophage monolayers prepared (as described in section 5.26). The cell number was adjusted to $1.0 \times 10^7$ $ml^{-1}$ in Lebovitz medium (L-15)(Sigma) containing 1% (v/v) penicillin/streptomycin and 0.1% (v/v) foetal calf serum (FCS). The cell preparation was used immediately.

### 6.2.3 ECP preparation

ECPs from mycobacteria were cultured for 12 days at 28°C in modified Sauton's medium, then raised from 28°C to 37°C for 48 h (as described in section 3.2.1).

### 6.2.4 Fish immunisation

Rainbow trout were immunised as described in section 5.2.2a.

### 6.2.5. Monoclonal antibody (MAbs) production

MAbs were produced as described in section 4.2.4.

### 6.2.6. Ascites production

Six male BALB/C mice ranging from 6-8 weeks were injected IP with 0.5 ml of pristane. After 7 days, the mice were injected IP with 0.5 ml of hybridoma suspension ($3x10^6$ cells in PBS). Ascites developed 10-14 days later and was drained from the peritoneal cavity of the mouse with a 19 gauge needle after having first anaesthetised the animal. The ascitic fluid was centrifuged at 400 x g for 10mins at 4°C and supernatants collected and stored at -70°C until used.

### 6.2.7 Opsonisation of bacteria

Bacteria ($2 x10^9$ bacteria $ml^{-1}$) were pre-treated with different preparations of opsonins; either 1 ml of PBS; 0.5 ml of PBS containing 20 % (v/v) fresh serum from rainbow trout (FS); 1ml 20% (v/v) fresh serum from rainbow trout which had been heat inactivated (HIS) and diluted in PBS; rainbow trout antisera against the ECP from TB267 or *M.*

*marinum* diluted five times with PBS (Ab); or ascites fluid containing MAb against the 65 kDa protein of TB267 (cell line 4B9) diluted five times with PBS. The bacteria were incubated with the different preparations for 1 h at 18°C, after which they were washed three times with PBS and resuspended in 0.5 ml PBS.

### 6.2.8 Phagocytic assay

The concentration of macrophages was adjusted to $1x10^7$ cells $ml^{-1}$ in L-15 with additives (see in section 6.2.2). One ml aliquots of the macrophage suspension were placed in polystyrene culture tubes and 100 µl of opsonised bacteria ($2x10^9$ cells $ml^{-1}$) were added and gently mixed. The tubes were incubated for either 0.5, 1, 2, 4, or 6 h at 18°C with intermittent shaking. The macrophages were then rinsed with cacodylate solution by centrifuging the tubes at 400 x *g* for 7 min at 4°C. The resulting pellets were fixed with 2% (v/v) glutaraldehyde diluted in cacodylate buffer for 1 h and the tubes were centrifuged again. The pellets were dehydrated with acetone, before embedding them in Araldite CY212 (Agar Scientific LTD). Thin sections of the pellets were prepared and stained with uranyl acetate and lead citrate. These were observed with a Philips EM301 electron microscope.

### 6.2.9 Ferritin labelling

Macrophage monolayers were prepared by adhering the cells to coverslips for 24 h in order to induce an abundance of lysosomes. The coverslips were washed three times with PBS, then covered with 0.5 ml L-15 medium containing 5% FCS and ferritin at a final concentration of 20 mg/ml and incubated for 3 h at 16°C. The coverslips were again

washed three times with L-15 medium to remove free ferritin from the cell layers, then further L-15 medium containing 5% FCS was added to the coverslips which were incubated for a further 3 h to allow the ingestion of ferritin to be completed. Macrophages were then incubated with TB267 for 2 and 4 hr at 18°C. Cells were harvested using a pasteur pipette with cold L15 medium and the cell suspensions were collected by centrifugation at 400 x *g* for 7 min at 4°C. The pellets were fixed and examined by EM, as described previously. The technique of labelling macrophage lysosomes with electron dense ferritin allowed lysosomal fusion to be examined by EM because of the presence of the dark electron dense labelling of the phagolysosome.

### 6.2.10 Assessment of damage

The organisms were considered to be damaged if there was any appearance of abnormality such as breaks in cell wall or membrane disorganization of cytoplasm or disorganization of the nuclear region. Myelin figure formation involves the bacterial cell wall and its surrounding electron transparent zone. Bacteria that were not considered damaged were classified as intact or obscure cases as doubtful.

### 6.2.11 Assessment of fusion

Lysosomal fusion was identified by the presence a dark electron dense label within the phagolysosome.

### 6.2.12 Examination of infected fish tissue

Tissue sections used to examine macrophage ultra-structure by EM were cut into small pieces (1 $mm^3$) and immersed in karnovsky fixative overnight at 4°C. These were processed for EM using a standard protocol (Appendix 1.).

## 6.3 Results

Ultra-thin sections of macrophages incubated with the pre-opsonised bacteria were examined by EM. *Mycobacterium* strains TB267 and *M. marinum* were used in the study. The basic features of structurally intact bacteria after phagocytosis by rainbow trout head kidney macrophages are illustrated in Figure 6.1 and 6.2. These are characterised by low electron density of the inner most region of the bacteria surrounded by a highly stained bacterial cytoplasm. The latter was rich in ribosomes and encircled by a bacterial plasma membrane. Outside this membrane a cell wall complex was apparent, the outer edge of which appears to be continuous with a distinctive electron transparent capsule. The capsule is a regular feature of the bacteria, but is variable in thickness and its outer surface appears to be in direct contact with the membrane of the phagosome wall. The shape of macrophages varies as does the number of indentations they possess. At some times are monopodic (Figure 6.3), while others are multipodic (Figure 6.4) with natatory appendices gathering together to form a foot structure. Macrophages were characterised by their abundant cytoplasm, eccentric nucleoli, large golgi region, numerous mitochondria and moderately abundant rough endoplasmic reticulum. The extensive cytoplasm of the macrophages contain a full complement of organelles associated with the synthesis and export of proteins.

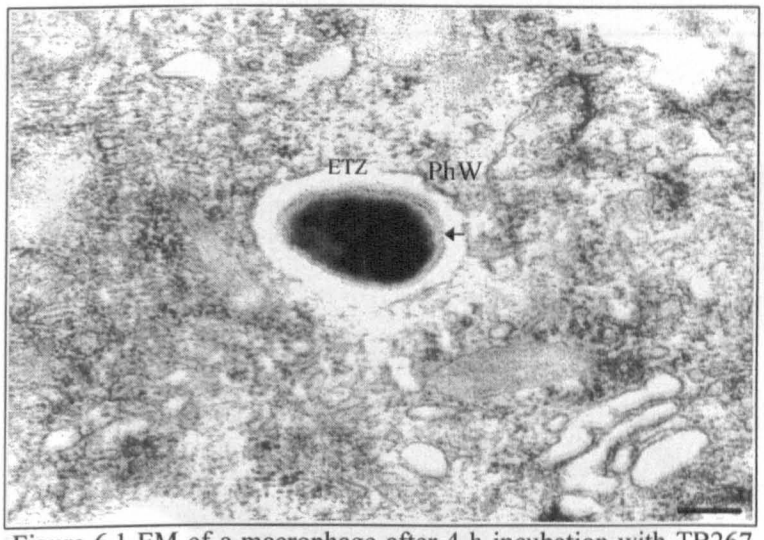


Figure 6.1 EM of a macrophage after 4 h incubation with TB267 (preincubated with fresh serum). An intracytoplasmic phagosome containing intact bacterium after ingestion is observed, illustrating some of the details of bacterial morphology. The organism is bounded by a typical plasma membrane (arrow) and a morphological cell wall. An irregular electron-transparent zone (ETZ) separates each organism from the phagosome wall (PhW). X 59,000. Bar=0.17 μm

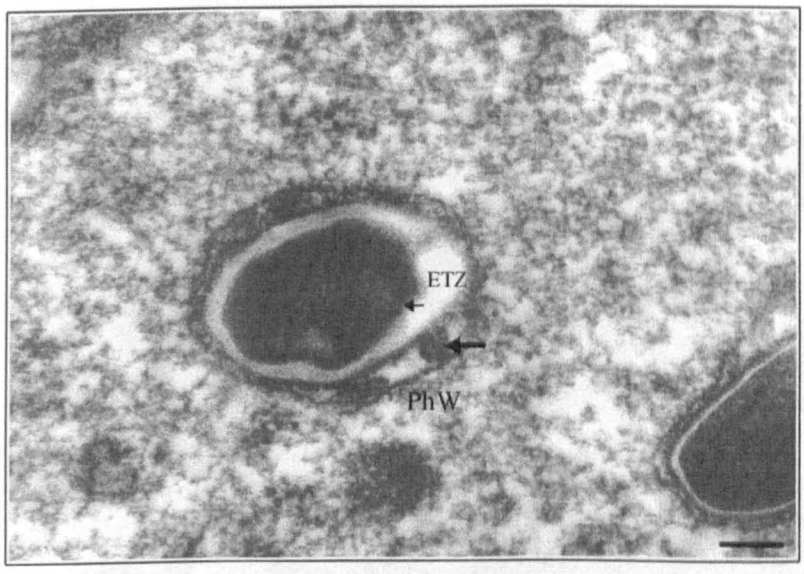


Figure 6.2 EM of a macrophage after 4 h incubation with *M. marinum* (1298) (preincubated with fresh serum). An intracytoplasmic phagosome containing intact *M marinum* after ingestion is observed as in Figure 6.1 Lysosomes (large arrow) are present around the phagosome but have not fused with it. X 59,000. Bar=0.17 μm

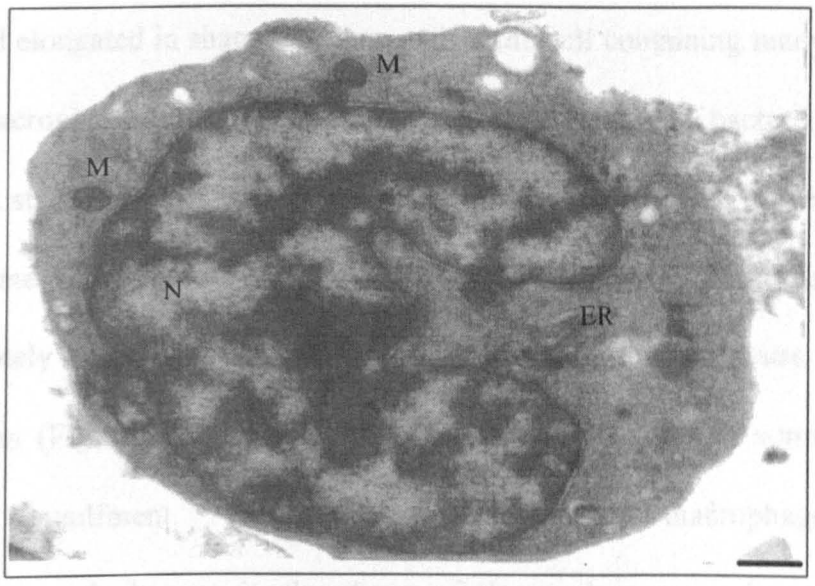


Figure 6.3 EM of a macrophage showing the nucleus (N), granules, endoplasmic reticulum (ER) and mitochondria (M). X 9,800. Bar=1.0 μm

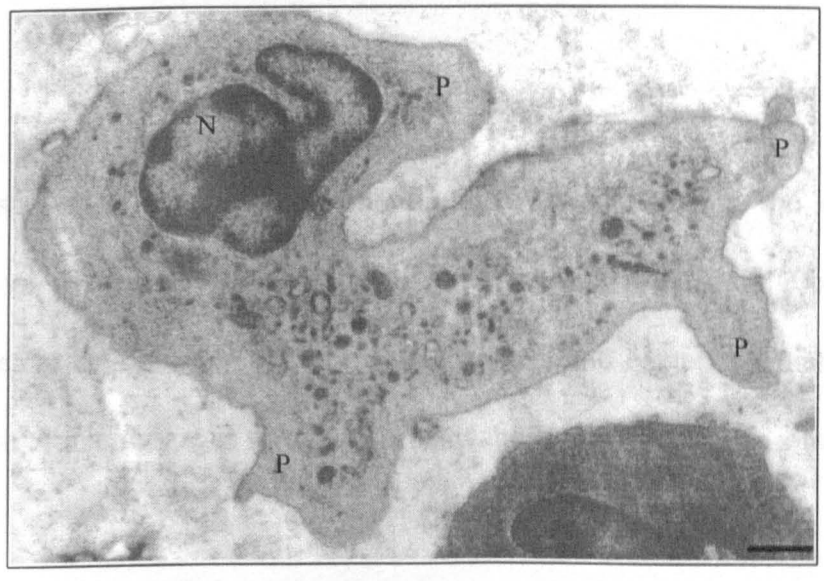


Figure 6.4 The spreading and changing macrophage's plasma membranes show an indented outline which is multipodic (P), the natatory appendices being gathered into a sort of pesudopodia. One pole of the cell contains the nucleus (N), while the other pole contains many mitochondria and lysosomes. X 9,800. Bar=1.0 μm

Macrophages incubated for 0.5 h with TB267 or *M. marinum*, pre-treated with MAb, appeared elongated in shape with one end of the cell containing many more mitochondria. Some macrophages appeared capable of phagocytosing many bacteria, all of which were at different stages of ingestion. Some of the phagocytosed bacteria were surrounded by the phagosome wall, while others were attached to or fused with the plasma membrane. Immediately adjacent to these attached bacteria are several dense granules resembling lysosomes (Figure 6.5). The morphology of the macrophage sometimes changed after bacterial engulfment. Figure 6.6a, and 6.6b show macrophages with a flattened appearance and changes in the shape of the nucleus were dependent on the plasma membrane morphology (Figure 6.7). The membrane of the phagosomes was either in close contact with bacteria or else there was a wide space between the bacteria and the phagosomal wall (Figure 6.8).

Distinct stages of phagosome formation were evident during the early stages of the ingestion of *M. marinum*, with the bacteria eventually becoming completely enclosed within membrane-bound vacuoles or phagosomes, and bacteria, which adhered tightly to the surface of macrophages showed more advanced stages of phagosome formation.

Phagocytosis appears to involve a two stage process. Firstly, there is the extension of macrophage pseudopodia (chopstick-like) (Figure 6.8) and secondly these surround the organism at the distal side and displace the cytoplasm at the region of contact between the macrophage and bacterium so that the bacterium appears to sink into the macrophage.

