

THE EFFECT OF SEA STAR COELOMOCYTE EXTRACT ON CELL-MEDIATED RESISTANCE TO *LISTERIA MONOCYTOGENES* IN MICE*

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Resistance to infection with intracellular bacteria such as *Listeria monocytogenes* is cell-mediated and requires the participation of both lymphoid cells and mononuclear phagocytes (1). During the course of a nonlethal infection there arises a population of specifically sensitized lymphocytes that are capable of passively transferring immunity to normal mice (2). This passive transfer requires the accumulation of recipient macrophages at the sites of *Listeria* infection, for it is the macrophage which is the ultimate effector cell that rids the host of the bacteria (3). Once activated by the specifically sensitized lymphocytes in the presence of *Listeria* antigen, the macrophages act nonspecifically, i.e., they will protect against infection with an unrelated bacterial pathogen (3, 4). The mechanism of activation of macrophages by lymphocytes is not known, but may be through the release of one or more of the lymphokines, as described for in vitro correlates of delayed hypersensitivity (5, 6).

Prendergast and Suzuki (7) have described a protein having a mol wt of 32,000, isolated from the coelomocytes of the sea star (*Asterias forbesi*), that acts in a manner analogous to the mediators of delayed hypersensitivity. This sea star factor (SSF)¹ has the following characteristics: (a) it causes a skin reaction in nonsensitized guinea pigs which is identical both temporally and cytologically to a delayed hypersensitivity-type skin reaction; (b) it inhibits the migration of normal guinea pig macrophages from capillary tubes; (c) it is chemotactic for macrophages; and (d) macrophages treated either in vivo or in vitro with SSF are morphologically altered to an "activated" form (8, 9).

Because of these macrophage-activating properties, we examined the effect of SSF on mice infected with *Listeria* where activation of macrophages is important as a defense mechanism. We postulated that enhanced resistances of

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¹ Abbreviations used in this paper: C, complement; Con A, concanavalin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LCM, lymphocytic choriomeningitis; MEM, Eagle's minimal essential medium; NRS, normal rabbit serum; SSF, sea star factor.

mice to *Listeria* would result after nonspecifically activating their macrophages with SSF. Mice treated with SSF, however, become more susceptible to *Listeria* infection rather than more resistant. The results of these experiments suggest that, rather than activating macrophages, SSF inhibits the immune response to *Listeria* and does so at the level of the lymphocyte.

Materials and Methods

Animals.—Female pathogen-free outbred Swiss-Webster mice aged 4–6 wk were used (Flow Laboratories, Inc., Rockville, Md.)

Organisms.—*Listeria monocytogenes*, strain EGD, was obtained from Dr. George Mackaness of the Trudeau Institute, and was maintained by passage in ICR mice. Organisms for injection were obtained from an 18–24 h culture of infected spleen grown in trypticase soy broth (Difco Laboratories, Detroit, Mich.). Organisms were counted in a Petroff-Hausser counting chamber (Arthur H. Thomas Co., Philadelphia, Pa.) and diluted in Hanks' balanced salt solution (HBSS) for injection. Viable counts were confirmed by plating an aliquot of the culture on trypticase soy agar.

Sea Star Factor (SSF).—SSF was prepared as described previously (7). Briefly, coelomocytes collected from the sea star (*Asterias forbesi*) were lysed by three cycles of freeze-thawing, followed by brief ultrasonication. This product was centrifuged at 60,000 g for 90 min and the clear supernate removed and dialyzed against phosphate buffered saline pH 7.2 for 24 h, then centrifuged again at 60,000 g for 50 min, and the supernate recovered and sterilized by passage through a 0.45 micron Millipore filter (Millipore Corp., Bedford, Mass.). SSF was stored at -20°C until use.

Measurement of Immunity.—Immunity, active or passive, was measured by enumerating the number of viable *Listeria* in livers and spleens of mice at various times after infection. Initial implantation was determined by assaying the number of *Listeria* in livers and spleens harvested at 15–30 min after inoculation. Three–five mice per group were sacrificed at each time period and organs from individual mice homogenized in 5 ml HBSS using a Ten Broek grinder. Homogenates were diluted in HBSS and duplicate pour plates were made using trypticase soy agar. Plates were counted after 24-h incubation at 37°C .

