IMMUNOLOGICAL INTERACTION BETWEEN THE FISH LOUSE *Lepeophtheirus salmonis* AND THE HOST ATLANTIC SALMON *Salmo salar* L.

Chandravathany Devadason#

Department of Cell Biology and Molecular Biology, Marischal College, University of Aberdeen, UK

ABSTRACT

The salmon louse, *Lepeophtheirus salmonis*, is a crustacean ectoparasite of salmonids and causes significant economic losses in Atlantic salmon farms. This study investigated the cellular inflammatory response, specifically macrophage migration, to the attachment of salmon louse to the epidermis of Atlantic salmon (*Salmo salar*). During the inflammatory response, macrophage infiltration is common at the site of infection in the host macrophages possessing phagocytic activity. The aim of the study was to investigate the immunological response of macrophages to the parasitic excretory and secretory product (E/S). The E/S of the parasites collected from the explants and *in vitro* cultures of parasites and macrophages isolated from fish blood were used. A macrophage migration and a phagocytosis assay were performed to study the inflammatory response. The inhibition of the macrophage migration response ($p<0.05$) and a significantly lower level of phagocytosis ($p<0.05$) were observed. The results indicate that the anti-inflammatory response of the host is elicited during louse infection.

* Corresponding author

E-mail: chand_oo@yahoo.com (Chandravathany Devadason)
Current Address: Department of Zoology, Eastern University, Sri Lanka, Chenkalady 30350

1 Introduction

The salmon louse, *Lepeophtheirus salmonis*, is a crustacean ectoparasite of salmonids and causes significant economic losses in salmon farms (Rae, 1979; Pike, 1989). The Atlantic salmon is more susceptible than other salmon species to *Lepeophtheirus* infections (Johnson & Albright, 1992a; Johnson & Albright, 1992b). Coho salmons treated with corticosteroids are susceptible to infection by sea lice due to a reduced inflammatory response in the fins and gills (Johnson & Albright, 1992a; Johnson & Albright, 1992b). Parasites have a complex lifecycle and have different defense strategies to successfully survive in the host via immunosuppressive tactics (Ko, 1989). Cell culture is generally considered to be a useful technique for biochemical (Kusuda et al., 1991) and biotechnological studies related to invertebrate pathology (Maramorosch, 1987), pharmacology, and immunology (Taylor & Baker, 1978). The development of *in vitro* cell cultures was established using tissues from shrimp (Luedeman & Lightner, 1992), lepidopteran, and dipteran insect cultures (Lee & Hou, 1992), as well as tick cultures (Munderloh et al., 1994). Most *in vitro* cell culture experiments were performed with the helminth parasite *Schistosoma mansoni* (Bayne et al., 1994). The host signals for the attachment of various ectoparasites and endoparasites to host explants have been studied in the fish parasite copepodid (*Salmincola edwardsii*), as well as cercaria of a fish parasite (*Acanthostomum japanicum*), a duck parasite (*Trichobilharzia ocellata*), and a human parasite (*Schistosoma japonicum*). In previous studies, the primary culture of normal pituitary cells of carp (*Cyprinus carpio*) was found to release gonadotropin during *in vitro* culture (Ribeiro et al., 1983) and the synthesis of *Sarcophaga* lectin and sarcotoxins in NIH-SAPE-4 was observed in a *Sarcophaga peregrina* embryonic cell line (Komano et al., 1988). The adhesion of parasites on to host surfaces has been widely reported in mammalian cell lines (Kirkpatrick & Svilenov, 1987; Lumb et al., 1988). For endoparasitic protozoans, the attachment of *Cryptosporidium* sporozoites to Madin-Darby canine kidney (MDCK) cells (Lumb et al., 1988) was demonstrated. Furthermore, it has been reported that *Eimeria magna* sporozoites attach to embryonic bovine trachea cells (Jensen & Edger, 1976) and that *Eimeriatenella* develops in Japanese quail embryos (Nakai et al., 1992). *Sarcocystis cruzi*s porozoites have been found to develop in both bovine monocytes and bovine endothelial cells (Speer & Dubey, 1986). Primary cell cultures and immortal cell lines grown in a bovine fetal kidney cell culture (Chang & Gabrielson, 1984) have been established for *Toxoplasma gondii* (Hughes, 1986). The penetration of *Schistosoma mansoni* cercaria into a host material, equivalent to living skin, has been previously demonstrated by Fusco et al. (1986). An inflammatory response and extensive cellular infiltration has been observed during parasitic infection (Paperna & Zwerner, 1982; Johnson & Albright, 1992a). Hosts

