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Inflammatory Response in Indian Major Carp, *Catla catla* and Barb, *Puntius cauveriensis*, to *Aphanomyces invadans* and Freund's Complete Adjuvant

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Abstract

The objective of the present study is to determine the pattern of inflammatory response in two different susceptible fish species Indian major carp, *Catla catla* and barb, *Puntius cauveriensis* of Epizootic Ulcerative Syndrome (EUS) to two different antigen models (*Aphanomyces invadans* and Freund's complete adjuvant). Inflammatory response studies with *A. invadans* indicated that in the case of barb there was very weak or no inflammatory response. The severity of myonecrosis was so high that in the moribund fish, virtually no normal muscle fibres were observed in the lesion area. On the other hand, in the case of Indian major carp (catla), there was an active host inflammatory response (particularly at the central part of the lesion) and the severity of myonecrosis was less than that of the barb. Studies on inflammatory response to Freund's complete adjuvant indicated that similar to *A. invadans* experiment, the degree of inflammatory cellular infiltration was very low in the case of barb and no inflammatory cells were observed

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around most of the adjuvant droplets. On the other hand, in the case of Indian major carp, there was a very active inflammatory response and most of the adjuvant droplets were encapsulated by a thick layer of epithelioid cells. Hence, it was considered that due to very poor inflammatory response, the barb might be offering a very insignificant or no resistance to *A. invadans* infection and that might be one of the major reasons for its high susceptibility to Epizootic Ulcerative Syndrome.

Introduction

Epizootic Ulcerative Syndrome (EUS) is one of the most destructive diseases of farmed and wild fishes in fresh and brackish water in the Asia-Pacific region (Lilley et al. 1998). The causative agent of EUS is an oomycete fungus, *Aphanomyces invadans* (Baldock et al. 2005). The disease has been reported over more than 100 fish species (Lilley et al. 1998). The range of incidence of disease recorded from various species of fish and different types of water bodies reveals that barb is highly susceptible (Roberts et al. 1994; Lilley et al. 1998) and Indian major carps are mildly susceptible (Das 1997) or refractory (Kumar et al. 1991; Vishwanath et al. 1997a; 1997b; 1998; Jayaraman 1991). It is well established that inflammation is the basic protective response of an organism and is the starting point which ultimately decides the overall resistance to any infection or injury. Therefore, it was felt that it would be meaningful to compare the inflammatory response between these two groups of fish i.e. highly susceptible (barb) and mildly susceptible (Indian major carp) against an infectious agent, *Aphanomyces invadans* and an inert material, Freund's complete adjuvant.

Materials and Methods

Forty eight fingerlings of catla (8.4 ±0.8 cm) and barb (8.1 ±0.9 cm) were used for artificial infection test. Catla was collected from Karnataka State Government Bhadra reservoir project fish hatchery and the barb was collected from the riverine source. Both fish species were divided into four groups (one for *A. invadans*, one for adjuvant and two control groups, one for each) having 12 fish each. In the case of *A. invadans*, each experimental fish was injected intramuscularly with 0.1 ml of spore suspension ($6x10^4$ spores$\cdot ml^{-1}$) of *A. invadans* (strain B99C) as described by Chinabut et al. (1995). Suspension of motile secondary zoospores was prepared as

described by Lilley et al. (1998). In the case of adjuvant, each experimental fish was injected with 0.1 ml of solution as described by Sobhana et al. (2002). The control fish group of both the experiments received 0.1 ml autoclaved pond water at the same time as the test fish.

After injection, each fish species of experimental and control groups were kept separately, in 500 L capacity fiberglass tubs containing 400 L of water. Aeration was maintained with replenishment of 50% of water daily. Water temperature of the experimental tanks ranged from 26 to 29°C as measured twice daily in the morning and evening. The experimental period was for a period of 10 days and two fish each from the experimental and control groups were sampled at 1, 2, 4, 6, 8 and 10 days post injection.

After gross observation, blocks of muscle and skin were excised from the area of injection and preserved in 10% buffered formalin for histopathological analysis. All the histopathological analyses were carried out as described by Chinabut and Roberts (1999).

