PERGAMON

Developmental and Comparative Immunology 27 (2003) 105–110

Developmental & Comparative Immunology

www.elsevier.com/locate/devcompimm

Lipopolysaccharides induce intestinal serum amyloid A expression in the sea cucumber *Holothuria glaberrima*

Pedro G. Santiago-Cardona, César A. Berríos, Francisco Ramírez, José E. García-Arrarás*

Department of Biology, University of Puerto Rico, P.O. Box 23360, UPR Station, Río Piedras, PR 00931-3360, Puerto Rico

Received 14 March 2002; revised 18 July 2002; accepted 20 July 2002

Abstract

We have previously characterized the first invertebrate homolog of serum amyloid A (SAA) proteins in the sea cucumber *Holothuria glaberrima*, where its expression is associated with intestinal regeneration, suggesting a possible involvement of SAA proteins in intestinal morphogenesis. Here we show that bacterial lipopolysaccharides (LPS) trigger a coelomocyte-mediated immune response in *H. glaberrima*, inducing an approximately threefold increase in coelomocyte phagocytic activity. Furthermore, LPS induces an approximately fourfold increase in SAA mRNA levels in non-regenerating intestines. These results show that in *H. glaberrima*, LPS act as an immune activator and that SAA expression can be modulated by immune-associated processes.

Keywords: Echinoderms; Lipopolysaccharides; Serum amyloid A; Intestinal regeneration; Immune activation; Phagocytosis; Coelomocytes; Holothuroids

1. Introduction

Serum amyloid A (SAA) proteins comprises a superfamily of highly conserved apolipoproteins found primarily in vertebrates, especially in mammals [1], and in some non-mammalian vertebrates such as the duck [2,3] and in salmonid fish species [4]. In vertebrates, SAA protein synthesis is dramatically induced during the acute-phase response, which is an immune-mediated systemic response aimed at counteracting challenges such as infection, trauma or tissue injury [5–7]. The acute-phase response promotes the rapid restoration of normal homeostasis and physiological functions through the isolation and neutralization of pathogens, the minimization of tissue damage, and the activation of repair processes [7]. Although SAA proteins are clearly implicated in the acute-phase response and may be part of a well-conserved anti-inflammatory mechanism, their precise physiological roles have not been clearly established.

We have recently identified and characterized the first invertebrate homolog of SAA proteins in an echinoderm, the sea cucumber *Holothuria glaberrima* [8]. Sea cucumbers undergo regenerative processes

Abbreviations: LPS, lipopolysaccharides; SAA, serum amyloid A.

* Corresponding author. Tel.: +1-787-764-000 x2596; fax: +1-787-764-3875.

E-mail address: jegarcia@upracd.upr.clu.edu (J.E. García-Arrarás).

0145-305X/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0145-305X(02)00068-X

where most of their digestive tract can be regenerated [9]. In *H. glaberrima*, SAA expression increases during the morphogenetic stages of intestinal regeneration, where the lumen and the muscular layers of the regenerating intestine are being formed [8]. This close association between regeneration processes and SAA expression has served to postulate the involvement of SAA proteins in intestinal morphogenesis, thus proposing a non-traditional role for these proteins [8]. Such a role does not preclude that SAA can also participate in inflammatory and immune-mediated processes. Therefore, the present study is aimed at testing the possibility that the holothuroid SAA proteins, like their vertebrate counterparts, may participate in immune-related mechanisms, particularly those that occur during activation of the immune system.

2. Materials and methods

2.1. Animals

Specimens of *H. glaberrima* (10–15 cm) were collected in the northeast coast of Puerto Rico and kept in seawater aquaria at 20–24 °C. Sea cucumbers can undergo a process, named evisceration, where most of the viscera are expelled from the body cavity and eventually regenerated [9]. Evisceration was induced by injecting 3–5 ml of 0.35 M KCl into the coelomic cavity of adult animals. Eviscerated and non-eviscerated animals were kept in the aquaria.

