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The macrophage chemotactic activity of *Streptococcus agalactiae* and *Streptococcus iniae* extracellular products (ECP)

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Abstract

The ability of *Streptococcus agalactiae* and *Streptococcus iniae* to attract macrophages of Nile tilapia (*Oreochromis niloticus*) was investigated. The extracellular products (ECP) from *S. agalactiae* and *S. iniae* were tested *in vitro* for macrophage chemotaxis using blind-well chambers. The macrophages were obtained from the peritoneal cavity 4–5 days after intraperitoneal injection of squalene. Both macrophage chemotactic and chemokinetic activities were demonstrated using the *S. agalactiae* ECP. However, only chemotactic activity was shown for *S. iniae* ECP. High-pressure liquid chromatography fractionation revealed that semi-purified *S. agalactiae* and *S. iniae* ECPs had estimated molecular weights of 7.54 and 19.2 kDa, respectively. The prominent chemotactic activities of ECP from *S. agalactiae* and *S. iniae* are likely to be involved in the proinflammatory responses of macrophages to *S. agalactiae* and *S. iniae* infections.

Keywords: *Streptococcus agalactiae*; *Streptococcus iniae*; Chemotaxis; Macrophage; Extracellular products; Inflammation; Nile tilapia

1. Introduction

Streptococcus agalactiae and *Streptococcus iniae* are major bacterial pathogens of tilapia as well as other cultured and wild fish species from fresh and marine water [1,2]. An early event of inflammation in fish involves the movement of macrophages from blood and surrounding tissues at sites of *S. agalactiae* and *S. iniae* invasion [3,4]. Macrophages accumulate at the sites of microbial invasion in response to chemical mediators (chemoattractants) produced by the bacteria and/or the host [5–7]. The directional movement of macrophages induced by a chemoattractant gradient is chemotaxis. On the other hand, chemokinesis is random migration that is not truly directional [5,6]. *In vitro* chemotaxis of fish leukocytes to a variety of microbial chemotactic factors has been described [5,6,8–12].

Previously, Klesius and Sealey [5] reported that extracellular products (ECP) of *Edwardsiella ictaluri* were chemotactic and chemokinetic for macrophages of channel catfish. Through both *in vitro* and *in vivo* chemotaxis assays, they

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concluded that excreted or secreted ECP from *E. ictaluri* possess macrophage chemoattractant properties. Knowledge regarding the effects of ECP from *S. agalactiae* and *S. iniae* on macrophage chemotaxis and chemokinesis is lacking. The objective of the present study was to determine if ECP from *S. agalactiae* and *S. iniae* had chemotactic and chemokinetic effects on peritoneal macrophages from tilapia *in vitro*.

2. Materials and methods

2.1. Fish

Nile tilapia (*Oreochromis niloticus*) (60 ± 5 g) were produced from one spawn and reared at the USDA, ARS, Aquatic Animal Health Research Laboratory at Auburn, AL on a commercial diet (Aquamax Grower 400, Brentwood, MO). The fish were fed daily to satiation. Two weeks prior to experimentation, the fish were acclimated in 57 L glass aquaria. The aquaria were supplied with flow-through dechlorinated water at 27 ± 1 °C. The water was continuously aerated and a photoperiod of 12 h light/12 h dark was used. Feeding was discontinued 1 day prior to collection of squalene-induced peritoneal macrophages.

2.2. Preparation of S. agalactiae and S. iniae crude extracellular products

S. agalactiae ARS-MU11B and *S. iniae* ARS-60 were used for the preparation of their extracellular product (ECP). These bacteria were cultured in tryptic soy broth (TSB) as previously described by Klesius et al. [13] and Evans et al. [14]. Briefly, two 5 L flasks of TSB were inoculated with a −70 °C stored and thawed aliquot of each isolate and each inoculated flask was grown at 27 °C for 72 h. Neutral buffered formalin was added to give a final concentration of 3% for 24 h at 27 °C. The formalin treated cultures were centrifuged at $7000 \times g$ for 30 min and the cell pellet and culture fluid separated. The cell-free, supernatants (ECP) were concentrated 20 times using a 2 kDa hollow fiber concentrator, filter sterilized (0.2 μm) and stored frozen at −70 °C in aliquots until used. The protein concentrations of the crude ECP were determined by the BCA™ Protein Assay Kit (Pierce, Rockford, IL). The protein concentrations of the crude ECP from *S. agalactiae* and *S. iniae* were standardized at 400 μg mL^{-1}.