Immunization.—Mice were immunized with a sublethal dose of *Listeria* (2×10^3 – 5×10^3) given intravenously. Splenic lymphocytes for transfer were harvested 8 days after inoculation with bacteria.

Preparation of Spleen Cell Suspensions.—Single cell suspensions were prepared by gently pressing spleens through stainless steel mesh screens into HBSS containing 1% fetal calf serum (FCS). Particulate material was allowed to sediment and the supernate removed and centrifuged at 300 g for 5 min. The pellet was resuspended in 5 volumes of 0.83% NH_4Cl and incubated at 37°C for 15 min. This procedure lysed the red blood cells without affecting the viability of the lymphocytes. The cells were then washed twice and resuspended in HBSS with 1% FCS.

Cells to be incubated with SSF were resuspended in 5% FCS in minimal essential medium (MEM) at a concentration of 5×10^7 cells/ml. SSF was then added at a concentration of 1–2 mg/ml and the cultures incubated at 37°C for 2 h. They were then washed twice and resuspended in HBSS with 1% FCS for injection. The standard inoculum was 1×10^8 cells given intravenously in a volume of 0.5 ml. Viability was tested by trypan blue exclusion and was consistently greater than 85%.

Rabbit Anti-SSF Serum.—New Zealand white rabbits were immunized with 2 ml SSF (1 mg/ml) emulsified in an equal volume of complete Freund's adjuvant. Three weekly injections were administered intramuscularly in both thighs and subcutaneously in the nape of the neck. Rabbits were bled 7–10 days after the last injection. Serum was inactivated by heating to

56°C for 30 min, and assayed for precipitating antibody by immunodiffusion. The serum showed a strong precipitin line at a 1:80 dilution when reacted with SSF at a concentration of 0.5 mg/ml.

RESULTS

Enhancement of Listeria Infection by Treatment with SSF.—Preliminary experiments indicated that SSF treatment did not protect mice against a lethal challenge with *Listeria* and, in fact, may have increased their susceptibility. To determine if this were indeed the case, mice were injected with 0.5 mg SSF intravenously 24 h before infection with a sublethal dose (6×10^3) of *Listeria*. As shown in Fig. 1, normal mice began to clear the infection at 48 h. SSF-treated mice, however, failed to clear the infection, and at 72 h there were 3 log units more organisms in the livers and 1.5 log units more in

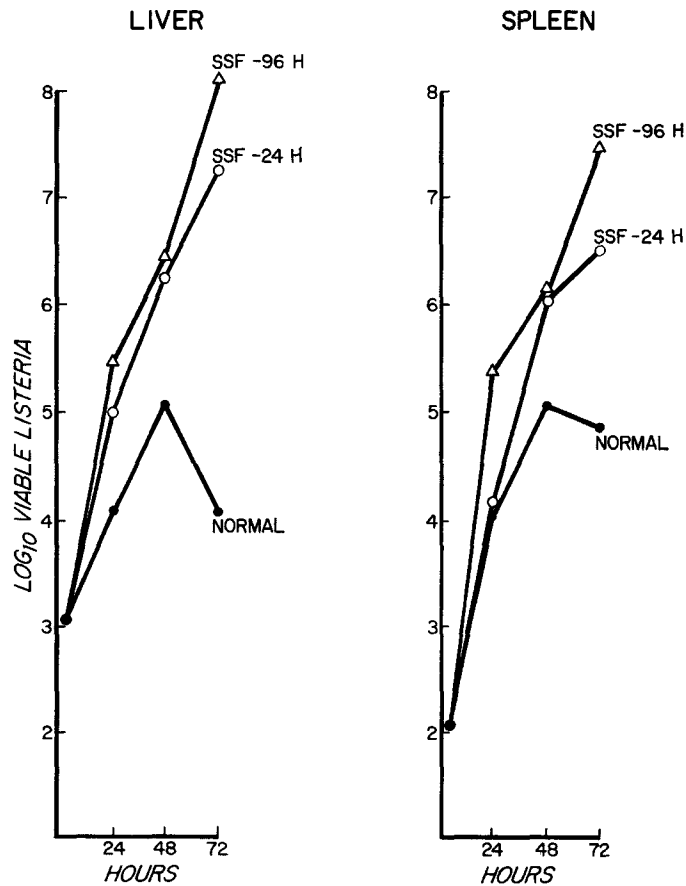


FIG. 1. Growth curves of a sublethal dose (6×10^3) of *Listeria* in livers and spleens of mice given 5 mg SSF 24 or 96 h before infection. Means of three mice per time point.

the spleens of treated mice than in normal mice. By 96 h, SSF-treated mice had succumbed to the infection. Fig. 1 also shows that this enhancing effect of SSF was seen even when it was given 4 days before infection.

