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Comparative Study of Experimental Mycobacteriosis Between Snakehead Fish (*Chana striata*) and Frog (*Rana tigrina*)

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Abstract

In many of the culture areas in Thailand, snakehead fish suffer mortality from septicemic mycobacterium infections (Chinabut et. al., 1990). The contaminated drainage from fish farms may spread into the environment and potentially cause problems to frog hatcheries and growout farms.

A total of 80 fish and 160 frogs were intramuscularly injected with a pure cultured virulent strain of *M. marinum*, isolated from the kidney of a diseased snakehead fish with clinical signs of mycobacteriosis. The inoculated animals were observed daily for the appearance of clinical signs of infection and were sacrificed routinely for the pathological study.

After 15 days of injection, the infected fish showed emaciation, lack of coordination, erosion of fins, scale loss and severe ulceration, whereas the infected frogs did not show any gross pathological lesions throughout the experiment. Gray-white miliary tubercles were observed in the spleen, kidney and liver of the infected fish whereas these tubercles were never found in any organs of infected frogs. Histologically, the granulomatous inflammation was observed both in fish and frogs. Granulomas found in the visceral organs, dermis and injected areas of fish and in the injected areas of frogs revealed the composition of epithelioid and phagocytic cells surrounded by whorls of fibroblastic cells. Central caseous necrosis was observed only in fish granulomas. Two different cell types were noticed in frog skin in the early period of the experiment. The dark elongated nuclear cells resembling mammalian Langerhans cells were observed in the suprabasal epidermis, and the stellate shaped dendritic cells were observed both in the dermis and hypodermis. Both cell types are reported with reference to delayed-hypersensitivity in humans.

Introduction

Snakehead fish (*Channa striata*) and frogs (*Rana tigrina*) are cultured throughout Thailand. Snakehead fish in many culture areas suffer mortality from mycobacteriosis, a septicemic chronic Mycobacterium infection (Chinabut et. al., 1990). The pathogenesis of mycobacterium infection is a complex and

multifactorial process involving the organism and host immune response (Lungworth 1993; Theon and Chiodini 1993).

The microscopic lesions observed in fish and amphibians are generally described as tubercles which are different from those in warm-blooded animals. Central caseous necrosis may be present in fish granulomas, but is not prominent in amphibian lesions (Marcus 1981; Hines et al., 1995). Histological examination of a classic tuberculosis granulomas reveals a composition of epithelioid and phagocytic cells, occupying a central position surrounded by whorls of fibroblastic cells (Samuelson 1997).

The contaminated drainage from the diseased snakehead fish ponds may spread into the environment and potentially cause problems to frog hatcheries and growout farms. The susceptibility of *R. tigrina* to *Mycobacterium marinum* was investigated in this study.

Materials and Methods

Live young fish and frogs were obtained from farms and were acclimatized in laboratory glass aquaria for at least one month before experimentation. The animals were fed once daily with a nutritionally complete floating pellet. Healthy fish weighing about 100g and 10-15 cm long, and healthy frogs weighing between 25-35 g were used. A total of 80 fish and 160 frogs were intramuscularly injected with a pure culture of a virulent strain of *M. marinum* (Shotts and Teska 1989) isolated from the kidney of a diseased snakehead fish with clinical signs of mycobacteriosis.

Fish were injected with $3.0x10^7$ bacteria/ml, while frogs were injected with $5.8x10^7$ cells/ml. Bacterial suspensions were intramuscularly injected 0.1 ml per 100 g body weight of experimental animals into the left side near the dorsal fin and into the ventral left thigh of the frogs.

Control animals, 20 fish and 40 frogs, were injected with the same amount of 0.85% sterile isotonic saline. Animals in each treatment were kept in separate aquaria which contained 28°C water for further examination.

Eight infected and two control fish were sacrificed routinely at 1, 3, 6 and 24 hrs and on day 3, 5, 7, 14, 21 and 28 after injection. Eight infected and two control frogs were sacrificed routinely as follows: every hour from 1 to 6 hrs and then 12 hrs, every day from day 1 to day 7, then 1, 3, 6 and 24 hrs and then day 14, 21 28, 38, 48 and 58. Small blocks of tissues encompassing the area under investigation were dissected out, including liver, kidney, spleen, stomach, intestine and muscle at the site of injection. These tissues were fixed in 10% buffered formalin for at least 24 hrs, processed and embedded in paraffin wax and sectioned at 5 μm. The sections were stained with hematoxylin and eosin (H&E).

Results

The fish injected with bacteria were seen lying at the bottom of the aquaria by 15 day post injection. At the site of the bacterial injection, swollen

hemorrhagic lesion in the skin was observed. The injected fish showed emaciation, lack of coordination, sometimes floating under the surface of water. Fin erosion, scale loss and many foci of hemorrhagic skin lesions were also observed with severe ulceration near the caudal fin. In contrast, no gross lesions were observed clinically in frogs injected with bacteria throughout the experiment. None of the control animals died during the course of experiments.

