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Chapter 10

The Uniqueness of *Achatina fulica* in its Evolutionary Success

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http://dx.doi.org/10.5772/68134

Abstract

The increasing load of environmental pollutants poses a serious threat over the globe. In this vulnerable situation, it is essential to have alternative sources of medicines, may be from invertebrates. Among invertebrates, although molluscs are known for their consumption as food and ethno-medicinal use, the importance of these animals is still overlooked. Presently attention has been geared toward molluscs including *Achatina fulica* which are now considered as one of the most evolutionary successful animals. During the last few decades, researchers are trying to decipher their complex immune system to harvest valuable molecules to treat human diseases. In the present review, the existence of important immunological factors in *Achatina* is discussed addressing the coagulation system, innate immune molecules, bioactive proteins and lastly the enigmatic C-reactive proteins.

Keywords: *Achatina fulica*, innate immunity, antibacterial activity

1. Introduction

Extensive research on invertebrate immune system for the last few decades, including molluscs, revealed that invertebrates contain peptides which are endowed with anti-microbial activity [1]. These peptides can trigger specific anti-bacterial reaction by producing different isoforms specific for each bacterial species. Among immunological molecules of invertebrates, Toll-like receptor 4 (TLR4) gained much attention, though its essentiality happens to be more pronounced in vertebrates [1]. Gastropod diversity is well documented, recording 40,000–150,000 species with size variance of 1 mm to 1 m and indicating a strong immune system in gastropods [1–3].

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The giant African snail, *Achatina fulica*, is one of the large and most widely distributed land snails, considered as an agrihorticultural pest [4].Since *Achatina* develops rapidly and produces large numbers of offspring, it is now listed as one of the top 100 invasive species in the world [5]. Moreover, *Achatina* is a unique species and maintains three different life cycle stages in the same individual, surviving in the environment for millions of years. Apart from maintaining the critical life cycle stages, *A. fulica* has survived successfully, consequently, gaining the disrepute as an agricultural pest in India. In addition, these snails are considered as bio-indicators of ecosystem health. Although they do not possess immunoglobulins, they have evolved unique modalities to detect and respond to microbial surface antigens such as lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, peptidoglycans and (1→3) β-D-glucans [6].

Terrestrial snails are well known for accumulating heavy metals in their tissues and serve as a pertinent species for monitoring trace metals, agrochemicals, urban pollution and electromagnetic exposures [7]. The effect of accumulated heavy metals in different molluscan tissues and possible use of such alterations as biomarkers of exposure to xenobiotics has been investigated in some detail [8, 9]. Although snails are considered as alleged pest they are used by humans for various purposes including vigorous consumption of mollusc meat in several countries around the globe, including tribal and urban populations of India and Bangladesh [10]. Another important aspect is the ethno-medicinal use of several mollusc species highlighted by several authors [11, 12]. Pharmacological application of different body parts of mollusc are used to treat several diseases which suggests its potential to act as a source of drug [12]. In the present chapter, various characters of *Achatina* will be described including their unique immune system that contributes toward the evolutionary success of *A. fulica* in the terrestrial ecosystem.

2. Molecules in the Innate Immune System of *A. fulica*

2.1. Coagulation system in *A. fulica*

Invertebrates are not able to synthesize immunoglobulins, rather they have developed a potential defense system against microbial surface antigens such as lipopolysaccharides (LPS)/endotoxins and glucans [13]. Among various kinds of innate immune mechanisms in invertebrates, two types of coagulation mechanisms are on record: (i) in crustaceans such as lobster, crayfish [14] and insects [15] clotting occurs through Ca-dependent transglutaminase, (ii) serine protease zymogens dependent coagulation system is reported which is similar to mammalian system [13]. In *Limulus polyphemus*, commonly known as the horseshoe crab, endotoxins are sensed by amoebocytes. In invertebrates, amoebocytes are known to be associated in both hemostasis and innate or nonadaptive immune responses against microbial infections [16]. Amoebocytes behave like macrophages in mammals and can either bind pathogens directly or recognize and engulf pathogens that have been opsonized by serum proteins. This direct recognition plays a major role in host defense [17]. It has been proposed that activation of the innate immune system is initiated when pathogens bind to nonclonally distributed pattern recognition receptors on immune cells [18]. In *Limulus*, the ancient horse shoe crab, the

