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Immunostimulatory effect of azadirachtin in *Oreochromis mossambicus* (Peters)

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The effect of azadirachtin, a triterpenoid derived from *Azadirachta indica* on the immune response was studied in the freshwater teleost, *O. mossambicus*. Bovine serum albumin (BSA) and sheep erythrocytes (SRBC) were used as antigens to evoke immune response. The immune responses in fish were measured by quantifying antibodies produced and counting the peripheral blood leucocytes in control and experimental fish. In general, azadirachtin significantly enhanced the antibody response and leucocyte count in a dose dependant manner. An inverse relationship was observed between the dose of azadirachtin and the degree of immunostimulation. Timing of azadirachtin administration in relation to immunization revealed that the maximum enhancement of antibody response was observed when the stimulant was given two days prior to immunization. The observed immunostimulatory property of azadirachtin has an implication in the maintenance of finfish health in freshwater intensive aquaculture practices.

In intensive aquaculture farms, fish populations are continuously challenged by both natural (like temperature, crowding etc.) and artificial (like pollutants) stress factors. Such stressors often interfere with functions of innate (nonspecific) and adaptive arms of the immune system, which results in immunosuppression. Such compromise in the immunological status of the fish results in increased susceptibility to a wide variety of biological stressors, such as bacteria, viruses and parasites which could potentially lead to population reductions[1] and were reported for sporadic episode of mass mortality. The accelerating pace at which man made changes are occurring in the aquatic environment seems to have channeled substantial interest for immediate and efficacious solutions[1]. Problems with present antibiotics, drugs and chemical treatment to prevent disease in fish set the stage for the new concept in disease prevention–immunostimulants. As far as fish are considered immunostimulants seem to be more important as they depend more heavily on nonspecific defense mechanisms[2]. Though it has been shown that a variety of chemical and biological agents such as levamisole[3,4], muramyldipeptide[5], β-glucans[6], etc. could increase immune response, the present study is the first observation of its kind that a product from a medicinal plant *Azadirachta indica* produced a similar effect on both specific and nonspecific immunity in fish.

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Azadirachta indica (Neem) is a widely prevalent and highly esteemed wonder tree of the Indian subcontinent and several of its beneficial properties are reported[7]. The neem tree has been in use for ages and ayurveda regards this as *sarvaroga nivarini*, which means 'cure for all diseases'. The use of neem products has been reported in ancient medicine and modern medical applications are receiving widespread attention as its mammalian and environmental safety is well recognized[8] . Biomedical research has shown that *A. indica* possess anti-HIV[9], anti- tumour and anti-microbial[10] activities. In an attempt to find the possible immunostimulatory effect of the extract of plants known for medicinal properties, the present study on the effect of azadirachtin—a triterpenoid derived from *A. indica* on the immune response of *Oreochromis mossambicus* has been undertaken. Antibody responses (primary and secondary) to a protein antigen, bovine serum albumin (BSA) are used as index to assess specific immunity while peripheral blood leucocyte count is used as a reference to examine both specific and nonspecific immunity. In addition, the timing of azadirachtin administration for immunization with BSA is also studied.

Materials and Methods

Experimental animals—Oreochromis mossambicus (Tilapia), of either sex weighing 25 g were used. The experiments were carried out in circular cement tanks (60 cm diam.; vol 150 l). The water temperature of

the fish holding tanks was not controlled as only minor daily fluctuations were (28°± 1.5°C) observed.

Immunostimulant - Azadirachtin (sonnet 105/1030), a triterpenoid derived from neem seed kernel was obtained from Prof.Dr.H.Rembold, Institute for Biochemistry, Martinsried, Germany.