2.3. Macrophage collection

For the collection of macrophage enriched peritoneal exudate cells, five fish per experiment were intraperitoneally (IP) injected with 250 μL of squalene (Sigma, St. Louis, MO) as described by Klesius and Sealey [5]. The fish were placed in 57 L aquaria and maintained as described above for 4–5 days. At the end of this period, fish were removed and killed with 200 mg mL^{-1} buffered tricaine methane-sulfonate (MS-222, Finquel, Agrent Chemical Lab, Redmond, CA, USA). The peritoneal cavity was washed three times with 15 mL^{-1} of sterile, cold phosphate buffered saline (PBS) using a 20-gauge needle attached to a three way valve. The peritoneal exudates of the fish were combined into a 50 mL^{-1} centrifuge tube and centrifuged at $300 \times g$ for 10 min at 4 °C. The supernatant was removed, discarded, and cells were suspended in Ca- and Mg-free Hank's balanced salt solution without phenol red (HBSS, GIBCO, Grand Inland, NY). The cells were washed once in the HBSS at $300 \times g$ for 10 min at 4 °C. The cell pellet was suspended in 2 mL^{-1} of HBSS and diluted with 9 mL^{-1} of sterile deionized water to lyse the red blood cells for 20 s, then 1 mL^{-1} of 10X HBSS was added. The cells were washed twice in the above Hank's balanced salt solution at $300 \times g$ for 10 min at 4 °C. Cell count and viability were established following enumeration with a haemocytometer by trypan blue exclusion assay.

2.4. Alpha-naphthyl acetate esterase (ANAE) assay

The peritoneal exudates cells were stained for ANAE activity according to the procedure described by Sigma Chemical Company, St Louis, MO. Suspensions of exuduate cells were air-dried and fixed on clean glass microscope slides and stained with ANAE according to Procedure No. 91 (Sigma Chemical Company).

2.5. Chemotaxis and chemokinetic assays

Chemotaxis was determined by a modification of the lower-surface as previously described by Klesius and Sealey [5]. Assays were done in duplicate using blind-well chemotactic chambers (Corning CoStar, Cambridge, MA) and 8 μm-pore polycarbonate-membrane filters (Nuleopore, Pleasanton, CA) presoaked in RPMI-1640 (Gibco BRL, Grand Island, NY) containing 1% horse serum. The lower compartment was filled with 200 μL^{-1} of RPMI-1640 containing 1% horse serum or various percentages of sterile, filtered ECP (assess directional movement), and the upper compartment was filled with 5.0×10^6 mL^{-1} of exudate cells to give a final volume of 200 μL^{-1} compartment. The percent concentration of ECP in the lower compartments was 0, 10, 30, 50, 70, or 90%.

The checkerboard assay (chemokinetic and chemotactic determination) described by Zigmond and Hirsch [15] was performed by placing ECP with the exudates cells in the upper compartment, as well as placing 0, 10, 30, 50, and 70% of ECP in the upper, lower, or both compartments. The final volume was 200 μL^{-1} compartment. The chambers were incubated on a horizontal platform shaker (100 revolutions min^{-1}) for 90 min at 25 °C. Following incubation, the filters were removed, inverted, placed on precleaned slide, attached with clear fingernail polish, and stained with Leukostat [8]. Enumeration of migrating cells per field of view was done in five fields on the bottom surface of the filters using a light microscope at 400 × magnification. The mean number and standard error of only migrating macrophages per field of view were determined and recorded. The experiments were repeated twice for a total of 10 fields.

