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Research Article

Studies on modulation of hemocyte surface antigen through agglutination reaction under arsenic toxicity in edible mudcrab (*Scylla serrata*)

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Abstract
Scylla serrata (Crustacea: Decapoda), which is widely spread on the intertidal mudflat of West Bengal, India's Sundarbans Biosphere Reserves, is a potential aqua crop and an economically significant edible species. One of the larger crab groups in the mangrove swamp of the Sundarbans is thought to be this one. The *S. serrata's* multifaceted immune response is directly tied to its diverse habitat and survival technique. It lives in dangerous surroundings and is constantly in danger of physiological stress brought on by various xenobiotics, such as arsenic. By producing a number of polyclonal antisera in rabbits (New Zealand White, albino), the study attempted to evaluate the surface antigen against crab hemocytes and murine lymphocytes. Control hemocytes and hemocytes treated to 1 ppm expressed very identical reactivity to antihemocyte sera for the agglutination reaction. The control results, however, shifted when exposed to 2 and 3 ppm of sodium arsenite, indicating arsenic-induced hemocyte surface modification. The agglutination reaction from the control sets of hemocytes that reacted with murine antilymphocyte sera gradually, shifted as the quantity of sodium arsenite in the medium of the treatment sets increased. The maximum equivalence zone of murine lymphocyte and hemocyte agglutination 98.6% and 99% respectively suggested a potential epitope sharing between two phylogenetically separate species. The situation may lead to a possible alteration of immune status and make opportunity for pathogenic foreign invaders within the mud crab body. Chronic arsenic exposure indicated a steady decline of edible and demandable *S. serrata* in the natural habitat of Sundarbans.

Keywords: Agglutination, Antisera, Hemocyte Antigen, *Scylla serrata*, Sodium arsenite

INTRODUCTION

Arsenic is a metalloid that causes a toxic effect on invertebrates, including decapods crustaceans, at cellular and sub-cellular levels (Saha and Ray, 2014). In nature, arsenopyrite is the most common arsenic-based mineral and the natural environment. Arsenic-containing minerals undergo oxidation in different ways and release arsenic into water (Gomez - Caminero *et al.*, 2001). According to the environmental theory there are different concepts present in nature regarding arsenic contamination in ground water - 'Oxyhydroxide Reduction' and 'Pyrite Oxidation' (Acharyya, 2002; Gomez - Caminero, 2001). Apart from the agricultural activities other anthropogenic activities for arsenic contamination are preservation of timber, electronics manufacturing (microwave devices, lasers, light-emitting bodies, photoelectric cells and semiconductor devices), purifying industrial gases (removal of sulfur) mining, smelting of non-ferrous metals and burning of fossil fuels etc. (Saha and Ray, 2014; Chakraborty *et al.*, 2021; Rahaman *et al.*, 2021). Many of the districts of West Bengal are under the stress of arsenic toxicity and more than 1 million people use arsenic-contaminated water ranges from 0.05 ppm to 3.7 ppm (mg/lit.) (Das and Roy Chowdhury, 2006; Chakraborty *et al.*, 2021; Rahaman *et al.*, 2021).

In India, the coastal region consists of large numbers of estuaries, creeks, tidal mud flat, bheries, mangrove swamp that support a good number of aquacultural shellfish including *Scylla* sp which is economically (foreign exchange), nutritionally (protein and minerals

rich flesh of muscle) and ecologically (estuarine detritus feeder) important (Ray and Saha, 2011; Saha *et al.*, 2022). Among mud crabs, *Scylla serrata* is considered the most valuable mudcrab species for capture and culture in India (Saha *et al.*, 2022). Seeds are harvested from its natural habitat, and juvenile crabs are cultured in a saline pond in Sundarbans of West Bengal (Saha and Ray, 2014; Saha et al., 2022). The biodiverse Sundarbans ecosystems are today exposed to a range of xenobiotic pollutants, including arsenic, that has both anthropogenic and natural origins (Saha, 2015; Saha *et al.*, 2022). Due to exposure to metalloid arsenic, *S. serrata's* natural habitat and growing pond are also at risk (Saha and Ray, 2014).

