Enhancement of Immunity and Antigen Clearance in *Cyprinus carpio* and *Labeo rohita*

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ABSTRACT

Achyranthes aspera L. an Indian medicinal plant (Family: Amaranthaceae), was incorporated in the artificial fish diets. Cyprinus carpio L. (90 ± 17 g) and Labeo rohita S. (200 ± 27 g) were fed with the diets containing Achyranthes aspera and control groups were fed with normal diet without Achyranthes aspera. After 4 weeks of feeding, fishes were intraperitoneally immunized with bovine serum albumin. C. carpio were sampled for serum, 4 times on weekly intervals after immunization; and spleen were sampled from both C. carpio and L. rohita on second week alone. Antibody response specific to BSA was determined by ELISA in C. carpio; antigen clearance was determined by immuno-electron microscopy in spleen of both C. carpio and L rohita. Achyranthes aspera significantly (P<0.05) enhanced the antigen-specific antibody response in C. carpio. Both C. carpio and L. rohita, treated with Achyranthes aspera, efficiently cleared the injected antigen, i.e. BSA.

1. Introduction

Immunostimulants increase resistance to infectious diseases by enhancing non-specific and/or specific defense mechanisms. Use of these immunostimulants is an effective means of increasing the immunocompetency and disease resistance. Several immunostimulants stimulate phagocytes, natural killer cells, complement, lysozyme, and antibody responses. Activation of these immunological functions is associated with increased protection against infectious disease (Sakai, 1999). Several reports are available on immunostimulants originated from plant, animal and bacterial sources. Agglutinins derived from Abrus precatorius were tested on the murine macrophages in-vitro. Both heat-treated and native agglutinins have exhibited immunostimulatory properties by enhancing macrophage activities, such as increased production of nitric oxide and hydrogen peroxide, and high phagocytic and bactericidal activities, and also production of IL-1 (Tripathi and Maiti, 2003). The effect of an ethanolic extract of Aconitum heterophyllum was studied on delayed type hypersensitivity, humoral responses to sheep red blood cells, skin allograft rejection, and phagocytic activity of the reticuloendothelial system in mice. Aconitum heterophyllum was appeared to stimulate phagocytic function while inhibiting the humoral component of the immune system (Atal et al., 1986). Bath treatment of spawn of Catla catla with 1% crude extracts of neem, garlic and turmeric (1:1:1) increased disease resistance against experimental infection with Aeromonas hydrophila (Dey and Chandra, 1995). Feeding of glycyrrhizin enhanced the complement activity and increased the resistance against experimental infection with Edwardsiella seriola in yellowtail, Seriola quinqueradiata (Edahiro et al., 1990). Ethanolic plant extract of Clinacanthus nutans or Phyllanthus spp., complexed with polyvinylpyrrolidine (PVP), when fed to Penaeus monodon, enhanced the survival rate and provided resistance against yellow head virus (Direkbusarakom et al., 1995, 1998). Feeding with Catharanthus roseus plant extract incorporated diet enhanced the immune response of Labeo rohita (Thuy et al., 2002). Treatment with the extract of tunicate (Ete) enhanced the phagocytosis and protection against Aeromonas hydrophila in American eel, Anguilla rostrata (Davis and Hayasaka, 1984). Injection of extracellular products of Mycobacterium sp. mixed with Freund's adjuvant stimulated non-specific immune response in Nile tilapia, Oreochromis nilotica (Chen et al., 1998). Plasmid DNA and synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG enhanced the serum lysozyme, phagocytic and NBT responses in common carp, Cyprinus carpio (Asmi et al., 2002) and induced production of anti-viral cytokine activity in Atlantic salmon, Salmo salar leucocytes, thus proved to be potent immune activator (Jorgensen et al., 2001). Oral treatment of peptidoglycan (Matsuo and Miyazono, 1993), lactoferrin (Sakai et al., 1993) and injection of yeast glucan (Thompson et al., 1995) to rainbow trout Oncorhynchus mykiss, increased lysozyme level, phagocytosis and resistance against Vibrio anguillarum.