2.6. Semi-purification of extracellular products

The ECP was semi-purified using high-pressure chromatography system (Waters, Milford, MA) equipped with a Shodex® Protein KW-804 column (Showa Denko K.K., Tokyo, Japan). The total and the void volumes were 24 mL^{-1} and 4.5 mL^{-1}, respectively. The crude EPC was concentrated twentyfold by lyophilization and reconstitution in 0.1 M phosphate buffered saline (PBS). Tryptic soy broth (TSB) was used as a control. Elution was done in PBS at flow rate of 0.5 mL^{-1} min. Detection was done at a wavelength of 280 nm (Waters 996 Photodiode Array Detection). Each of the semi-purified ECPs was collected from peaks observed at 280 nm absorbance during the 60 min elution. The molecular weights of the semi-purified ECP were estimated from molecular weight standards, 1.35–670 kDa range (Biorad 151-1091). The semi-purified ECP and TSB fractions were stored until used in the migration assays at −70 °C.

2.7. Statistical analysis

Chemotactic and chemokinetic data were subjected to analysis of variance [16], and means were separated by Duncan's multiple-range test. Differences were considered significant at the 95% level.

3. Results

3.1. Alpha-naphthyl acetate esterase (ANAE) activity of peritoneal exudate cells

ANAE-positive macrophages were readily identified by intense dark (re-brown) staining distributed throughout the cytoplasm (Fig. 1). In the staining procedure, ANAE is hydrolyzed to alpha-naphthol that is simultaneously bound to hexazonium pararosaniline to produce the red-brown azo dye product in the cytoplasm of the macrophages. In contrast, the cytoplasm of lymphocytes is less intensively stained. The cytoplasm of neutrophils is either unstained or faintly stained. The majority of the peritoneal exudates cell population were ANAE-positive macrophages. The other white blood cells were lymphocytes and neutrophils.

3.2. Chemokinetic activities to crude extracellular product from S. agalactiae and S. iniae

The checkboard assay was used to compare *S. agalactiae* crude ECP to those of *S. iniae* for both chemokinetic and chemotactic activities. The *S. agalactiae* crude ECP had both chemokinetic and chemotactic activities, because macrophage migration (three- to fourfold increase in migration over the control: 0% concentration) occurred when the ECP were either in the upper or lower compartment only (Table 1). Additionally, the increased migration was not

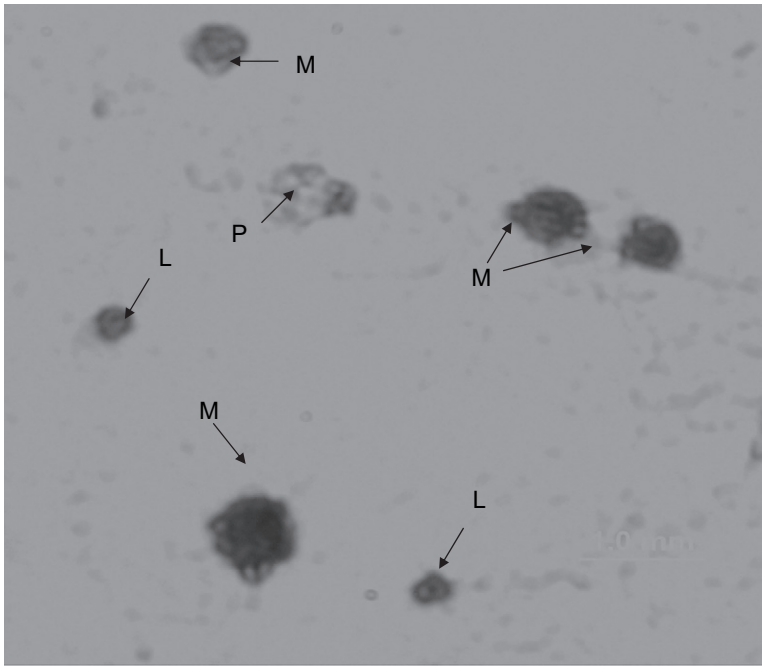

Fig. 1. Squalene-induced peritoneal exudate cells stained for alpha-naphthyl esterase (ANAE) activity (×600 magnification). The large dark stained cells are macrophage (M). Lymphocytes (L) are slightly stained and neutrophils (N) are more faintly stained.