Hemocytes, the circulating chief immuno effector blood cells of *S. serrata* perform diverse immunological functions including agglutination (Ray and Saha, 2011). Agglutination is the clumping of biological material suspended in a liquid, usually in response to a particular antibody. A standard technique for measuring the responsiveness of invertebrate hemolymph in putative defense responses is the agglutination assay and agglutinins have been involved as an opsonin (Ray and Chottopadhya, 2002 and 2003). A report on the toxicity of arsenic in modulating the surface epitope of selected hemocytes of *S. serrata* through antisera-mediated agglutination is not available and demands a thorough investigation. The present study aimed to detect the alteration of *S. serrata* hemocyte surface antigen through antigen-antibody agglutination reaction against sublethal concentration of sodium arsenite toxicity. Further, the study was to give baseline information to establish a suitable immuno-marker of aquatic toxicity in contaminated aquatic habitats.

MATERIALS AND METHODS

Experimental design

Healthy adult specimens of *S. serrata* weighing (70 ± 10 g) were collected from the selected habitat of the district of south 24 Parganas and the area of relatively heavy metal-free zones of Sundarbans biosphere reserve (intertidal mudflat of river Malta). The average length and breadth of the carapace of the specimen were 6 ± 0.5 cm and 9 ± 0.5 cm, respectively. All the crabs were carried alive in jute bags to the Department of Zoology, University of Calcutta (Aquatic Toxicology Laboratory). Live animals were maintained in standard glass aquaria in batches and fed with fresh flesh of molluscs (*Bellamya bengalensis*). The water of the static water environment was replenished every 24 hours to avoid residual toxicity. Animals were acclimatized in laboratory conditions for two weeks prior to experimentation. The experiments were done in airy and well-illuminated laboratory conditions and the water temperature was kept constant at 25 ± 5 ^{o} C (Saha and Ray, 2014).

Ethical approval

The work was conducted in the Department of Zoology, Aquatic Toxicology Laboratory, following the animal ethical guidelines and the approval number of the University is: 885/GO/Re/S/05/C PCSEA (Department of Zoology, College of Science & Technology, University of Calcutta, No.35, Ballygunge).

Sodium arsenite treatment

In Borosilicate glass containers, a standard solution containing 100 ppm of sodium arsenite (E. Merck, Germany; 99% pure; Chemical Abstracts Service number [CAS no.] 7784-46-5) was created using arsenic-free distilled water. Under pH 7.4 two sets of laboratory experiments were conducted under sodium arsenite solution. For determining the arsenic toxicity, LC-50 bioassay was conducted in *Scylla serrata* in controlled laboratory conditions after 'Behrens-Karber method' (Klassen, 1991).

$LC_{50} = LC_{100} - (ab + \text{---------} + ab) / n$ Eq. 1

Lethal dosages for 50% and 100% of the samples are shown here by the symbols LC_{50} and LC_{100}. The mortality brought on by two successive dosages is represented by "a" as the difference between them, "b" as the arithmetic mean, and "n" as the number of samples in each group. Based on LC_{50} values three sublethal concentrations of sodium arsenite (1, 2 and 3 ppm) were derived to conduct all experiments (Shibu Vardhanan and Radhakrishnan, 2002). Each experimental set (1, 2, and 3 ppm) had 10 animals of the same age and sex in triplicate forms, along with the control group. In a static water environment, biochemical tests were conducted for 15 days, replenishing fresh sodium arsenite solutions every 24 hours. Animals under treatment were given daylight and had routine checks for death and morbidity. Dead crabs were taken out of the test medium right away. The water temperature was kept between 26^{0}C and 30^{0}C (Saha and Ray, 2014).

Rationale for selecting the experimental concentrations

In controlled laboratory conditions, LC-50 of sodium arsenite value is 15 ppm and 10 ppm for adult and juvenile crabs, respectively, under 96 hours of duration. All the sodium arsenite concentrations of less than 50% of LC_{50} value were maintained because it is reported that water concentration of arsenic in West Bengal ranges elevates up to 3.2 ppm with seasonal variations (Das and Roy Chowdhury, 2006; Saha and Ray, 2014). All the experimental concentrations of sodium arsenite were less than the highest reported natural concentration of arsenic (Saha and Ray, 2014).