Results and Discussion

In the case of *A. invadans*, the injected spores were able to germinate in the muscle tissue of both the fish species within one day after injection (Figs. 1 and 2). Two days after injection, many hyphae were observed on the injected side of both the species but in the case of IMC at the centre of the lesion, there was extensive infiltration of inflammatory cells and most of the hyphae were encapsulated by inflammatory foci of macrophages (Fig. 3). Whereas, in the case of barb, very few inflammatory cellular infiltrate had migrated into the lesion area (Fig. 4) and no inflammatory foci of macrophages around the fungal hyphae was observed.

Four days after injection, mycotic lesion had extended to both injected and non-injected sides in both the species. But, in the case of catla, hyphae were encapsulated by several layers of epithelioid cells at the centre of the lesion (Fig. 5). On the other hand, in the case of barb, very less inflammatory cells had migrated into the lesion area and no encapsulatory response by inflammatory foci of macrophages or epithelioid cell granulomata (Fig. 6) as seen in the case of catla were observed. In addition, in the

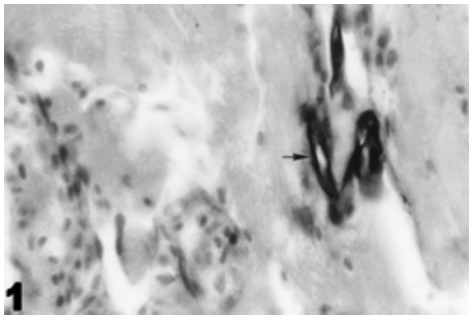


Figure 1. A few fungal hyphae (arrow) in the myotome area of Indian major carp (IMC) at one day post injection (dpi) (Grocott – H&E, x1000).

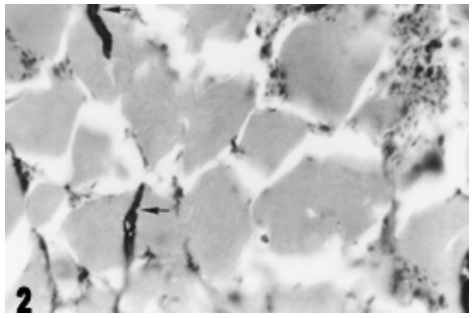


Figure 2. Few fungal hyphae (arrows) in the myotome area of barb at one dpi (Grocott -H&E, x 400).

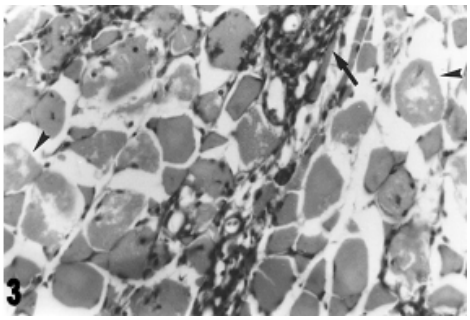


Figure 3. Development of an inflammatory focus of macrophages around the hyphae (arrow) and degenerated muscle fibres (arrow heads) in the lesion area of IMC at two dpi (H&E, x400).

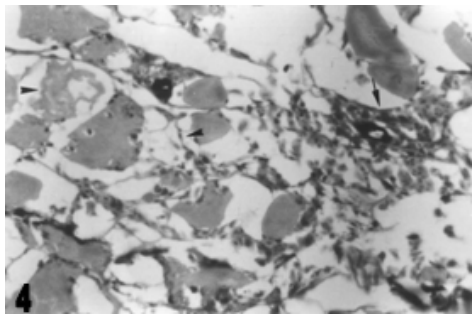


Figure 4. Mycotic lesion area of barb at two dpi (arrows) showing degenerated muscle fibers (arrow heads) and very less or no inflammatory cellular infiltration (Grocott – H & E, x 400).

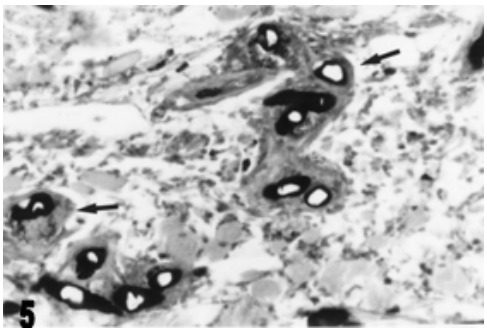


Figure 5. Mycotic lesion area of IMC at four dpi showing well developed encapsulatory response by the epithelioid cells around hyphae (arrows) (Grocott -H&E,x 400).