Achyranthes aspera L., a herb belonging to family Amaran-

thaceae, is widely available and distributed throughout India. The pharmacological properties of this plant are thermogenic, expectorant, revulsive, carminative, digestive, stomachic, laxative, anodyne, depurative, nthelminthic, diuretic, linthontriptic, sudorific, demulcent, hematinic and anti-inflammatory; it is useful in treating cough, asthma, bronchitis, dyspepsia, flatulence, colic, painful inflammations, dropsy, ophthalmopathy, vomiting, leprosy, skin diseases, pruritus, helminthiasis, strangury, renal and vesical calculi, cardiac disorders, anaemia, vitiated conditions of kapha and vata and general debility (Warrier et al., 1996). This plant has been shown to be immunostimulatory in mammals, as the aqueous extract of Achyranthes enhanced the antigen-specific antibody response in mice of different genetic background, namely H-2b, H-2d and H-2q (Vasudeva et al., 2002). Among the different parts of the plant, the seed and root possess greater stimulatory activity. Also Achyranthes enhanced cRBC-specific antibody response, and other components of non-specific immunity of carps (Vasudeva et al., 2004, Vasudeva and Chakrabarti, 2004, 2005a, b). In this study we have tested the effect of Achyranthes aspera on the specific immunity against injected antigen and its' clearance in Cyprinus carpio L. and Labeo rohita S.

2. Materials and methods

2.1. Animals

Cyprinus carpio (90 ± 17 g) and *Labeo rohita* (200 ± 27 g) were obtained from the local fish market, Delhi and were acclimatized for three weeks separately. Fish were cultured in outdoor cemented tanks (170 L). Two feeding regimes were used in each experiment; control and *Achyranthes aspera* incorporated diets. Four replicates (7 fish/group) were used for each feeding condition. Temperature and pH ranged between 27 - 33^{0}C and 7.4 - 8.0, respectively during the experiment. Dissolved oxygen level was maintained above 5 mg/L with the help of aerators throughout the experiment.

2.2. Experimental diet and feeding

Control diet (without *Achyranthes aspera*), and test diet containing 0.5% *Achyranthes aspera* seed were prepared and used in the experiment. Feeding was started 4 weeks prior to immunization at the rate of 1% of body weight/day, once at 09:00 AM, and continued till the end of the experiment.

2.3. Antigen and immunization

After four weeks (28 days) of feeding, fishes were anaesthetized with MS-222 and injected intraperitoneally with 500 µl of Bovine serum albumin (BSA, Fraction-V, Merck) solution in phosphate buffered saline (i.e. 10 mg of BSA/fish).

2.4. Sampling

Blood was collected from 4 fish of each group of *C. carpio* on days 7, 14, 21 and 28 after immunization and allowed to clot at room temperature. Serum was obtained by centrifugation. Spleen samples were aseptically dissected out from *C. carpio* and *L. rohita*, on day-14, for immuno-electron microscopy.

2.5. Determination of antigen-specific antibody titers in immunized fish serum by enzyme linked immunosorbent assay (ELISA)

The wells of the microtiter plate (greiner bio-one, Germany, ELISA plate, Microlon, 96W, Flat-bott, High binding) were coated with 100 µl of fish serum (2-fold serial dilutions) diluted in phosphate buffered saline (pH 7.4). The plates were incubated for 12 h at 4^{0C}. After incubation the wells were washed three times with PBS containing tween-20 (0.05%). The free binding sites of the wells were blocked by adding 300 µl of 5% gelatin dissolved in PBS per well and incubated for 12 h at 40C. Following incubation, the wells were washed with PBS-tween, and 100 µl of BSA dissolved in PBS was added to each well (100 ng/well) and incubated for 2 h at 40C. Later the wells were washed with PBS-tween, and 100 µl of rabbit anti-BSA serum diluted to 1:500 in PBS was added to each well and incubated for 1 h at 370C. After incubation, the wells were washed with PBS-tween, and goat anti-rabbit Ig-G antibodies conjugated to horseradish peroxidase, diluted to 1:1000 in PBS, was added to all wells (100 µl/ well) and incubated for 1 h at 370C. Following incubation, the wells were washed with PBS-tween, and 100 µl of substrate (containing 13 mg of o-phenylenediamine dihydrochloride and 10 µl of hydrogen peroxide in 10 ml of citrate-phosphate buffer, pH 5.0) was added to all wells and incubated for 10 min. After the development of color, the reaction was terminated by adding 50 µl of 1M oxalic acid per well. The optical density was measured at 492 nm in an automatic microplate reader (Microscan - MS5605A, Electronic Corporation of India Ltd.). The highest dilution of the serum that gave the OD>0.1 was taken as the titer.

2.6. Determination of antigen clearance by immuno-electron microscopy (IEM)

Since the spleen of *L. rohita* was diffused in to small pieces, these pieces were directly fixed, but in *C. carpio* the spleen is intact one, and it was cut into small pieces of minimum 1 mm size before fixation.