Collection and preparation of hemocyte suspension:

The external surface of the crabs was carefully cleaned by placing them under running tap water for 10 minutes. Following Ray and Saha (2011), fresh blood (Hemolymph) of *S. serrata* was collected aseptically from the base of the second walking legs of the control and post-treated animals using a sterile syringe and a 23-gauge needle. To prevent cells from aggregating with anticoagulant, the blood was then stored in pre-chilled glass vials (Ray and Saha, 2011). Each animal's blood sample did not include more than 2 ml (Yildiz and Atar, 2002). The hemolymph was centrifuged at 3000 rpm for 5 minutes to separate the hemocytes. Cell density was maintained by phosphate buffer solution (PBS) according to unit volume. Non-aggregated live blood cells were examined as a suspension under an inverted microscope.

Testing of hemocyte viability

Cell viability was determined by diluting 50 µl of cell suspension with 50 µl of 0.4% trypan blue (Himedia) following the principle of dye exclusion and introducing this cell suspension into an improved Neubauer hemocytometer. The total number of hemocytes and initial cell viability were determined microscopically by measuring the percentage of stained and unstained cells (Sauve *et al.*, 2002). Six hundred cells were evaluated for each specimen. Experiments were carried out with cell suspension with more than 95 % viable hemocytes (Ray and Saha, 2011).

Immunoagglutination responses

Raising of anti-sera

Antihemocyte sera were raised in rabbits against live control hemocytes of *S. serrata* (Ray and Chattopadhyay, 2002), whereas antilymphocyte sera were raised in rabbits against live lymphocytes of swiss mice (Honigberg *et al.*, 1971; Ray and Chattopadhyay, 2003).

Preparation of cellular antigen

S. serrata were bled aseptically and hemocyte suspensions with > 95% viable hemocytes were used as a source of antigen (2 x 10^6 cells / ml) for antihemocyte sera test. White swiss albino mice were sacrificed by cervical dislocation. Then spleen was dissected and perfused with chilled PBS. Lymphocyte suspension with > 95% viable cells were used as a source of antigen (3 X 10^6 cells/ml) for the antilymphocyte sera test (Ray and Saha, 2011, Van de Braak *et al.*, 2000).

Immunization of rabbits

Four-month-old, female albino New Zealand rabbits weighing 6 – 6.5 lbs were used for immunization. Booster doses of live cellular antigen (2 x 10^6 hemocytes/ml and 3 X 10^6 cells/ml) were injected in separate rabbits over a span of 8 weeks through various routes, i.e. intravenously through marginal ear vein, intramuscularly through thigh muscle and subcutaneously through the skin along vertebral column following a fixed schedule. Control sets of animals were injected with sterile saline through the routes described. During immunization, rabbits received a balanced natural diet and multivitamin formulation (Ray and Saha, 2011).

Collection, preservation and storage of antisera

The hair of the pinna was removed by a sterile razor blade and the marginal ear vein was punctured aseptically by the needle to collect the blood in sterile glass tubes and left overnight at 4°C separately. After 12 hours, the antihemocyte and antilymphocyte serum were isolated from the clotted blood by Pasteur pipette and stored in an aliquot at – 20 °C. The purification of antilymphocyte sera was carried out by exhaustive absorption against 10000 x g supernatant of liver homogenate, mouse RBC and rabbit RBC to yield monospecific antiserum (Ray and Chattopadhyay, 2002). Each serum aliquot of 1 ml was mixed with 2 mg of thiomersal (Sisco Research Laboratories Pvt. Ltd., India) for preservation.

Testing and titration of antisera

Preimmune and antisera (antihemocyte and antilymphocyte) were diluted 100 times with sterile phosphate buffer saline and were subsequently diluted serially (double dilution). The serum with buffer was mixed thoroughly in microfuge tubes. A serum of 10µl volume was mixed with a fixed number of viable hemocytes (10^6 cells) represented in the unit volume of buffer. Cell mixtures were incubated separately for 30 min at 37°C and screened for cell agglutination under the light microscope (Axiostar, Zeiss, Germany) (Ray and Saha, 2011).