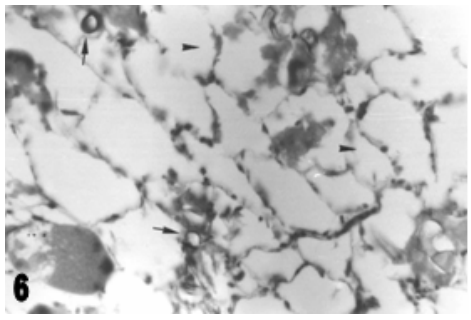


Figure 6. Mycotic lesion area showing no or very weak inflammatory cells around the hyphae (arrows) and extensive necrosis of muscle fibers (arrow heads) in barb at four dpi (Grocott -H&E,x400).

case of barb, the severity of myonecrosis in the lesion area was very high (Fig. 6) compared to catla.

Six days after injection, in both species, both the injected and non-injected sides and almost all the internal organs were extensively occupied by mycotic lesions. But, in the case of catla, at the centre of the lesion, hyphae surrounded by epithelioid cell granulomata were fused with each other to form large proliferative lesions (Fig. 7) and outside the centre of the lesion area, hyphae had penetrated most of the muscle fibers and these muscle fibers showed extensive degeneration and necrosis (Fig. 8). Whereas, in the case of barb, around most of the hyphae at the centre of lesion (Fig. 9) and all the hyphae outside the centre of lesion (Fig. 10) no inflammatory cellular response was found. The severity of myonecrosis was so high that virtually no muscle fibres were observed in the lesion area. In the case of barb, mortality started 5 days after injection and all the fish had died by day 7. In the case of IMC, mortality started 6 days after injection and all the fish had died by day 9.

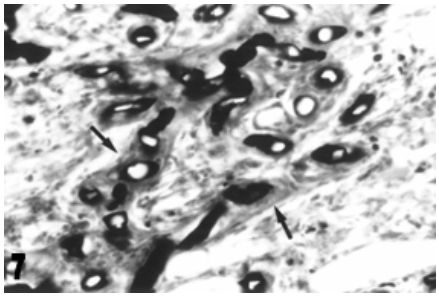


Figure 7. Large proliferative lesions (arrows) at the center of mycotic lesion area of IMC at six dpi (Grocott – H&E,x 400).

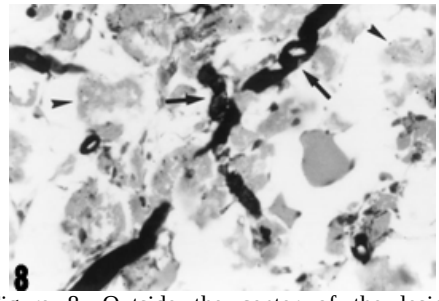


Figure 8. Outside the center of the lesion showing an extension of fungal hyphae (arrows) and adjacent degenerated muscle fibers (arrow-heads) in IMC at six dpi (Grocott -H&E,x400).

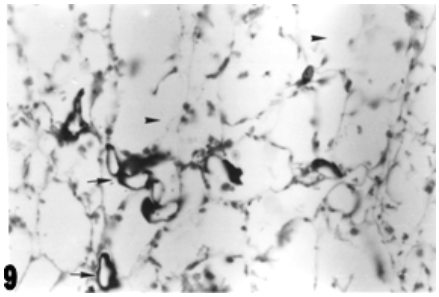


Figure 9. Center of mycotic lesion area of barb at six dpi showing very less or no inflammatory cells around the hyphae (arrows) and extensive myonecrosis (Grocott – H&E, x400).

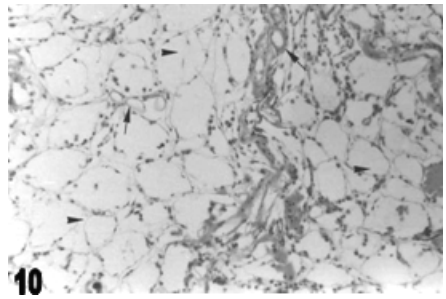


Figure 10. Extensive mycotic lesion area of barb at six dpi showing necrotized muscle fibers (arrow heads) and fungal hyphae (arrows) with very few/no inflammatory cells around (H&E, x 200).