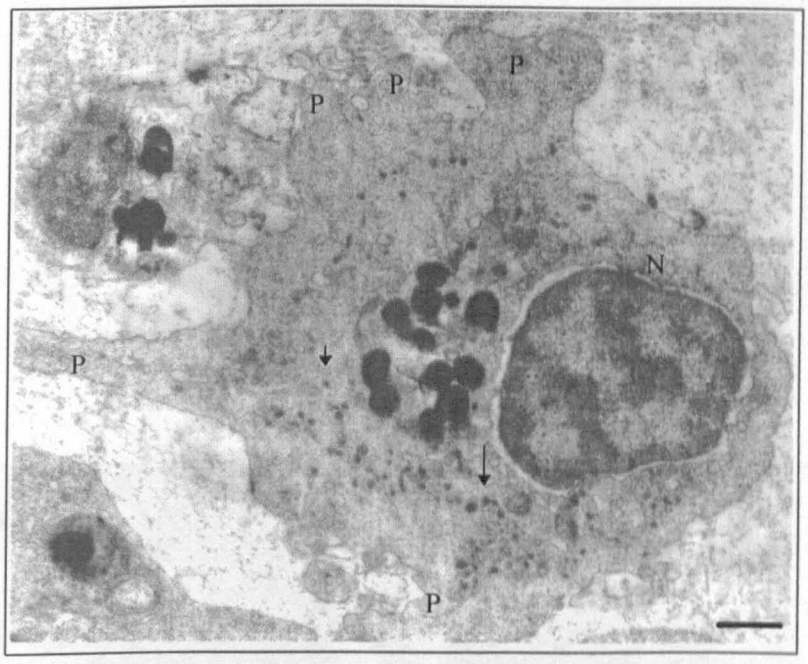


Figure 6.5 EM of a macrophage after 30 min incubation with TB267. The spread and changing plasma membrane shows a much indented outline, multipodic (P), the natatory appendices being gathered as a sort of foot. One pole of cell contain the nucleus (N), when the other pole contains many mitochondria and lysosome (arrow). Some bacteria have been engulfed by the macrophage. X 9,800. Bar=1 μm

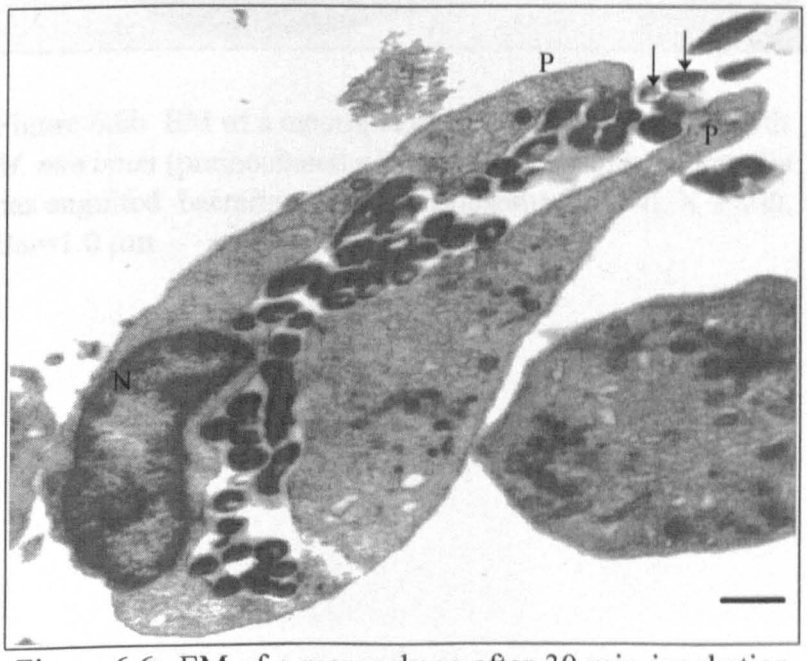


Figure 6.6a EM of a macrophage after 30 min incubation with *M. marinum* (preincubated with PBS) showing an elongated shape. Bacteria are being engulfed by two pseudopodia (P). X 5,900. Bar=1.7 μm

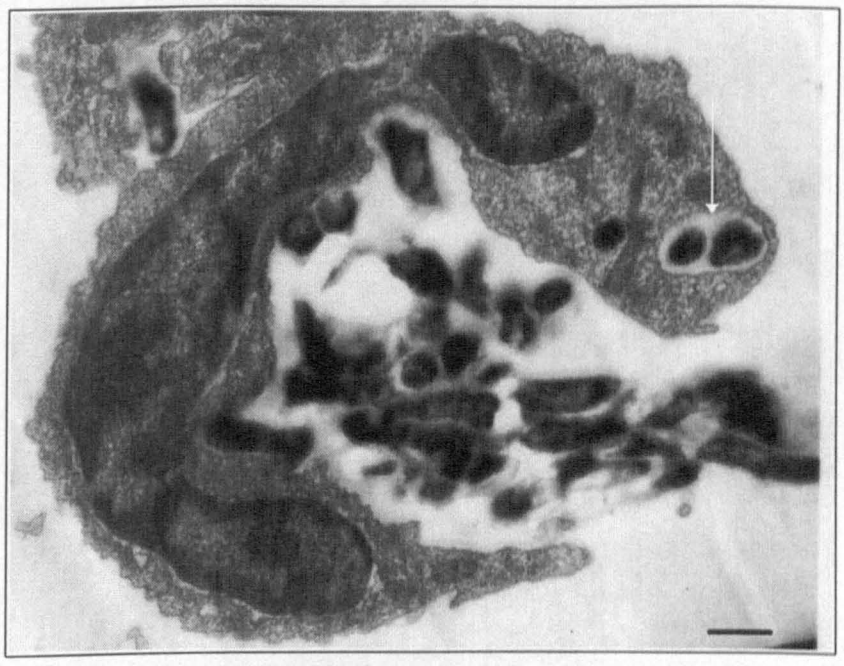

Figure 6.6b EM of a macrophage after 0.5 h incubated with *M. marinum* (preincubated with PBS). The cell pseudopodia has engulfed bacteria and one phagosome (arrow). X 9,800. Bar=1.0 μm

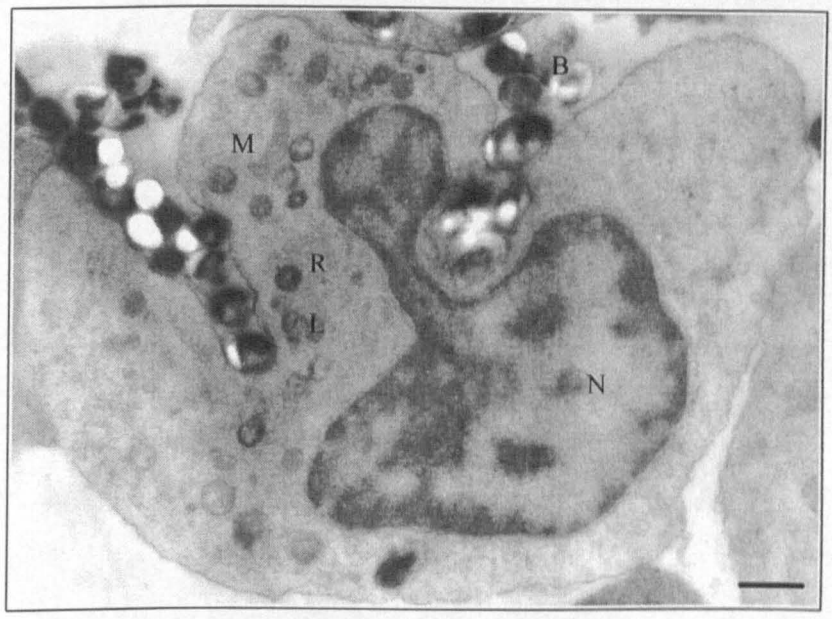


Figure 6.7 EM of a macrophage after 30 min incubation with TB267 (preincubated with MAb) showing an elongated shape. One pole of the cell contains the nucleus (N) , while the other pole contains many mitochondria (M) and lysosomes. The spreading and changing plasma membranes are engulfing the bacteria (B). The changing nucleus morphology is dependent on plasma membrane morphology. X 9,800. Bar=1 μm

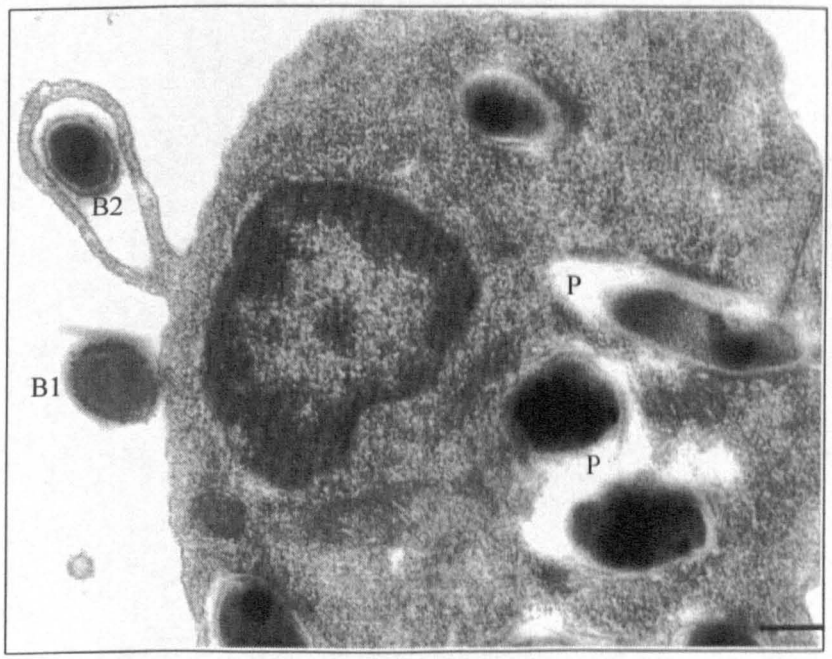


Figure 6.8 EM of a macrophage after 1 h incubation with *M. marinum*, (preincubated with fish fresh serum). Bacterium (B1) has attached to the plasma membrane of the macrophage. Bacterium (B2) has been engulfed by two tiny pseudopodia. Some bacteria have also been engulfed in the phagosome (P). X 22,000. Bar=0.45 μm

After 0.5 h incubation, the majority of engulfed bacteria (both TB267 and *M. marinum*) as well as all extracellular bacteria appeared morphologically normal. The number of engulfed bacteria was usually around three to five bacteria per macrophage. The percentage of macrophages which phagocytosed bacteria seemed to be greater after pre-treating the bacteria with FS, Ab or MAb (46%, 53.5%, 41.1% for TB267 and 43.6%, 41.3%, 36% for *M. marinum*, respectively) than bacteria pre-treated with PBS or HIS (26.3%, 25.6% for TB267 and 24.3%, 25.7% for *M. marinum*, respectively) (Table 6.1). Phagosomes were observed to vary in size containing both intact and damaged bacteria. Usually the phagosome contained either one or two bacteria per phagosome (Table 6.2), but larger phagosomes contained several bacteria. Bacteria were never observed to be free in the cytoplasm after 0.5 h incubation. After 1 h incubation with TB267, macrophages were found to contain between 5 and 10 bacteria per cell (Figure 6.9). A few of the macrophages contained between 50 to 60 bacteria and the phagosomes varied greatly in size between the macrophages. There was a slight increase in the percentage of phagocytic cells (Table 6.1) and the number of phagosomes at this incubation time (Table 6.2) compared with 0.5 h, especially with FS, Ab and MAb-treated bacteria. Macrophages cultured for 2 h with *M. marinum* showed even more engulfed bacteria per macrophage (10-20) than cells cultured for a shorter time. Some of the mycobacteria-laden macrophages had extensive vacuolation and contained very few organelles (Figure 6.10). The macrophages contained large numbers of phagosomes, greater than was found within macrophages incubated for 1 h, and again these varied in size and in bacteria content.

Figure 6.1. Percentage of rainbow trout macrophage phagocytic cells after different incubation times with *Mycobacterium* spp.

| Strains | Incubation time (h) | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 4 | 6 |
| 267+PBS | 26.3±6.1 | 26.3±3.8 | 28.0±6.2 | 36.0±3.0 | 36.5±1.9 |
| 267+FS | 46.0±4.0 | 50.6±8.1 | 41.5±5.0 | 52.0±8.0 | 50.2±2.6 |
| 267+HIS | 25.6±7.3 | 33.3±10.0 | 30.5±9.1 | 37.0±6.0 | 36.3±2.5 |
| 267+267 Ab | 53.5±3.5 | 54.6±5.9 | 54.8±5.7 | 63.0±5.0 | 55.5±5.1 |
| 267+65K MAb | 41.1±3.2 | 43.3±6.5 | 42.6±12.7 | 45.0±8.0 | 41.7±4.0 |
| Mm + PBS | 24.3±5.5 | 21.6±2.1 | 24.0±3.5 | 20.0+5.3 | 23.6±3.1 |
| Mm+FS | 43.6±10.0 | 43.0±8.9 | 40.2±10.0 | 41.3±2.6 | 46.5±4.9 |
| Mm+HIS | 25.7±5.5 | 27.3±1.8 | 23.8±1.2 | 28.0±2.5 | 22.9±1.6 |
| Mm+Mm Ab | 41.3±10.0 | 48.3±11.0 | 45.5±15.2 | 54.1±2.5 | 42.4±1.2 |
| Mm+65K MAb | 36.0±4.0 | 39.0±3.0 | 39.3±2.0 | 39.0±7.1 | 39.3±6.0 |

(Data are from three samples)

* Percentage= No. of macrophage phagocytosed bacteria / 100 macrophages

PBS: Phosphate buffered saline

FS: Rainbow trout fresh serum

HIS: Rainbow trout heat inactivated serum

Ab: Rainbow trout anti-267 or *M. marinum* NCIMB 1298 ECP antibody

65K MAb: Mouse anti-TB267 ECP-65 kDa protein monoclonal antibody ascites

Mm: *M. marinum* NCIMB 1298

Table 6.2. Number of phagosomes produced by rainbow trout macrophage after different incubation times

| Strain | Incubation time (h) | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 4 | 6 |
| 267+PBS | 1.4±0.7 | 1.9±1.0 | 2.6±2.0 | 2.5±1.3 | 3.0±2.1 |
| 267+FS | 2.0±1.1 | 2.6±1.1 | 2.6±1.6 | 4.0±2.4 | 3.9±1.2 |
| 267+HIS | 1.7±0.9 | 2.0±1.1 | 3.0±2.4 | 2.3±1.5 | 2.5±0.6 |
| 267+267 Ab | 1.9±0.8 | 3.4±1.9 | 3.2±1.9 | 2.9±2.0 | 3.1±1.3 |
| 267+65K MAb | 2.4±1.2 | 2.9±2.0 | 4.0±2.6 | 3.6±1.4 | 3.7+0.6 |
| Mm+PBS | 1.4±0.8 | 1.5+0.7 | 3.4±2.6 | 2.5+1.5 | 2.8+1.1 |
| Mm+FS | 1.9±1.1 | 2.9±1.8 | 4.1±1.5 | 4.0+1.5 | 4.9±2.4 |
| Mm+HIS | 1.6±0.7 | 2.3±1.3 | 2.3±1.2 | 2.3±1.4 | 2.6±1.8 |
| Mm+ Mm Ab | 2.7±1.3 | 3.0±1.4 | 3.6±1.8 | 3.9±2.0 | 3.3±1.2 |
| Mm+65K MAb | 2.5±1.5 | 3.1±1.7 | 3.6±1.6 | 3.8±1.5 | 3.2+1.3 |

(Data are from three samples)

*Counted 50 phagocytosed cells for each sample

PBS: Phosphate buffered saline

FS: Rainbow trout fresh serum

HIS: Rainbow trout heat inactivated serum

Ab : Rainbow trout anti-TB267 or *M. marinum* (NCIMB 1298) ECP antibody

65K MAb: Mouse anti-TB267 ECP-65 kDa protein monoclonal antibody ascites

Mm: *M. marinum* NCIMB 1298

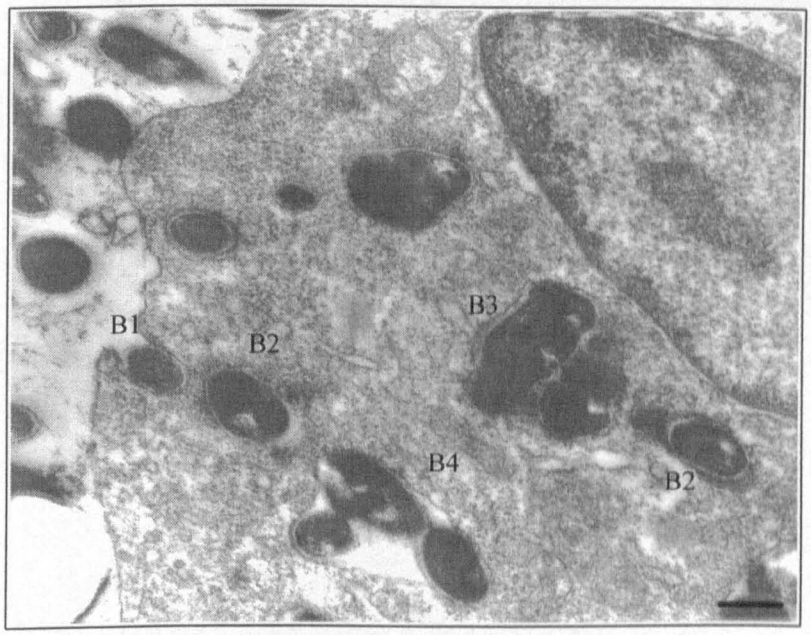


Figure 6.9 EM of a macrophage after 1 hr incubation with TB267 (preincubated with PBS ) showing that one pole of the cell contains many mitochondria. Some bacteria (B1) are in the process of being engulfed with the plasma membrane of the macrophage forming the wall of the phagosome. The cell has phagocytozed many bacteria, phagosomes containing one bacterium (B2), two bacteria (B3), and three bacteria (B4) are visible at different stages of ingestion. X 18,000. Bar=0.55 μm.

