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Research Paper

Studies on innate immune response of *Duttaphrynus himalayanus* (Gunther) to combat microbial infection

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Abstract

***Duttaphrynus himalaynaus* is a unique high altitude amphibian species, adapted to cold climatic conditions of the hills of Darjeeling in West Bengal and Sikkim in India. How these poikilotherms respond to bacterial (*Aeromonus caviae*) infection was studied in the present investigation. *D. himalayanus* has all the immunecompetent cell types to trigger humoral or cell mediated immune response to bacterial infection, but interestingly, they prefer to combat through their innate immune mechanism to eradicate the bacteria. Apparently it appears that the innate immune system in amphibians plays a proactive role in combating microbes at least during the initial phase of infection.**

Keywords: Immune response, Amphibia, Complement, Serum

Introduction

Evolution of vertebrate immune system can be traced down to amphibians, who dared to take the giant leap to conquer the land some three hundred million years ago. Primitive amphibians had to interact with completely new world of pathogens, antigens and allergens, to which the evolutionary force of aquatic immune system was never exposed. Modern day amphibians must be carrying the evolution legacy of the immune system from their predecessors and thus appear to be an excellent model to study the evolution of the complex immune system of higher vertebrates. Analysis of the extant amphibian immune system may provide glimpses of the modification that took place some 300 million years ago.

In higher vertebrates, lymphocytes[1-3] and monocytes[4,5] constitute the arms of the immune system and play an extremely important role in the process of eliciting an effective acquired immune response. We investigated the immune response of a unique species, *Bufo himalayanus* that survives in relative isolation, adapted to cold climatic conditions of the Eastern Himalayas. This species displays significant morphological, behavioral and physiological differences from other members of Bufonidae. They have a very limited distribution and are restricted to 5000 ft. above sea level, in the hills of Darjeeling and Sikkim in India and in parts of southern China.

The amphibian counterpart of the circulatory leukocytes of mammals have been reported and reviewed by many authors[6-10]. Earlier we had reported the presence of five major classes of immune

competent cells in the peripheral blood and spleen of *B. himalayanus*[11]. Presence of immunoglobulins, the most studied of all molecules in amphibians was detected by several authord[12-14]. The presence of immunoglobulins was also noted in *D. Himalayanus* [15]. How this poikilotherm, with low metabolic activity manages to combat foreign pathogens in the high altitude cold climatic conditions was not known. In the present investigation, the immune response of *Duttaphrynus himalaynau*s to *Aeromonus caviae* were studied to get the perspective of how the species living in a different climatic condition responds to the environmental pathogens, especially bacteria in its natural habitat.

Materials and Methods

Animal Model

Both the sexes of *D. himalayanus* were collected from the wild and used for the study. The animals were kept in plastic cages and fed with chicken liver extract. During blood collection, the animals were anaesthetized with mild chloroform exposure and approximately 0.5-1.0 ml blood was collected by ventricular puncture.

Immunizing Agents and Immunization Schedule

Aeromonus caviae were used to immunize *D. himalayanus* and *D. melanosictus* in the study. Bacterial cell suspension from overnight culture at 37°C was taken and cell number adjusted to 1×10^6 cells/ml as described by Zasloff and Magainins, 1987 [0.8 OD at 600 nm corresponds to approximately 10^9 Colony Forming Units (CFU)/ml] [16]. Bacteria were attenuated prior to immunization. Freshly cultured bacteria were attenuated by 0.05 % formalin treatment. The cells were washed and suspended at a concentration of 1 x 10^6/ml in amphibian phosphate buffered saline (APBS). The bacterial suspension was then thoroughly mixed with Freund's complete adjuvant and 0.1 ml of the mixture was injected subcutaneously in the axial region of the toads. A booster dose of the antigen with incomplete adjuvant was given 5 days after the first injection. 2 days after the last injection, 0.5-1.0 ml blood samples were collected by ventricular puncture in sterilized tubes and allowed to clot at room temperature for 1 hour. Blood was then centrifuged at 5000 rpm for 10 mins. Serum was then collected and used immediately or stored at -20^0C centigrade until further use.

Treatment of *Aeromonus caviae* with immunized serum:

Approximately 5 x 10^4 of *Aeromonus caviae* cells in 50 µl medium (Nutrient broth, Himedia) was pretreated with 50 µl of immunized serum collected from immunized *D. himalayanus*. The triplicate samples were subjected to incubation for 30 min. at 28 degree centigrade after which fresh 3 ml sterile nutrient broth was added to each tube and then further incubated for 24 hours. The growth of bacteria was monitored at every 2 hrs interval by noting the change in the OD of the medium. For control experiments, 50 µl of serum from non-immunized toads were used for all the experiments.