In the case of adjuvant, in both the species, one day after injection of adjuvant, the degenerative changes in the lesion area consisted of myonecrosis, inflammatory cellular infiltration, myophagia and hemorrhages due to the passage of needle inserting the inoculums. Two days after injection, in the case of catla, the degree of cellular infiltration into the lesion area was comparatively more than that of the barb (Figs. 11 and 12). With time course, the degree of cellular infiltration into the lesion area in the case of catla had increased substantially and there was very well developed encapsulatory response by the macrophages around the adjuvant droplets (Fig. 13). Where as, in the case of barb, around most of the adjuvant droplets very poorly developed or no encapsulatory response was observed. (Fig. 14). At the end of the experimental period of 10 days, in the case of catla, most of the droplets were encapsulated by very well developed /thick epithelioid cell layers (Fig. 15) and the lesion area appeared repaired with regenerated muscle fibres. On the other hand, in the case of barb, the typical encapsulatory response as observed in the case of catla was not observed and large adjuvant droplets were encapsulated by very thin layers of epithelioid cells (Fig. 16).

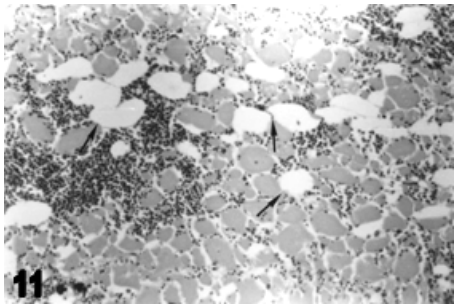


Figure 11. Lesion area of IMC showing adjuvant droplets (arrows) and a large number of inflammatory cells at two dpi with adjutants (H&E, x200).

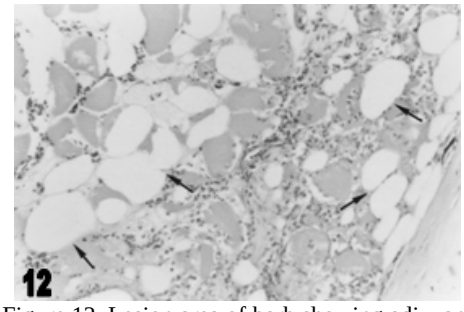


Figure 12. Lesion area of barb showing adjuvant droplets (arrows) and very less inflammatory cellular infiltration compared to IMC at two dpi (H&E,x200).

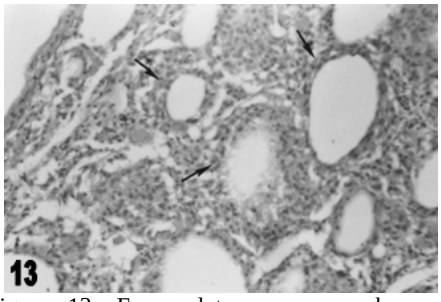


Figure 13. Encapsulatory response by epithelioid cells around the adjuvant droplets (arrows) at six dpi in IMC (H&E, x200).

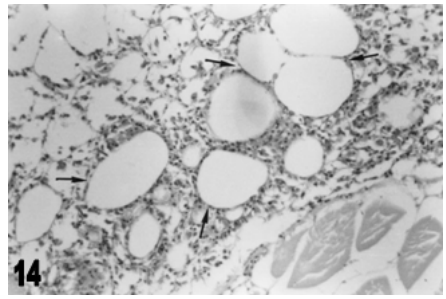


Figure 14. Note poorly developed encapsulatory response around the adjuvant droplets (arrows) at six dpi in barb (H&E, x200).

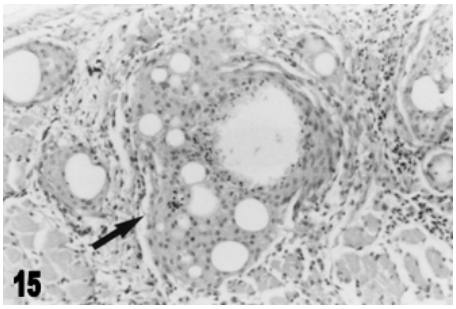


Figure 15. Adjuvant droplets encapsulated by thick layers (arrow) of epithelioid cells in IMC at ten dpi (H&E, x200).