RESULTS AND DISCUSSION

The present study observed that median lethal concentration LC_{50}) value of sodium arsenite in edible mud crabs (*Scylla serrata*) was 15 ppm for 96 hours of exposure. Polyclonal anti-hemocyte antisera were raised in rabbit model against live hemocytes of *S. serrata*. Cellular antigen was titrated against serially diluted antisera to determine zone of equivalence following the principle of cell aggregation reaction. *S. serrata* hemocytes were exposed to 1, 2, and 3 ppm of arsenic against antisera and a change in agglutination maxima against titers 5 and 6 & 4 and 5 was seen in hemocytes exposed to 2 and 3 ppm, respectively (Fig. 1 and 3). The agglutination did not change after exposure to 1 ppm of arsenic (Fig. 1 and 3). One ppm of sodium arsenite exposure did not significantly alter the agglutina-

tion reaction from the control; instead, the largest overlapping zone of agglutination was seen between the two concentrations, indicating epitope sharing (Fig. 1). A change in maximal agglutination was seen in hemocytes exposed to 2 and 3 ppm of arsenite compared to controls. When reacted with antihemocyte, hemocyte expressed intercellular agglutination, as shown in Fig. 3 and 4.

Antimurine lymphocyte sera were developed in rabbits by injecting B cell-rich spleenocytes suspension and were titrated with hemocytes of crab, *S. serrata* exposed to 1, 2 and 3 ppm of sodium arsenite along with the control. Antisera were made specific by exhaustive treatment of antisera with similar antigens. Live lymphocytes were titrated with serially diluted antisera to determine agglutination maximum (Fig. 2 and 3). Untreated hemocytes yielded agglutination maxima against titer 2 and 3 (Fig. 2). Hemocytes exposed to 1, 2 and 3 ppm of sodium arsenite expressed a dose-dependent decrease percentage of agglutination reaction but the control agglutination reaction percentage similar to the percentage of the anti-lymphocyte sera but varies in their zone of equivalence (Fig. 2). Ray and Chattopadhyay (2003) reported similar observation in the hemocyte of *Bellamya bengalensis* collected from polluted habitat of West Bengal mainly Medinipur district. Recognition of cell surface epitopes of hemocytes by murine antilymphocyte serum was indicative of sharing common epitopes among immunocompetent blood cells of crab and mammal.

S. serrata is an important estuarine species harvested indiscriminately and unscientifically from its natural habitat by professional collectors for human consumption (Saha and Dash, 2021a and b). On the other hand, the natural habitat of *S. serrata* is under constant threat of contamination by diverse xenobiotics and pathogens, including arsenic (Saha and Dash, 2021b; Saha *et al.*, 2022). Aquatic invertebrates are efficient bioaccumulation through which persistent xenobiotics move to the higher trophic level through the food web and affect non-target organisms, including humans (Ray and Chattopadhyay, 2003). Thus, propagation of *Scylla* in a bio-unsafe environment poses a potential threat to self and its humans consumers. Ray and Chattopadhyay (2002) and (2003) raised antisera against molluscans hemocyte and murine lymphocytes in rabbits. They reported high titer of hemocyte agglutination was due to sharing of common surface epitopes by molluscans hemocytes and murine lymphocytes against pesticides. A shift of agglutination response of hemocyte exposed to sodium arsenite was suggestive of a definite modulation of hemocyte surface antigen that occurred due to exposure (Fig. 1 and 2). Data is indicative of possible alteration of surface characteristics as well as membrane epitope of hemocyte of *S. serrata* from arsenic-contaminated habitat (Fig. 3 and 4).

Hemocytes, the chief mediator of crab immunity, travel extensively in hemocoel and thus can encounter external pathogens and xenobiotics (Ray and Chattopadhyay, 2003; Saha *et al.*, 2008; Saha and Ray, 2014). The immunocompetent hemocyte population is capable of lectins-mediated recognition and subsequent attachment of self and non-self surfaces along with pathogens (Marques and Barracco, 2000; Saha *et al.*, 2015). Therefore, alteration of surface property may lead to a state of immune alteration of the animal distributed in a biounsafe environment. Arsenic-induced impairment of immune response may initiate an opportunistic growth of microbes in the different body parts of crab (Saha and Dash, 2021b). Such a situation may lead to the decline of economically important species in its natural habitat. Recognition of surface epitopes of hemocytes of murine antilymphocyte sera was indicative of sharing of common epitopes between hemocytes and murine