Agar diffusion test in lawn cultures

Nutrient agar Petri plates were prepared as per standard protocol. Cultures of *Aeromonus caviae* were spread into the plates using a smooth-surfaced glass rod and were allowed to adhere to the agar surface. Immediately after the bacterial cells adhered on the agar surface, wells were cut into the agar plate for loading toad serum. Serial double dilution of immunized serum was prepared. 20 µl of the immunized serum fractions were loaded into the wells marked 1-4 and control non-immunized serum was loaded in the well marked 'C', 30 minutes after the bacteria lawn was prepared. The plates were then incubated at 37 ºC in a humid environment overnight for optimal bacterial growth.

Complement Inactivation of Serum

Complements are serum proteins which are required to perform antibody mediated lysis. The complement proteins are very heat sensitive and to inactivate complement components, serum collected from experimental and control toads were incubated at 55 degree, in water bath for 45 mins. The samples were then allowed to cool down to room temperature and stored in aliquots in –20 degree refrigerator.

Results and Discussion

Bacterial Attenuation

Aeromonus caviae were attenuated with different concentration of formalin in Luria broth to determine the effective concentration of formalin that will inhibit bacterial growth. The growth of bacteria after 12

hrs is shown in figure 1. It is evident that 0.025% formalin was sufficient to curve the bacterial growth. To be on the safer side, 0.05% was chosen to be the optimum dose for attenuation of the bacterial population under consideration before injecting the bacteria subcutaneously for immunization.

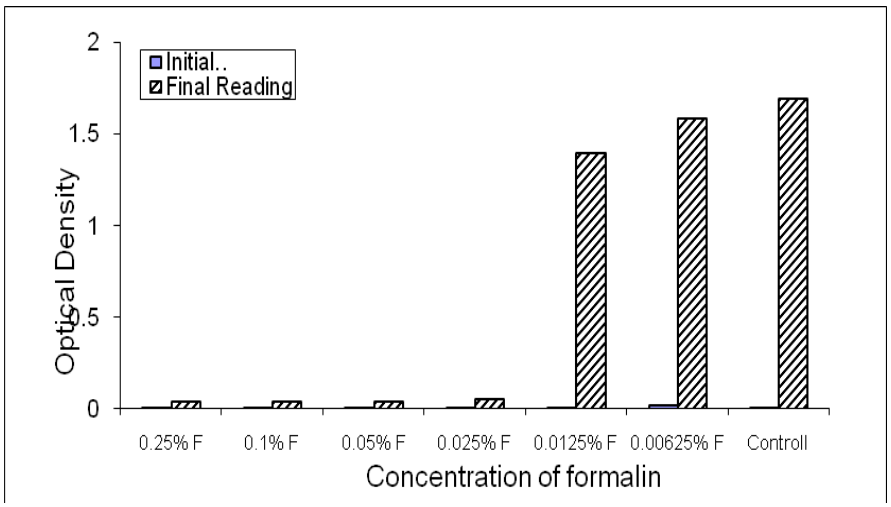

Figure 1: Growth of *Aeromonus caviae* after attenuation with different concentration of formalin

Growth retardation of *Aeromonus caviae* in suspension culture pre-treated with immunized serum

The growth pattern of Aeromonas bacteria pretreated with immunized and non-immunized serum from *B. himalayanus* is shown in Figure 2. Immunized serum treated Bacteria exhibits reluctance to divide and proliferate in comparison to the control experiments. Cell division in the treated cells do not cease completely instead, a slow rate of cell division is maintained all through the culture period. Inhibitory effect on cell division by immunized serum indicates the presence of some factor in the serum that influences the rate of bacterial cell division. Even after 15 to 16 hrs of incubation, the bacteria are unable to overcome the effect of the factors present in the immunized serum. In comparison, the control experiments, bacterial growth enters the log phase just after 3 hrs of incubation, reflecting a significant difference in the growth pattern. There was a continuous increment in the optical density of the control serum which indicates that normal non-immunized serum does not affect the bacterial growth.