Internally, the kidney of the affected fish showed marked swelling and pale colour. The spleen was enlarged. Whitish tubercles were observed in these organs and also in the liver, whereas there were no histopathological changes in the internal organs of injected frogs.

Histopathologically, many developing granulomas were observed in the skin and the muscle at the site of injection as well as in the spleen, kidney and liver. There were two types of granulomas, hard and soft granulomas, found in the injected fish. Both of these comprised epithelioid and phagocytic cells surrounded by whorls of fibroblastic cells. In addition, caseous necrosis was found at the center of the soft granulomas. By comparison with injected fish, granulomas were observed only in the muscle at the sites of injection of frogs and all granulomas were of the hard type (Fig. 1). Giant cells were also observed in frog granulomas, whereas they could not be observed in fish granulomas.

Two different cell types were noticed in the skin of infected frogs at the site of injection by 3 hrs post injection and began to decrease by day 2. Such cells were not observed in the skin of control frogs. The first cell type noticed was only their dark elongated nuclei which was obvious in the suprabasal epidermis. The second type found in the dermis and hypodermis were stellate shaped dendritic cells (Figs. 2 and 3). These cells were first observed in both dermis and hypodermis 1 hr post injection, and began to decrease in number by day 1. After day 1 these cells were located in the spongiosum much more than in the hypodermis. These cell types were never observed in the infected fish. Many subepidermal blisters were formed. By 3 hrs, many large foci of well defined dermal fiber nests composed of fragments of dermal fibers themselves and encapsulated by their fibers were observed. By day 29 post infection the dermal nests found in the spongiosum were transformed to blood vessels (Fig. 4).

Discussion

This comparative experiment showed that *Mycobacterium marinum* is a pathogen in both snakehead fish and the frog, because the experimental animals given intramuscular injection with bacteria showed a chronic inflammatory response. This bacterium caused more severe response in fish than in frogs. Clinical signs observed in fish including emaciation (Chinabut et al., 1990), immobility (Gomez et al., 1993), focal skin lesions such as scale loss (Hatai et al., 1988), paleness, and lack of appetite (van Duijn 1981) have been reported in tropical fish with mycobacterium infection. The clinical signs observed in the snakehead fish were similar to these symptoms.

The typical granulomas found in the experimental fish were similar to those observed in natural infections in the snakehead fish (Chinabut et al.,

1990). Hines and co-workers (1995) reported that amphibians appeared to be relatively resistant to mycobacterial infections. Lesions usually consisted of grayish nodules in the skin and abdominal viscera, including extensive ulceration of the skin. Miliary lesions were found throughout the visceral organs. Lesions were often most severe in the liver, with variable severity in the spleen and kidney (Reichenbach-Kline and Elkan 1965; Marcus 1981). In this study the lesions found in the infected frogs were granulomas in the muscle at the site of injection, including giant cells in some granulomas which could not be observed in fish granulomas. Foci of caseous necrosis were present in fish granulomas whereas this kind of necrosis was not observed in frog granulomas. These results support the report of Marcus (1981).

Infection with *Mycobacterium* typically leads to the development of delayed- hypersensitivity (Kumar et al., 1997) which is a prerequisite for granuloma formation and for enhanced resistance to *Mycobacterium* (Samuelson 1997). The sequence of events in delayed-hypersensitivity in humans, as these occur in the tuberculin reaction, begins with the first exposure of the individual to tubercle bacilli. In humans, $CD4^+$ lymphocytes recognize peptide antigens of *Mycobacterium* in association with class II antigens on the surface of monocytes of cutaneous dendritic, or Langerhans cells that have processed mycobacterial antigens. These changes are accompanied by the secretion of a number of cytokines which are responsible for the expression of delayed-type hypersensitivity (Botham et al., 1978; Kolde and Knop 1987; Picut et al., 1987; Hanau et al., 1989; Kumar et al., 1997). Bos and Kapsenberg (1986) described that human Langerhans cells were suprabasal dendritic cells found in the epidermis where they formed an evenly distributed network of cells with long dendrites that almost seem to touch each other. Langerhans cells can pass through the epidermal basement membrane into the dermis (Hashimoto and Tarnowski 1968; Silberberg et al., 1976; Larsen et al., 1990). The finding of these two cell types in the cutaneous layer in this study, which have not been observed in other experiments, may have something to do with the delayed-hypersensitivity and the resistance to Mycobacterium infection of the infected frogs. These cells resemble the Langerhans cell of mammals which may be in-