Spleen samples (about 1 mm thickness) were fixed in a solution containing 1% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer for overnight. After washing with 0.1M phosphate buffer, tissues were dehydrated with gradient ethanol (30-100%) at 40C. The dehydrated tissues were first infiltrated with ethanol and LR White (1:1) followed by pure LR White (TAAB). Tissues were embedded in beam capsule filled with LR White and polymerized at 550C. Ultra thin sections (70 nm) were cut in a microtome (Reichert Jung Ultracut-E) and grids were prepared. The sections on the grid were blocked with 2% fish gelatin in 0.1M phosphate buffer for 2 h. After blocking, the sections were washed and labeled by incubation with rabbit anti-BSA polyclonal antibodies (ICN Biochemicals, USA) diluted to 1:500 in phosphate buffer containing 1% fish gelatin and incubated for 12 h. Subsequently, the grids were washed thoroughly with 1% fish gelatin in phosphate buffer and labeled with secondary antibody, i.e. goat anti-rabbit-IgG conjugated with 15 nm gold particles (TAAB), diluted to 1:100 in phosphate buffer containing 1% fish gelatin, and incubated for 2 h. The grids were washed with phosphate buffer containing 1% fish gelatin, and finally washed with double distilled water. After labeling, the sections were stained with uranyl acetate and lead citrate. Stained sections were viewed for labeled particles under transmission electron microscope (Fei-Philips Morgagni 268D).

2.7. Statistics

The data was statistically analyzed using students' t-test in Microsoft Excel. The level of significance was $P<0.05$.

3. Results

3.1. Antibody response

In *C. carpio*, BSA-specific antibody level was peaked on day-7 and then gradually decreased up to day-28. The trend was similar in both control and *Achyranthes* treated groups. The anti-BSA antibody level was always higher in *Achyranthes* treated group than the control throughout the study period (Fig. 1). The difference was significant ($P<0.05$) throughout the study period, except on day-28. The titers reduced by 59% in control group

and 62% in test group on day-28, compared with their respective titers of day-7. In control group, the average antibody titer was 800×10^3, 640×103, 485×10^3 and 330×10^3 on days 7, 14, 21 and 28, respectively. In *Achyranthes* treated group, the average titer was 1280×10^3, 1120×10^3, 960×10^3 and 480×103 on days 7, 14, 21 and 28, respectively. The anti-BSA antibody titers of the *Achyranthes* treated group was 1.45 - 1.97x times higher than the control in various days of sampling.

3.2. Antigen clearance

Spleen from *C. carpio* and *L. rohita* were sampled on day-14, after immunization with BSA. Immuno-labeling was performed to locate the BSA molecules in the spleen under electron microscope, which were shown in Figs. 2 - 5. The presence of gold particles locates the BSA in the sections. In control group *C. carpio*, crowded particles appeared in few samples and few particles appeared in other samples (Fig. 2). In *Achyranthes* treated group, the particles were found rarely, which can be clearly seen in Fig. 3. This indicates that the injected antigen was still present in control group, and in *Achyranthes* treated group antigen was efficiently and almost completely cleared. In control group *L. rohita*, the particles were heavily crowded and found abundantly (Fig. 4), which indicates the abundant presence of BSA. In *Achyranthes* treated sample, the particles were found significantly lower than the control group, which was evident from Fig. 5. This indicate that the injected antigen was efficiently cleared in *Achyranthes* treated group than the control *L. rohita*.

4. Discussion

4.1. Effect of Achyranthes aspera on antibody response

Antibody is the major humoral component of the specific immune system, which plays an adaptive role in neutralizing, killing and clearing of invaded pathogens with the help of other humoral and cellular components of the immune system. *Achyranthes aspera has been found to increase the antibody response against chicken RBC, also it enhanced the nonspecific immunity, in different carps species (Vasudeva et al., 2004; Vasudeva and Chakrabarti, 2004, 2005a, b). In this present study the effect of Achyranthes aspera on the specific immunity against BSA, and its clearance from the spleen were studied. Achyranthes incorporated diets were fed to fishes before immunization as a prophylactic treatment. It was found that Achyranthes has significantly enhanced the specific antibody response against BSA in C. carpio. This result can be correlated with the earlier results studied in different carp species including C. carpio. Achyranthes aspera has demonstrated similar results in mammalian immune system. Intraperitoneal treatment of Achyranthes enhanced antigen-specific antibody responses in different genetic strains of mice, also with different doses of antigen and different doses of Achyranthes itself (Vasudeva et al., 2002).*