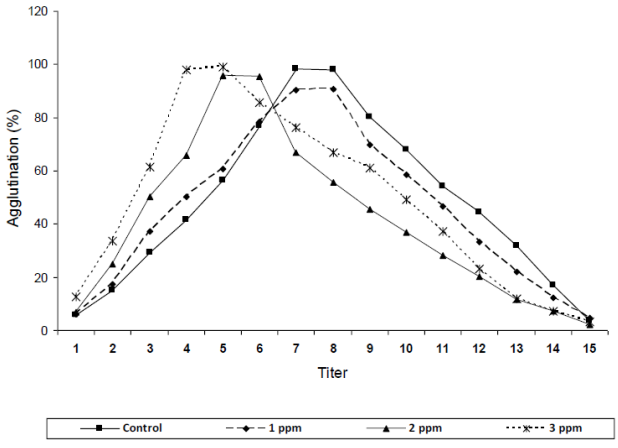

Fig. 1. *Alteration of Antihemocyte antisera mediated hemocyte agglutination reaction of Scylla serrata under exposure of 1, 2 and 3 ppm of sodium arsenite (in vivo) for 30 days. Data expressed as percentage (%) (n =5)*

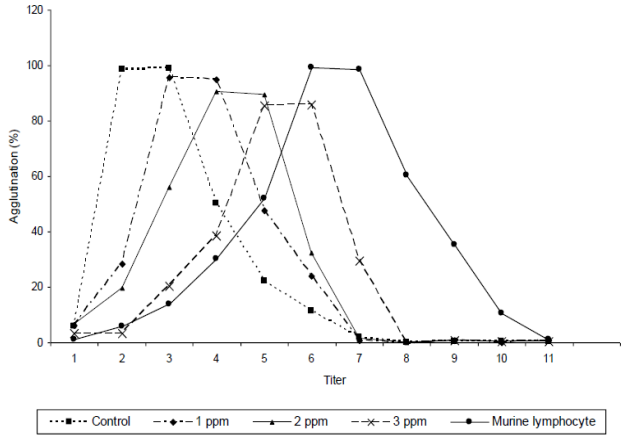

Fig. 2. *Alteration of Antilymphocyte antisera mediated hemocyte agglutination reaction of Scylla serrata under exposure of 1, 2 and 3 ppm of sodium arsenite (in vivo) for 30 days. Data expressed as percentage (%) (n =5).*