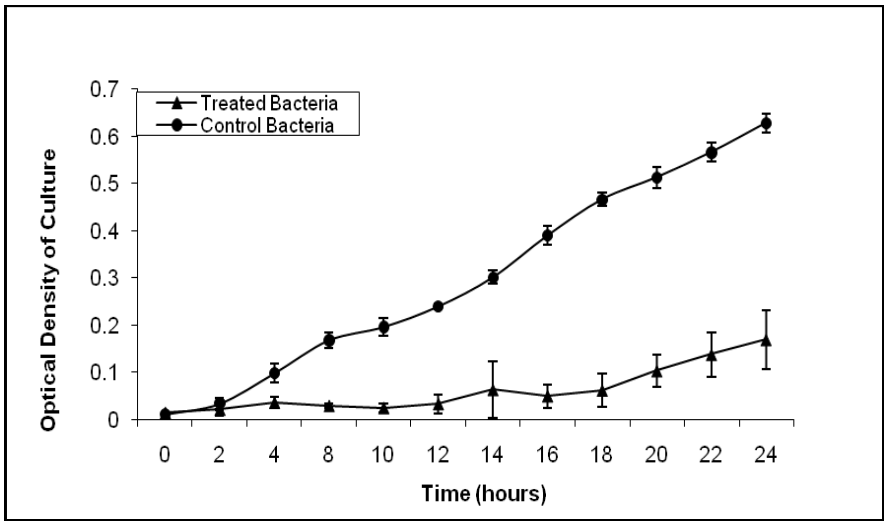

Figure 2: Development of serum antimicrobial in *B. himalayanus* after immunization with attenuated *Aeromonas caviae* that retards the growth of Aeromonas in suspension cultures

Growth of Aeromonas bateria treated with complement inactivated immunized serum

Complement inactivated immunized and non-immunized serum treated Aeormonas showed a growth pattern comparable to the growth pattern observed in previous experiments (Figure 2). Inhibitory effect of the immunized serum still persisted even after heat inactivation of the complement system. This indicates that antibody mediated lysis is not the cause for the sluggish growth of the treated bacteria. Hereto, significant difference is observed between the control and experimental bacterial growth.

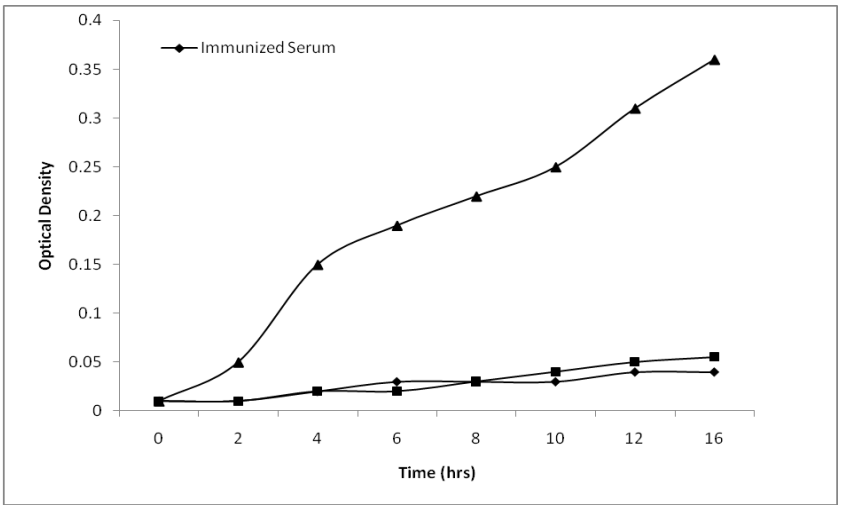

Figure 3: Persistence of growth retardation of Aeromonas in culture by complement inactivated immunized serum from *B. himalayanus*

Growth Inhibition Assay In Lawn Cultures

The aim of this assay was to visualize the inhibition of bacterial growth, which was seen in broth cultures and also to determine the lowest concentration of the assayed antimicrobial agent. Serum isolated from immunized toads of *D. himalayanus* exhibited bacteriostatic effect as seen in Figure 4. The zone of inhibition was maximum in well 1 loaded with raw serum. There was a gradual decrease in the zone of inhibition as the serum got diluted and minimum bacteriostatic effect was seen in well 4 where the 1/8 dilution of the immunized serum was loaded,

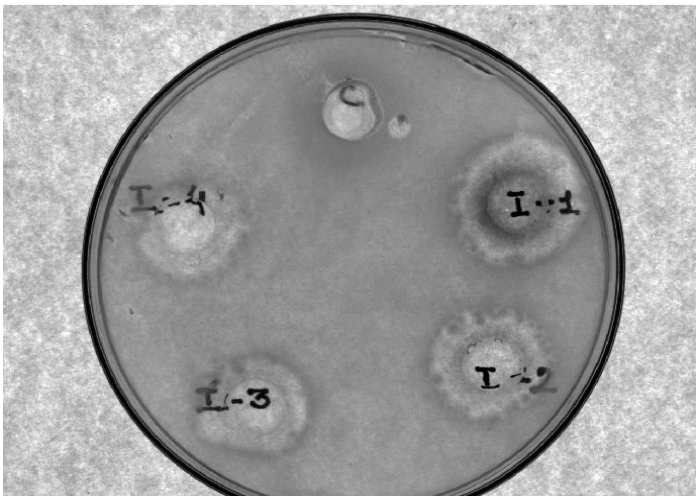

Figure 4: Dose dependent growth inhibition of Aeromonus *caviae* in lawan culture by immunizes serum from *B. himalayanus*