rejected the parasitic copepods *Lernaea cyprinacea* and *Lernaea polymorpha* (Shariff & Roberts, 1989). In fish, inflammatory responseshave been observed upon injection of inactivated bacteria (Bartos & Sommer, 1981), live bacteria (MacArthur & Fletcher, 1985), extracellular products of *Aeromonas salmonicida* (Ellis et al., 1976), and parasitic diseases (Cawthorn et al., 1990).

Macrophages and neutrophils secrete a variety of products with defensive functions, such as reactive oxygen species (Secombes et al., 1988), cytokines (Clem et al., 1985), leukotriene B (Knight & Rowley, 1995), and lipoxins (Rowley, 1991). Several mediators are released at the site of inflammation, namely C5a-like chemotactic complement protein, arachidonic metabolites, such as leukotriene B4 (Samuelsson, 1983), cytokines, interleukin-1β, tumor necrosis factor-α (Dinarello, 1991), IL-8 (Ribeiro et al., 1983), macrophage derived chemotactic factor (MNCF) (Cuha & Ferreira, 1986), bacterial peptides, and LPS (Creamer et al., 1991). *Glugea* parasitic infection in the three spine stickle back (Weissenberg, 1968) and plaice (McVicar, 1975), and *Cryptobiasalm ositica* infection in rain bow trout (Jones & Woo, 1986) have also been previously investigated. The aim of this study was to understand the immunosuppressive effect of *L. salmonis* cultured *in vitro*, and to determine whether a leucocyte migratory inhibitory factor could be obtained in the supernatant.

2 Materials and Methods

2.1 Experimental Fish

Atlantic salmon (*Salmo salar* L.) weighing 1 kg and 200 g were obtained from the SOAEFD marine fish farm and maintained in glass fiber tanks with a constant flow (4 l/min) of continuously aerated sea water at 18°C and fed an appropriate daily diet of BP commercial pelleted food (2% w/w/day). Experimental animals were anaesthetized with benzocaine (Sigma) and euthanized by a sharp blow to the head.

2.2 *In vitro* culture of *L. salmonis*

To allow for copepodid attachment to the *in vitro* host cell line, the culture medium was optimized by determining the copepodid survival. This was measured as activity and cell viability, determined as the spread of confluent cells and the appearance of cells in different media with varying osmolarity. Mean copepodid survival in percentage was calculated by comparing the number of wells showing copepodid survival to total cells containing copepodids. The mean survival of cells was calculated based on the number of cells showing confluent cell monolayers exposed to copepodid in the wells. The media used were minimum essential medium (MEM) (Gibco), Leibovitz (L-15) (Gibco), sterile water, hosphate buffered saline (PBS), and insect medium (Sigma) at 10°C. Copepodids were sterilized prior to carrying out the *in vitro*

attachment assay. Copepodids were harvested and surface sterilized by washing with sterile culture medium (MEM) three times. Following this, the copepodids were incubated in sterile culture medium (MEM) containing gentamicin (10000 units/ml), penicillin-streptomycin (1000 units/ml), and amphotericin (1000 units /ml) (Sigma, UK)for 3 h at 18℃ under aseptic conditions.