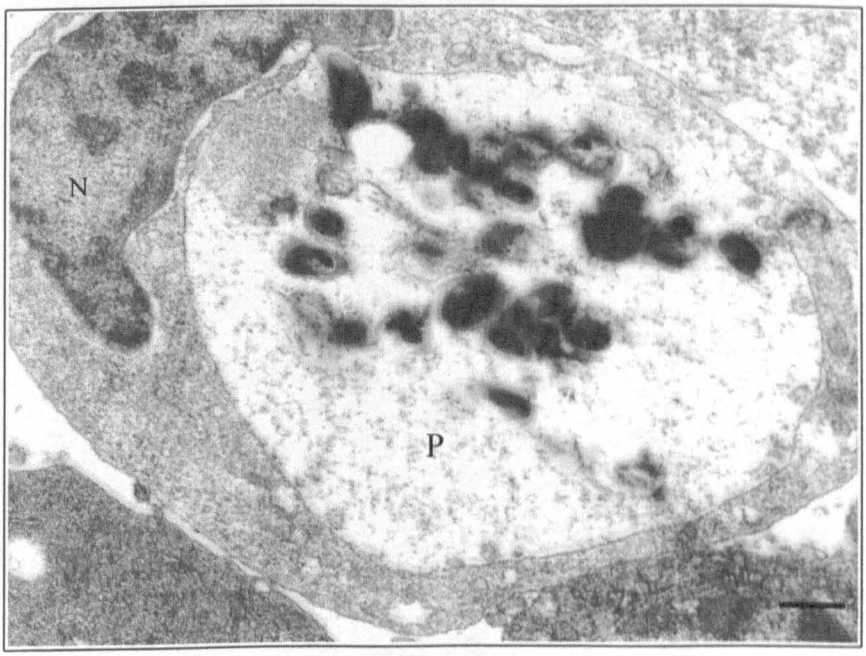


Figure 6.10 EM of a macrophage after 2 h incubation with *M. marinum* (preincubated with MAb) lacking almost all the cytoplasm granules and containing only one large phagosome (P). X 13,000. Bar=0.77 μm.

There were also some inactive macrophages which had not engulfed any bacteria. Numerous granules occupied the peri bacterial space, while other bacteria had lost their transparent capsule and had broken cell walls and membranes. Most bacteria pre-opsonised with either HIS and PBS remained intact. Bacterial damage appeared greater in the Ab and MAb-opsonised groups of bacteria than occurred with the other three groups of bacteria, for both strains of mycobacteria. Moreover, macrophages incubated with bacteria opsonised with Ab and MAb possessed a large number of phagosomes containing damaged bacteria with Ab and MAb (52% and 66% for TB267 and 49% and 63% for *M. marinum*, respectively) (Table 6.3). Few bacteria were damaged when they were pretreated with PBS, FS or HIS (8%, 9% and 4% of TB267 were damaged and 36%, 19% and 33% of *M. marinum* were damaged when treated with PBS, FS or HIS, respectively, and 91%, 90% and 93% of TB267 and 64%, 77% and 61% of *M. marinum* remained undamaged) (Table 6.3).

After 4 h incubation, the nucleus, mitochondria and rough endoplasmic reticulum of the macrophage appeared to be unaffected by the phagocytosed bacteria. In general, between 20 to 25 bacteria were seen in each phagocytic macrophage, but some macrophages were able to engulf more than 100 bacteria (Figure 6.11). The frequency of phagosomes varied from cell to cell and each phagosome contained approximately three bacteria, although larger phagosomes containing more bacteria were also observed. In some cases the cell wall of engulfed bacteria had joined with the wall of the phagosome to form a double walled phagosome (Figure. 6.12), while in other macrophages, the phagosomes

Table 6.3. Proportions of intact and damaged bacteria in the macrophage 2 h after ingestion of *Mycobacterium* spp. assessed by EM.

| Strains | Bacteria encountered | Structure appearance of bacteria | | |
|---|---|---|---|---|
| | | Intact | Damaged | Doubtful |
| | No. | % | % | % |
| 267+PBS | 120 | 91 | 8 | 1 |
| 267+FS | 208 | 90 | 9 | 1 |
| 267+HIS | 123 | 93 | 4 | 3 |
| 267+267Ab | 178 | 41 | 52 | 7 |
| 267+65K MAb | 101 | 28 | 66 | 6 |
| Mm+PBS | 59 | 64 | 36 | 0 |
| Mm+ FS | 164 | 77 | 19 | 4 |
| Mm+ HIS | 107 | 61 | 33 | 6 |
| Mm+ Mm Ab | 216 | 47 | 49 | 8 |
| Mm+65K MAb | 132 | 32 | 63 | 7 |

PBS: Phosphate buffered saline

FS: Rainbow trout fresh serum

HIS: Rainbow trout heat inactivated serum

Ab: Rainbow trout anti-TB267 or *M. marinum* (NCIMB 1298) ECP antibody

65K MAb: Mouse anti-TB267 ECP-65 kDa protein monoclonal antibody

Mm: *M. marinum* NCIMB

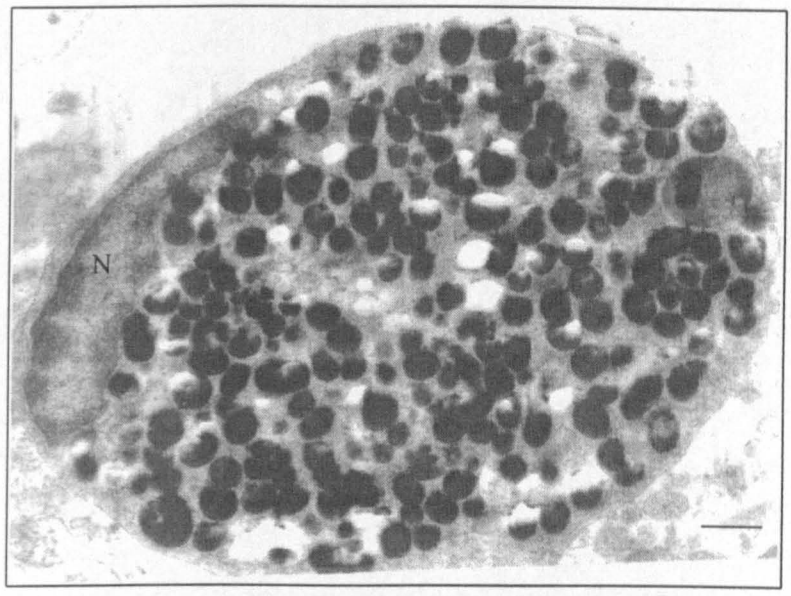


Figure 6.11 EM of a macrophage after 2 h incubation with *M. marinum*, (preincubated with MAb) showing that more than 100 bacteria have been engulfed. The cell lacks almost all the cytoplasmic granules and organelles. X 9,800. Bar=1.02 μm

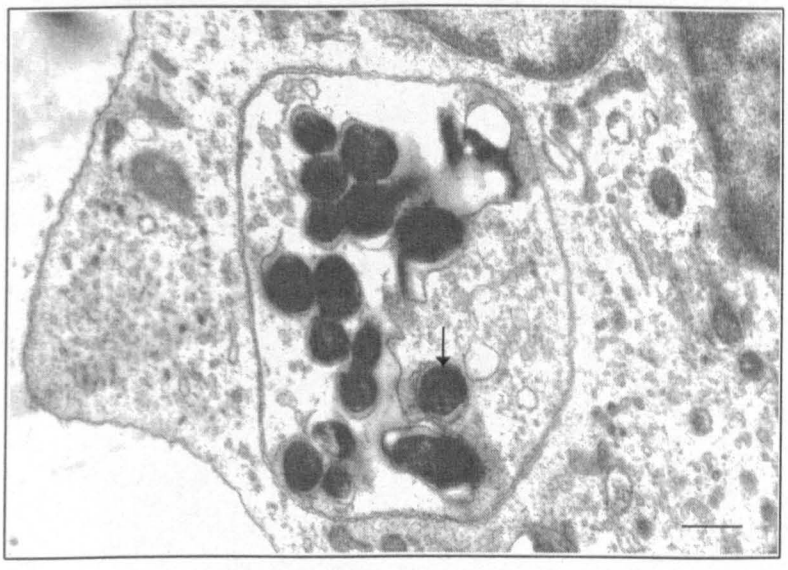

Figure 6.12 EM of a macrophage after 2 h incubation with TB267 (preincubated with fish anti-TB267 ECP antibody). The cell has phagocytosed many bacteria. One of bacteria in the phagosome has a double phagosomal wall (Arrow) X 18,000. Bar=0.55 μm

had fused with lysosomes (Figure 6.13 and 6.14). A few bacteria could be found free in cytoplasm, outside the phagosome. Bacterial damage was still higher for bacteria pre-treated with Ab and MAb than for the other three groups of bacteria, for both *M. marinum* and TB267, although damage to the former was found to be greater than to TB267. Furthermore, in macrophages inoculated with FS, Ab and MAb-treated bacteria, the majority of phagosomes contained damaged bacteria (56%, 63% and 76% for TB267 and 54%, 59% and 67% for *M. marinum*), whereas bacteria treated with PBS and HIS had very few damaged bacteria (5% and 11% for TB267 and 30% and 37% for *M. marinum*) (Table 6.4).

Macrophages incubated for more than 6 h with bacteria contained numerous intra-phagosomal bacteria. There was a marked increase in the extent of phagosomal lysosome fusion and the degree of bacteria damage with myelin figure development. Partially digested bacteria were observed in lysosomes as lysozyme granules which have entered the phagosome, and bacteria were observed also being digested in the phagolysosome (Figure 6.15). The degree of phagosome-lysosome fusion within a single cell was also found to vary (Figure 6.16, 6.17, 6.18, 6.19 and 6.20). For example in one macrophage, one of the phagosomes had changed to a residual body-like structure (bacteria had disappeared and there was marked myelin formation) (Figure 6.17) while another phagosome had already fused with a lysosome (see Figure 6.19). Figure 6.19 shows a phagosome that has not yet fused with the lysosome. Increased *Mycobacterium* damage occurred with the myelin figure development (Figure 6.20). Bacterial damage after 6 h incubation with macrophages from the various pre-treatment groups is shown in Table 6.5.

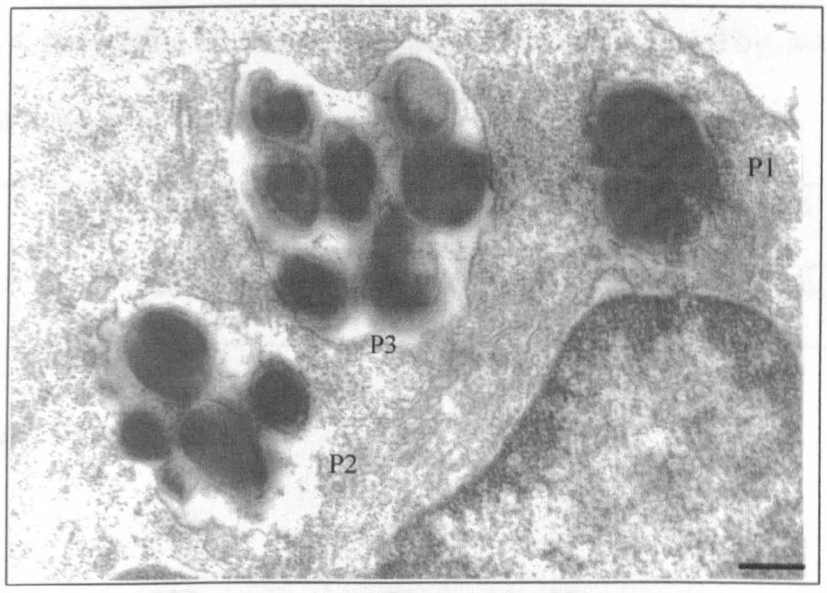


Figure 6.13 EM of a macrophage after 4 h incubation with *M. marinum*, 1298 (preincubated with heat-inactivated fish serum). The cell has phagocytosed many bacteria. A two bacteria phagosome (P1), a five bacteria phagosome (P2) and a 7 bacteria phagosome (P3) are visible at different stages of ingestion. X 22,000. Bar=0.45 μm

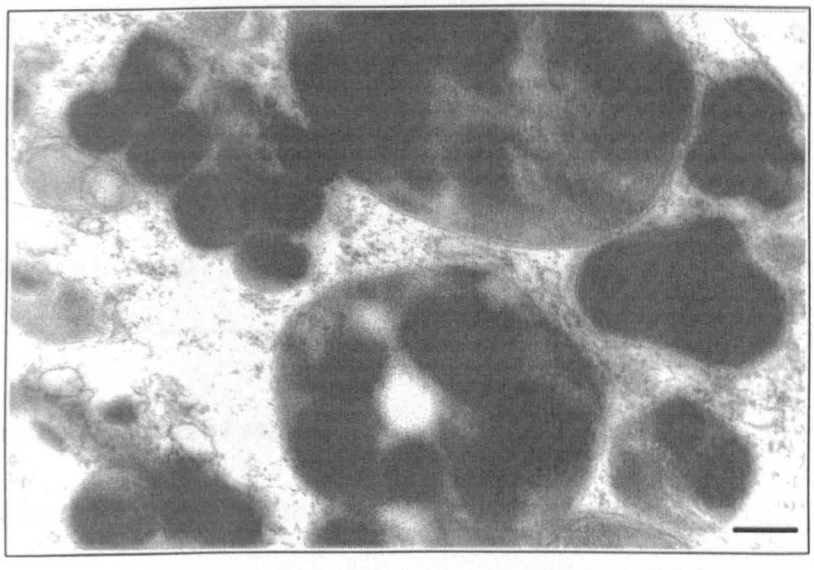

Figure 6.14 EM of a macrophage after 4 h incubation with *M. marinum* (preincubated with trout fresh serum). Lysosomes have fused with phagosomes. Most bacteria have lost the transparent zone. X 22,000. Bar=0.45 μm

Table 6.4. Proportion of intact and damaged bacteria ingested by macrophages 4 h post incubation with *Mycobacterium* spp., assessed by EM.

| Strains | Bacteria encountered | Structure appearance of bacteria | | |
|---|---|---|---|---|
| | | Intact | Damaged | Doubtful |
| | No. | % | % | % |
| 267+PBS | 173 | 88 | 5 | 7 |
| 267+ FS | 223 | 22 | 76 | 2 |
| 267+ HIS | 228 | 76 | 11 | 13 |
| 267+ 267Ab | 228 | 34 | 63 | 3 |
| 267+65K MAb | 228 | 15 | 76 | 9 |
| Mm+PBS | 226 | 63 | 30 | 7 |
| Mm+ FS | 167 | 40 | 54 | 6 |
| Mm+ HIS | 160 | 60 | 37 | 3 |
| Mm+ 1298 Ab | 100 | 39 | 59 | 2 |
| Mm+65K MAb | 70 | 24 | 67 | 9 |

PBS: Phosphate buffered saline

FS: Rainbow trout fresh serum

HI: Rainbow trout heat inactivated serum

Ab: Rainbow trout anti-TB267 or NCIMB 1298 ECP antibody

65K MAb: Mouse anti-TB267 ECP-65 kDa protein monoclonal antibody ascites

Mm: *M. marinum* NCIMB 1298

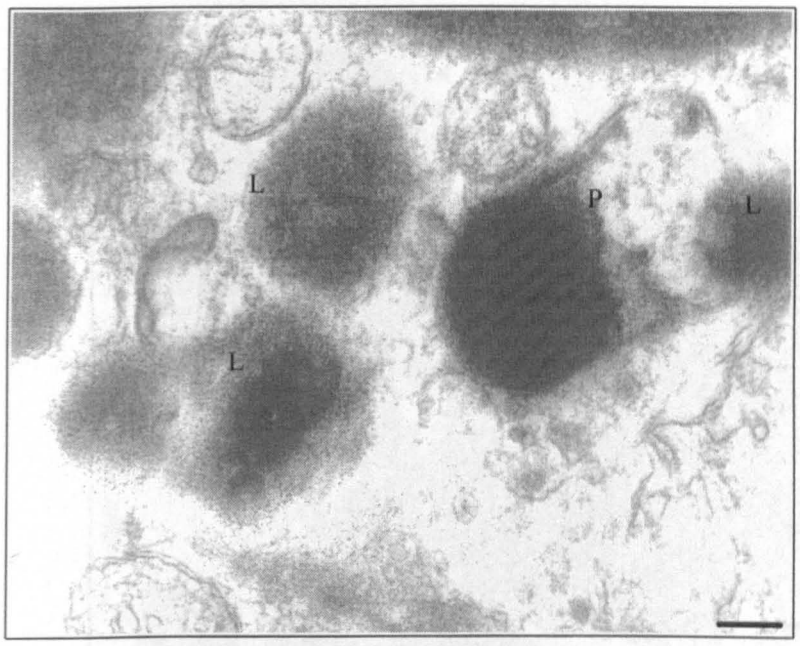


Figure 6.15 EM of a macrophage after 4 h incubation with TB267 (preincubated with PBS). The phagosome (p) has fused with the lysosome (L). The bacterium is in an advanced stage of disintegration within phagocytic vacuoles. X 36,000. Bar=0.28 μm.