Similarly several sources were reported to enhance the antibody response in fishes. Intraperitoneal administration of leaf extract of Acalypha indica or Phyllanthus niruri has enhanced the antibody response in tilapia (Oreochromis mossambicus) against sheep red blood cells (SRBC) (Hemapriya et al., 1997). Administration of azadirachtin, a triterpenoid derived from seed kernel of neem (Azadirachta indica), resulted in the enhancement of antibody response in tilapia (Hemapriya et al., 1997). Spirulina had significantly increased antibody titers to keyhole limpet hemocyanin (KLH) in channel catfish (Duncan and Phillip, 1996a, b). Bath treatment of rainbow trout for 30 min in levamisole/QAC/ISK solutions before a 2 min bath in Aeromonas salmonicida O antigen bacterin elevated specific immune response. The increased activity of the specific immune response was monitored by counting numbers of plaque-forming cells, and by demonstrating elevated circulatory antibody titers (Jeney and Anderson, 1993).

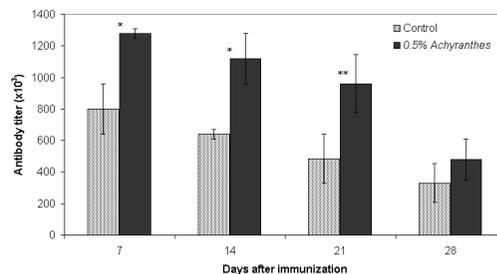### 4.2. Effect of Achyranthes aspera on antigen clearance

The host's immune system recognizes the invaded antigens, and mounts its immune response against it to clear the antigen from the body of the host as quickly as possible to confer safety of the host and to protect the host from the onset of

infection. As the BSA was injected into the fish, the immune system should mount a response and eliminate it from the system of the host. It was observed from the immuno-electron microscopic studies that *L. rohita* could not able to clear the injected BSA from the system. From the electron micrographs it was clearly noticed that, high amount of BSA was still present in the spleen of untreated control *L. rohita* even 14 days after immunization. Compared with *L. rohita*, the immune system of *C. carpio* was more efficient in the clearance of the antigen from the system. From the electron-micrographs of untreated control group *L. rohita* and *C. carpio*, it could be clearly seen that in almost all samples of *L. rohita* the particles were heavily crowded and difficult to count, this indicates the presence of high amounts of BSA (Fig. 4); whereas in untreated control *C. carpio*, though the particles were crowded in some samples, the density was low, i.e. the amount of the BSA in the spleen was also low (Fig. 2). These results indicate that *C. carpio* is efficient in clearing the injected antigen over *L. rohita*. *C. carpio* is said to be hardy fish, withstanding to adverse conditions. The present study proves the same. These studies indicate that the immune system of Indian major carp *L. rohita* was poor in clearing the invaded antigen, hence, high percentage of mortalities have been seen in *L. rohita* during disease outbreaks. Hence, the immune system of *L. rohita has to be boosted to enhance the efficiency of antigen clearance.* Treatment with *Achyranthes* has enhanced the immunity of *L. rohita* that has efficiency eliminated the BSA from the system, compared with untreated control *L. rohita*, which was evident from electron micrographs (Fig. 5). In *C. carpio* also *Achyranthes* treatment has further enhanced the antigen clearance. From electron micrographs it was seen that the antigen was completely cleared (Fig. 3) and the particles were rarely found. This enhanced antigen clearance by the fish treated with *Achyranthes* can be correlated with our earlier results, in which *Achyranthes* has significantly reduced the mortality in *L. rohita* infected with *Aeromonas hydrophila* (Vasudeva et al., 2005c). In the present study it was confirmed that *Achyranthes* enhances the efficiency of antigen clearance in carps, hence, *L. rohita* treated with *Achyranthes* showed high survival by efficiently clearing pathogen *A. hydrophila*.