neutralized at equal quantities of 10, 30, and 50% of *S. agalactiae* ECP in both the upper and lower compartments (Table 1). The increased macrophage migration was neutralized at 30%, 50% and 70% quantities of *S. iniae* ECP (Table 2).

3.3. Estimated molecular weights of extracellular products from S. agalactiae and S. iniae

As shown in Fig. 3a, the elution profile of *S. agalactiae* ECP revealed that the estimated molecular weight of the chemotactic and chemokinetic activities were observed at estimated molecular weight of 7.54 KDa. In contrast, the chemotactic activity of the *S. iniae* ECP was observed at an estimated molecular weight of 19.2 KDa (Fig. 3b). The molecular weights were estimated on elution times of the five molecular standards (1.3–670 kDa). The peaks were collected at the proper elution times and used to identify chemotactic and chemotactic activities of semi-purified ECP. Tryptic soy broth peaks lacked both activities (Fig. 3c). No detectable protein was found in either of the semi-purified peaks by the BCA protein assay.

Table 1
Effect of varying the concentrations of extracellular product (ECP) from *Streptococcus agalactiae* in both the upper and lower compartments on Nile tilapia macrophages migration to the lower compartment

Percent concentration of ECP in the lower compartment	Percent concentration of ECP in the upper compartment				
	0	10	30	50	70
0	4.6 ± 0.6^{n}	$16.4 \pm 2.6^{g,h}$	17.0 ± 2.4^{g}	27.2 ± 4.6^{d}	$12.0 \pm 3.5^{j,k,l}$
10	$9.4 \pm 1.1^{l,m}$	$11.6 \pm 2.1^{j,k,l}$	$10.5 \pm 4.0^{k,l}$	$13.4 \pm 1.7^{j,i}$	$12.5 \pm 2.3^{j,k}$
30	33.0 ± 3.2^{b}	Nd	$10.2 \pm 2^{k,l,m}$	$12.0 \pm 1.5^{j,k,l}$	$13.2 \pm 1.6^{j,i}$
50	45.6 ± 1.4^{a}	$15.8 \pm 3.1^{g,h,i}$	$13.2 \pm 3.7^{f,g}$	$14.0 \pm 4.8^{j,h,i}$	7.8 ± 1.3^{m}
70	20.0 ± 1.6^{f}	30.0 ± 4.9^{c}	22.4 ± 4.0^{e}	23.2 ± 2.8^{e}	4.8 ± 1.8^{n}

Mean of duplicate filters, each read in five fields of view using light microscope at 400×. Macrophages added to upper chamber and migration to ECP in lower chamber was measured. Nd indicates not done. Macrophage values without a letter in common are significantly different ($P < 0.05$).

Table 2
Effect of varying the concentrations of extracellular product (ECP) from *Streptococcus iniae* in both the upper and lower compartments on Nile tilapia macrophages migration to the lower compartment