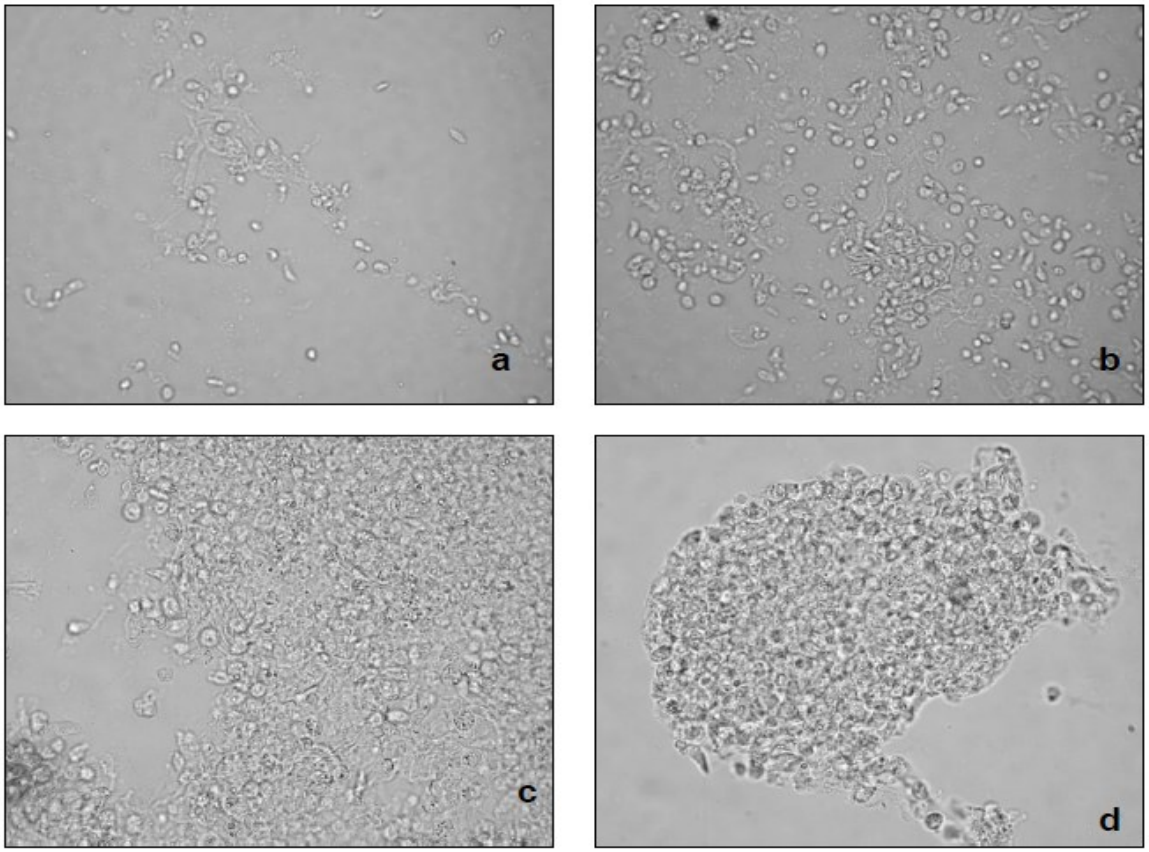

Fig. 3. *Antisera mediated agglutination of hemocytes of Scylla serrata: mild agglutination response of hemocytes titrated with antilymphocyte (a) and antihemocyte (b) serum; Hemocyte clumping showing strong agglutination response against antilymphocyte (c) and antihemocyte (d) serum. (Magnification 100 x).*

lymphocytes. Despite being distant phylogenetically, the identical pattern of cell agglutination response bears the evolutionary significance of immunity in aquatic invertebrates.

Conclusion

Arsenic, a toxic metalloid of the environment, bears the potential to affect the normal physiology of animals, including mudcrabs. Arsenic-contaminated mud flats of Sundarbans are the natural habitat of mud crabs, including *S. serrata*, an important biotic member of the Sundarbans ecosystem. It is already recorded that a large amount of this arsenic residue accumulates in aquatic *S. serrata*. The situation may result in alteration, impairment of immune function, and dysfunction of immune cells and hemocytes of *S. serrata*. In present study, a shift of agglutination response of hemocyte exposed to sodium arsenite was suggestive of a definite modulation of hemocyte surface antigen that occurred due to its exposure. Data is indicative of possible alteration of surface characteristics as well as membrane epitope of hemocyte of *S. serrata* from arsenic-contaminated habitat. As a result, crabs may become

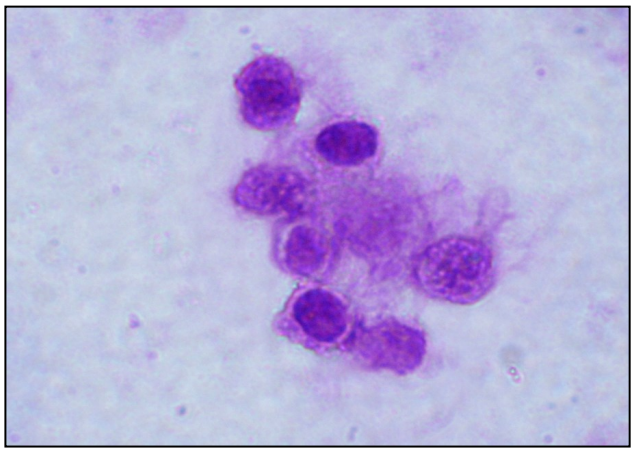

Fig. 4. *Agglutinating tendency of hemocytes of Scylla serrata (H&E, 400 x)*

vulnerable to microbial infection due to alteration activity or inactive interaction of epitopes, which could cause inactiveness of immune response and cause mortality. Prolonged residual toxicity in mud crabs could cause this economically important species to decline in the Sundarbans delta and imbalance of the ecosystem. Further investigation and in-depth research are needed to protect biodiversity from arsenic contamination in Sundarbans.

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Conflict of interest

The authors declare that they have no conflict of interest.

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