To further investigate the relationship between time of SSF treatment and enhancing effect, mice were injected with 8×10^8 *Listeria*, and then given 0.5 mg SSF either 24 or 48 h after injection. Again, the growth of organisms in normal mice peaked at 48 h and then began to decline (Fig. 2). In SSF-treated mice the organisms continued to grow, and all were dead by 96 h. Thus, 0.5 mg SSF given to mice 96 h before to 48 h after infection with a sublethal dose of *Listeria* led to uncontrolled growth of the organisms and death.

Dose-Response Relationship.—Previous studies in this laboratory indicated that high concentrations of SSF would destroy cultured macrophages, either through a toxic effect or by activating the cells to the extent that they under-

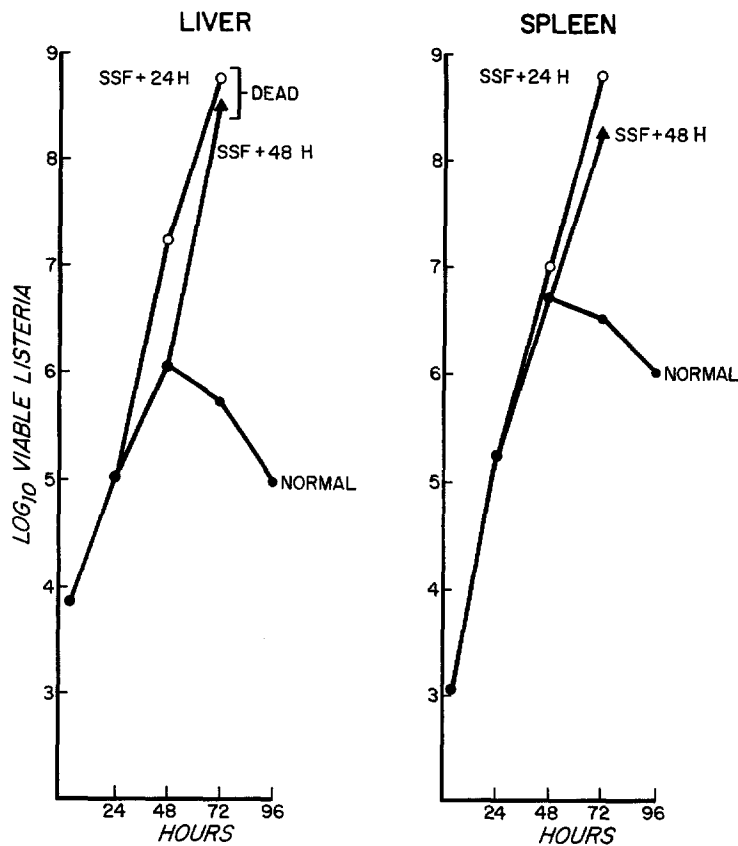


FIG. 2. Growth curves of a sublethal dose (8×10^8) of *Listeria* in livers and spleens of mice given 0.5 mg SSF 24 or 48 h after infection. All SSF-treated mice died after 72 h. Means of three mice.

went autolysis. We therefore examined the effect of various doses of SSF on resistance to infection.

Mice were given 2.0, 0.5, 0.25, 0.1, or 0.01 mg SSF intravenously 24 h before infection with a sublethal dose of *Listeria*. Livers and spleens were harvested and assayed for bacteria at 24, 48, and 72 h postinfection. The results shown in Fig. 3 demonstrate that doses of SSF of 0.1 mg or greater enhanced infection. Treated mice began dying between 48 and 72 h, and only one mouse in the groups receiving 0.25 and 0.1 mg SSF was alive for assay at 72 h. Mice treated with 0.01 mg SSF did not differ significantly from normal mice. All doses of SSF employed in this experiment were below that which had been shown to destroy macrophages *in vitro*. It is unlikely, therefore, that the enhancing effect of SSF on *Listeria* infection was through the destruction of macrophages.