Percent concentration of ECP in the lower compartment	Percent concentration of ECP in the upper compartment				
	0	10	30	50	70
0	$4.2 \pm 0.6^{j,k}$	$8.7 \pm 2.8^{f,g,h}$	$6.9 \pm 1.4^{h,i}$	$7.1 \pm 1.1^{g,h,i}$	$7.8 \pm 1.0^{g,h}$
10	15.7 ± 3.0^{d}	$9.6 \pm 1.1^{f,g}$	$7.6 \pm 2.4^{g,h}$	$4.3 \pm 1.3^{k,j}$	12.5 ± 2.3^{e}
30	24.4 ± 3.8^{b}	$11.1 \pm 3.4^{e,f}$	$4.3 \pm 2.5^{j,k}$	$3.9 \pm 1.3^{j,k}$	$7.0 \pm 2.1^{g,h,i}$
50	27.6 ± 3.1^{a}	23.9 ± 2.0^{b}	Nd	$3.8 \pm 1.2^{j,k}$	$6.4 \pm 0.7^{h,i,j}$
70	22.6 ± 2.2^{b}	28.5 ± 2.3^{a}	19.5 ± 1.8^{c}	$4.7 \pm 0.8^{j,i,k}$	3.7 ± 0.5^{k}

Mean of duplicate filters, each read in five fields of view using light microscope at 400×. Macrophages added to upper chamber and migration to ECP in lower chamber was measured. Nd indicates not done. Macrophage values without a letter in common are significantly different ($P < 0.05$).

3.4. Chemotactic and chemokinetic activities of semi-purified extracellular product from S. agalactiae and S. iniae

As shown in Fig. 4, chemotactic acitivities were observed for both *S. agalactiae* and *S. iniae* semi-purified ECPs at estimated molecular weights of 7.5 and 19.2 KDa, respectively. Chemokinetic activity was only observed at estimated molecular weight of 7.5 KDa for *S. agalactiae* ECP. Chemotactic activity was observed in the 19.2 KDa fraction of *S. iniae* ECP (result not shown).

4. Discussion

This is the first report that evaluates macrophage chemotactic activities in the fish response to *S. agalactiae* and *S. iniae* ECP. In order to determine the ability of the ECP to attract macrophages, we examined the effect of various quantities of the ECP on macrophage chemotaxis in blind-well assay chambers. Significant enhancement of macrophage activities was demonstrated for both *S. agalactiae* and *S. iniae* ECP in the lower compartment (Fig. 2). The type of macrophage migration was next determined in blind-well chambers using a checkerboard pattern of the ECP in upper and lower chambers. The results presented in Table 1 showed that *S. agalactiae* crude ECP was both chemokinetic and chemotactic for tilapia macrophages. Extracellular product in the upper compartment enhanced the macrophage migration as the quantity of ECP increased. The migration was not neutralized when equal quantities of ECP were placed in both upper and lower compartments. Additionally, dose-dependent enhanced migration was

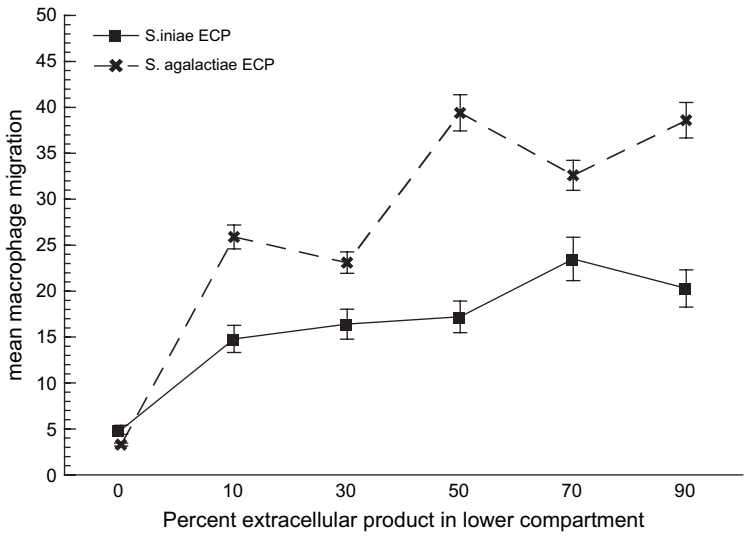

Fig. 2. Macrophage chemotaxis in response to extracellular products from *Steptococcus agalactiae* (**x**) and *Streptococcus iniae* (■). Each value represents the mean macrophage migration (±S.D.).