http://dx.doi.org/10.5772/68134

components, termed as Factor C [19, 20], Factor B [21] and pro-clotting enzyme [22], undergo endotoxin-mediated sequential limiting proteolysis/activation followed by irreversible conversion of clottable protein (coagulogen) into insoluble gel (coagulin) [23]. A similar pattern of coagulation system is also reported in *A. fulica* akin to endotoxin-mediated coagulation system in the circulating amoebocytes. An endotoxin-sensitive factor (ESF) available in the *Achatina* amoebocyte lysate (AAL) has been purified and characterized to be a serine protease type. These factors undergo a series of events such as aggregation and rapid degranulation leading to coagulation [24]. The aggregation mechanism causes bacterial sequestration, while degranulation results in secretion of serine protease zymogens [16]. Although the molecular basis of coagulation in *A. fulica* and further characterization of AAL remains to be determined, amoebocytes are considered as one of the primary immune cells in innate immune system in *A. fulica*.

3. Acharan sulfate, the new glycosaminoglycan from *A. fulica*

Acharan sulfate, a glycosaminoglycan isolated from *A. fulica*, has a major disaccharide repeating unit of 2-acetyl,2-deoxy-a-D-glucopyranose-2-sulfo-a-L-idopyranosyluronic acid, which is structurally related to both heparin and heparin sulfate. Acharan sulfate is known to be a polydisperse, with an average molecular mass of 29 kDa that contain un-sulfated iduronic acid. This glycosaminoglycan was found to be located in the body of this species and considered to be the major constituent of the mucus and the structure and compartmental distribution of acharan sulfate in the snail body [25]. Different populations of acharan sulfate having charge and/or molecular mass heterogeneities were isolated from *Achatina* whole body, mucus and the organic shell matrix. A minor glycosaminoglycan fraction was also purified which appeared to be susceptible to degradation by nitrous acid confirming the presence of N-sulfated glycosaminoglycan molecules. Furthermore, application of histochemical techniques of metachromatic staining and histoautoradiography (following metabolic radiolabeling with [^{35}S] sulfate) was evident that acharan sulfate is of wide distribution in the snail body.

4. Anti-bacterial protein from mucus of *A. fulica*

Achacin is an antibacterial glycoprotein obtained from the mucus present on the body surface of *A. fulica*. Achacin is known not only to inhibit growth of both Gram-positive and Gram-negative bacteria [26], but also appeared to attack the bacterial plasma membranes [27]. It is hypothesized that achacin is an active molecule although its role in controlling innate immunity warrants further research. However, the sequence of achacin has reported [28] its ability to catalyze oxidative deamination producing ketoacids, hydrogen peroxide (H_2O_2) and ammonia (NH_3). The antibacterial activity of achacin was found to be dependent on H_2O_2 production which is produced by the oxidative deamination reaction. Interestingly, LAOs in vertebrates also have antibacterial activity [29] which effects are most likely due to H_2O_2 formation. However, the concentration of achacin-generated H_2O_2 in the culture medium was

not sufficient to inhibit bacterial growth [28]. Bacteria in their growth phase appeared to play an important role in the antibacterial activity of achacin. These data illustrate that when snails are infected by pathogens, achacin should bind to the plasma membranes of those that are proliferating. Achacin may attack pathogens during other growth phases too by increasing the local concentration of H_2O_2 so as not to harm neighboring host cells. Thus, LAOs, which are widely distributed in living organisms, appeared to be of import in both vertebrate and invertebrate host defenses.