Effect of azadirachtin on primary and secondary antibody responses to BSA—Preliminary studies were conducted and the 96 hr median lethal dose (LD_{50}) of azadirachtin in water was found to be 5260 ng. From this a range of sublethal doses of 526, 52.6, 5.26 or 0.526 ng/fish which correspond to 10, 1, 0.1 or 0.01% LD_{50} of azadirachtin, respectively were administered to fish groups (n=8/group). After 2 days fish in all the groups were immunized with 5 mg soluble BSA (S-BSA, bovine serum albumin, Fraction V-powder, Sigma Chemical Co, St. Louis, USA). An untreated immunized control group (n=8) was maintained. A booster injection was given on day 35 post immunization with the same dose of antigen to both the control and experimental groups. Azadirachtin and antigen administration and serial bleeding were done between 1400 hrs and 1600 hrs throughout the investigation to avoid the possible influence of daily rhythmic variations[11]. Fish were bled serially from the common cardinal vein[12] at a regular interval of 5 days and the serum was separated and decomplemented (classical pathway). The decomplemented sera were stored at -20°C for further use. Anti-BSA antibodies were titrated using passive haemagglutination assay. The highest dilution of the serum giving detectable macroscopic agglutination was recorded and expressed as $\log_2$ antibody titre of the serum.

Effect of azadirachtin on primary antibody response to SRBC—A range of sublethal doses of 526, 52.6, 5.26 or 0.526 ng/fish which correspond to 10, 1, 0.1 or 0.01% LD_{50} of azadirachtin, respectively were administered to fish groups (n=5/group). After 2 days fish in all the groups were immunized with 0.1 ml of 5% SRBC and on the 5th day with 0.1ml of 25% SRBC (7×10^8 cells). An untreated immunized control group (n=5) was maintained. Azadirachtin and antigen administration, serial bleeding, decomplementation and storage of antisera were done as described earlier. Anti-SRBC antibodies were titrated using direct haemagglutination assay. The highest dilution of the serum giving detectable macroscopic agglutination was recorded and expressed as $\log_2$ antibody titre of the serum.

Timing of azadirachtin administration to immunization—A dose of 0.526 ng azadirachtin which produced a maximal antibody response was injected to fish groups (n=8/group) two or four days before (–4 days, –2 days), on (0 day) or after (+2, +4 days) the day of antigen (5mg S-BSA/fish) administration. An untreated immunized control group (n=8) was maintained simultaneously. Anti-BSA antibodies were titrated using passive haemagglutination assay.

Peripheral blood cell count—Two days prior to immunization with 5 mg S-BSA, fish were administered with selected sublethal doses of azadirachtin. Fish were bled in a syringe rinsed with 1% EDTA[13], diluted eight times with Natt-Herrig's solution and the total leucocytes were counted using Neubauer's counting chamber. To count individual type of leucocytes, blood smears were prepared on clean slides and stained with Leishman's stain. Monocytes, granulocytes (nonspecific immunity) and lymphocytes (specific immunity) were counted using a cell counter (Systronics 191, Bombay, India) and expressed in percentage.

Results and Discussion

Azadirachtin in general enhanced significantly ($P < 0.05$) both the primary and secondary antibody responses to BSA compared to the control (Fig. 1). In other such studies using levamisole[3,4] and ascorbic acid[14] lower doses of immunostimulants have been found to enhance the immune response and higher doses were found to be suppressive. However in the present study, the highest dose of 526 ng that did not produce any effect ($P > 0.05$) in the primary antibody response (Fig. 1a) was found to evoke a minimal stimulatory effect ($P < 0.05$) in the secondary antibody response (Fig. 1b) when compared to other doses ($P < 0.005$).