2.2. Immune response activation and coelomocyte cell counts

H. glaberrima received intracoelomic injections of *E. coli* lipopolysaccharides (LPS, Sigma Chemical Co., St Louis, MO). LPS was diluted in filtered seawater at concentrations of 0.1 and 1 μg/μl. Five hundred microliters of LPS solution were injected per animal. Since *H. glaberrima* specimens contain an average of 7.5 ± 2.2 ml of coelomic fluid (as measured by obtaining the coelomic fluid from 10 animals), LPS concentrations of 0.1 and 1 μg/μl correspond to approximately 7 and 70 μg of LPS per ml of coelomic fluid, respectively. These concentrations are similar to those used in sea urchins, where LPS concentrations ranging from 2 to 140 μg/ml of coelomic fluid were able to activate the sea urchin immune system [10]. Control animals were injected with 500 μl of filtered seawater. Forty-eight hours after LPS injection, the coelomic fluid of the animals was obtained by making a longitudinal incision in the right lateral side of the body. The coelomic fluid was collected in sterile 15 ml graduated tubes and centrifuged at $450 \times g$ for 7–10 min at room temperature. The pelleted coelomocytes were resuspended in 5 ml of sterile calcium- and magnesium-free tyrode medium (NaCl 137 mM, KCl 2.7 mM, $NaHCO_3$ 12 mM, $NaHPO_4$ 0.48 mM, glucose 0.6 mM). A 1:1 dilution of this cell suspension was done with 0.4% trypan blue dye in saline (Biowhittaker, MD). The number of coelomocytes in 10 μl of this mixture was counted on a bright line hemacytometer under a microscope and the number of coelomocytes per ml of coelomic fluid was calculated for each animal.

2.3. Phagocytosis assays

H. glaberrima specimens were injected with LPS as described earlier. Forty-eight hours after immune activation, 500 μl of a 1:1000 dilution (in filtered seawater) of fluorescent microbeads 0.77 μm in diameter (Polysciences Inc., Warrington, PA) were injected into the coelomic cavity of animals. Two hours after microbead injection, coelomocytes were obtained from the coelomic fluid, fixed in 4% paraformaldehyde, spread unto a microscope slide, and viewed under both fluorescence and light microscopy. The total number of cells in four different visual fields was determined and the percent of cells with ingested fluorescent microbeads was calculated for each field.

2.4. RNA isolation and northern blot analysis

Animals were anesthetized by immersion in 6% $MgCl_2$ for 1 h before dissection. Intestines were excised through a longitudinal slit of the body wall, briefly rinsed in ice-cold, filtered seawater, and immediately processed for RNA isolation according to the method of Chomczynski and Sacchi [11]. Northern blot analysis was done as previously described [8]. SAA levels were quantified by volume

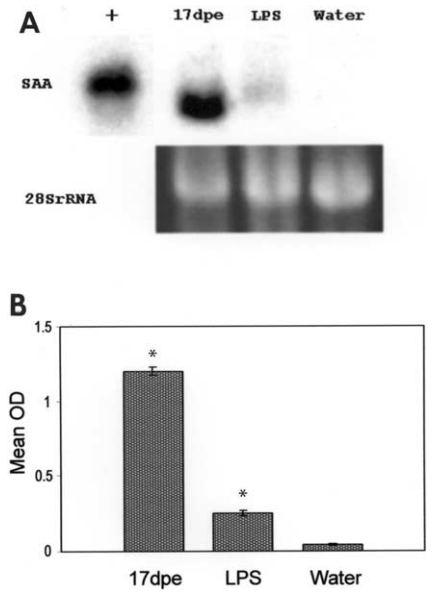

Fig. 1. Northern blot analysis of SAA mRNA levels following LPS treatment. (A) Representative northern blot of SAA mRNA levels in intestinal tissue from animals treated with LPS (LPS), sea water control (water) or regenerating animals (17 dpe). Control animals were injected with filtered sea-water. Regenerating animals 17 days post-evisceration were used for comparison. The SAA mRNA levels are compared to the 28s rRNA in the ethidium bromide stained gel prior to transfer to nylon membrane to show equal loading of the lanes. The first lane shows the positive control (+) amplification. (B) SAA mRNA levels with different treatments were quantified by volume analysis of band intensity and normalized against the 28s rRNA values. Mean OD refers to the mean intensity of the SAA band in relation to the mean intensity of the 28S rRNA band. Each bar represents the mean of at least three Northern blots ± SE Different from control (*t*-test) $*P < 0.001$.

analysis of band intensity using the Multi Analyst software from Bio-Rad (Hercules, CA). Expression levels for SAA mRNA were normalized by calculating the ratio of SAA mRNA levels to 28s rRNA levels. Statistical analyses were done with Statworks and GraphPad Prism softwares.

3. Results

3.1. LPS induces coelomocyte phagocytic activity in H. glaberrima

The effect of LPS on coelomocytes was assessed by determining the number of coelomocytes and their phagocytic capacity. Injection of 1 μg/μl LPS did not affect the number of coelomocytes per ml of coelomic fluid, as compared with seawater-injected controls ($8.6 \pm 1.5 \times 10^6$ cells per ml in controls versus $9.1 \pm 1.5 \times 10^6$ cells per ml in LPS injected).