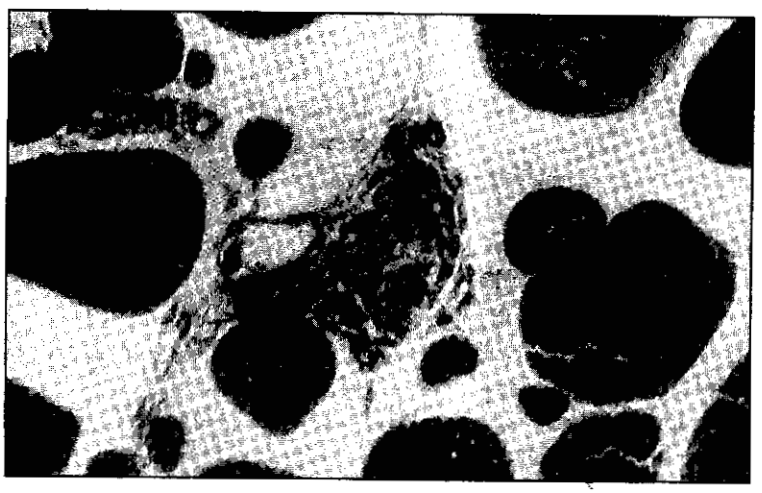

Fig. 1. Granuloma (GR) in the muscle of the frog at the site of injection H&E x520.

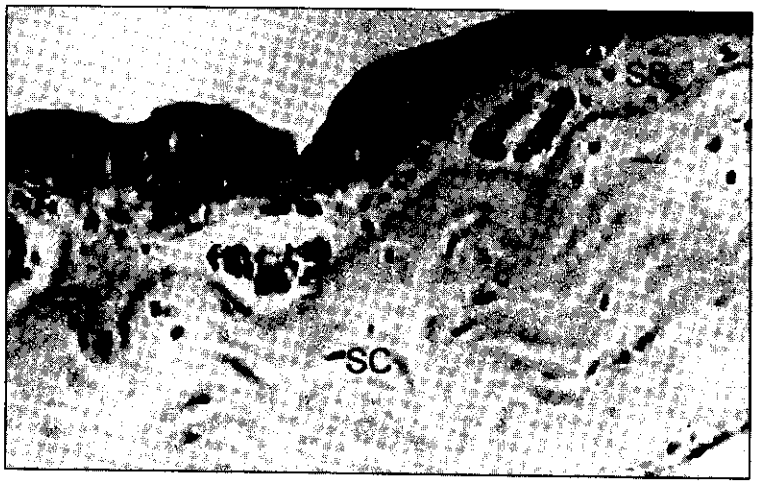

Fig. 2. Skin of the control frog shows normal stratum spongiosum and stratum compactum (SC). H&E x360.

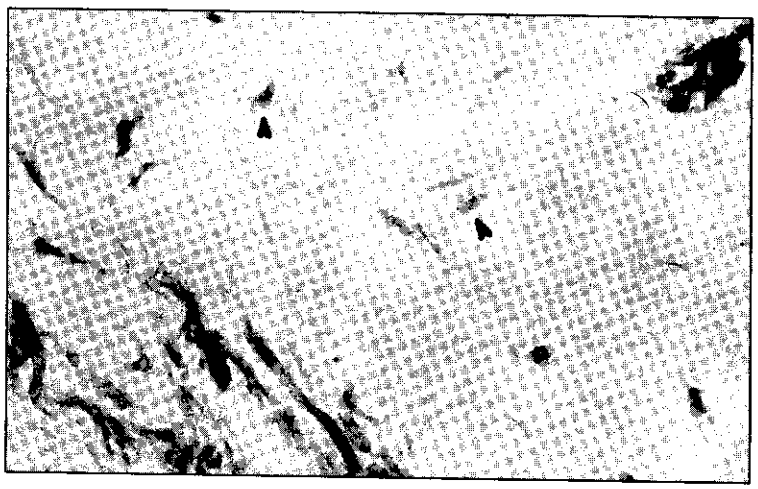

Fig. 3. Dendritic cell (arrow head) in the stratum spongiosum of the infected frog. H&E x520

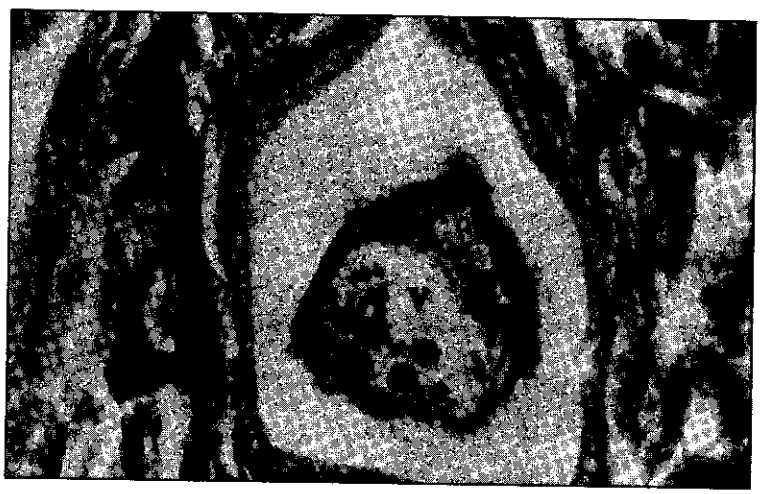

Fig. 4. The transformed blood vessels (V) with erythrocytes and thrombocytes in the lumen. H&E x520

volved with the interaction between the surface antigens of these cells and mycobacterial antigens as described by Kumar and his co-workers (1997).

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