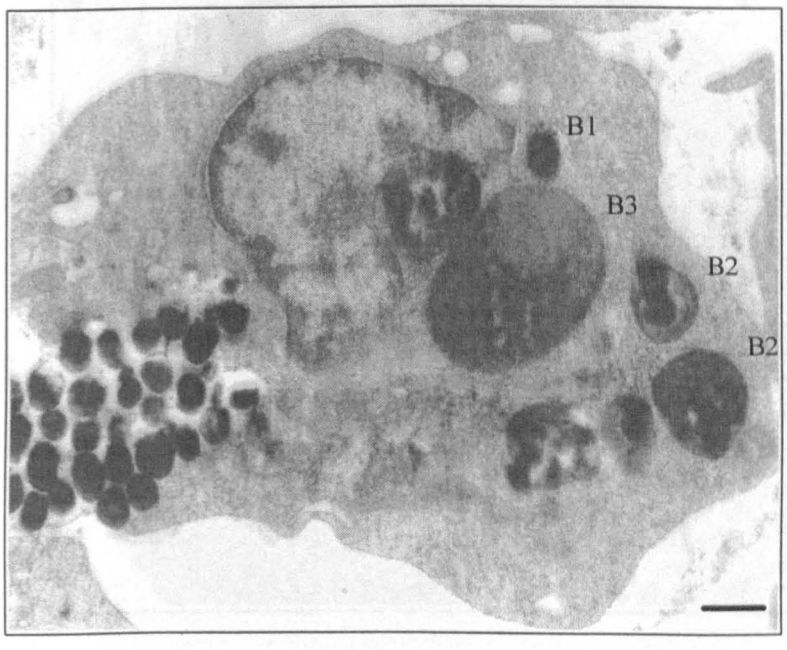


Figure 6.16 EM of a macrophage after 6 h incubation with *M. marinum* (preincubaed with PBS). The macrophage has phagocytosed many bacteria (B1-B3), which are visible at different stages of ingestion. B1 early stage of phagosome, B2, degeneration phagosome, B3, phagosomal residual body. X 9,800. Bar=1 μm.

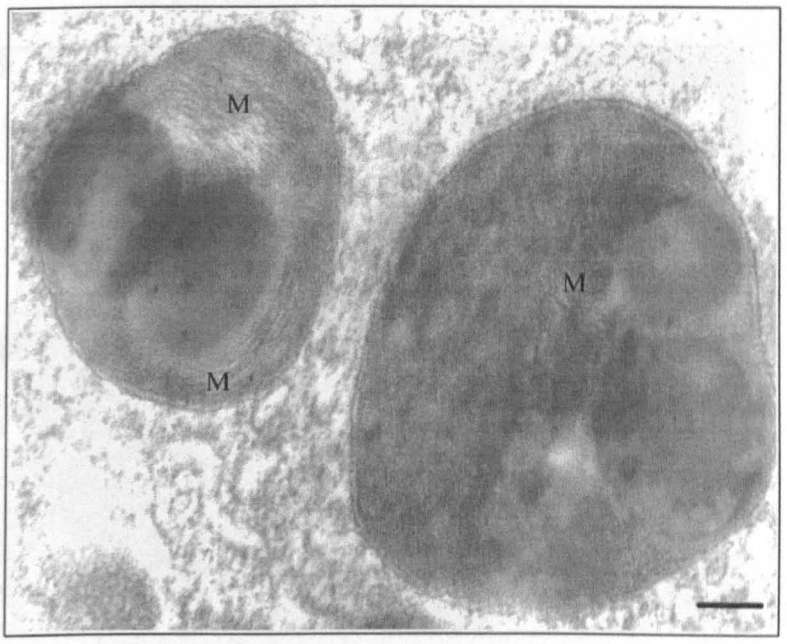


Figure 6.17 Magnification from Figure 6.16. The phagosomes with myelin (M) figures have fused with a lysosome. Bacteria are partially digested by lysozyme. X 36,000. Bar=0.28 μm

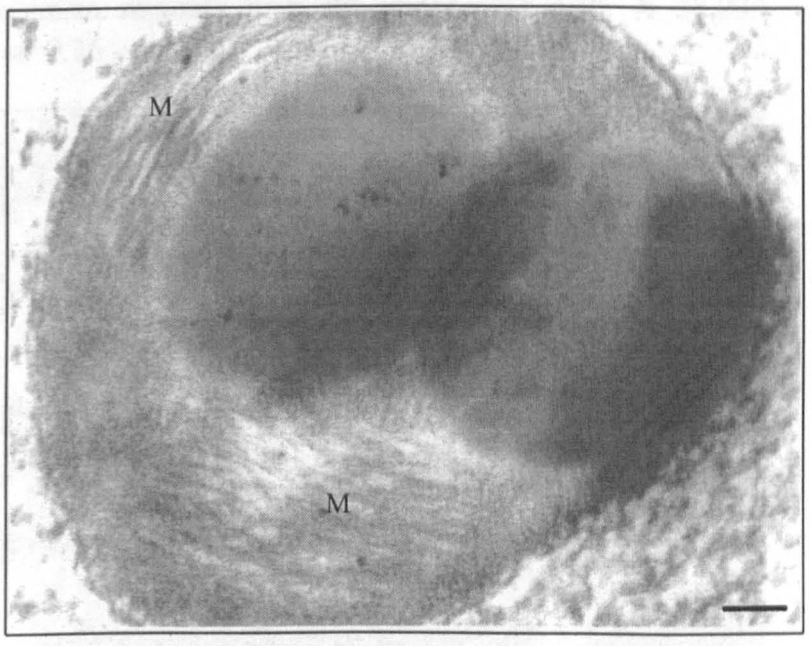


Figure 6.18 Magnification from Figure 6.17. This shows marked lysosome-phagosome fusion. Increased degrees of *Mycobacterium* damage are observed, with myelin (M) figure development. X 75,000. Bar=0.14 μm.

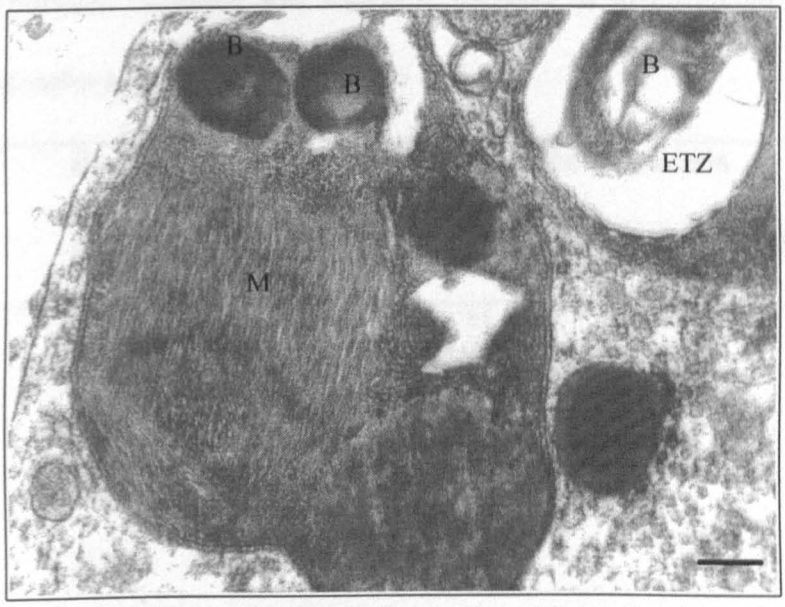


Figure 6.19 EM of a macrophage after 6 h incubation with *M. marinum* (B), (preincubated trout fresh serum). Marked phagosome-lysosome fusion has occured. Increased degrees of *Mycobacterium* damage, with myelin (M) figure development. Another phagosome has an irregular ETZ organism separate from the phagosome wall. X 28,000. Bar=0.35 μm

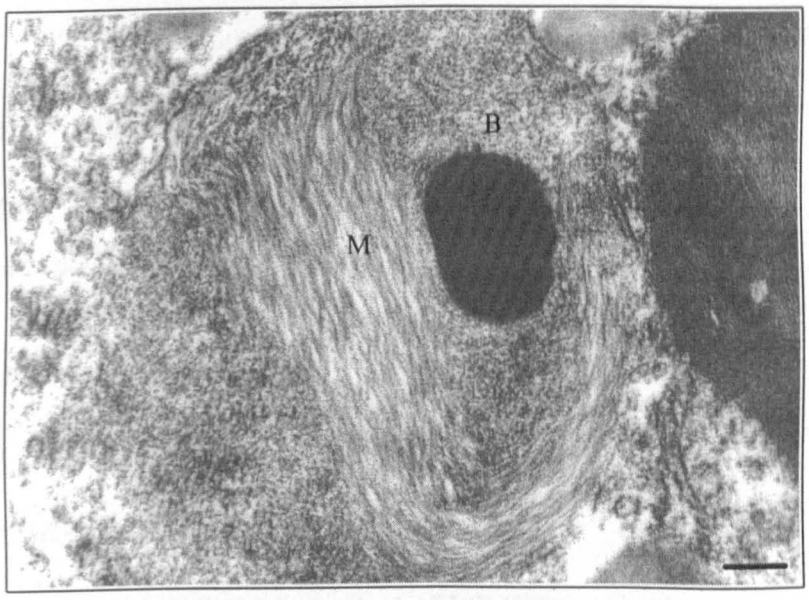


Figure 6.20 EM of a macrophage after 6 h incubation with *M. marinum* (B), (preincubated with trout fresh serum). The same feature as described in Figure 6.19. is observed. The phagosome-lysosomal wall has ruptured. X 36,000. Bar=0.28 μm.

Table 6.5 Proportions of intact and damaged bacteria in macrophage profile 6 h after ingestion of *Mycobacterium* spp. assessed by EM.

| Strains | Bacteria | Structure appearance of bacteria | | |
|---|---|---|---|---|
| | encountered | Intact | Damaged | Doubtful |
| | No | % | % | % |
| 267+PBS | 203 | 67 | 23 | 10 |
| 267+ FS | 60 | 28 | 65 | 7 |
| 267+ HIS | 123 | 79 | 18 | 3 |
| 267+ 267Ab | 127 | 15 | 82 | 3 |
| 267+65K MAb | 100 | 17 | 80 | 3 |
| Mm+PBS | 125 | 50 | 39 | 11 |
| Mm+ FS | 142 | 25 | 49 | 6 |
| Mm+ HIS | 150 | 69 | 28 | 3 |
| Mm+ Mm Ab | 174 | 8 | 85 | 7 |
| Mm+65 K MAb | 182 | 20 | 78 | 2 |

PBS: Phosphate buffered saline

FS: Rainbow trout fresh serum

HIS: Rainbow trout heat inactivated serum

Ab : Rainbow trout anti-267 or *M. marinum* 1298 ECP antibody

65K MAb: Mouse anti-TB267 ECP-65 kDa protein monoclonal antibody ascites

Mm: *M. marinum* NCIMB 1298.

Electron dense markers (ie. staining with ferritin) could been found in the macrophage lysosomes before incubating with bacteria. Occurrence of the phagosome lysosome fusion in bacteria-ingested macrophages was then detected by EM using the pre-labelled lysosomes (Figure 6.21, 6.22). Phago-lysosome fusion in ferritin labelled macrophages exhibited a lack of fusion when incubated with live TB267 (Figure 6.23, 6.24, 6.25). In contrast, Figure 6.26 illustrates phagosomes in which macrophages had engulfed formalin killed organisms. There appeared to be no inhibition of their fusion with lysosomes (Figure 6.26, 6.27, 6.28) and some of these bacteria were observed to be digested by lysozyme.

Tissue from Siamese fighting fish, infected with *Mycobacterium* spp. was also studied by EM. Most of the bacteria were found in macrophage phagosomes in the spleen. The majority of bacteria within the phagosomes were intact (Figure 6.29), some of which appeared to be surrounded by lysosomes (Figure 6.30, 6.31), and some of which had fused with the lysosomes and contained degenerate or lysed bacteria (Figure 6.32)

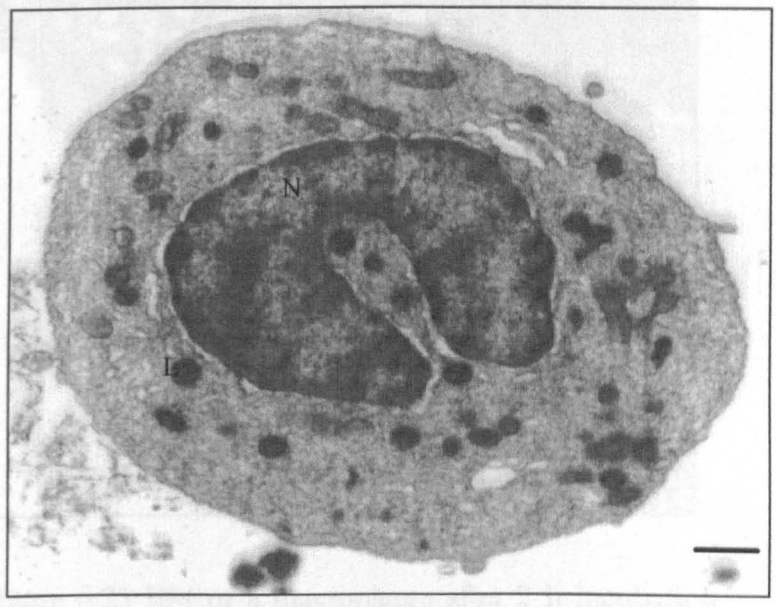


Figure 6.21 EM of macrophage from a 1 day culture in L-15 medium. Dense granules, or secondary lysosomes (L) are abundant, varying both in size and in content density. X 75,00. Bar=1.4 μm

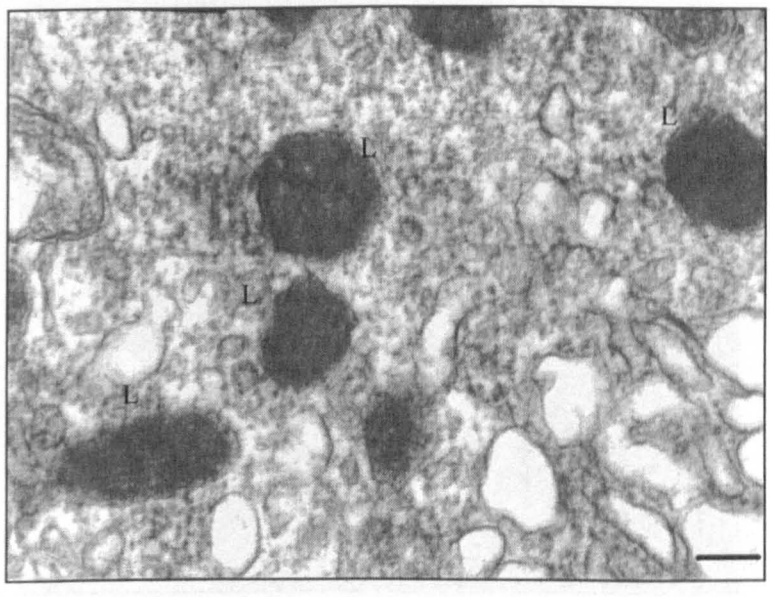


Figure 6.22 Four dense granules at higher magnification, showing the limiting unit membrane. X 59,000. Bar=0.17 μm

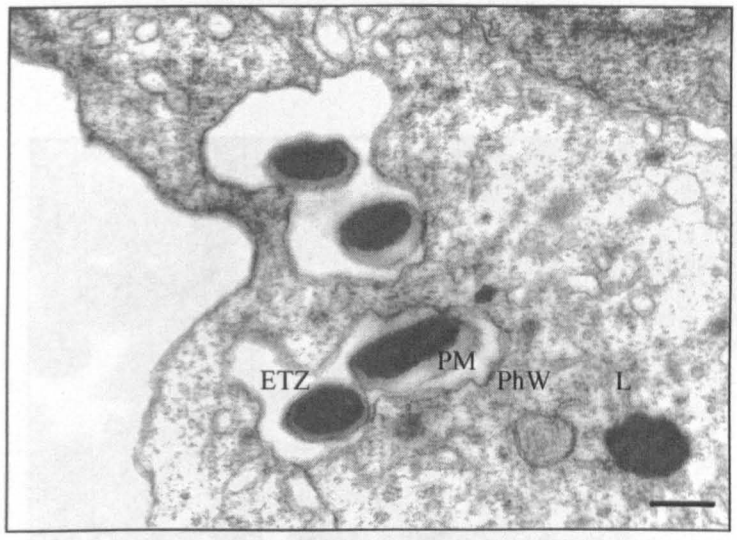


Figure 6.23 EM of a macrophage after 2 h incubation with live *Mycobacterium* TB267. The macrophage received a standard ferritin pulse before infection. The organism is bounded by a typical plasma membrane (PM) and a morphological cell wall. An irregular electron-transparent zone (ETZ) separates each organism from the phagosome wall (PhW). Lysosomes (L) are observed around the phagosome but have not fused with it. X 28,000. Bar=0.35 μm