Studies on timing of azadirachtin administration in relation to immunization revealed that irrespective of the day of administration, azadirachtin caused a significant enhancement ($P < 0.01$) of antibody response on all days tested (Fig. 2) compared to control. A similar enhancement in rainbow trout was observed when the immunostimulant was injected before immunization[3]. It has been suggested that such an elevation in immune response may prepare the fish for exposure to any immunogen[3]. In the fish administered with azadirachtin 4 days prior to immunization the peak antibody response occurred 5 days earlier than the control. An earlier increase in the rate of prolif-

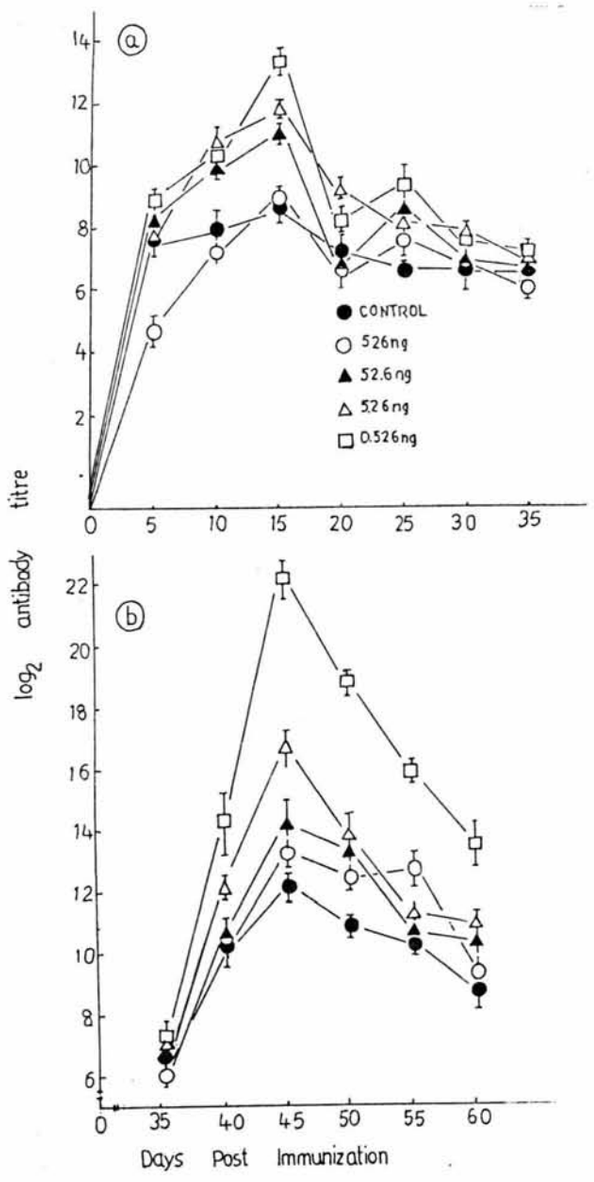

Fig. 1—Effect of azadirachtin on (a) primary and (b) secondary antibody response to S-BSA (Each point represents the mean ± SE of 8 fish).

eration of antibody producing cells may be the reason for the advancement of peak antibody response. Azadirachtin when administered 4 days after or on the day of immunization was found to have less immunostimulatory effect. This may be due to the reason that administering some immunostimulants after the antigen exposure may be suppressive sometimes by interfering with some of the delicate communications needed between cell populations in the physiological pathway[2].

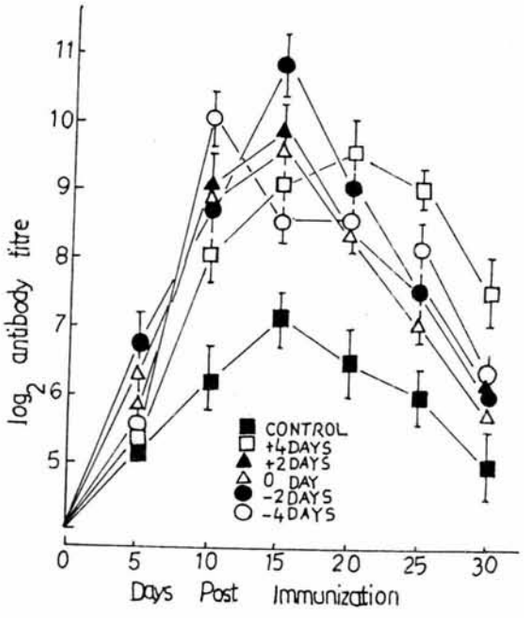

Fig. 2—Temporal relationship between the administration of azadirachtin and immunization (Each point represents the mean ± SE of 8 fish).