A tissue culture plate containing confluent cell monolayers on the collagen matrix was prepared as described above. MEM (150 μl, 550 mml/kg) was added to each well. The actively sterilized copepodids were then picked up using a pipette. Two copepodids per well were transferred into the culture medium and incubated at 10℃ in a cooled incubator to enable attachment. Observations were carried out at 12 hrs intervals and the attachment was recorded using a camera. The culture medium was replaced with fresh medium every 48 hrs carefully to avoid disturbing the copepodids or the cell layer.

2.3 Isolation of leucocytes

Leucocytes were isolated from the head kidney (HK), for this, pronephros tissue of the HK was aseptically removed and separated using a 100 μm size nylon mesh (Nitex) with 5 ml 1-15 (Gibco) containing 2% fetal bovine serum (FBS) (Gibco), penicillin/streptomycin (100 units/ml)(Gibco), and 10 units/ml of heparin (Sigma). The cell suspension was placed on a 37-51% Percoll (Pharmacia) discontinuous density gradient and centrifuged at 400 g and 4℃ for 35 minutes. The leucocyte cell fraction was separated from the 37-51% Percoll interface was resuspended in L-15 (Gibco) containing 1% FBS (Gibco) and centrifuged at 400 g for 15 minutes. The cell pellet was collected and resuspended in the same medium and the cell number was adjusted to 1×10^7 cells/100 μl with L-15 medium [containing 5% FBS and penicillin/streptomycin (100 units/ml)] and dispersed into each well of a 96-well tissue culture plate (Nunc). After 3 hrs incubation at 18℃, non-adherent cells were removed by washing the cell layer with sterile phenol red free HBSS, pH 7.4 (Gibco). The remaining cells were resuspended in L-15 containing 2% FBS with vigorous pipetting.

2.4 Production of host chemoattractant containing supernatant

A chemoattractant containing supernatant was generated from the macrophage monolayer, as described in the section above, by stimulation with a mixture of 50 μl of 10 μmol calcinophore (Sigma) and 50 μl of 50 μg/ml sea lice culture supernatant (SLSN). Likewise, the series of wells containing leucocyte monolayers were also stimulated with control tissue culture supernatant (TCSN) (80μg/ml), copepodid crude extract (COPE) (50 μg/ml), or Hank's balanced salt solution (HBSS), added together with the above mentioned volumes and concentrations of

calcinophore. The plate was incubated for 2 hrs at 18℃. The leucocyte supernatant was harvested and centrifuged at 1000 g for 25 min to remove the leucocytes, then the supernatant was aliquoted and stored at -20℃ until further use. Atlantic salmon serum and LPS were also used as the host chemoattractant.

2.5 Preparation of macrophages treated with parasite products

The macrophages (HK) of Atlantic salmon were isolated as described previously (section 2.3) and washed twice with sterile HBSS. The cell viability was determined using the 0.1 (w/v) trypan blue exclusion method (Phillip, 1973) and the cell number was adjusted to 1×10^6 cells/well (1×10^7 cell/ml) diluted with HBSS. The cells were then incubated with 100 μl copepodid culture supernatant (SLSN) and 100 μl tissue culture supernatant (80 μg/ml) or copepodid crude protein extract (50 μg/ml) for 2 h at 180℃. Afterwards, the cells were washed thoroughly with sterile HBSS and the viability of cells was observed again as described previously.