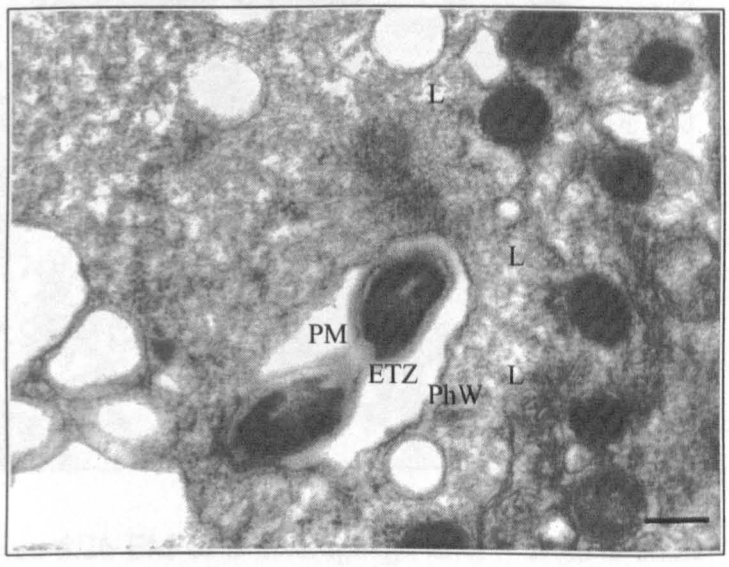


Figure 6.24 EM of a macrophage after 2 h incubation with formalin killed *Mycobacterium* TB267. The macrophage received a standard ferritin pulse before infection. The same illustrated in Figure 6.23. are shown. X 36,000. Bar= 0.28μm

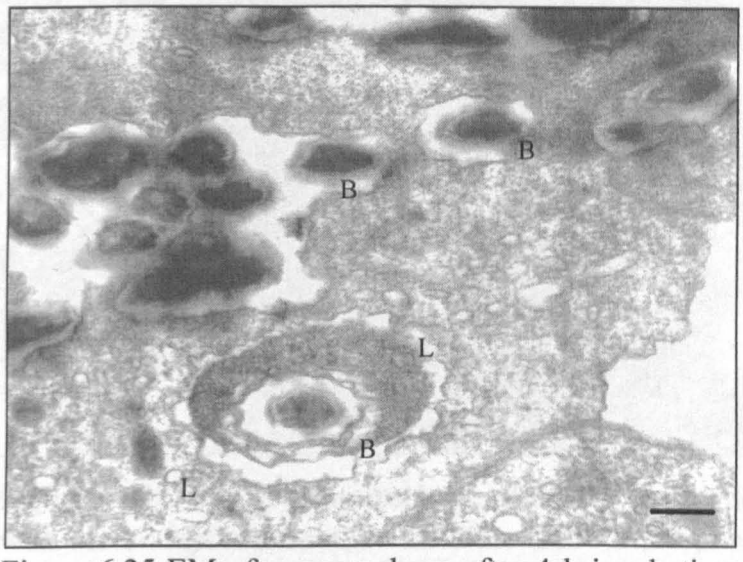


Figure 6.25 EM of a macrophage after 4 h incubation with live *Mycobacterium* TB267. The macrophage received a standard ferritin pulse before infection. Lysosomes (L) are observed around the phagosome but have not fused with it. X 22,000. Bar=0.45 μm

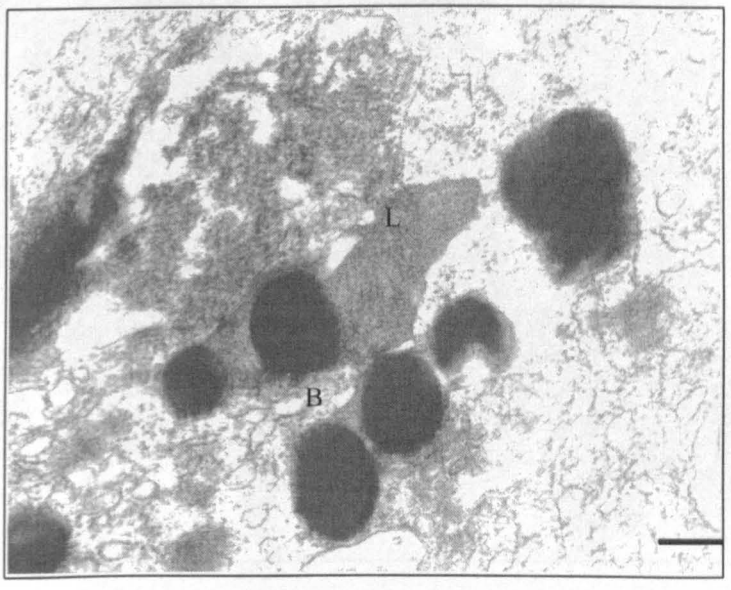


Figure 6.26 EM of a macrophage after 4 h incubation with formalin killed *Mycobacterium* TB267. The macrophage received a standard ferritin pulse before infection. Lysosomes (L) have fused with phagosomes. and the bacteria have lost their transparent zone. X 36,000. Bar=0.28 μm

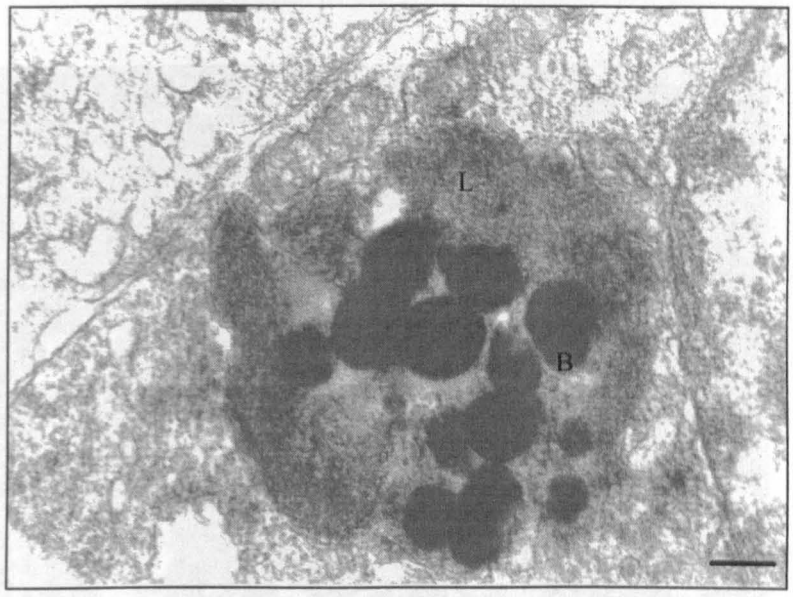


Figure 6.27 EM of a macrophage after 4 h incubation with live *Mycobacterium* TB267. The macrophage received a standard ferritin pulse before infection. The lysosome (L) has fused with the phagosome. Bacteria (B) in the phagolysosome have been damaged by lysozyme. X 28,000. Bar=0.36 μm

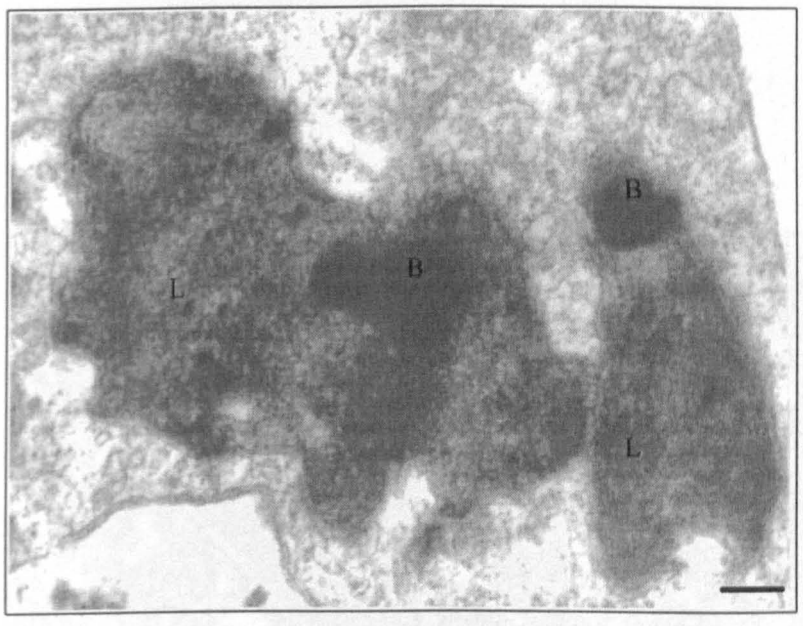


Figure 6.28. EM of a macrophage after 4 h incubation with formalin killed *Mycobacterium* TB267. The macrophage received a standard ferritin pulse before infection. The lysosomes (L) have fused with the phagosome. Bacteria (B) in the phagolysosome have been damaged by lysozyme. Some of bacteria have been digested. X 28,000. Bar=0.36 μm

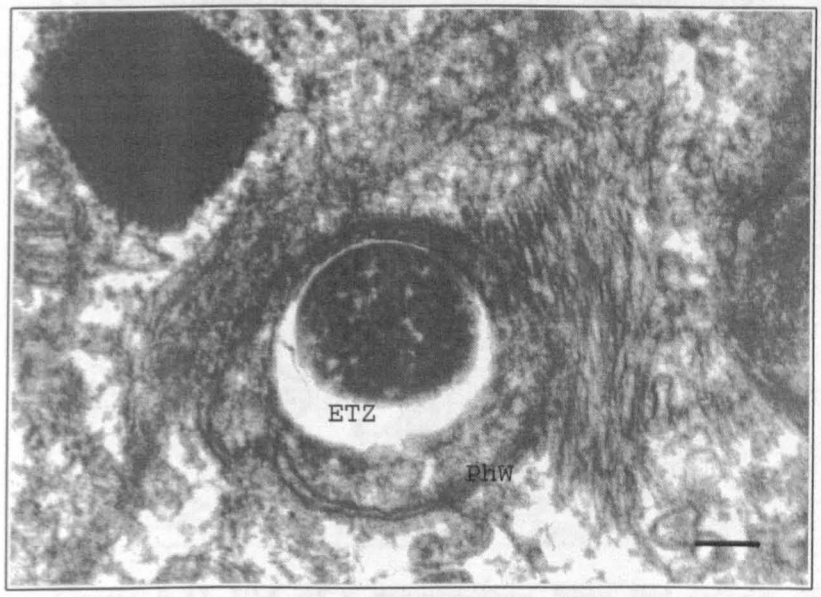


Figure 6.29 EM of a macrophage from the spleen of Siamese fighting fish. The lysosome (L) has fused with the phagosome. The bacterium are still intact within the phagosome. X 36,000. Bar=0.28 μm.

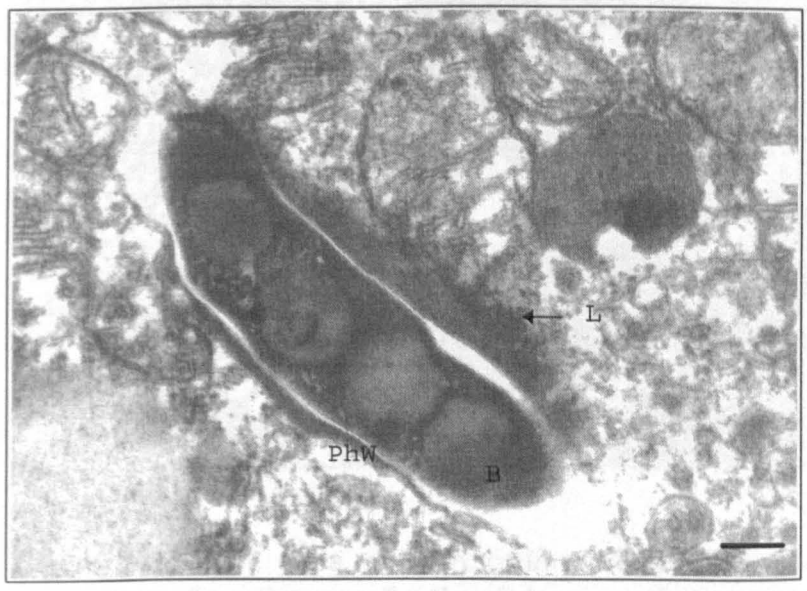


Figure 6.30 EM of a macrophage from the spleen from Siamese fighting fish. The lysosome (L) has fused with the phagosome. The bacteria (B) are still intact within the phagosome. Phagosomal wall (phw) X 28,000. Bar=0.28 μm.

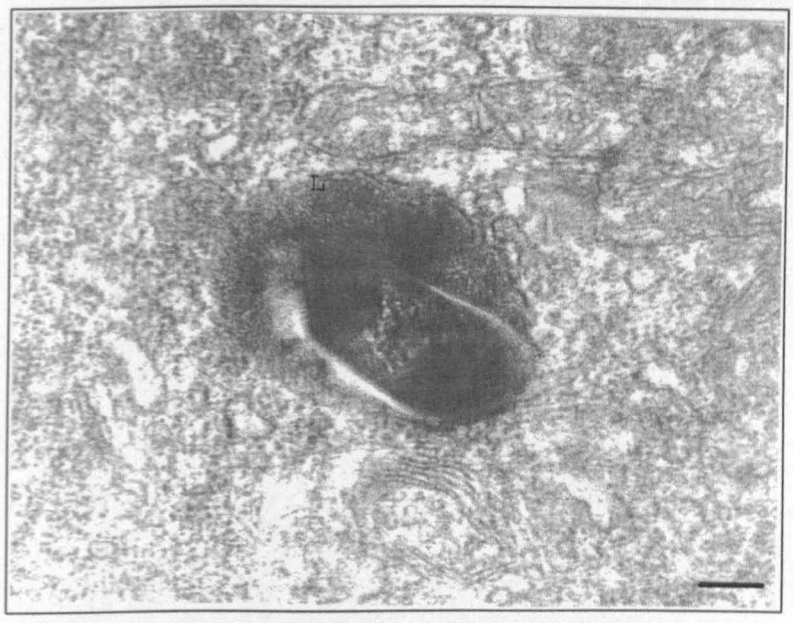


Figure 6.31 EM of a macrophage from the spleen of Siamese fighting fish. A lysosome (L) has fused with a phagosome and the bacteria have lost the transparent zone. X 36,000. Bar=0.28 μm.

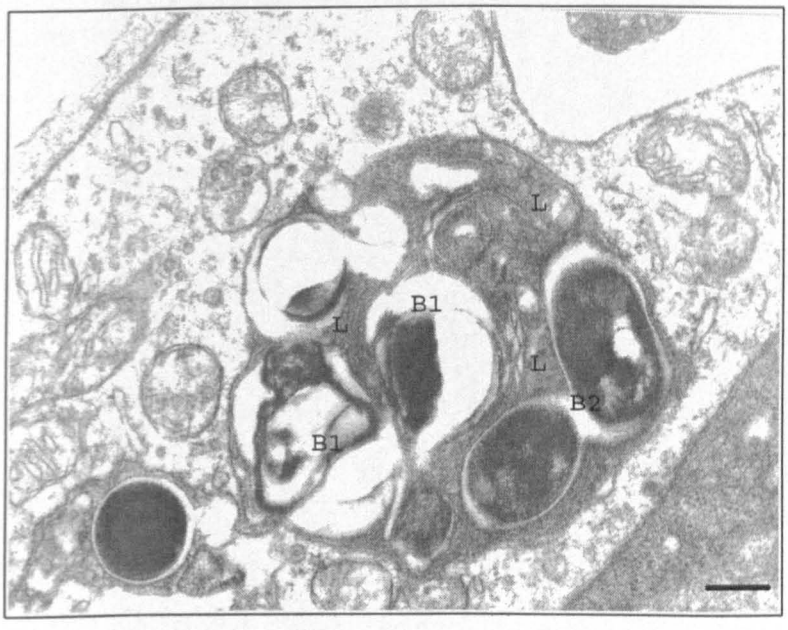


Figure 6.32 EM of a phagolysosome observed in a macrophage from Siamese fighting fish spleen. Lysosomes (L) have fused with phagosome. Some bacteria (B1) have lost the transparent zone. Other bacteria (B2) are still intact. X 22,000. Bar=0.45 μm.