Changes in phagocytic activity were determined by measuring internalization of fluorescent microbeads. Phagocytic coelomocytes were identified by the presence of ingested fluorescent particles in their cytoplasm. The number of cells that had ingested fluorescent beads was compared with the number of cells with no ingested fluorescent beads. We did not take into account differences in the number of beads ingested per cell. In seawater-injected controls 14.3 ± 2.2% of the coelomocytes showed phagocytic activity, while 22.4 ± 1.3% of the coelomocytes from animals injected with 0.1 μg/μl and 23.7 ± 3.1% of animals injected with 1 μg/μl LPS showed phagocytic activity. Both LPS treatments are significantly different from controls $p < 0.001$ (*t*-test).

3.2. LPS induces intestinal SAA mRNA levels in H. glaberrima

Northern blot analyses were done in order to determine if LPS was able to induce SAA mRNA expression in the intestinal tissues of normal (non-eviscerated) animals. Since we have previously shown that SAA expression increases during intestinal regeneration [8], animals undergoing intestinal regeneration were used for comparison. Animals injected with 1 μg/μl LPS showed a fourfold increase in intestinal SAA mRNA levels over filtered seawater-injected controls. Nevertheless, this induction, while significant, falls short of the twenty-fourfold increase in intestinal SAA mRNA levels observed in regenerating animals 17 days post-evisceration (dpe) (Fig. 1).

3.3. Phagocytic activity during intestinal regeneration in H. glaberrima

Our results show that immune activation by LPS is able to increase both the phagocytic activity of coelomocytes and intestinal SAA mRNA levels. Furthermore, we have previously shown that SAA mRNA levels increase during intestinal regeneration peaking at 14–20 dpe [8]. These observations led us to investigate whether immune activation, as assessed

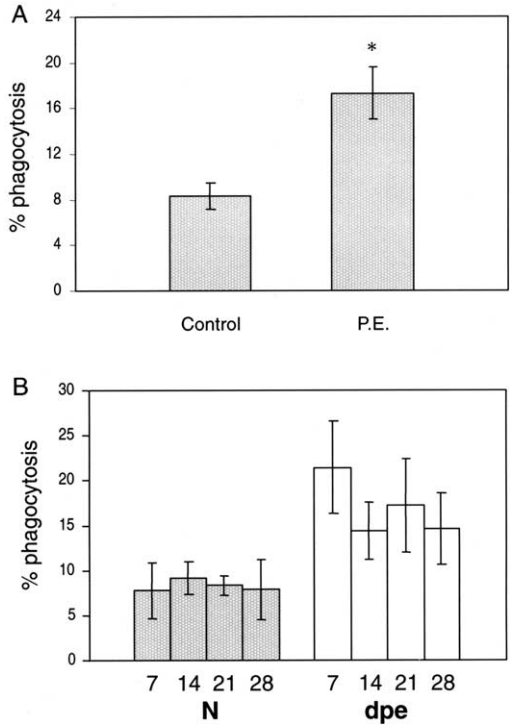

Fig. 2. Coelomocyte phagocytosis during intestinal regeneration. (A) A doubling in the phagocytic activity of coelomocytes is observed in animals pooled at various days (7–28 days) post-evisceration (P.E.). The bar incorporates the data from all regenerative stages. Controls are non-eviscerated animals housed within the same aquaria as the regenerating animals. Each bar represents the mean ± SE of twelve animals. Different from control (*t*-test) $*P < 0.001$. (B) The percentage of phagocytic coelomocytes was determined in animals at different regenerative stages. Each bar represents the mean ± SE of at least three independent experiments. Although regenerating animals have a higher percentage of phagocytosis than non-eviscerated animals, no difference was found among regenerating animals at different regenerating stages (7 days post-evisceration-28 dpe). Similarly non-eviscerated animals kept in the aquarium for different lengths of time (7N–28N) do not show differences in the phagocytic activity of their coelomocytes.

by coelomocyte phagocytic activity, occurs during the process of intestinal regeneration. Animals were injected with fluorescent beads at different time-periods after evisceration and their coelomocyte phagocytic activity was determined. Control non-eviscerated animals were housed within the same aquaria and for the same time-period as the regenerating ones. Our results show that an increase in the phagocytic activity of coelomocytes does occur during regeneration (Fig. 2). When regenerating animals are grouped together, there is a doubling in the percentage of phagocytic coelomocytes, increasing from a basal 8% in non-regenerating animals to close to 17% in animals undergoing regeneration (Fig. 2). When specific regeneration stages are analyzed, the data shows an overall increase in the amount of phagocytosis at all regenerating stages with no statistical difference among them.