D. himalayanus is adapted to cold climatic conditions of the hills of Darjeeling and adjoining ranges of the Himalaya in India. As revealed from our previous finding, the species have a well organized immune system to accomplish both innate and acquired immune responses[15]. The expectation was that the species will respond to Aeromonas inoculation by eliciting antibody or cell mediated immune

response. The results of the first experiment (Figure 2) clearly demonstrate that the toads develop and display anti microbicidal activity after inoculation with Aeromonas. Immunized serum of the species inhibited the growth of Aeromonas in broth cultures, whereas non-immunized serum could not inhibit the bacterial growth. Apparently it appeared that the bacteria might have been subjected to antibody mediated lysis that caused the delay in bacterial growth entering the log phage. However, when complement inactivated immunized serum continued to display microbicidal activity (Figure 3), it became evident that the response to Aeromonus was not being mediated by humoral arm of the immune system simply because antibody mediated lysis require the presence of complement. Growth inhibition assay in lawn cultures enabled to visualize the effect of the microbicidal property of the immunized serum and the zone of inhibition was proportional to the concentration of the serum. Microbicidal properties from the skin extract and gastric glands has been reported in anurans[17,18]. In toads, information of the presence of antimicrobial components is only available from few species like *Bufo rubescens*[23], *Bombina orientalis*[19] and *Bufo arenarum*[20]. BM-ANF1, a compound isolated and purified from the skin extract of *B. melanostictus* has been shown to induce apoptosis in cancer cell lines by degrading DNA[22]. But this is the first time we have detected inducible microbicidial properties in the serum of toads. Earlier studies have shown that macrophages get activated against mycobacteria infection[21]. It appears that inoculation of Aeromonas in *B. himalayanus* activates the macrophages that synthesizes and release certain microbicidal factors in the serum and these factors most likely inhibit the proliferation of Aeromonas in cultures. However, several questions still remain to be answered and this laboratory is actively pursuing to isolate and purify the microbicidal factor from the serum of amphibians.

Conclusion

D. himalayanus is adapted to cold climatic conditions of the hills of Darjeeling and adjoining ranges of the Himalaya in India. As revealed from our previous finding, the species have a well organized immune system to accomplish both innate and acquired immune responses (Bhattacharjee, 2010)[16]. The expectation was that the species will respond to Aeromonas inoculation by eliciting antibody or cell mediated immune response. The results of the first experiment clearly demonstrate that the toads develop and display anti microbicidal activity after inoculation with Aeromonas. Immunized serum of the species inhibited the growth of Aeromonas in broth cultures, whereas non-immunized serum could not inhibit the bacterial growth. Apparently it appeared that the bacteria might have been subjected to antibody mediated lysis that caused the delay in bacterial growth entering the log phage. However, when complement inactivated immunized serum continued to display microbicidal activity, it became evident that the response to Aeromonus was not being mediated by humoral arm of the immune system simply because antibody mediated lysis require the presence of complement. Growth inhibition assay in lawn cultures enabled to visualize the effect of the microbicidal property of the immunized serum and the zone of inhibition was proportional to the concentration of the serum.

Microbicidal properties from the skin extract and gastric glands have been reported in anurans by Rinaldi [17] and Conlon and Sonnevend[18]**.** In toads, information of the presence of antimicrobial components is only available from few species like *Bufo rubescens* [23], *Bombina orientalis* [19] and *Bufo arenarum*[20]. BM-ANF1, a compound isolated and purified from the skin extract of *B. melanostictus* has been shown to induce apoptosis in cancer cell lines by degrading DNA[22]. But this is the first time we have detected inducible microbicidial properties in the serum of toads. Earlier studies have shown that macrophages get activated against mycobacteria infection[21]. It appears that inoculation of Aeromonas in *B. himalayanus* activates the macrophages that synthesizes and release certain microbicidal factors in the serum and these factors most likely inhibit the proliferation of Aeromonas in cultures. However, several questions still remain to be answered and this laboratory is actively pursuing to isolate and purify the microbicidal factor from the serum of amphibians.

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Conflict of Interest
The authors declare that they have no competing interests.

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