2.6 Leucocyte migration assay

The migratory responsiveness of freshly isolated Atlantic salmon macrophages (HK) and sea lice culture supernatant-treated macrophages to the abovementioned host chemoattractant was investigated using a technique initially developed by Boydon (1962). A volume of 200 μl/well chemoattractant was dispensed into triplicate wells of the 24-well chemotaxis chamber (Nucleopore Corp., Cambridge, MA, USA). The chemoattractants were fresh Atlantic salmon serum (1:4 dilution), calcium ionophore stimulated leucocyte supernatant (1:2 dilution), LPS (10 μg/ml), sea lice culture supernatant (SLSN) (80 μg/ml), copepodid crude protein extracts (COPE) (50 μg/ml), and tissue culture supernatant (TCSN) (80 μg/ml). A 3 μm pore size polyvinyl pyrrolidone free polycarbonate filter (Millipore Corp., Bedford, MA, USA) was then placed above the cells. Subsequently, the upper chamber was placed on top of the filter. A volume of 100 μl of 2×10^6 cells/well macrophages or macrophages treated with the parasitic supernatants were dispensed into triplicate wells of the upper chamber. The extracted supernatants were added to the lower chamber. The chemotaxis chamber was incubated at 18℃ for 3 hrs. This assay was also carried out with untreated macrophages in the upper chamber as well as the sea lice culture supernatant (80 μg/ml) and the copepodid crude protein extract (50 μg/ml) in the respective lower chambers. After the incubation period, the filter was removed and the upper surface was thoroughly washed with HBSS three times to remove any non-adherent cells on the upper surface. The number of the cells attached to the lower surface of the filter showing directional or chemotactic migratory responsiveness under a microscope (400× magnification). The mean of the data was used to calculate the migratory index.

2.7 Statistical analysis

The chemotaxis assay was performed in triplicate on at least six fish. The data were analyzed using analysis of variance (ANOVA). Mini-tab 10 software was used to analyze the raw data.

3 Results and Discussion

Macrophages and cell viability

The number of macrophages collected from the Percoll gradient did not vary significantly between the fish, with a concentration of 1×10^{10} cells/ml/fish. The contaminating cells were red blood cells and thrombocytes. In these cells, less than 5% of neutrophils were found. Macrophage viability was determined immediately after isolation, as well as at 2, 6, 12, and 24 hrs using the trypan blue exclusion method. Both immediately after isolation and 2 hrs later, the cell viability was found to be greater than 80%. After 24 hrs, approximately 70% of the macrophages were viable. After 12 hrs, the cells showed a greatly reduced migratory response compared to that after 6 hrs. The cells isolated after 24 hrs did not migrate whereas the cells collected immediately after isolation showed an extensive migratory response after 2 hrs. This observation must be compared with the observations of Atlantic salmon, coho salmon, and chinook salmon experimentally infected with *L. salmonis*. This demonstrates that Atlantic salmon is more susceptible to *L. salmonis* infection than coho salmon (Johnson & Albright 1992a; Johnson & Albright 1992b). In addition, hydrocortisol-treated coho salmon showed an enhanced susceptibility to *L. salmonis* infection (Johnson & Albright 1992a; Johnson & Albright 1992b). This supports that the copepodid or chalimus might possess an immunosuppressive or anti-inflammatory chemotactic factor, apparently inhibiting cell migration (Figure 4). The chemotactic inhibitory factor may be found in soluble form in the secretory product of sea lice, resulting in a significantly lower migratory responsiveness, when macrophages from sea lice culture supernatant were compared to controls (tissue culture supernatant) in the leucocyte migration assay.

3.2 Treated macrophages and cell viability

The size of the treated macrophages was enlarged compared to untreated macrophages, with a vacuolated cytoplasm, as seen in Figure 1. Some of the macrophages seemed to have a slightly extended cytoplasmic tail. The cell viability was approximately 60-70% and lower than that of untreated cells.