4. Discussion

We have previously characterized an invertebrate homolog of SAA proteins in the sea cucumber *H. glaberrima*, where both the SAA mRNA and protein are expressed in regenerating intestinal tissue [8]. The fact that SAA expression during intestinal regeneration peaks during the morphogenetic stages has lead us to propose a possible involvement of the holothuroid SAA protein in intestinal morphogenesis. However, this does not exclude a possible participation of holothuroid SAA proteins in immune-associated processes. In fact, the participation of SAA proteins in such processes has been extensively characterized in vertebrates, where they are part of immune-mediated anti-inflammatory mechanisms associated with infection and tissue injury [5–7]. For that reason, we were interested in elucidating a possible association between the holothuroid SAA protein and immune-associated responses in *H. glaberrima*, and in particular to determine whether SAA expression can be modulated by the activation of the immune system.

A challenge to the immune system can be experimentally induced by administration of bacterial LPS, and such injections are able to dramatically induce SAA expression in various vertebrate species [7,12–14]. In echinoderms, studies of immune activation by LPS have been done mainly in sea urchins, where intracoelomic LPS injection triggers a coelomocyte-mediated immune response characterized by the expression and synthesis of various immune response-related molecules. For example, LPS-activated coelomocytes have been shown to produce proteins of the invertebrate complement system and other immune effector proteins [15–20]. LPS have also been shown to increase the levels of

profilin mRNA in sea urchin coelomocytes. Profilin is a major actin binding and cytoskeletal modifying protein that might play a central role in the cytoskeletal organization that occurs during chemiotaxis, phagocytosis and the formation of cellular clots [10,18,21]. An increase in the levels of profilin mRNA has been suggested as an indicator of coelomocyte activation during immune responses [10,21]. Another feature of echinoderm immunity is coelomocyte-mediated phagocytosis of foreign particles, including bacteria and foreign non-cellular particles and proteins [18]. The association between phagocytosis and immune activation has been shown in sea urchins, where coelomocytes respond to inflammatory processes by acquiring migratory and phagocytic activity [22].

Our studies of immune activation in *H. glaberrima* show that intracoelomic LPS injection induces coelomocyte activation, as assessed by the almost twofold increase in phagocytic activity observed in coelomocytes from LPS-injected animals compared to coelomocytes from filtered seawater-injected controls. Interestingly, LPS injection did not affect the total number of coelomocytes per ml of coelomic fluid 48 h after injection, suggesting that the observed increase in phagocytic activity is not due to an increase in the number of coelomocytes in the coelomic fluid. Although the possibility that changes in cell number occur at other time periods, cannot be discarded.

After determining that LPS is able to activate the holothuroid immune system, the next step in our investigations was to determine if LPS is also able to regulate the expression of SAA mRNA. We found that 1 μg/μl LPS is able to induce a fourfold increase in intestinal SAA mRNA levels over water-injected controls, showing that SAA expression in *H. glaberrima*, as in vertebrates, can be modulated by immune activation. However, in these experiments SAA mRNA levels in LPS-injected animals were approximately six times lower than the SAA mRNA levels induced during intestinal regeneration [8]. Thus, our experiments of immune activation showed that SAA expression can be modulated by immune-associated processes, however, there is no strict correlation between SAA expression and immune activation. The activation induced by LPS treatment causes the highest levels of phagocytosis but a small, by comparison, activation of SAA mRNA expression. On the other hand, in 14–20 dpe regenerating animals, where the expression of SAA mRNA levels are the highest, phagocytic activity is not significantly different from that of other regenerating stages. These results suggest a dissociation between immune-associated processes and SAA expression during intestinal regeneration in *H. glaberrima*. Nonetheless, the fact that SAA expression is indeed increased by LPS-induced immune system activation is important to the evolution of the acute phase response, as has been discussed recently by Beck and colleagues [20].

Taken together, these observations suggest that in these echinoderms, SAA expression may be triggered, at least in part, by morphogenetic-related processes. This brings forth the possibility that SAA proteins, although structurally conserved, may posses enough functional diversity to participate in processes that include both immune response and morphogenesis.

Acknowledgements

The authors are grateful to Dr Maria Lazaro for help in setting up the phagocytosis assays. PSC and CAB are recipient of fellowships from the Alliance for Graduate Education and the Professorate (AGEP) and the Minority Access to Research Careers (MARC) Programs, respectively. The present study was supported by grants from Sea Grant, NIH-RCMI (RRO-3641-01), NSF (IBN-0110692), NIH-MBRS (S06GMO8102) and the University of Puerto Rico.