## 6.4 Discussion

Macrophages are important cells in the disease resistance of the fish. They are an integral part of the specific immune response, although they do not elicit a specific immune response themselves. As accessory cells, they are involved in the recognition of invading particles and thus play a significant role in the initiation of the specific immune response. They do this by phagocytosing and killing microorganisms intracellularly, degrading them, then presenting processed material to cells which deliver a specific response (Clem *et al.*, 1985). Numerous factors can affect any or all of these stages. MacArthur and Fletcher, (1985) suggest that the killing mechanisms of fish macrophages are affected by more than the particle uptake. Evidence of this can be seen with a number of fish diseases, such as bacterial kidney disease of salmonids (Young and Chapman, 1978; Garduno, Thornton and Kay, 1993), *Edwardsiella ictaluri* in channel catfish (Blazer *et al*, 1985), and with *Piscirickettsia salmonis* or rickettsia like organisms (Fryer, Lannan, Giovanonni and Wood, 1992; Chen *et al.*, 1994). It has been established that bacteria causing these diseases can remain viable and are able to divide within the macrophages of infected fish.

The macrophage usually has an indented outline, and is multipodic or monopodic in nature, with natatory appendices being gathered as a sort of foot. The earliest investigations of phagocytosis with amoeba and polymorphonuclear leucocytes have shown that invagination is triggered by the contact of phagocytes with their prey. Phagocytosis is a complex process involving attachment of a particle to the cell surface, followed by ingestion (the formation of micro-projections of plasma membrane around the particle and membrane fusion). Different type of phagosomes were observed within the

macrophages examined in this study. In the first type, one or more bacteria were all within the intracellular vacuoles and no lysosomes were present, therefore no phagolysosome fusion occurred. With the second type of vacuole, phagosomes were surrounded by lysosomes but no fusion occured, and in the third type of vacuole, phagosomes had fused with the lysosomes, but bacteria were intact. In the fourth vacuole type, phagosomes had fused with the lysosomes and bacteria were damaged by lysosome. Examination of the fifth type of vacuoles showed that phagosomes had fused with the lysosomes and bacteria were damaged by lysosome with myelin surrounding. Brown and Draper (1970) showed that the electron-transparent zone (ETZ), now identified as a mycoside capsule, appeared to be a space separating intracellular *M. lepraemurium* from lysosomal enzymes of the host macrophage. It is possible that the material in the ETZ offers some protection to the bacteria (Draper and Rees, 1970 and 1973). On the other hand, virulent tubercle bacilli are capable of surviving and multiplying in the macrophages and seem to do so mostly in phagosomes that have not fused with the secondary lysosomes, thus escaping direct exposure to the lysozyme.

In the present study, macrophages were observed to undergo morphological changes consisting of elongation and segregation of the nucleus and organelles to one end of the cell. The level of phagocytosis of bacteria which occurred after 0.5 h incubation was low (3 to 5 bacteria per macrophages), but bacteria opsonised with FS, Ab and MAb had a higher percentage of engulfment than bacteria coated with PBS or HIS.

It has been reported that macrophage monolayers from normal mice infected *in vitro*, exhibited little ability to kill *Mycobacterium microti*. In contrast, if bacteria had been maintained first in the presence of supernatants from immunologically-activated spleen cells, they killed > 90% of the phagocytosed bacilli within 24 h (Lowrie, 1983).

Quantitative electronmicroscopic evaluation of both the occurrence of phagosome-lysosome fusion and morphology of *Nocardia* sp. infected macrophages showed that most of the organisms of the less-virulent strains of this bacteria were damaged, and there was evidence of phagolysosomal fusion. In contrast, virulent strains of the bacteria were neither damaged nor were phagosomes evident. Results of this study indicate that the most virulent strain, with the highest viability, was also the strain with the fewest number of damaged cells (Davis-Scibienski and Beaman, 1980).

Opsonisation of foreign particles by serum factors, such as specific antibodies, and complement such as nonspecific opsonins, is often necessary for optimal ingestion. Griffin (1983) showed that the presence of specific antibody greatly enhanced the phagocytosis of *Yersinia ruckeri* by rainbow trout leucocytes; however, it did not affect intracellular killing. Conversely, the phagocytic activity of salmonid peritoneal exudate cells against *A. salmonicida* was only slightly enhanced by specific antibody. The addition of both specific antibody and complement greatly increased phagocytosis (Sakai, 1984; Olivier *et al.*, 1986). Blazer *et al*, 1991 have also found only slight increases in the phagocytosis of *E. ictaluri* by head kidney macrophages from immunised channel catfish.

However, opsonisation with immune sera (antibody and complement) again greatly enhanced intracellular killing (Blazer *et al.*, 1989).

In this study, it was observed that not only does phagocytosis greatly increase in the FS, Ab, MAb groups, but intracellular killing was also increased after 2 h incubation. After 4 and 6 h incubation, the results showed that the presence of FS, Ab and MAb greatly enhanced phagocytosis and intracellular killing ability.

How can the bacteria survive in the fish macrophage? Some bacteria may maintain their viability by escaping from the phagosome into the macrophage cytoplasm. On the other hand, *Mycobacterium* spp. secrete materials inhibiting phagosome lysosome fusion. Some *Mycobacteria* spp. survived in the phagosome which has fused with the lysosome. Fujii, (1981) reported that phagocytic activity of polymorphonuclear leucocytes was enhanced by a specific antibody in the lamprey. Honda *et al.*, (1985, 1986) observed an increased phagocytosis of *Vibrio anguillarum* by macrophages of rainbow trout after opsonisation of bacteria with antibody and complement component.

The proportion of phagocytic cells in fish able to phagocytose non-opsonized foreign microorganisms has been reported as follows: 40.5% of macrophages from peritoneal exudate cells (PEC) of rainbow trout phagocytosed latex particles and 26.4% phagocytosed *V. anguillarum* (Honda *et al.*, 1985); 3% of phagocytes from PEC of rainbow trout phagocytosed *A. salmonicida* (Sakai, 1984); 90-98% phagocytosed latex particles, fixed SRBC, yeast and bacteria (Braun-Nesje *et al.*, 1981) and 59.0, 22.5 and

47.0% of macrophages from rainbow trout phagocytosed Candida, yeast and *Staphylococcus epidermis* (Mckinney *et al.*, 1977).

Thus, the results differ, depending on the species, the type of foreign microorganism and the origin of the phagocytes. Kaige *et al.*, 1990 stated that phagocytosis of Japanese eel (*Anguilla japonica*) macrophages was high, because, out of 500 macrophages, the number of cells phagocytosing non-opsonized SRBC was 339.7 (67.9%) and the number phagocytosing more than five non-opsonized SRBC was 149.3 (29.9%).

Griffin (1983) observed that phagocytosis by macrophages was enhanced by a specific antibody in rainbow trout. Homologous antibody and complement were found to enhance the phagocytic activity of macrophage-like cells from salmonid fish, but only weakly effected the activity in other kinds of fish (Sakai, 1984). Honda *et al.*, (1985, 1986) also demonstrated that the phagocytic activity of macrophages from rainbow trout was increased by homologous antibody and complement. Moreover, both Sakai (1984) and Honda *et al.*, 1985,1986) observed that the number of macrophages phagocytosing more than five or ten bacteria was increased by treatment with both antiserum and complement. However, they did not detect an opsonic effect with rainbow trout complement when the antigen was treated with complement alone, indicating that complement was not activated through the alternative pathway. It was suggested that macrophage-like cells might bear Fc and C receptors (Sakai, 1984). On the other hand, Wrathmell and Parish (1980) maintained that the a lack of opsonic effect by fish antibody and complement was due to an absence of Fc and C receptors on fish macrophages.

In this study, the bacteria were damaged in the PBS and HIS groups at 2, 4 and 6 h, suggesting that these two strains may be of low virulence. TB267 has been subcultured in our laboratory more than 4 years and, therefore, may have lost its virulence. Jackett, Aber and Lowrie (1978;1980;1989) studied three different strains of *M. tuberculosis* in which low virulence was associated with (i) laboratory attenuation, (ii) loss of catalase activity, (iii) peroxide susceptibility with loss of catalase activity. They compared the three for susceptibility to killing by macrophages and the studies suggest that leucocytes microbiocidal biochemistry may be relevant to the cells killing activity (Jackett *et al.*, 1978; 1980).

Observation of the ultrastructure of phagosome-lysosome fusion utilising ferritin labelling indicated that after a 4 h infection, almost all of the phagosomes containing formalin killed *Mycobacterium* TB267 were heavily labelled, thus indicating a high incidence of fusion. In contrast, only 24% of phagosomes fused with lysosomes when live *Mycobacterium* TB267 was used. Formalin killed *Mycobacterium* TB267 appeared to have lost their ability to secrete products which inhibit phagosome-lysosome fusion and thus protect themselves.

In fish infected with mycobacteriosis, most of the bacteria within the macrophages were in the phagosome. Inhibition of phagosome-lysosome fusion was indicated and only a few cells were damaged. It is very interesting that some bacteria were damaged while other bacteria were still intact within the same phagolysosome. Some bacteria remained intact after phagolysosome fusion and not all bacteria were killed.

Numberous reports in the literature have established that many pathogenic organisms including mycobacteria, can maintain their viability and function as intracellular parasites in mammalian macrophages (Armstrong and Hart, 1971; Davis-Scibienski and Beaman, 1980).

Previous reports on *M. tuberculosis*, *M. lepraemurium* and *Listeria monocytogenes* suggested that the level of interaction between the alveolar macrophage and the intracellular parasites determines the extent of infection within the mammalian lung (Patterson and Youmans 1970; Wilder and Edberg, 1973). Several possible mechanisms for the intracellular viability and integrity of this virulent strain were suggested by comparing it to other intracellular parasites. Reports on *Trypanosoma cruzi* and *M. leprae* have suggested that these organisms are able maintain their viability by escaping from the phagosome into the macrophage cytoplasm (Evans and Levy, 1972; Nogueira and Cohn, 1976). Another possible mechanism for bacterial survival was suggested from previous studies with *M. tuberculosis* where it was shown that it is able to inhibit the fusion of lysosomes with phagosomes (Hart, Young, Gordon, and Sullivan, 1987).

Great effort has been directed towards understanding the mechanisms underling this intriguing biological phenomenon and the benefits which are raised from avoiding contact with lysosomal contents. Three different, but possibly complementary mechanisms have been proposed. Firstly, bacterial release of poly anionic cell wall components (Goren, Hart Young and Armstrong, 1976); secondly, the release of ammonia, and the release of cyclic AMP or stimulation of cyclic AMP synthesis (Lowrie, Jackett and Ratcliffe, 1975; Lowrie,

Aber and Jackett, 1979; Lowrie *et al.*, 1980); and thirdly, sulfatides from *M. tuberculosis* are inhibitors of phagolysosomes in cultured macrophages (Goren *et al.*, 1976).

Clearly further studies are required to investigate the virulence of *Mycobacterium* spp. in snakehead fish and Siameses fighting fish, and their interaction with fish macrophages.

## Chapter 7 General discussion

Shinnick (1996) stated that human tuberculosis results from an infection with *Mycobacterium tuberculosis*, and the World Health Organization estimate that perhaps as much as one-third of the world's population or approximately 1.9 billion persons have been infected with tuberculosis. Each year, there are 8-10 million new cases of tuberculosis and about 3 million deaths due to it (Shinnick, 1996). The recognised fish pathogens are *M. marinum*, *M. fortuitum* and *M. chelonae*. These strains also infect man. Puttinaowarat (1995) reported that in Nahorn Pathom, a province in the central part of Thailand, fish infected with aquatic *Mycobacterium* spp. were detected in up to 45% of Siamese fighting fish farms in Thailand. Human (in Thailand people working on fish farms and attending home aquaria) infections with aquatic mycobacteria have been reported with increased frequency (T. Somsiri, personal communication). Mycobacteriosis (fish tuberculosis) is a chronic bacterial disease which infects fresh water and marine fish world wide (Nigrell and Vogel, 1963). To date, there is no effective vaccine against the disease. Treatment of fish with antibiotics is not economical and long-term (more than 1-2 months) for the farmers.

A project was initiated at Stirling in 1992 to develop MAbs to whole cells of *Mycobacterium* spp. isolated from snakehead and Siamese fighting fish. The aim was to utilise the probes to develop rapid diagnostic tests to monitor the spread of mycobacteriosis in Thailand (Adams *et al.*, 1996). The data demonstrated that *Mycobacterium* spp. could be detected in the spleen and kidney of fish by using a sandwich ELISA providing the samples were pretreated to disrupt

bacteria. Immunohistochemical analysis proved less conclusive with only a very weak staining of the bacteria being observed.

Aquatic mycobacteria were grown on the surface of media as pellicles after 7-10 days of culture in different media. The MSM, SM and Long's medium changed to a brown colour under the pellicle and this diffused into the medium if the culture was more than 16 weeks old. The dead bacteria sank down to the bottom of the culture flask. The production of ECP from *Mycobacterium* cultured in all media used was very low. The ECP from *Mycobacterium* spp. was also difficult to prepare for electrophoresis because large volumes had to be concentrated to obtain a sufficient quantity. The ECP was very adherent resulting in poor resolution on the gels.

Of the media examined, modified Sauton's medium appeared to be the optimal culture medium for growth of fish mycobacteria. After 14 days of culture, supernatants from all mycobacterial strains tested by SDS-PAGE showed major bands at 65 and <14 kDa. The major bands were similar to those observed with *M. tuberculosis* although many minor bands indicated strain differences (Verbon, *et al.*, 1990). More protein appeared to be secreted by the bacteria under heat shock conditions and the 65 kDa protein appeared to be the major heat shock protein secreted by the mycobacteria. *M. tuberculosis* ECP, however have 10, 14 and 65 kDa proteins which are heat shock proteins (Verbon *et al.*, 1991 and 1992; Young *et al.*, 1987).

The ECP secreted by some fish pathogens has been reported to be toxic when administered to fish. Lee (1995), found that the ECP of *Vibrio alginolyticus* was lethal to grouper, *Epinephelus malabaricus*, with a minimum lethal dose of 0.52μg/g fish body weight. Fouz

*et al.* (1993) reported that the ECP from all *Vibrio damsela* was lethal for fish with an LD50 ranging from 0.06-3.7 μg/g fish. Lee and Ellis (1989) reported that the LD50 of protease and haemolysin from extracellular products of *A. salmonicida* when injected separately was 2400 ng protein/g fish and 44 ng/g fish, respectively.

Mycobacterial ECP examined in this project, on the other hand, appeared to be of low toxicity in trout and tilapia as doses of 400 μg/ fish resulted in low mortalities. Some enzyme activity may have been inactivated however, after incubation at 75°C for 15 min during preparation of the ECP. Research on other intracellular fish pathogens (e.g. *R. salmoninarum* which causes BKD) showed that all strains of bacteria from different geographic origins appeared to produce a low concentration of extracellular proteins and the results confirmed that most of the isolates were poor producers of proteolytic enzymes. None of the ECP samples displayed cytotoxic activities regardless of the origin of the cell line employed [e.g chinook salmon embryo-214 (CHSE-214 ), rainbow trout gonad (RTG2)] employed and did not contain substances lethal for fish. In this study, the ECP from *Mycobacterium* spp. isolated from infected fish did not contain substances lethal for fish and also produced low concentrations of ECP (Badin *et al.*, 1991). In general, the ECP of chronic disease pathogens, including, *N. asteroides* isolated from snakehead fish and large mouth bass, also do not appear to contain substances lethal for fish (Chen, unpublished data). Toxicity may depend on the virulence of strains used. In this study, although mycobacteria were originally isolated from disease outbreaks, the virulence after subculture was not determined due to contraints on fish species and numbers. Further research is clearly required to determine if lethal factors are produced *in vivo* with virulent strains. McIntosh (1996) pointed out that there have been some notable examples of how attempts to create conditions which more closely resemble the in *vivo* environment have led to the

discovery of previously unrecognised virulence factors. Hirst and Ellis (1994) reported that iron regulated outer membrane proteins of *A. salmonicida* are important protective antigens in Atlantic salmon against furunculoisis. Farias (1995) reported on the characterisation of *R. salmoninarum* using API ZYM. The API ZYM profile of bacteria cultured in kidney disease medium 2 (KDM-2) without fetal calf serum were identical in all isolates tested with only slight differences in the intensity of the reaction. However, bacteria cultured in KDM-charcoal showed a notable reduction in the production of butyrate esterase, caprylate esterase and α-glucisidase, while bacteria grown in Muller Hinton cysteine (MHC) failed to produce acid phosphatase. Thus it appears that culture conditions affect the expression of antigens and this may also be true for mycobacteria.