5. Role of Snail Hemocytes in Innate Immunity

Circulating blood cells known as hemocytes represent the main cellular component of the molluscan immune system. Hemocytes are composed of a mixture of different subpopulations of cells, for example, flow cytometric analyses of hemocytes from the freshwater snails *Biomphalaria glabrata* [30] and *Planorbarius corneus* [31] confirmed two types of circulating cells with two distinct functions [31]. Large granular hemocytes of *B. glabrata*, characterized by the absence of the monoclonal antibody BGH1 surface marker [32], are highly phagocytic in nature, while the $BGH1^+$ is nonphagocytic. *Lymnaea stagnalis* also possess two subtypes having specific surface epitopes such as the mature LS1 and nondifferentiated $LS1^+$ hemocytes [29]. It is presumed that hemocyte subpopulations that differ both chemically and functionally are regulated in their activities or behaviors through specific receptors and the signals conveyed by their interaction with appropriate ligands. It was further concluded [33] that there are five types of cells in the hemolymph of *B glabrata* and *Biomphalaria straminea* which contributes to the knowledge base for studies on hemocytes and their involvement in controlling *Schistosoma mansoni* infection.

If attention is focused on the functional attributes of hemocytes, several reports in this direction revealed diverse immunological functions such as phagocytosis [34], cytotoxicity [35], aggregation [36] and pathogen encapsulation [37, 38]. In addition to hemocytes, hemolymph, the humoral component of the molluscan immune system, is reported to exhibit the activities of superoxide dismutase [39], catalase [40] and acid [41] and alkaline phosphatases [42]. Total hemocyte count in mollusc has been considered as an important immune parameter [43]. Elevation of the total hemocyte count indicates augmentation of immunity of invertebrates [44]. Phagocytosis is an established strategy of immune defense in invertebrates including mollusc. It is considered as the major immunological activity evidenced in many molluscan species [45]. Major cytotoxic molecules such as superoxide anion and nitric oxide generated by the circulatory hemocytes of molluscs are functionally associated with the destruction of pathogens [46, 47]. Phenoloxidase is reported to be functionally associated with phagocytosis, self-nonself discrimination, cytotoxicity and melanization response [48]. Superoxide dismutase and catalase play a significant antioxidation role in the cellular physiology of molluscs. In addition, glutathione-S-transferase is functionally associated with general detoxification response of xenobiotics and anti oxidation activity [49]. All these enzymes are involved in scavenging and deactivating the toxic oxidative radicals and protect the tissue from oxidative damage [46]. Acid and alkaline phosphatases are functionally involved in pathogen

http://dx.doi.org/10.5772/68134

destruction in phagolysosome which bear immunological significance [50]. Several reports also demonstrated a range of receptors which bind carbohydrates, extracellular matrix proteins, hormones, growth factors and cytokines resulting specific immunocyte signals not only in vertebrates but also in molluscs [37]. Thus, it can be surmised that signaling systems are evolutionary conserved functions of immunocytes in the animal kingdom.

Apart from the above-mentioned defense mechanisms, snails also undergo starvation and aestivation under any stress condition. Though several reports are available on starvation and aestivation of snails, information on immune-related parameter of Indian mollusc is scant. In *Helix pomatia*, antioxidant enzymes are stimulated during aestivation [51] and physiological correlation exists between antioxidant defense and metabolic depression [52]. Starvation is reported to compromise the immunological activity of a land snail, *Helix aspersa* [53]. As per these reports, several immunological parameters are shown to be influenced by nutrition; some of these parameters are hemocyte count, phenol oxidase activity and phagocytosis. One of the elegant reports [54] in this perspective showed modulation of the innate immune parameters during experimental aestivation and starvation in *Parashorea globosa*. The parameters studied by this group included generation of cytotoxic molecules like superoxide anion, nitric oxide and phenoloxidase and the activities of superoxide dismutase, catalase, glutathione-S-transferase, acid phosphatase, alkaline phosphatase and total protein in hemocytes and hemolymph of *P. globosa* during activity, aestivation, arousal and starvation. This finding appears to be important in the field of comparative immunity and physiology for *P. globosa* which is considered as a commercially important mollusc in India.