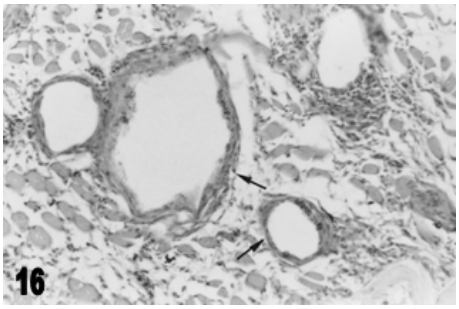


Figure 16. Encapsulation of adjuvant droplets by thin layers (arrows) of epithelioid cells in barb at ten dpi (H&E, x200).

The present study clearly indicated that zoospores of the fungal pathogen *A. invadans* were able to germinate in the muscle tissue of both fish species. But, in the case of catla, the degree of inflammatory cellular infiltration was comparatively high. *In-vitro* studies by Thompson et al. (1999) have indicated that macrophages were getting clumped around the growing hyphal tips. Hence, it appears that a similar phenomenon might be occurring in *in vivo* conditions and that infiltration of large number of inflammatory cells in the case of IMC, might be providing resistance for the invasive spread of fungal hyphae. As a result, severity of myonecrosis was less and onset of mortality was also later than that of the barb. Similar to the present study findings, Wada et al. (1996) in their artificial infection studies found that intense inflammatory response in common carp played an important role in preventing fungal growth and restricting the mycotic lesion area. On the other hand, in the case of barb, the degree of inflammatory cellular infiltration was very low. It was thus assumed that the degree of resistance offered to the invasive spread of hyphae was also very low or insignificant. As a result, the extent of myonecrosis caused by the fungal hyphae was very high and the onset of mortality was earlier than catla. Similar to the *A. invadans* experiment, in the case of adjuvant experiment also consistently, in the case of barb the degree of inflammatory response was very weak or insignificant compared to catla. Similar to the present study findings, depending on the fish species, variation in the intensity of inflammatory response to antigens has also been reported by Sommerville (1981).

Comparison of inflammatory response between the antigen models i.e. *A. invadans* and FCA within species indicated that the sequence of host response i.e. inflammatory cellular infiltration, development of inflamma-

tory foci of macrophages and finally epithelioid cell granulomata were similar in both the antigen models. But, in the case of FCA experiment, at the end of the experimental period of 10 days, a large number of inflammatory cells were present in the lesion area. Hence, it was assumed that the combined effect of persistence of oil droplets plus the mycobacterial toxin in the adjuvant might have imparted a more intense inflammatory response compared to *A. invadans*. Similarly, Sobhana et al. (2002) studied the effect of vitamin C on inflammatory response to Freund's complete adjuvant and reported that at the end of the experimental period of 9 days, a large number of inflammatory cells were present in the lesion area. A comparison of the nature of granulomatous response between FCA and *A. invadans* indicated that, in the case of adjuvant, the granuloma was in the form of circular granuloma and that the superficial section was looking like a mass of cells. In the cross section, it looked like an empty space surrounded by an aggregation of macrophages. On the other hand, in the case of *A. invadans*, it was in the form of an organized tube of macrophages encasing the growing fungus (as it extended through the tissue) and was in the form of linear granuloma.

Based on the observations from natural outbreaks of EUS, Chinabut and Roberts (1999) grouped the Indian major carps and barb fingerlings under a similar category i.e. per-acute infection category, where the acuteness and severity of the infection is so high that virtually, there is no host inflammatory response. In contrast to the findings of Chinabut and Roberts (1999), the histopathological features of catla fingerlings in the present investigation were of higher category i.e. acute category, where there is significant host response to the fungus. This was supported by the findings of adjuvant experiment, where consistently, in the Indian major carp, the degree of cellular infiltration was higher than that of the barb. However, in the case of barb, in both the experiments (*A. invadans* and adjuvant) the degree of inflammatory response was very weak or insignificant and was similar to the findings of Chinabut and Roberts (1999). Hence, from the present study, it was considered that infiltration of inflammatory cells played an important role in resisting the *A. invadans* infection and due to very poor inflammatory response, barb might be offering a very insignificant resistance to *A. invadans* infection and that might be one of the major reasons for its high susceptibility to EUS.

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