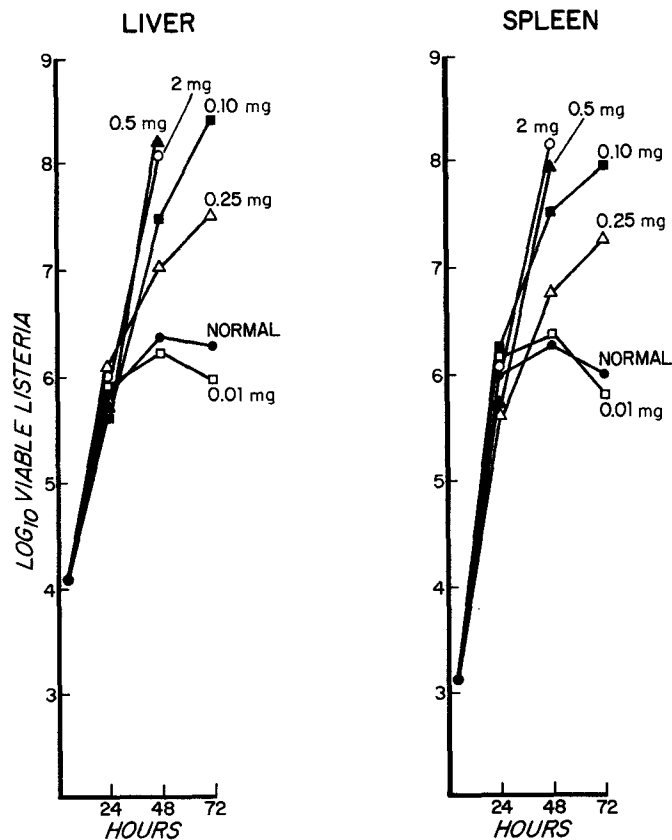


FIG. 3. Curves showing the effect of various doses of SSF on the growth of a sublethal dose of *Listeria*. SSF was given 24 h before infection. Mice receiving 0.5 or 2.0 mg SSF died after 48 h. Means of three mice.

Effect of SSF on Established Immunity.—In order to further examine the effect of SSF on macrophages, the following experiment was performed. Mice were immunized with a sublethal (5×10^8) dose of *Listeria*. 7 days later, one half the mice were given 0.5 mg SSF intravenously. On day 8, the SSF-treated and the untreated mice were challenged with a lethal dose of *Listeria* (2×10^5). The results are shown in Fig. 4. Immune mice given SSF before challenge were equally as efficient in clearing the infection as were immune mice not given SSF.

If the enhancing effect of SSF were due to destruction of macrophages, then its effect should be independent of the immune status of the mice; i.e., without macrophages, immune mice should be just as susceptible as non-immune mice. The present experiment demonstrates that this is not the case.