References

[1] Uhlar CM, Burgess CJ, Sharp PM, Whitehead AS. Evolution of the serum amyloid A (SAA) protein superfamily. Genomics 1994;19:228–35.

[2] Gorevic PD, Greenwald M, Frangione B, Pras M, Franklin EC. The amino acid sequence of duck amyloid A (AA) protein. J Immunol 1977;118:1113–8.

[3] Ericsson LH, Eriksen N, Walsh KA, Benditt EP. Primary structure of duck amyloid protein A. The form deposited in tissues may be identical to its serum precursor. FEBS Lett 1987;218:11–16.

[4] Jensen LE, Hiney MP, Shields DC, Uhlar CM, Lindsay AJ, Whitehead AS. Acute phase proteins in salmonids: evolutionary analyses and acute phase response. J Immunol 1997;158:384–92.

[5] Malle E, De Beer FC. Human serum amyloid A protein: a prominent acute-phase reactant for clinical practice. Eur J Clin Invest 1996;26(6):427–35.
[6] Jensen LE, Whitehead AS. Regulation of serum amyloid A protein expression during the acute-phase response. Biochem J 1998;334:489–503.
[7] Uhlar CM, Whitehead AS. Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem 1999;265(2): 501–23.
[8] Santiago P, Roig-López JL, Santiago C, García-Arrarás JE. Serum amyloid A protein in an echinoderm: its primary structure and expression during intestinal regeneration in the sea cucumber *Holothuria glaberrima*. J Exp Zool (Mol Dev Evol) 2000;288:335–44.
[9] García-Arrarás JE, Estrada-Rodgers L, Santiago R, Torres II, Díaz-Miranda L, Torres-Avillán I. Cellular mechanisms of intestine regeneration in the sea cucumber, *Holothuria glaberrima* Selenka. J Exp Zool 1998;281:288–304.
[10] Smith LC, Britten RJ, Davidson EH. Lipopolysaccharide activates the sea urchin immune system. Dev Comp Immunol 1995;19:217–24.
[11] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
[12] Lowell CA, Stearman RS, Morrow JF. Transcriptional regulation of serum amyloid A gene expression. J Biol Chem 1986;261:8453–61.
[13] De Beer MC, Kindy MS, Lane WS, De Beer FC. Mouse serum amyloid A protein (SAA5) structure and expression. J Biol Chem 1994;269(6):4661–7.
[14] Jorgensen JB, Lunde H, Jensen L, Whitehead AS, Robertsen B. Serum amyloid A transcription in Atlantic salmon (*Salmo salar* L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1 beta, IL-6 and TNF alpha or bacterial lipopolysaccharide. Dev Comp Immunol 2000;24:553–63.
[15] Smith LC, Chang L, Britten RJ, Davidson EH. Sea urchin genes expressed in activated coelomocytes are identified by expressed sequence tags. Complement homologues and other putative immune response genes suggest immune system homology within the deuterostomes. J Immunol 1996;156: 593–602.
[16] Smith LC, Shih CS, Dachenhausen SG. Coelomocytes express SpBf, a homologue of factor B, the second component in the sea urchin complement system. J Immunol 1998;161: 6784–93.
[17] Al-Sharif WZ, Sunyer JO, Lambris JD, Smith LC. Sea urchin coelomocytes specifically express a homologue of the complement component C3. J Immunol 1998;160:2983–97.
[18] Gross PS, Al-Sharif WZ, Clow LA, Smith LC. Echinoderm immunity and the evolution of the complement system. Dev Comp Immunol 1999;23:429–42.
[19] Clow LA, Gross PS, Shih CS, Smith LC. Expression of SpC3, the sea urchin complement component, in response to lipopolysaccharide. Immunogenetics 2000;51(12):1021–33.
[20] Beck G, Ellis TW, Habicht GS, Schluter SF, Marchalonis JJ. Evolution of the acute phase response: iron release by echinoderm (*Asterias forbesi*) coelomocytes, and cloning of an echinoderm ferritin molecule. Dev Comp Immunol 2002; 26:11–26.
[21] Smith LC, Britten RJ, Davidson EH. SpCoel1: a sea urchin profiling gene expressed specifically in coelomocytes in response to injury. Mol Biol Cell 1992;3:403–14.
[22] Mangiaterra MBBCD, Silva JRMC. Induced inflammatory process in the sea urchin *Lytechinus variegatus*. Invert Biol 2001;120:178–84.