Fibronectin on the surface of human oropharyngeal cells is thought to be important in promoting adhesion of certain gram positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus* as well as preventing attachment of several gram negative organisms. Binding of fibronectin to *S. pyogenes* and *S. aureus* is dependent on the interaction of a specific site located on the fibronectin molecules with bacterial cell surface receptors (Speziale *et al.*, 1984; Nealon *et al.*, 1986). Mycobacteria are believed to use fibronectin to avoid immune detection or as a bridge to interact with molecules and cells of the host (Proctor, 1987).

Espitia *et al.*, (1992) using culture filtrate antigens of *Mycobacterium tuberculosis* showed strong reactivity with fibronectin-binding protein at 30-31 kDa (Fn30-31). This was demonstrated in 55.9% of tuberculosis sera and in 56.5% of lepromatous leprosy sera by Western blot. Positive titres were found in 63% of 65 tuberculosis sera and in 60.5% of 43

lepromatous leprosy sera. Espitia *et al.*,(1992) found that direct evidence for the presence of this antigen in *M. leprae* was obtained by immunochemistry of lepromatous leprosy lesions with a monospecific antibody raised against *M. tuberculosis* Fn 30-31. In the present study, major fibrionectin binding proteins were observed at 21-25 kDa in all the mycobacterial ECPs tested. The >210 kDa fibronectin binding protein was, however, only detected in *Mycobacterium* spp. isolated from Siamese fighting fish. The Western blot pattern of fibronectin binding protein differs between *M. tuberculosis* and *Mycobacterium* spp. isolated from fish. It would be interesting to investigate the Western blot pattern of serum from snakehead fish naturally infected with *Mycobacterium* spp to establish which components of the ECP from *Mycobacterium* spp. are recognised.

Cross reactivity between the rabbit anti-65 kDa antibody and antigen from many bacterial species pathogenic to fish (other than *Mycobacterium* spp.) suggests that the 65 kDa antigen may have a possible role in the pathogenesis of mycobacterial infections and other diseases (Chapter 3, Young *et al.*, 1987). Young *et al.*, (1987) speculated upon why the 65 kDa antigen elicits such a strong immune response. They suggested that this may be a direct consequence of its presence in a diverse range of bacteria with constant restimulation of T helper cells during contact with normal bacterial flora. If the immune response is due to priming by a cross-reactive bacterial antigen it would be appropriate to consider what effect exposure to other bacteria in endemic areas might have on the immune response to mycobacterial infection and how such factors might influence the effectiveness of BCG vaccination. It should also be asked whether priming of responses to the 65 kDa antigen during mycobacterial infections or BCG vaccination has an effect on the immune response to challenge with other environmental bacteria.

The six MAbs produced in a parallel project (Adams *et al.*, 1995 and 1996) directed against *Mycobacterium* spp. (strain TB1, isolated from Siamese fighting fish) recognized the *Mycobacterium* type strains *M. marinum, M. fortuitum, or M. chelonae* as well as all the *Mycobacterium* spp. isolated from snakehead and Siamese fighting fish tested. Thus, they are not species specific (Adams *et al.*, 1995). These MAbs are therefore useful for the diagnosis of mycobacteriosis in general but are unable to differentiate among the type strains or between the mycobacteria isolated from snakeheads or from Siamese fighting fish. Definitive classification (as a particular type strain) of mycobacteria isolated from these fish in Thailand has been unsuccessful using MAbs. However, their biochemical characteristics are most similar to *M. marinum*. Recently PCR analysis has shown that the strains TB267 and TB268 used in this study were indeed *M. marinum* (Puttinaowarat, 1995) and others are currently being tested.

The response of fresh isolates (whole bacteria from snakehead and Siamese fighting fish) in the sandwich ELISA ranged from 15.0% to 128.8%. When bacterial lysates were introduced, the absorbances of the ELISA increased, thus improving the sensitivity of the assay from $1 \times 10^6$ to $1 \times 10^5$ bacteria/ml (Adams *et al.*, 1996). The cell wall of a *Mycobacterium* is constructed from a complex mixture of mycolic acids, glycolopids, mycosides and phospholipids (Goren *et al.*, 1972). It would appear that the MAbs recognised epitopes masked by this thick waxy coat, and that rupturing the bacteria effectively exposed them. In agreement with these findings it has been shown that the MAbs did not react with whole bacteria when an indirect fluorescent antibody technique was used (Fridman, 1995). Further MAbs to the surface of *Mycobacterium* spp. are currently being developed

Adams *et al.*, (1995) reported that some of the MAbs cross reacted with species other than mycobacteria. In particular all the MAbs to WCS of *Mycobacterium* spp. recognised *Micrococcus luteus* when these bacteria were introduced at high concentrations. The response, although positive, was low and as this bacteria would normally be found on the surface of fish, it was not considered to be a potential problem in the diagnosis of mycobacteriosis from tissue samples.

Rabbit anti-65 kDa protein polyclonal serum was shown to react with a diverse range of bacterial genera and it appears to be a common bacterial protein found in many fish bacterial pathogens (Chapter 3 ). MAb 7B8, 12C6, 4H10, 11B11, 4H5, 7B7 and 9C2 (against 65 kDa ) only reacted with *Mycobacterium* sp. and these MAbs may prove useful in the differentiation of mycobacteria from other pathogenic bacteria in fish due to their specificity. It is intended that the MAbs in the ELISA will provide a rapid alternative to the time-consuming methods of diagnosis currently in use.

Because of sensitivity of the immune system, immunological biomarkers can play an important role in monitoring the health of a population as well as indicating potential harm from environment contaminants. Numberous tests have been devised to study various aspects of the immune system (Weeks *et al.*, 1992). One method of monitoring the non-specific response is through the metabolic activity of neutrophils using a NBT test. The activity of neutrophil populations can give clues to general health. The NBT activity is positively correlated with phagocytosis and killing activity of neutrophils and macrophages. Detection of neutrophil activation by the NBT staining method after injection could help fishery biologists confirm the effectiveness of immunisation

(Anderson *et al.*, 1992). The NBT test appears to have potential as an effective field assay because it is quick, uncomplicated, and inexpensive. Peripheral blood monocytes and tissue macrophages also become activated after antigenic stimulation. A rise in the numbers of NBT positive cells to *Y. ruckeri* O antigen bacterin was reported by Anderson *et al.*, (1992). The highest response for the NBT-positive neutrophil assay occurred on day 2 or 3 after injection. The fish injected with PBS also showed a slight rise in the number of NBT positive cells. Fish not injected but merely transferred to control tanks also showed a slight rise in the number of NBT positive neutrophils. The NBT test was used in the present study to determine the response of rainbow trout and tilapia to ECP from *Mycobacterium* spp. The results indicated that NBT positive cells appeared to peak every second week both in experimental and control rainbow trout. This may be a physiological phenomena in rainbow trout, with NBT cells having a 4 week cycle. Nevertheless, a significant difference between immunised and control groups was observed. Therefore, ECP from *Mycobacterium* spp. is a good immunogen in rainbow trout and tilapia. It would be also interesting to repeat this experiment in snakehead and Siamese fighting fish.

An increase in lysozyme serum levels has also been correlated with non-specific immune response in fish. Seta and Yamanouchi (1994) reported that lysozyme activity of an experimental group after 4 weeks administration of Beta-1,3 glucan was significantly higher compared with that of the control group. Roed *et al.* (1993) found that statistically significant effects on the lysozyme activity provide evidence of significant additive genetic variation in lysozyme activity in rainbow trout. Jorgensen *et al.*, (1993) found that injection of glucan into rainbow trout also resulted in enhanced serum lysozyme activity throughout the 3 week experimental period. The level of serum lysozyme was also significantly increased by *Aeromonas salmonicida* infection (Moeyner *et al.* 1993).

Lysozyme levels were also significantly higher in fish with acute furunculosis when compared with the levels in fish with chronic disease. There were, however, no significant results for the serum lysozyme data after oral or intraperitoneal vaccination with particulate or soluble *E. ictaruli* antigen. In contrast, lysozyme concentrations were highest in the gut and skin mucus having the next highest concentration (Anisworth, Rice, and Xue, 1995). In the present study, lysozyme activity in serum peaked 2 weeks after primary vaccination. The activity dropped at 6 weeks but then increased again at 8 and 10 weeks. The vaccinated groups showed significally higher lysozyme activity than the controls.

The swim bladder was also used in this study as a site for administration of antigens (Endo *et al.*, 1995) and the immune response determined. The injection, sited above the of pelvic fin and midway between the pelvic fin and laternal line, allowed entry into the swim bladder without injury to the other visceral organs. This method was used as it is easier to collect exudative cells from the swim bladder than the intraperitoneal cavity.

Endo *et al.*, (1995) stated that swim bladder administration has the advantage of allowing a precise dose to be delivered, reducing injury to the other organs and a slow absorption. The characteristics of the slow absorption in the swim bladder administration, however produced inflammation to the swim bladder wall. This was not due to injection but as the effects of ECP, the PBS control should no signs of inflammation. Swim bladder administration can deliver 1 ml of PBS into the tilapia without any abnormal behaviour.

Nile tilapia were immunised by injecting extracellular products (ECP) alone from *Mycobacterium* spp. (strain TB40, TB267 or the type strain *M. marinum*) into their swimbladders. A variety of adjuvants; FCA, FIA, Titre max or phosphate buffered saline

(PBS), respectively used as a control, were similarly injected into additional groups of tilapia. The ECPs from *Mycobacterium* spp. were shown to be good immunogens to tilapia. Further studies in snakehead clearly need to be performed and the exact nature of the antibodies established (ie, are they protective or not ?).

A comparison of response of fish, rabbits and mice sera to ECP or WCS antigens indicated differences between the species by Western blot, More bands were recognized by rabbit and mouse serum than with fish serum. This phenomena has also been reported for other fish pathogens (e.g *A. salmonicida*, Hastings and Ellis, 1988). and is probably due to, the fact that the immune system of the rabbits and mice are more developed than that of the fish. Such differences are important for the development of an effective fish vaccine. The immune response in snakehead fish also need to be determined as this may differ from trout and tilapia.

The course of tuberculosis infection has been extensively studied in man and in experimental animals (Dannenberg, 1994; Balasubramanian *et al.*, 1994; Orme and Collin 1994 and is quite different to that in fish. It includes several stages; stage 1: initial uptake of bacteria; stage 2: T-cell response; stage 3: dormancy, stage 4: liquefaction, extracellular multiplication and transmission (Dannenberg, 1991). In stage 1, bacteria were killed in activated macrophages and bacteria also multiplied in non-activated macrophages. Bacteria were also released by cytotoxic T lymphocytes (Lurie, 1964; Dannenberg, 1991). Bacterial killing, antibody response and recruitment and activation of monocyte and macrophages occurred in stage 2 (Lurie, 1964). Activated macrophages engulf bacteria and released liparabino-mannan from the bacterial cell wall which encouraged production of various cytokines. These cytokines, in particular tumor necrosis factor, encourage conversion of

monocytes into epithelioid cells and Langhan's giant cells forming a granuloma in stage 3 (Dannenberg, 1991). The liquefied material is an excellent growth medium for bacteria and bacterial multiplication, once they have been released from macrophages. The factors that cause liquefaction are largely unknown but hydrolytic enzyme and delayed type hypersensitivity to the tuberculin products of bacteria are involved (stage 4). In stage 4, the stage of liquefaction, the bacillus evades these host defenses. Liquefaction of the caseous center is frequently followed by cavity formation. In the liquefied caseous center, the bacilli multiply extracellularly for the first time, frequently reaching tremendous numbers. They enter the bronchial tree and spread to the other parts of the lung and to the outside environment (Young and Duncan, 1995).

Piscine tubercle granuloma has been described in gold fish, *C. auratus* (Majeed *et al.*, 1981) Striped bass, *M. saxatilis* (Hedrick *et al.*, 1987) and Siamese fighting fish, *B. splendens* (Pungkachonboon *et al.*, 1994).

Pungkachonboon *et al.*, 1994 reported that granuloma formation of fish infected with *Mycobacterium* sp. isolated from Siamese fighting fish, was observed at the injection site by day four and spread to haemopoietic organs by day 5. They developed into typical granuloma within one day. Foreign type giant cells developed in the lesion only on day 5. This is much faster than development of Langhan's type giant cells in plaice which are produced at only days 24 and 28. (Pungkachonboon *et al.*, 1994). The quicker rate of lesion formation in Siamese fighting fish may be due to the characteristically rapid growth and virulence of this specific isolate of *Mycobacterium* sp. (5-7 day at 30°C). However, it

is most likely due to water temperature which is an important factor in influencing the fish imflammatory response.

The interaction between mycobacteria and rainbow trout macrophages was investigated in this thesis. Macrophages provide a major line of defence against microbial invasion. They take up large particles such as bacteria and protozoa and destroy them. They move towards the microbial particles guided by a gradient of chemotactic molecules. Once particles are bound to the surface of the macrophage, the plasma membrane expands along the surface of particles and engulfs them. The process requires the active participation of actin-containing microfilaments present under the cell surface. Attachment of the particles to the cell surface is mediated by specific macrophage receptors and is dependent upon the nature of the bacterial surface.

In the present study, bacteria were engulfed with macrophage plasma membranes to form the wall of the phagosome. Attachment, engulfment and phagosome development appeared in the macrophage after 0.5 and 1 hr incubation. The cells had phagocytosed many bacteria, and phagosomes containing one to several bacteria were visible at different stages of ingestion. Different types and numbers of phagosomes were observed within the macrophages examined at this stage.

Once the bacterial particles are taken up by macrophages, it has been shown that the vesicles containing the particles (phagosomes) fuse with primary lysosomes, forming secondary lysosomes (phagolysosomes) (Nichols *et al.*, 1971). Degradation of the ingested particles takes place within the lysosome, which contains hydrolases and other enzyme that generate substance which are essential for killing the bacteria. Some bacteria are taken up but not killed

by macrophages. *M. tuberculosis* prevents fusion of the phagosome with lysosomes, thereby avoiding killing by lysosomal enzymes (Shurin and Stossel 1978). Other bacteria, such as *Mycobacterium leprae*, resist degradation of lysosomal enzymes (Nathan *et al.*, 1980) and other not only resist, but even require an acidified phagolysosome for growth (Hackstadt and William, 1981). *Mycobacterium* spp. isolated from diseased fish also prevent phagosome-lysosome fusion in macrophages and some bacteria can escape from the phagolysome into the macrophage cytoplasm.

Enhanced phagocytic activity of macrophages is due to opsonization of the foreign particle by serum factors, such as specific antibodies, or complement. Griffin (1983) showed that the presence of specific antibody greatly enhanced the phagocytosis of *Y. ruckeri* by rainbow trout leukocytes; however, it did not affect intracellular killing. Conversely, the phagocytic activity of salmonid peritoneal exudate cells against *A. salmonicida* was only slightly enhanced by specific antibody. The addition of both specific antibody and complement greatly increased phagocytosis (Sakai, 1984; Olivier *et al.*, 1986). Blazer *et al.*, (1985) also found only slight increases in phagocytosis of *E. ictaluri* by head kidney macrophages from immunised channel catfish. However, opsonization with non-immune sera (antibody and complement) greatly enhanced intracellular killing (Blazer *et al.*, 1989).

In the current study, not only enhanced phagocytosis with bacteria opsonized with FS, Ab, or MAb was observed but also enhanced intracellular killing activity after 2 h incubation with the bacteria. Increasing the incubation times to 4 or 6 h greatly enhanced phagocytosis and intracellular killing ability of the macrophages.

The results of opsonization differ, depending on fish species, the type of foreign microorganism and the origin of phagocytes. Kaige *et al.*, 1990 stated that phagocytosis of Japanese eel macrophages was high, because, out of 500 macrophages, 339.7 (67.9%) were able to phagocytose non-opsonized SRBC and the number able to phagocytose more than five non-opsonized SRBC was 149.3 (29.9%).

Intracellular micro-organisms can avoid death within the phagocytes by producing resistant cell surface molecules such as lipids and glycolipids, which prevent phagosome lysosome fusion, inhibit vacuolar acidification, and inhibit the release of reactive oxygen intermediates (Finlay and Falkow, 1989). The existence of such avoidance mechanisms has so far not been demonstrated in intracellular fish pathogens (such as *R. salmoninarum*, *Mycobacterium spp.* and *N. asteroides*) although an apparent resistance to reactive oxygen intermediates has been shown for *R. salmoninarum* (Kaattari *et al.*, 1987; Bandin *et al.*, 1993).