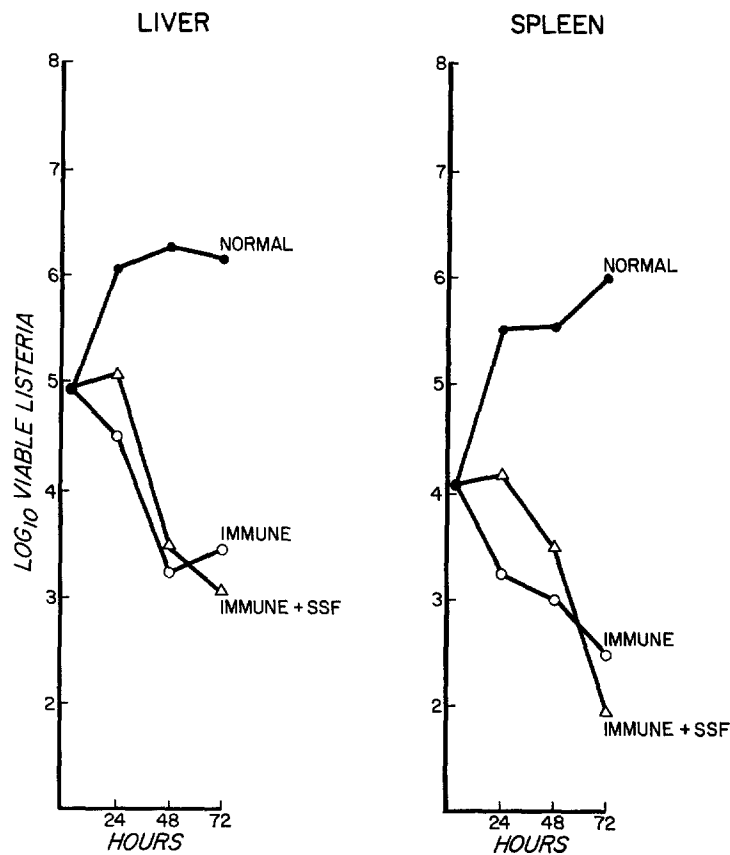


FIG. 4. Growth curves showing the inability of SSF to alter established immunity. Mice were immunized with a sublethal dose of *Listeria* on day 0. On day 7, one-half the immune mice were given 0.5 mg SSF intravenously. 24 h later, both treated (immune + SSF) and nontreated (immune) mice were challenged with a lethal dose of *Listeria*. Means of five mice.

Effect of SSF on the Afferent Limb of the Immune Response.—Whether SSF prevents immunization was next examined. Mice were given 0.5 mg SSF intravenously on day 0. 24 h later they were injected with a small dose (8×10^2) of *Listeria*. Another group of mice received only the 8×10^2 *Listeria*. On day 10, both groups were challenged with a lethal dose of organisms, as was a group of normal mice.

The results of this experiment are presented in Fig. 5. The organisms grew rapidly in normal mice as shown previously. Mice that had been immunized with 8×10^2 *Listeria* were immune to challenge, whereas those that had re-

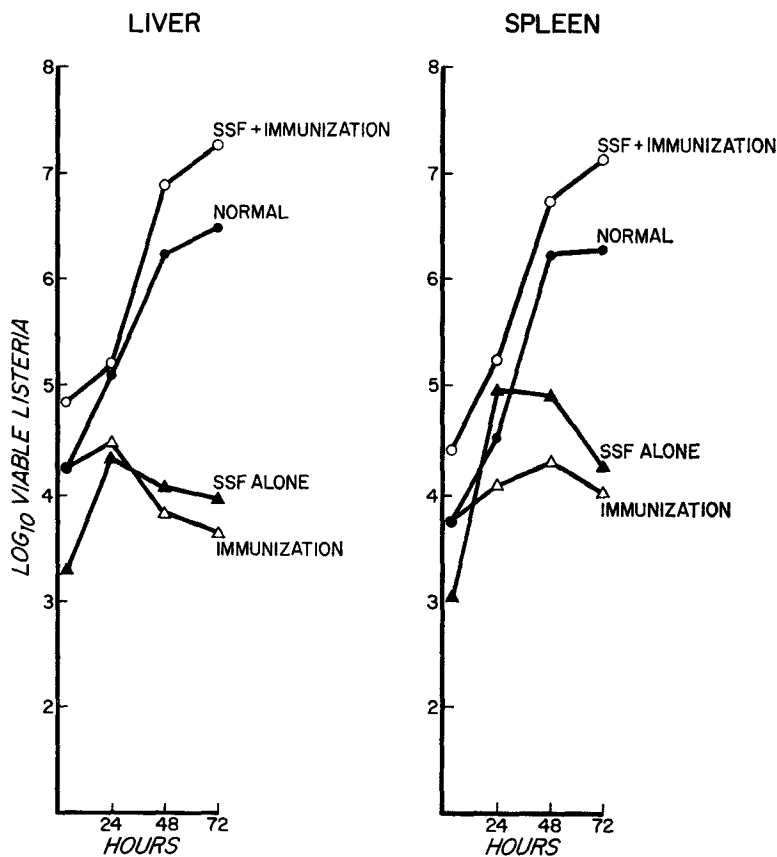


FIG. 5. Growth curves describing the inhibitory effect of SSF on immunization of mice with *Listeria*. Mice were given 0.5 mg SSF 24 h before an immunizing infection with 8×10^2 *Listeria* (SSF + immunization). Another group of mice received only the immunizing infection (immunization). 10 days later, both groups were challenged with a lethal dose of *Listeria* as was a group of normal mice. As a control for the persistence of SSF in quantities sufficient to enhance infection, another group of mice was given 0.5 mg SSF (SSF alone) and then challenged 10 days later with a sublethal dose of *Listeria*. Means of five mice.