Leucocyte response in fish appears to provide a rapid, sensitive and quantitative method for determining stressful/healthy status of fish[15]. The total leucocyte count increased in the azadirachtin-treated fish compared to the control. Maximal enhancement ($P < 0.01$) was observed in the group administered with 0.526 ng of azadirachtin (Fig. 3). The significant increase in the total peripheral blood leucocyte count substantiates the increase in antibody response. Among the different leucocyte populations, monocytes and granulocytes exhibited an apparent increase in the earlier phase (Fig. 4a and b) while the lymphocyte count (Fig. 4c) showed a marked increase in cell counts in the later phase. This may be attributed to the fact that during the initial stages of the immune response, the granulocytes and monocytes are the first line of defence and will be involved in antigen processing and presentation, whereas lymphocytes will be involved in antibody production which takes place later. The increase in lymphocyte count during the later phase (Fig. 4c) is well correlated with the peak in the primary antibody response (Fig. 1a). An inverse relationship between the dose of azadirachtin administered and the degree of stimulation of antibody response was observed when sheep erythrocytes (SRBC) was used as the antigen (Fig. 5). Antibody response to many antigens requires cooperation

among two types of lymphocytes (T and B cells and macrophages) for optimal expression. Modulation of the functions of any of these two cell types would influence the immune response[16]. *A. indica* is known to increase humoral and cell mediated immune responses in hen[17]. In the present study the proliferation of lymphocytes (Fig. 4c) may have increased antibody production to S-BSA in *O. mossambicus*.

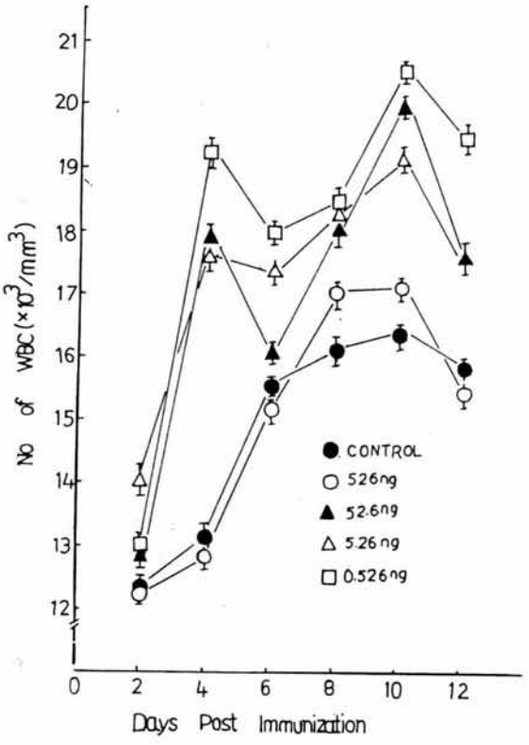

Fig. 3—Effect of dose of azadirachtin on total peripheral blood leucocyte count (Each point represents the mean ± SE of 5 fish).

One intention of studying the immunostimulatory capacity of azadirachtin was for future uses as an adjuvant in vaccines, administered either intraperitoneally or orally. However, the enhancement of the immune parameters assessed in the present study should be correlated with an increased protection against virulent pathogens. Further *in vitro* experiments will address on the actual mechanism (T and/or B cell stimulator) of azadirachtin at the cellular level. Earlier studies reported the reversal of immunosuppressed rat by *A.indica*. Hence, the encouraging results of the present investigation suggest the possibility of using azadirachtin in immunocompromised fishes raised in intensive aquaculture farms. However, the effective use of immunostimulants apart from the timing and dosage, also rely on the method of administration and physiological status of the fish.