3.3 Leucocyte migration assay

Viable macrophages were treated with SLSN, TCSN, and copepodid extract in order to confirm the effect on macrophage migration towards the chemoattractant. This assay was performed

by comparing treated and untreated macrophages in parallel. Untreated macrophages showed a higher migratory responsiveness compared to treated macrophages in all experiments. Untreated macrophages showed a high migratory responsiveness to fresh Atlantic salmon serum used as a chemoattractant, but a low responsiveness to heat-inactivated Atlantic salmon serum (Table 1). The chemotactic responsiveness of treated cells was significantly lower than that of untreated cells in all experiments when serum was used as a chemoattractant ($p<0.05$). However, there was no significant difference in the migration of cells treated with sea lice supernatant (SLSN) or tissue culture supernatant (TCSN) ($p>0.01$).
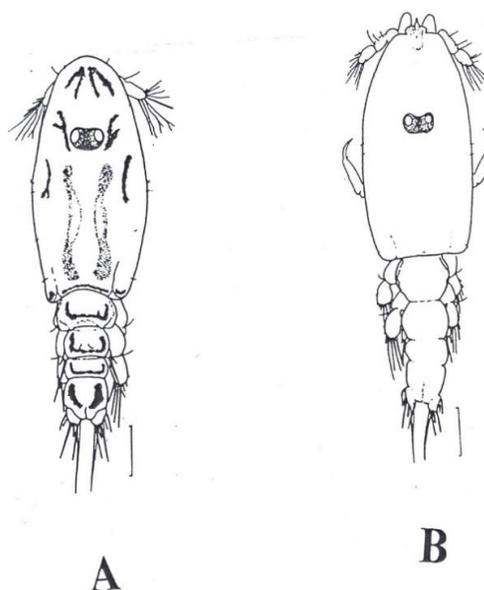

Figure 1 Schematic illustration of copepodid (A) Free living stage of copepodid (B) Attached copepodid.

Table 1 Chemotactic activity (number of macrophages (HK) showing migration) when fresh serum was used as the host chemoattractant at different times following isolation.

Incubation period (time in hours)	Chemotactic activity (mean number of cells/ microscopic field)
02	63 ± 12
06	28 ± 05
12	12 ± 03
24	00 ± 00

3.4 Ca^{++} ionophore stimulated leucocyte supernatant

The enhanced migratory responsiveness when the Ca^{++} ionophore-generated leucocyte supernatant was used as a chemoattractant is shown in Figure 2. In this case, macrophages were stimulated with a Ca^{++} ionophore as well as lice products. The supernatants generated were harvested and used as potential chemoattractants. However, they did not show any significant difference in responsiveness compared to the use of Ca^{++} ionophore alone (Figure 2).

The cells treated with copepodid crude protein extract showed a significantly lower responsiveness when LPS was used ($p<0005$) (Figure 3). There was no significant difference in migratory responsiveness between treatment and control groups.

Notably, the migratory responsiveness was significantly lower when only sea lice culture supernatant (SLSN) was used as a chemoattractant rather than the control tissue culture product (TCSN) ($p<0.01$). The macrophages in the presence of the sea lice culture product also showed significantly lower responsiveness compared to cells in the presence of copepodid crude protein product alone (Figure 4).

3.5 Migratory pattern of macrophages

After the macrophages migrated through the pores of the filter, the macrophages had a characteristic shape. The cells appeared elongated, with projecting cytoplasmic processes and greater plasticity. In most of the cells, the cellular body containing the nucleus preceded a highly elongated cytoplasmic tail. The migratory pattern of the untreated cells was found to differ from that of the treated cells. Untreated cells showed a highly extended cytoplasmic tail compared to treated cells, indicating that the macrophage receptors are blocked by the crude factors in the culture products.
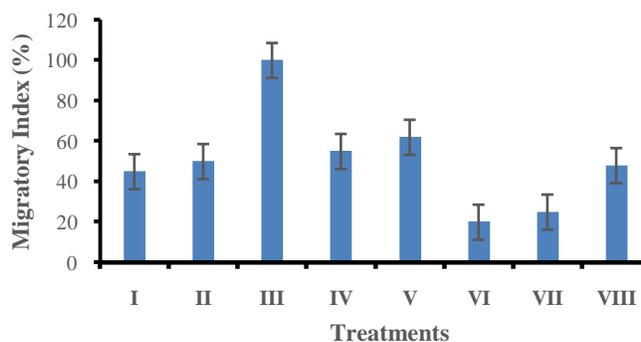