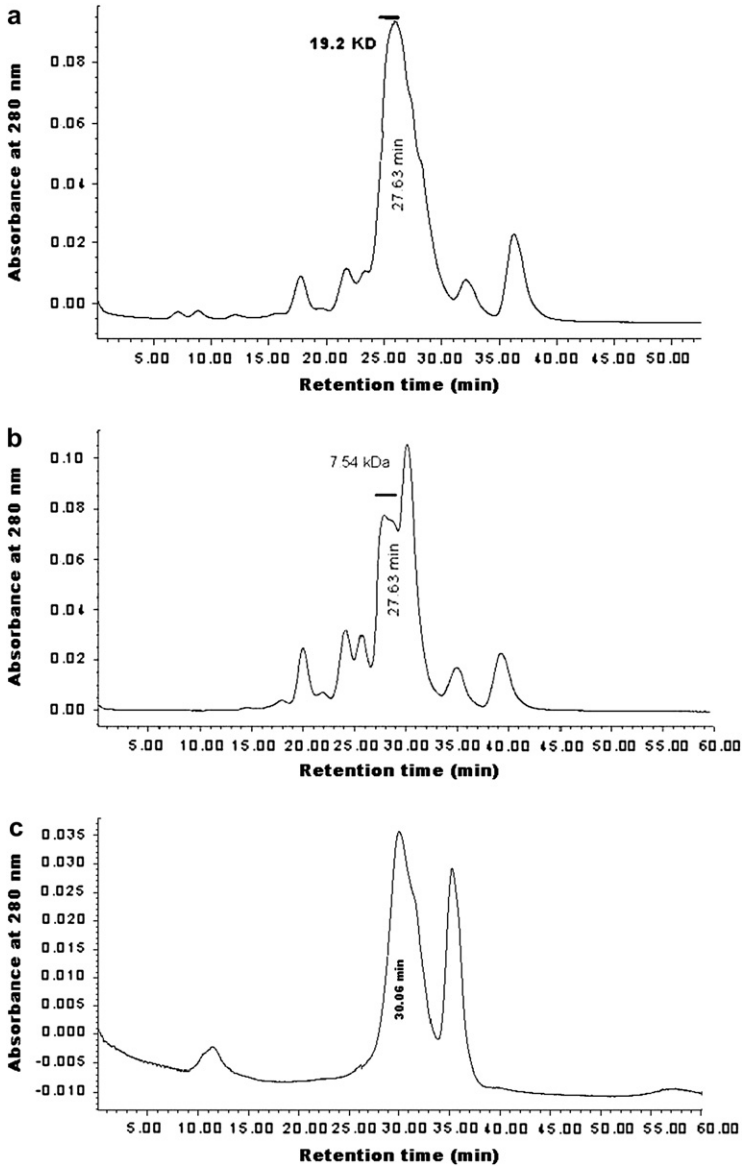

Fig. 3. (a) Retention profile (absorbance at 280 nm) of *Streptococcus agalactiae* ECP from a high-pressure liquid chromatography Shodex® Protein column in 0.1 M phosphate buffered saline. The bar indicates the *S. agalactiae* ECP eluted for chemotactic assay. (b) Retention profile (absorbance at 280 nm wavelength) of *Streptococcus iniae* ECP from a high-pressure liquid chromatography Shodex® Protein column in 0.1 M phosphate buffered saline. The bar indicates the *S. iniae* ECP eluted for chemotactic assay. (c) Retention profile (absorbance at 280 nm wavelength) of tryptic soy broth from a high-pressure liquid chromatography Shodex® Protein column in 0.1 M phosphate buffered saline.

also noted when increasing ECP quantities were placed in the lower compartment. In contrast, the effect of *S. iniae* crude ECP on macrophage migration was of the chemotactic type (Table 2).