5.1. C-reactive protein (CRP), a multifunctional player in Achatina

C-reactive protein (CRP) was first discovered in Oswald Avery's laboratory at the Rockefeller Institute for Medical Research [55]. CRP has evolved conservatively, and homologous proteins with similar functional attributes have been found in many other species. The stable preservation of this protein during evolution implies some biological significance. Thus, CRP is an ancient molecule discovered in humans only about 82 years ago. It belongs to a protein family called pentraxin (from the Greek words "penta" five and "ragos," berries) that constitutes a phylogenetically ancient family of proteins exhibiting a remarkable conservation of structure and binding reactivities. The presence of CRP has been reported from a wide range of different animals such as monkey, dog, goat, rabbit, rat, mice, domestic fowl, fish, shark and lumpsucker among vertebrates and horseshoe crab [56] and *A. fulica* [57] among the invertebrates. The finding that CRP is a major blood constituent of primitive animals, for example, horseshoe crab, *L. polyphemus* and dogfish argues strongly for an important role of this protein.

In *A. fulica*, induction of C-reactive protein (CRP) synthesis was triggered by exogenous administration of the steroid 4-androstenedione (4 AD) [58].Further, it has been suggested that the hepatopancreas is the main site of CRP expression and the CRP gene in the hepatopancreas is acutely responsive to Gram-negative bacterial infection [59]. Previously, a question had been raised on whether CRP is inducible in *Limulus* [60]. A search of the *Limulus* CRP promoter for the IL-6 response element and the Drosophila heat shock element in the human CRP promoter [61] revealed an absence of these cis-elements which led to the conclusion that *Limulus* CRP

expression is constitutive [60]. However in mammals, hepatic CRP is soluble in nature which is released into circulation [62] induced by proinflammatory cytokines. Recently, in an interesting study, the evolutionary significance of TNF, IFNγ and iNOS in immune response has been amply demonstrated in two Indian mollusc species [63]. Besides assessing different toxicological parameters, anti-bacterial property of the innate immune molecule, namely C-reactive protein (CRP) isolated from *A. fulica*, was also determined. CRP is a prototypic acute phase reactant, which is a phylogenetically conserved protein expressed in invertebrates such as arthropods [56], molluscs [58] and also in all vertebrates [64]. In *Limulus*, an arthropod, CRP acts as a main front-line innate immune molecule [59] which may be the key to a powerful defense mechanism of these animals against microbial infections that are potentially lethal in other organisms. Moreover, the presence of high level of endogenous CRP (2–4 mg/ml) in the hemolymph of *A. fulica* [58] might be the sole reason behind their effective survival in the environment.

Several authors reported that CRP can protect mice from infections caused by both Gram-positive *Streptococcus pneumoniae* [65] and Gram-negative *Neisseria elactamica* [66] and *Haemophilus influenzae* [67] bacteria via direct binding with repetitive phosphorylcholine moieties on the lipoteichoic acid or the lipopolysaccharide (LPS) of these pathogens, respectively. The level of CRP also increases dramatically during periods of immunological challenge and boosts the bactericidal activities of monocytes and neutrophils by enhancing the release of reactive oxygen intermediates [68]. CRP also induces oxidative stress in vitro in endothelial cells, smooth muscle cells and monocyte-macrophages [69, 70]. Although there are many reports on properties of CRP in a wide range of in vitro and in vivo model systems, clear understanding of the actual biological functions of this phylogenetically ancient and highly conserved molecule remains elusive.

It is also noted that bacterial cells are strongly dependent on metabolic cycles for their survival and pathogenicity [71, 72]. Therefore, effect of *Achatina* CRP (ACRP) on these bacterial metabolic cycles comprising key metabolic enzymes such as phosphofructokinase 1(PFK1) in glycolysis, isocitrate dehydrogenase (ICDH) and isocitrate lyase (IL) in TCA cycle and fructose-1,6-bis phosphatase (FBP1) in gluconeogenesis was also investigated. Various authors have reported the existence of eukaryote-like programmed cell death and the involvement of caspase-3-like proteins in bacteria [73]. Based on this information, it was attempted to delineate the anti-bacterial property of ACRP in terms of inhibition of salient metabolic enzymes which decrease bacterial infection accompanied by ROS generation and apoptosis-like phenotypes during bacterial cell death.