Similarly there were several reports that treatment with certain drugs enhanced the antigen clearance in various models. Itraconazole, 200 mg twice daily, is safe and effective in preventing relapse of disseminated histoplasmosis in patients with AIDS. Antigen clearance from blood and urine correlates with clinical efficacy (Joseph et al., 1993). Treatment with itraconazole reduces the concentration of *Histoplasma* antigen in blood and urine, suggesting the rapid clearance of fungemia (Joseph et al., 2002). In a clinical study it was found that liposomal amphotericin B enhances the antigen clearance (fungemia) in patients with AIDS (Joseph et al., 2001). Single dose of diethylcarbamazine-fortified salt (DEC-FS) is proved to be more efficacious than single-dose DEC in reducing the prevalence of antigenaemia with an overall high antigen clearance rate after 6 months (Sapak et al., 2000). Microfilariae were cleared promptly and permanently after CGP 20376 treatment, and no adult worm was recovered in jirds infected with *Brugia malayi, 20 weeks after treatment with CGP 20376 (Chandrashekar et al., 1990). PMA (panmalarial antigen) of Plasmodium falciparum and Plasmodium vivax was rapidly cleared following ART (artesunate plus sulfadoxine-pyrimethamine) + SP (sulfadoxine-pyrimethamine) treatment in association with rapid clearance of gametocytemia (Emilina et al., 2001).*
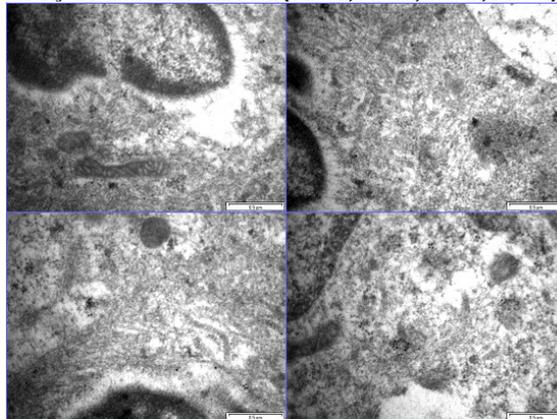
Acknowledgements

Fig. 1. Effect of *Achyranthes aspera* on BSA-specific serum antibody titers in *C. carpio*. Each value represent the mean ± SE of four fish (*$P<0.01$; **$P<0.05$).
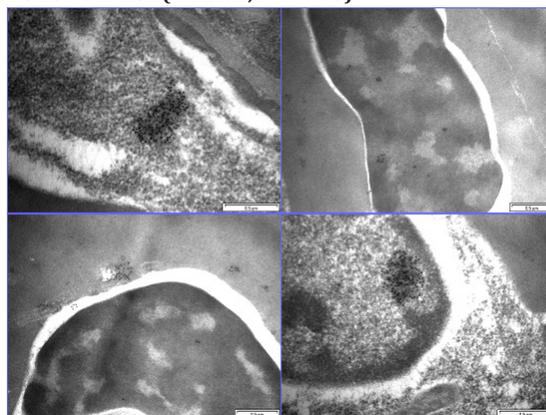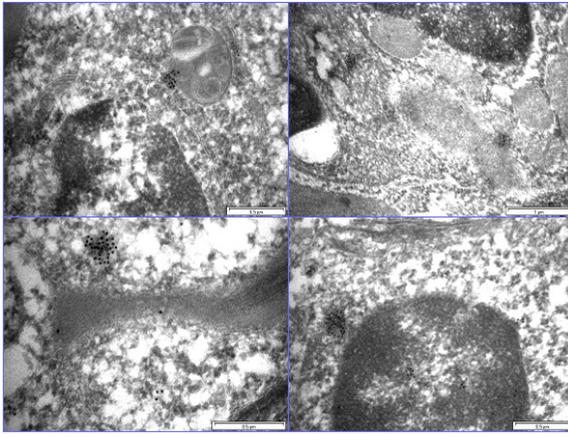

Fig. 2. Electron micrographs of spleen of control *Cyprinus carpio* on day-14 after immunization (7100x, 5600x, 5600x, 7100x).

Fig. 3. Electron micrographs of spleen of *Achyranthes* treated *Cyprinus carpio* on day-14 after immunization (7100x, 7100x, 7100x, 7100x).
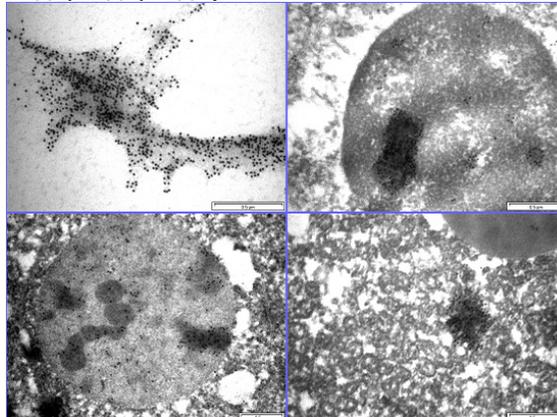
Fig. 4. Electron micrographs of spleen of control *Labeo rohita* on day-14 after immunization (8900x, 7100x, 5600x, 7100x).

Fig. 5. Electron micrographs of spleen of *Achyranthes* treated *Labeo rohita* on day-14 after immunization (7100x, 4400x, 8900x, 7100x).

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