Other studies on *R. salmoninarum* have involved electron microscopic analysis of naturally infected rainbow trout kidney tissue. These revealed *R. salmoninarum* inside phagosomes and also in the cytoplasm (Gutenberger, 1993). *R. salmoninarum* posseses an active membrane damaging factor which allows the bacterium to escape from the phagolysosome and live free in the cytoplasm. A similar membrane damaging factor has been identified in *Listeria monocytogenes*. This sulphur-dependant protein termed listeriolysin O is responsible for the escape of this pathogen from the phagosome (Portnoy *et al.*, 1992). It has been reported that both p57 and listeriolysin O possess the

same molecular mass and apparently the same sulphur dependant nature (Bandin *et al*,, 1992).

The macrophages from the head kidney of rainbow trout have different phagocytic and killing activity. The average number of phagocytic cells opsonized with FS, Ab or MAb was 40-60 % after 6 h incubation with *Mycobacterium* spp. The majority of bacteria opsonized with FS, Ab or MAb were damaged in the phagosome lysosome fusion within the macrophages. A minority of mycobacteria were still intact in the macrophages after phagosome lysosome fusion.

In general, it is interesting that the ECP from *Mycobacterium* spp isolated from fish induced high nonspecific and specific immune responses in rainbow trout and tilapia. It has been shown that opsonization of *Mycobacterium* spp. by rabbit polyclonal (anti-ECP) and monoclonal antibody (anti-65 kDa) makes the bacteria more susceptible to engulfment and killing by rainbow trout kidney macrophages. In human tuberculosis, Silva & Lowrie (1994) reported that a single mycobacterial protein (hsp 65) from *M. leprae* expressed by transgenic antigen-presenting cells in vaccinated mice have a high degree of protective immunity against subsequent challenge with either *M. bovis* BCG or *M. tuberculosis* H37 Rv.

Future research should include competitive binding assays to analyse individual epitopes on the 65 kDa protein and to examine the biological activity of fibronectin binding protein. The MAbs developed here (against ECP) should also be utilised in sandwich ELISA and immunohistochemistry to screen fish tissue (spleen, kidney and liver) for the presence of

mycobacteria. Further understanding of the role of macrophage function in the fish immune response against mycobacteriosis is also required. Culture of *Mycobacterium* spp. *in vitro* using a fish macrophage cell line would be very useful for this work. Further research should also concentrate on the virulence of strains used in these studies in snakehead fish and Siamese fighting fish. Furthermore, the 65 kDa protein of *Mycobacterium spp.* should be examined as a potential vaccine candidate to protect fish. Such a vaccine would probably be used with expensive aquarium fish but not in low price food fish.

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## Appendix 1

### 1. Phosphate buffered saline (PBS)

Disodium hydrogen orthophosphate-2-hydrate (2.56 g/l., BDH), sodium dihydrogen orthophosphate (0.87 g/l, BDH), sodium chloride (8.77g/l, Sigma) were dissolved in distilled water and the pH was adjust to 7.2. The solution was autoclaved at 121°C for 15 minutes.

### 2. Complement inactivation of foetal calf serum

FCS, thoroughly thawed, was placed in a water bath at 56°C for 30 minutes, mixing periodically. It was aliquoted and stored at -20° C.

### 3. ELISA

#### 3.1 Coating buffer

| Carbonate-bicarbonate buffer | | |
|---|---|---|
| $Na_2CO_3$ | 2. 59 g | Dissolved in 1 L distilled water |
| $NaHCO_3$ | 2.93 g | Adjusted to pH 9.6 and made freshly |

#### 3.2 Wash buffer(10x) (low salt)

| | | |
|---|---|---|
| Trisma base | 24.2 g | Dissolved in 1 L distilled water |
| NaCl | 222.2 g | pH adjusted to 7.3 |
| Merthiolate | 1 g | |
| Tween 20 | 5 ml | |

#### 3.3 Wash buffer(10x) (high salt)

| | | |
|---|---|---|
| Trisma base | 24.2 g | Dissolved in 1 L distilled water |
| NaCl | 292.2 g | pH adjusted to 7.7 |
| Merthiolate | 1 g | |
| Tween 20 | 10 ml | |

#### 3.4 Substrate buffer

| Sodium acetate/citric acid buffer | | |
|---|---|---|
| Citric acid | 21.0 g | pH adjusted to 5.4 with 1M NaOH |
| Sodium acetate | 8.2 g | |

5 µl of $H_2O_2$ was mixed with 15 ml substrate buffer

### 3.5 Substrate

3'3'5'5' Tetramethylbenidine dihydrochloride (TMB) (42 mM) was added to 1:2 acetic acid :distilled water. 150 µl of solution was added to 15 ml substrate buffer.

### 3.6 Stop reagent

2M $H_2SO_4$ in distilled water

## 4 Electrophoresis

(Nanopure water used throughout)

### 4.1 Sample buffer

| | | |
|---|---|---|
| Nanopure water | 4.0 ml | |
| 0.5 M Tris HCl pH 6.8 | 1.0 ml | |
| 2-Mercaptoethanol | 0.4 ml | Samples were diluted 1:1 in sample buffer |
| 10 % Sodium dodecyl sulphate (SDS) | 1.6 ml | |
| 0.05 % Bromophenol blue | 0.2 ml | |
| Glycerol | 0.8 ml | |

### 4.2 Electrode buffer

| | | |
|---|---|---|
| Glycine | 14.4 g | Dissolved in 1 l of nanopure water |
| Tris base | 3.30 g | pH 8.1-8.4 which must not be adjusted. |
| Methanol adjusted | 200.0 ml | |
| SDS | 1.0 g. | |

### 4.3 Separating gel

| | | |
|---|---|---|
| 3 M Tris HCl pH 8.8 | 1.25 ml | Recipe for two mini-gels |
| 30 % acrylamide and 0.8% bisacrylamide | 4.0 ml | de-aerated for 10 min undervacuum made prior to use at 0.1g $ml^{-1}$ |
| 10% SDS | 0.1 ml | |
| Ammonium persulphate | 50 µl | |
| N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) | 5 µl | |
| Nanopure water | 4.6 ml | |

### 4.4 Stacking gel

| | | |
|---|---|---|
| 3 M Tris HCl pH 6.8 | 0.4 ml | Recipe for two mini-gels |
| 30 % acrylamide and 0.8% bisacrylamide | 1.25 ml | de-aerated for 10 min under vacuum |
| 10 % SDS | 0.1 | |

| | | |
|---|---|---|
| Ammonium persulphate | 80 µl | made prior to use at 0.1 $gml^{-1}$ |
| N,N,N',N',-Tetramethyl-ethylenediamine (TEMED) | 8 µl | |
| Nanopure water | 8.2 ml | |

**5. Western blot:**

| | | |
|---|---|---|
| (1) Western blot buffer | | |
| Glycine Base 3.03 g | 14.4 g | Dissolved in 1 l nanopure water |
| Tris base | 3.03 g | |
| (2) Tris buffer saline (TBS) | | |
| Tris base | 6.07 g | Dissolved in 1 l nanopure water |
| Disodium EDTA | 0.41 g | pH 8.0 |
| NaCl | 8.7 g | |
| (3) Tween-Tris buffer saline | (TTBS) | |
| Tris base | 6.07 g | Dissolved in 1 l nanopure water |
| Disodium EDTA | 0.41 g | pH 8.0 |
| NaCl ml | 8.7 g | |
| Polyoxyethylene Sorbitan monolaurate | 1.0 | |
| (4) Substrate buffer (pH 7.5) | | |
| 3,3-diaminobenzidine tetrahydrochloride | 6.0 mg | Made freshly, dissolved in 10 ml TBS with 30 µl $H_2O_2$. |

**6. Histology**

**6.1 Fixation**

Material for histological examination was placed in fixative (10% neutral buffered formalin saline) for at least 24 hours prior to cassetting. Individual tissues must be of a suitable size to allow permeability of fixative.

**6.2 Cassetting**

The allocated case number was entered on the cassette using a pencil. Tissue samples were trimmed to a suitable size taking care not be overcrowd and the in cassettes as this can lead to ineffective dehydration and ultimately difficulty in sectioning. Small samples were wrapped in tissue paper before placing in the cassette. Soft and hard tissues were be kept separate. Cassetted samples were not allowed to dry out and were left in a bowl of water or fixative until loading onto the processor.

### 6.3 Tissue processing

This procedure was carried out by placing the cassettes into a basket which was moved round automatically by a tissue processor at the appropriate time intervals schedule

| | |
|---|---|
| 1. 50% Methylated spirit | 1h |
| 2. 80 Methylated spirit | 2hrs. |
| 3. 100% Methylated spirit | 2hrs. |
| 4. 100 Methylated spirit | 2hrs. |
| 5. 100 Methylated spirit | 2hrs. |
| 6. 100% Alcohol | 2hrs |
| 7. 100% alcohol | 2hrs |
| 8. Chloroform | 2hrs. |
| 9. Chloroform | 1hr. |
| 10. Molten wax | 1hr. |
| 11. Molten wax | 2hrs. |
| 12. Molten Wax | 2hrs. |

Cassettes were removed from the processors and placed in molten wax until ready to block out. Tissues were trimmed and sections cut by microtomy.

### 6.4 Staining

Haematoxylin and Eosin

| | |
|---|---|
| 1. Xylene | 5 min |
| 2.Alcohol I | 2 min |
| 3. Methylated spirits | 1.5 mins |
| 4. Running tap water | 10 min wash |
| 5. Haematoxylin | 5 mins |
| 6. Wash in tap water | 3 min |
| 7. Acid Alcohol | 3 quick dips |
| 8. Wash in tap water | 3 min |
| 9. Scott s tap water | 30 secs. Check staining microscopically at this stage |
| 10.Wash well | |
| 11. Eosin | 5 mins |
| 12. Quick wash in Tap water | 10 secs |
| 13. Methylated spirits | 30 secs. |
| 14. Alcohol II | 2 mins. |
| 15. Alcohol I | 1.5 mins |
| 16. Xylene | 5 mins |
| 17. Xylene | 10 mins |

Sections were coverslipped after the last xylene in the staining series. Once coverslipped, sections were labelled with the case number, and mounting fluid allowed to dry before examination.

## 7. Electron microscopy

### 7.1 Sodium cacodylate buffer (0.1M)

Sodium cacodylate (10.7g) was dissolved in 500 ml distilled water. The pH was adjusted to pH 7.2-7.4.

### 7.2 Karnovsky fixative (1.3 % paraformaldehyde, 1.6% glutaradehyde, Karnovsky, 1965)

Paraformaldehyde (2g) was added to 25 ml distilled water in a 100 ml conical flask. The mixture was heated and shaken constantly whist maintaining the temperature at 60-70°C for several minutes. Before the mixture was cooled, a few drops of 1N NaOH was added and the flask shaken well to dissolve the precipitate. The flask was left to shake to cool before adding 0.025g calcium chloride (anhydrous) and 10 ml 25% glutaraldehyde (or 5 ml of 50% glutaraldehyde). The total volume was made up to 150 ml with the cacodylate buffer. The pH readjusted to 7.2-7.4. The fixative was stored at 4°C for a maximum of 1 month.

### 7.3 Rinsing solution (sucrose 0.1M in cacodylate buffer)

Sucrose 6.84 g was mixed with in 200 ml of cacodylate buffer. Sucrose was added to the cacodylate buffer solution in order to present osmotic shock to the tissue or cells. Store at 4°C for a maximum of 1 month.

### 7.4 Osmium solution (1%)

A small brown bottle was labelled speciallly kept for osmium and a strip of hazard tape marked Toxic, Removed the label from one 0.25g ampoula of osmium tetroxide. Wrapped the ampoula in the black paper and broke the ampoula. Placed both halves of the ampoula, together with any small crystals which dropped out the paper, into the bottle. Immediately added 25 ml cacodylate buffer, stopper the bottle and shaken bottle carefully. Leave to dissolve at least overnight and shaken again carefully before use. do not attempt to pH this solution. This solution was toxic and must be stored in a fume cupboard. Discard if discoloured, by siding an equal volume of vegetable oil to the osmium and leaving for 24 hrs before disposing of down the sink with copious amounts of water.

### 7.5 Uranyl acetate (Watson 1958)

20% solution in absolute ethanol, stored at 4°C.

### 7.6 Lead citrate (Reynolds)

Lead nitrate (1.33g) and sodium citrate (1.76g) were mixed in 15 ml distilled water in a 50 ml volumetric flask. Stoppered and shaken for 1 min. was left to stand for 30 minutes, shaken at intervals, to ensure complete conversion of lead citrate to lead nitrite. Then add 8 ml of fresh

1N NaOH to dissolve the precipitate and made up to 50 ml distilled water. Store at 4°C. Centrifuged a small volume before use.

### 7.7 Tissue processing for ultra-structural examination

Karnovsky fixed samples were washed and left in 0.1 M cacodylate buffer at 4°C for 12 hrs , and then fixed in 1% osmium tetroxide in 0.1M cacodylate buffer at 4°C for one hour. After two further 15 min washed in buffer, samples were dehydrated by passing through graded acetone (60%, and 90% acetone each wash, then dehydrated two times in 100% acetone, 15 minute each wash).

Arodyte blocks were rough trimmed with a zazor blade. Final trimming, semi and ultra-thin section cutting were performed on a ultramicrotome (OM U3, Rechter-Jung, Slough, Berks) fitted with a glass knife made on an LKB knife maker (Bromma, Sweden), Semi-thin sections were picked up on glass slides, ultra-thin section sections were floated out onto uncoated cooper grids.

Semi thin sections of 0.5 μm thickness were stained with alkaline toluidine blue ( Bancroft and Stevens, (1977). Ultra-thin section were double stained in alcoholic uranyl acetate and lead citrate solution (Raynolds, 1963). The grids face up were submerged in drops of uranyl acetate on parafilm (2 mins) and rinsed in stream of distilled water. The grids were blotted on filter paper. Float grids face were down on small blob of lead citrate on parafilm (2 mins) and rinsed in a stream of distilled water. Slides were routinely viewed with a reichter-Jung polyvar wide field photomicroscope (Reichter-Jung, Slough, Berks). Photomicrographs were taken on Kodak Ektacrome (daylight) 200 film (Eastman Kodak Co., Rochester, N. Y.)

## Appendix 2 suppliers of materials

### Animals

Rainbow trout: Swanawater fish farm, Stirling.
Tilapia : Institute of Aquaculture, University of Stirling.
Rabbit : University of Stirling Animal House.
Mice : University of Stirling Animal House.

### Biorad Labs. Ltd., Watford, Hertfordshire

Protein dye concentrate reagent
Low molecular weight markers
Prestain protein markers
Nitrocellulose paper

**BDH poole, England**

$K_2HPO_4$

**Ewos Ltd., Westfield, West Lothian, UK**

Trout diets

**Gibco Ltd., Paisley, Scotland**

Tryptic soya broth
Trytic soya agar

**Lancer, Div. of Sherwood Medical, Athy, Eire**

Haematocrit capillary tubes

**Millipore Corp., Bedford**

0.22 μm filters Northumbria Biologicals Ltd. (NBL), England
96 well flat bottomed microtitre plate(Costa, NBL)
96 well round bottom microtitre plates
6 well tissue culture plate

**Rathburn Chemicals Ltd., Walkerburn, Scotland**

Acetone
Chloroform
Cristaseal Ethanol
Glacial acetic acid
Hexane Methanol
Toluene blue

**Scottish antibody production unit (SAPU), Carluke, Scotland**

Anti-rabbit IgG-HRP
Anti-mouse IgG-HRP

**Sigma chemical Co. Poole, Dorset**

Benzocaine (ethyl-p-aminobenzoate)
Bovine serum albumin
Citric acid
Con A
DMSO
Polyethylene glycol
Freund's incomplete adjuvant
Freund's complete adjuvant
Giemsa stain
Glutaraldehyde
Heparin
KOH
*Micrococcus lysodeikticus*
NaCl
$NaH_2PO_4$
$Na_2HPO_4$
Nitroblue tetrazolium
Percoll
Phorbol myristate acetate
Poly-lysine
SDS
APS
TEMED
Sodium sulphate
Sodium bicarbonate
Trypan blue
Tween 20

**Whatman, England**

No. 1 filter paper

**Agar Scientific LTD**

Paraformaldehyde
Glutaraldehyde
Uranyl acetate
Lead citrate
Sodium cacodylate
Osmium tetroxide
LR white resin
Copper grids
Nickel grids

**Bio Cell**

Gold probe
Silver probe