ceived SSF 24 h before the immunizing injection failed to develop immunity, as evidenced by the rapid growth of the organisms in these animals. Another group of mice that had received SSF alone on day 0 and challenged with a sublethal dose of organisms on day 10 behaved like untreated animals receiving a sublethal dose, indicating that SSF did not persist, for the 10 days of the experiment, in quantities sufficient to enhance infection.

It should be noted here that because of the enhancing effect of SSF, 20% of the SSF-treated mice that received the low immunizing dose of *Listeria* developed a lethal infection. The other 80% survived and managed to clear most of the infection by day 10. Clearance in these mice, however, was not complete, as evidenced by the higher initial implantation values determined 30 min after the challenge infection. It is unlikely, however, that this higher initial implantation value could in itself account for the extreme difference in bacterial growth between immunized mice and mice immunized in the presence of SSF.

Effect of SSF on the Adoptive Transfer of Immunity.—The previous experiment demonstrated that SSF inhibits immunization. In order to examine whether SSF modifies the action of effector lymphocytes, its action on the adoptive transfer of immunity was studied.

Spleen cells from 8-day *Listeria* immune mice were prepared as described in *Materials and Methods*. Cells were suspended in MEM containing 5% FCS at a concentration of 5×10^7 cells/ml. 1 mg/ml SSF was added and the cell suspension incubated at 37°C for 2 h. The cells were then washed twice, counted, and adjusted to a concentration of 2×10^8 cells/ml. Viability after incubation was always greater than 85%. Normal mice were given 1×10^8 immune or SSF-treated immune cells intravenously in a volume of 0.5 ml. 1 h later both groups, as well as a control group of mice receiving no cells, were given a lethal dose of *Listeria* (5×10^4). In earlier adoptive transfer experiments, control mice received normal spleen cells which regularly failed to transfer any immunity. For the sake of conserving mice, all future experiments employed normal mice without nonimmune cells as controls.

The results shown in Fig. 6 indicate that a considerable degree of immunity was conferred by both immune cells and SSF-treated immune cells. Thus it appears that once the lymphocytes have been sensitized, SSF is not able to shut off the expression of the immunity.

Inhibition of Adoptive Transfer by SSF-treated Immune Cells with Anti-SSF and Complement (C).—*Listeria* immune spleen cells were incubated at a concentration of 5×10^7 cells/ml in the presence of 1 mg/ml SSF for 1 h at 37°C. The cells were then washed twice with HBSS, adjusted to a concentration of 1×10^8 cells/ml divided into 1-ml aliquots, and centrifuged. They were then suspended in 1 ml of either anti-SSF and C or normal rabbit serum (NRS) and C. The final concentration of both serum and C was 1:10. After 1 h incubation at 37°C, the cells were washed twice in HBSS, tested for viability with trypan blue, and