Natural compounds like azadirachtin may have an advantage over chemical drugs because being constituents of living systems they may be less toxic and more acceptable. Thus the present study indicates the use of azadirachtin and possibly such other plant

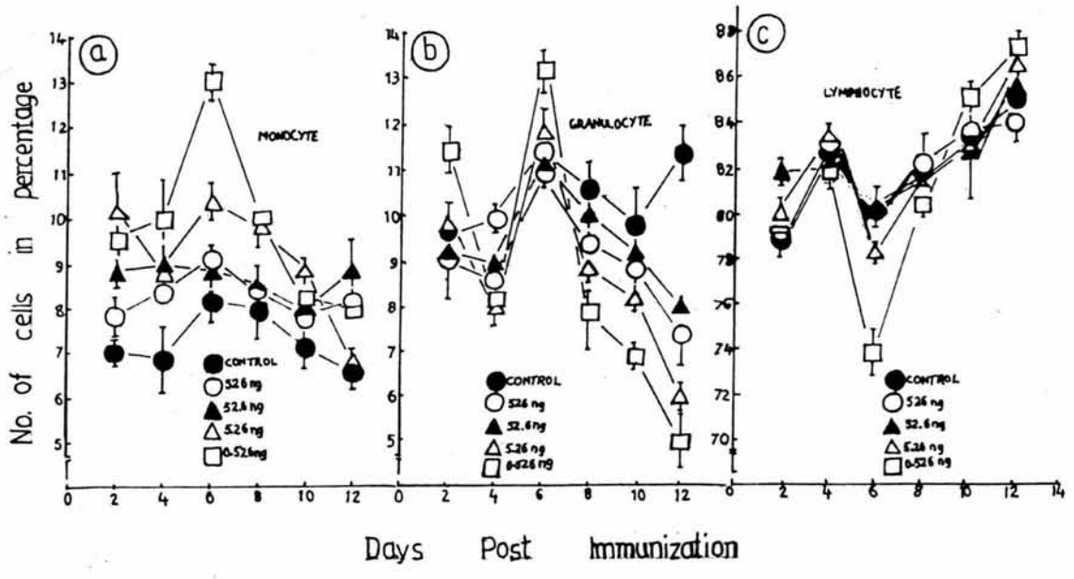

Fig. 4—Effect of dose of azadirachtin on a) monocyte b) granulocyte and c) lymphocyte count (Each point represents the mean ± SE of 5 fish).

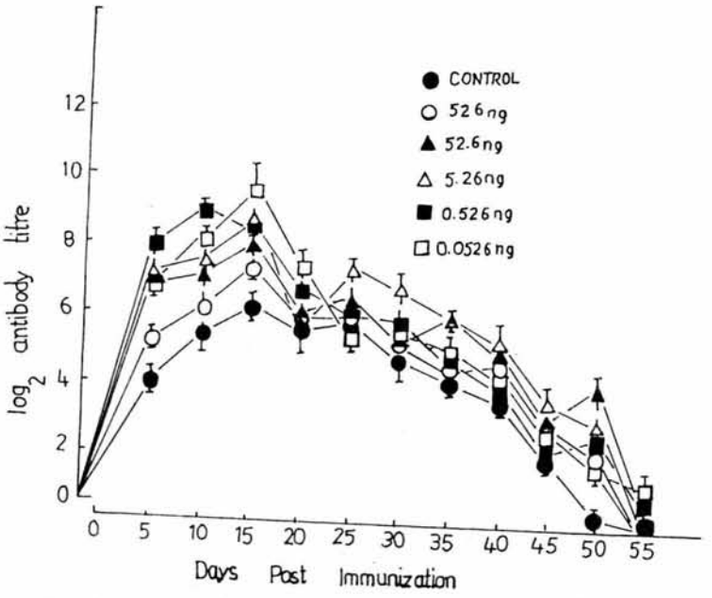

Fig. 5—Effect of azadirachtin on the primary antibody response to sheep erythrocytes (SRBC) (Each point represents the mean ± SE of 5 fish).

products to enhance overall resistance/immunity in fish and reduce the loss caused by disease in aquaculture.