Several authors [74] reported potentiality of human CRP to inhibit superoxide (O_2^-) generation and delay apoptosis in neutrophils [64]. Recently, it has been reported that immunepotent CRP modulates antioxidant and anti-inflammatory effects in LPS-stimulated human macrophages [75]. The anti-stress property of ACRP was tested in mice which are known to have a very low level of endogenous CRP (~2 μg/mL) even after an inflammatory stimulus [76]. In order to prove this hypothesis, lead nitrate was administered intraperitoneally at an environmentally relevant dose in mice, and the induced oxidative stress was found to be removed when ACRP was administered prior to treating with Pb. Furthermore, in an in vitro study, both native CRP and its subunits were found to accomplish reversal of lead-induced hepatotoxicity in *A. fulica* [77].

In molluscs, several anti-microbial peptide (AMP) genes are triggered during onset of a broad range of pathogenic infections. Furthermore, several categories of immune molecules are extracted from snails including glycosaminoglycans, peptides, proteins (glycoproteins) and enzymes which possess diverse biological activities [78, 79]. Interestingly, evolutionary success of *A. fulica* can be associated, in part, with their relatively simple and effective innate immune system comprised of defense molecules present in their hemolymph such as hemocyanins, lectins, C-reactive proteins and macroglobulins in addition to a large number of granular hemocytes or amoebocytes [78, 79].

It was earlier established that xenobiotics, like heavy metals, are successful in triggering the synthesis of CRP causing inflammatory condition, and in turn, CRP was found to be a very good scavenger in eliminating these heavy metals. In contrast to human and other higher level mammals, the normal fresh water teleost *Channa punctatus* has a high level of CRP [80]. The level of CRP was also found to be significantly high in the snail *A. fulica* [58] during rainy season which is nearer to the level of CRP in the horse-shoe crab, *Limulus*. It was clearly documented by several authors that level of CRP significantly increases in serum during onset of infection or inflammation and thereby CRP acts as an inducible protein in mammals. However, in invertebrates, CRP is constitutively expressed, as for example, *Achatina* hemolymph contains a higher level of CRP which is about 2 mg/ml and showed strong cross reaction with *Limulus* CRP antiserum [58].

Snails accumulate heavy metals more in their tissue inducing numerous acute and sublethal effects [81]. Due to this sensitivity, they are considered as excellent bioindicators of heavy metal contamination [82]. The effect of accumulated heavy metals on different molluscan tissues and possible use of such alterations as biomarkers of exposure to xenobiotics has been investigated [8, 9]. Molluscs have shown considerable promise as biomonitors of metal pollution [83], and an extensive literature has appeared concerning mechanisms of uptake, detoxification and storage of heavy metals [84]. Few studies on several fresh water and marine species further substantiate the role of molluscs as bioaccumulators [85]. Further, ecological and ecophysiological studies suggest that molluscs react to environmental stress and pollution by modifying their behavior [86]. It is reported that terrestrial snails might regulate some metals assimilated from food and xenobiotic exposure [7]. The kinetics of metal accumulation and detoxification are still a subject of discussion, and there is a lack of information regarding metal toxicity in snails [84, 87].

6. Conclusion

Presently immunological molecules in mollusc, especially *A. fulica* have gained much attention because they fail to synthesize immunoglobulins but possess a strong innate immune system comprised of several molecules such as microbial surface antigens lipopolysaccharides (LPS)/endotoxins, glucans, acharan sulfate-glycosaminoglycan, achacin-mucus–derived antibacterial protein, hemocyte-derived factors and C-reactive protein. Among these proteins, CRP not only acts as apotent defence molecule but also engages in several vital physiological

functions. Recently, elegant studies have clearly indicated the role of *Achatina* CRP in inhibiting growth of both Gram-positive and Gram-negative human pathogenic bacteria [88]. These investigations showed for the first time that CRP itself can trigger apoptotic like cell death in bacterial cells. Another important contribution from this group is that ACRP was found to cross species barrier and reduce metal toxicity in mammals (mice). However, more in depth study is warranted to exploit these molecules for the benefit of human beings.

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