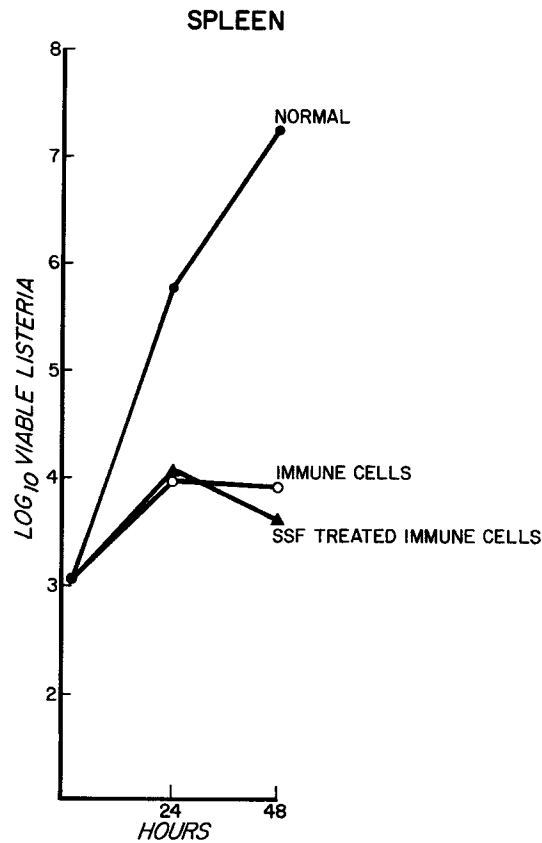


FIG. 6. Curves showing the effect of treatment of immune lymphocytes with SSF on adoptive transfer of immunity. Spleen cells from 8-day immune mice were incubated with 1 mg/ml SSF for 2 h at 37°C. They were washed and then transferred into normal recipients at a concentration of 1×10^8 cells/mouse. Recipients of immune cells or SSF-treated immune cells were challenged with a lethal dose of *Listeria* 1 h after cell transfer. The normal controls did not receive spleen cells. Means of five mice.

injected into normal recipients at a concentration of 1×10^8 cells/mouse. Nontreated immune cells were injected into another group of recipients, and all mice were challenged with 3×10^4 *Listeria* 1 h after cell injection.

The results, presented in Fig. 7, show that immune cells and SSF-treated immune cells treated with NRS and C both conferred a high degree of immunity on normal recipients. SSF-treated immune cells that had been incubated with anti-SSF and C lost their ability to transfer immunity, and growth of the organisms in recipients of these cells was similar to that in control animals which received no cells. Incubation of cells with NRS and C reduced the total viable cell number by about 10%. The same decrease was seen in nontreated cells after incubation. Incubation with anti-SSF and C, however, decreased the viable cell count by 35–40%.

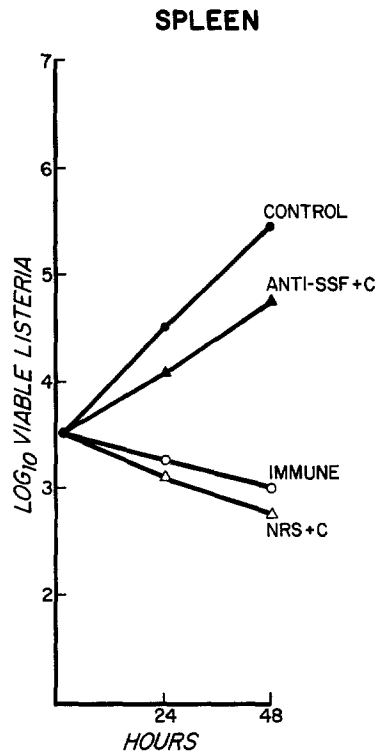


FIG. 7. Growth curves showing the inhibition of adoptive transfer by SSF-treated immune cells with anti-SSF and C. Spleen cells from 8-day immune mice were incubated with 1 mg/ml SSF for 1 h at 37°C, washed, and then incubated with either anti-SSF and C or normal rabbit serum and C for 1 h at 37°C. 1×10^8 viable cells were transferred to normal recipients. 1 h later, mice were challenged with a lethal dose of *Listeria*. IMMUNE indicates nontreated immune cells. Controls received no cells. Means of five mice.

DISCUSSION

Immunity to *Listeria* in mice has been shown to be ultimately a function of macrophages (1) which have become activated by a process that depends on specific interaction between immune lymphoid cells and the infecting organism (2, 10). However, host resistance may also be modified and strikingly increased by nonspecific means (11-14). The mechanism of macrophage activation, either specific or nonspecific, and the functional and biochemical changes accompanying activation remain largely unknown. One possible method of activation is through the release by lymphocytes of humoral substances, similar or identical to the lymphokines described for in vitro models of delayed hypersensitivity.

SSF is a protein isolated from the coelomocytes of *A. forbesi* which has macrophage-activating properties as well as other lymphokine-like activities (6, 7). We employed SSF as a macrophage-activating substance in an attempt

to increase the resistance of mice to listeriosis. Immunity to *Listeria* infection was severely depressed by treatment of mice with SSF. Injection with as little as 0.1 mg SSF produced uncontrolled growth of an otherwise sublethal dose of *Listeria* and resulted in death. This enhancing effect could be demonstrated when SSF was given from 96 h before to 48 h after infection.