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References

1 Zelikoff J T, *Impact of environmental changes on aquatic animal health*, paper presented to the third International symposium on aquatic animal health, Baltimore, August 30-September 3, (1998).

2 Anderson D P, Immunostimulants, Adjuvants and Vaccine carriers in fish: Applications to aquaculture *Ann Rev Fish Dis* 2 (1992) 281.

3 Anderson D P, Siwicki A K, Dixon O W & Lizzio E F, in *Viruses of lower vertebrates* edited by W Ahne & E Kurstak (Springer-Verlag, New York) (1989), 469.

4 Mulero V, Esteban M A, Munoz J K & Mesequer J, Dietary intake of levamisole enhances the immune response and disease resistance of the marine teleost gill head seabream (*Sparus aurata L*) *Fish Shellfish Immunol* 8 (1998) 49.

5 Kodama H, Hirota Y, Mukamoto M, Baba T & Azuma I, Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide *Dev Comp Immunol*, 17 (1993) 129.

6 Santarem M, Novoa B and Figueras A, Effects of β- glucans on the nonspecific immune responses of turbot (*Scophthalmus maximus* L.) *Fish and Shellfish Immunol*, 7 (1997) 429.

7 Govindachari T R, Chemical and biological investigations on *Azadirachta indica* (the neem tree) *Curr Sci*, 63 (1992) 117.

8 Ray A, Banerjee B D & Sen P, Modulation of humoral and cell mediated immune responses by *Azadirachta indica* (Neem) in mice *Indian J Exp Biol* 34(1996) 698.

9 Upadhyay S N, Dhawan S, Garg S, Wali N, Tucker L & Anderson D P, Immunomodulatory properties of *Azadirachta indica* in *World Neem Conference* edited by MS Chari, RP Sing, W Kraus & RC Saxena (Oxford & IBH Pub. Pvt. Ltd., London) (1993) 80.

10 Agarwal A, What's in neem? *Down To Earth*, 4 (1996) 21.

11 Michael R D & Priscilla A S, Diel variation in antibody response to bovine serum albumin in *Oreochromis mossambicus* (Peters) *Indian J Exp Biol* , 32 (1994) 474.

12 Michael R D, Srinivas S D, Sailendri K & Muthukkaruppan V R, A rapid method for repetitive bleeding in fish. *Indian J Exp Biol*, 32 (1994) 838.

13 Mainwaring G & Rowley A F, Separation of leucocyte in the dog fish, *Scyliorhinus canicula* using density gradience centrifugation and differential adherence to glass coverslips *Cell Tissue Res* 241 (1985) 283.

14 Michael R D, Quinn C G & Venkatalakshmi S, Modulation of humoral immune response by Ascorbic acid in *Oreochromis mossambicus* (Peters) *Indian J Exp Biol*, 36 (1998) 1038.

15 Rao C A P & Sharma S S S, Cytological shift between lymphocytes and thrombocytes in the freshwater fish, *Badis buctiani Curr Sci* 51 (1982) 947.

16 Michael R D & Meenal , Effect of the heavy metal, Cadmium on the antibody response to a protein and a cellular antigen in *Oreochromis mossambicus* (Peters) in *The Second Indian Fisheries Forum* edited by T J Varghese, P Keshavanth, K V Radhakrishnan & R R Lokeshwar (Saradha Press, Mangalore, India) 1993, 223.

17 Sadekar R D, Koller A Y, Barmase B S & Desai V F, Immunopotentiating effects of *Azadirachta indica* (Neem) dry leaves powder in broilers, naturally infected with IBD virus *Indian J Exp Biol* 36 (1998) 1151.