Figure 2 The effect of treatment (SLSN, seal lice culture supernatant) and control (TCSN, tissue culture supernatant) on leucocyte migration to serum attractant represented by the migratory index (%): I, treated MØ + SLSN/fresh serum; II, treated MØ + TCSN/fresh serum; III, MØ only/fresh serum; IV, treated MØ + SLSN/PBS; V, treated MØ + TCSN/PBS; VI, treated MØ + SLSN/inactivated serum; VII, treated MØ + TCSN/inactivated serum; VIII, MØ only/PBS. 2×10^6 cells/100 µl were used. Time of incubation for assay was 3 hrs at 18°C. Treated MØ were prepared by incubating with the appropriate supernatant for 2 hrs. Vertical bars represent the standard error of the migratory index.
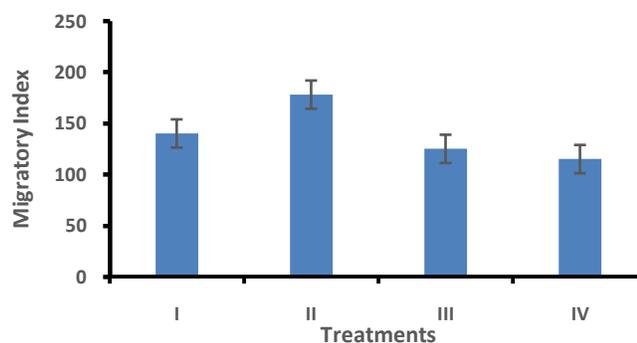

Figure 3 Macrophage migratory responsiveness to various Ca^{++} ionophore stimulated host chemoattractants (CIMS). I, attractant supernatants generated with SLSN + Ca^{++} ionophore; II, TCSN + Ca^{++} ionophore; III, HBSS + Ca^{++} ionophore; IV, copepodid crude extract + Ca^{++} ionophore. 2×10^6 cells/100 µl were used. Treated MØ were prepared by incubating with appropriate supernatant for 2 hrs. The incubation time for the assay was 3 h. Vertical bars represent the standard error of the migratory index.
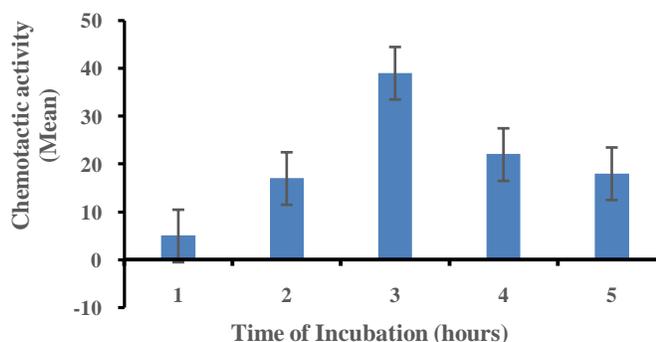