Another major difference between *S. agalactiae* and *S. iniae* ECP was elution times from a high-pressure chromatography column. Fig. 3a showed that chemotactic activity of *S. agalactiae* semi-purified ECP was contained in the 7.54 KDa peak eluted after 27.63 min compared to the estimated molecular weight of 19.15 kDa and retention time of 26.12 min for *S. iniae* semi-purified ECP (Fig. 3b). The smaller size of the *S. agalactiae* semi-purified ECP may explain both the greater chemotactic and chemokinetic activities than the chemotactic activity of the larger semi-purified ECP of *S. iniae*. Additionally, differences in the molecular structure and amount of glycosylation may possibly explain the observed differences in biological activities between *S. agalactiae* and *S. iniae* ECP. No protein components were

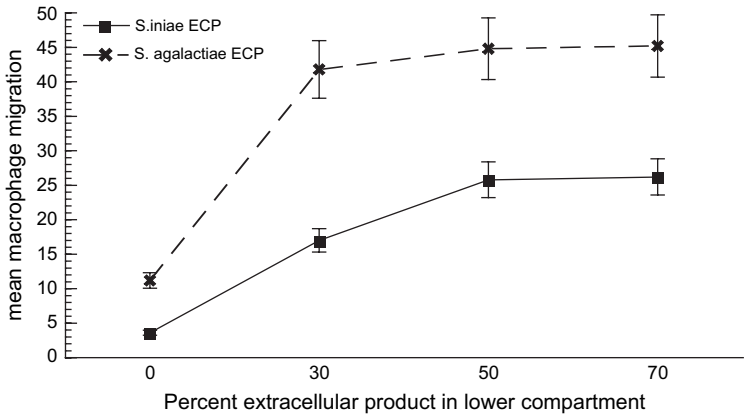

Fig. 4. Macrophage chemotaxis in response to semi-purified extracellular product from *Steptococcus agalactiae* (**x**) and *Streptococcus iniae* (■). Each value represents the mean macrophage migration (±S.D.).

detected in either of these peaks by BCA protein analysis. However, chemical analyses by means other than molecular weight estimates are needed to determine the chemical composition of the semi-purified ECP of *S. agalactiae* and *S. iniae*.

In our previous investigation of *S. agalactiae* ECP, 54 and 55 kDa antigens were revealed by Western blots using tilapia antiserum against *S. agalactiae* [17]. The antigenic properties of chemotactic compounds present in *S. agalactiae* and *S. iniae* ECPs remain to be determined. However, these antigens are considerably larger than those of the chemotactic components.

Accumulation of macrophages as part of an inflammatory response depends on the movement of phagocytes from the blood and other tissues to the site of bacterial infection [6]. The chemotactic activities of ECP from *Aeromonas salmonicida* has been described for Atlantic salmon macrophages [18]. The results of this study reported that ECP are proinflammatory and play an important role in the induction of early inflammatory reactions. However, the molecular weight of the chemotactic factor was not reported. In other studies, Weeks-Perkins and Ellis [6] reported that the chemotactic activities were caused by the A-layer. Additionally, the type of macrophage attraction for the 50 kDa A-layer was chemotactic activity rather than chemokinetic activity. The molecular weights of the *S. agalactiae* and *S. iniae* chemotactic ECP are smaller than that of the A-layer chemotactic factor. Furthermore, like the A-layer chemotactic factor, the *S. iniae* ECP was predominately chemotactic for macrophages. In contrast to the A-layer, the *S. agalactiae* had both chemotactic and chemokinetic activities for macrophages.

We believe that the secretion or excretion of *S. agalactiae* and *S. iniae* ECP is responsible, in part, for the attraction of macrophages to *S. agalactiae* or *S. iniae* localized in the organs. Tissue injury or host-derived factors may also be responsible for migration of macrophages. The interactions of macrophages with these pathogens may explain mechanisms of defence and pathogenesis. Further studies will be needed to determine the specificity of the reactions between these ECP and macrophages and how this affects macrophage migration, phagocytosis, and bactericidal activity.

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