Figure 4 Migratory activity of leucocytes with the time of incubation. Data are represented as the mean ± standard error (SE).
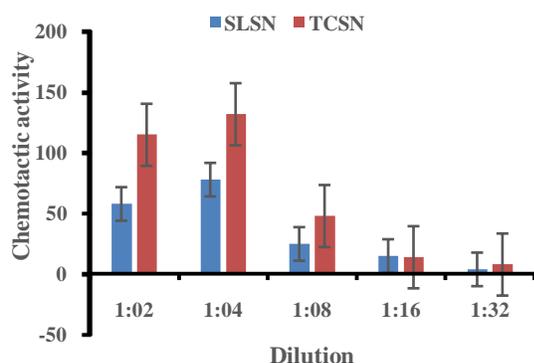
Figure 5 Migratory responsiveness of leucocytes (HK) to the test (blue bar) (SLSN, sea lice culture supernatant) and control (orange bar) (TCSN, tissue culture supernatant). The results represent the mean number of cells ± SE.
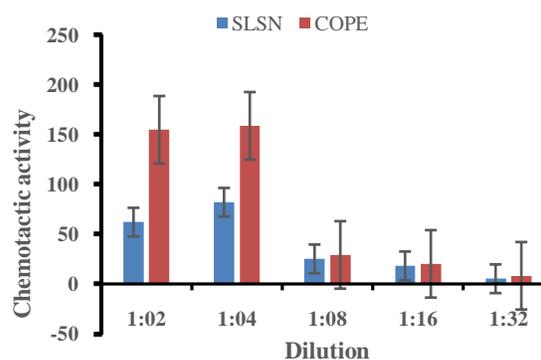
Figure 6 Migratory responsiveness of leucocytes (HK) in the assay. Blue bars represent sea lice supernatant-treated macrophage (SLSN) and orange bars denote copepodid crude extract (COPE). The results represent the mean number of cells ± SE.

The effect of sea lice secretory/excretory products or crude protein products on the macrophage (HK) migration suggests that the sea lice culture supernatant has chemotactic inhibitory characteristics, as demonstrated by the significantly lower migratory responsiveness towards the sea lice culture supernatant (p<0.01) (Figure 2). Of the different species of salmon experimentally infected with *L. salmonis*, the Atlantic salmon was found to be more susceptible to *L. salmonis* infection than the coho salmon (Johnson & Albright 1992a; Johnson & Albright 1992b). Hydrocortisol-implanted coho salmon also showed enhanced susceptibility to *L. salmonis* (Johnson & Albright 1992a; Johnson & Albright 1992b). These findings and the present results suggest that the chalimus secretes an immunosuppressive or anti-inflammatory chemotactic factor which seems to inhibit cell migration (Figure 5). In addition, the chemotactic inhibitory factor may be found in a soluble form in the secretory products of sea lice, which caused a significantly lower migratory responsiveness when macrophages were treated with sea lice culture supernatant alone compared to the control (TCSN) in the leucocyte migration assay (Figure 6).

Current study also assessed the migratory responsiveness of untreated and treated macrophages in Atlantic salmon serum, Ca^{++} ionophore stimulated supernatant, and LPS. An enhanced migratory response to the fresh serum and a significantly lower migratory response to the heated serum were found, suggesting that a chemotactic factor (C5a) in the serum can act as a chemoattractant in this experiment and could be denatured in the heated serum (Figure 4). The C5a-like complement chemotactic factor in the serum enhances vascular permeability and promotes cellular migration, aggregation, and enzyme release of neutrophils, in addition to having the capacity to activate leukocytes and induce leukocyte chemotaxis (Griffin, 1984). The

lower migratory response towards LPS exhibited by treated macrophages in response to the copepodid crude product suggests that inhibitory factors may be present in the copepodid (Figure 3).

Figure 5 shows that treated macrophages had a significantly lower migratory response to serum compared to that of untreated macrophages, possibly due to the irreversible blockage of cell receptors or by a direct suppressive affect. When the macrophages were treated with the sea lice supernatant, antigenic agents released into the supernatant were found on the macrophage receptors. In addition, cytokines such as IL-1 or similar compounds may be produced when the macrophages are treated with a sea lice secretory product, as suggested by Smith et al. (1980).

In conclusion, sea lice seem to have an immunosuppressive effect on the host immune system, triggering cell-mediated responses. Further studies also needed to identify the secretory/excretory products released by sea lice in the host as well as to identify the immune suppressive antigen found in the secretory excretory product. The findings presented here provide an insight into the mechanism of infection for the development of commercial antibiotics and vaccines against sea lice infection.

Acknowledgments

Conflict of interest

The corresponding author declares that there is no conflict of interest.

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