Previous observations in this laboratory² indicated that treatment of macrophages in vitro with high concentrations of SSF resulted in destruction of the cells. However, the effects of SSF described here were probably not due to the destruction of macrophages for several reasons. (a) The doses of SSF used in vivo were well below the concentration shown to cause destruction in vitro. The numbers of bacteria initially taken up by the livers and spleens of both normal and SSF-treated mice were identical, indicating that the phagocytic function of macrophages in SSF-treated mice was not impaired. (c) If the enhancing effect of SSF were due to the destruction of macrophages, then this effect should be independent of the immune status of the mice, since both normal and immune mice depend on functional, activated macrophages for their resistance. That this clearly was not the case is shown in Fig. 4, where treatment of immune mice with SSF before challenge with a lethal dose of *Listeria* did not alter their immune status.

Treatment of immune lymphocytes in vitro with SSF did not destroy their ability to adoptively transfer immunity to normal recipients (Fig. 6). However, if SSF was given to mice before infection with a small immunizing dose of *Listeria*, they failed to develop immunity, as evidenced by their susceptibility to a subsequent challenge. These data suggest that SSF acts as a suppressor of lymphocyte function, possibly by preventing replication and subsequent sensitization, but has no effect on committed, or effector, lymphocytes.

The above suggestion draws support from recent work demonstrating that the proliferative response of human peripheral blood lymphocytes stimulated by phytohemagglutinin and concanavalin A (Con A) was inhibited by microgram quantities of SSF added to the reaction mixture. In addition, it was shown that the effector function of splenic lymphocytes was not altered by treatment with SSF (15). The effector function measured was the ability of splenic lymphocytes from lymphocytic choriomeningitis (LCM) virus-immune mice to lyse target L cells infected with LCM virus. Incubation of immune lymphocytes with SSF did not inhibit their ability to lyse virus-infected cells.

A number of investigators (16–20) have demonstrated the thymus (T-cell) dependency of resistance to *Listeria* infection. Treatment of immune lymphocytes with anti- θ serum and C has been shown to destroy the ability of these cells to adoptively transfer immunity to normal recipients (16). In the present study, treatment of immune lymphocytes with SSF did not alter their ability

² Prendergast, R. A., unpublished observation.

to transfer immunity. However, if immune lymphocytes were treated with SSF and then incubated with anti-SSF and C, the ability to transfer immunity was lost. We interpret this observation to mean that SSF binds to T lymphocytes. We have no direct evidence of the T-cell specificity of binding in this experiment, and SSF may also bind to B lymphocytes. However, the viability studies indicated that, at most, 40% of the SSF-treated cells were destroyed by treatment with anti-SSF and C. This is approximately the percent of T cells one would expect to find in a spleen cell population. If SSF binds equally as well to B cells as to T cells, then a greater proportion of the total cell population should have been destroyed.

The T-cell specificity of SSF, however, is demonstrated in another system described by Prendergast et al. (15). Destruction of DBA/2 mastocytoma cells by spleen cells from C57Bl/6 mice immunized with the mastocytoma is a T-cell-dependent response (21, 22). SSF injected into C57Bl/6 mice at the time they received mastocytoma cells prevented the development of lymphocytes cytotoxic for these cells. If SSF-treated mice were immunized with pneumococcal polysaccharide, they developed a normal antibody response to this T-cell-independent antigen.

An apparent paradox exists with regard to the effect of SSF on macrophages. In vitro, SSF is a potent macrophage activator, while in vivo it appears to have little activity as evidenced by its inability to enhance protection against *Listeria*. One explanation could be that the concentration of SSF that contacts macrophages in vivo is not sufficient to cause activation. More important, however, is the effect of SSF on lymphocytes. SSF apparently binds to T lymphocytes, prevents their replication upon contact with *Listeria* antigens, and thus abrogates the generation of a population of sensitized lymphocytes that ultimately activate macrophages. This inhibition of the T-cell response apparently outweighs any primary macrophage-activating effect that SSF may have in vivo.

Studies are now in progress to more clearly define the T-cell specificity of SSF and to examine other systems where SSF may be used to inhibit T-lymphocyte-dependent immune responses, e.g., graft-vs.-host reactions and antibody production to T-cell-dependent antigens.

SUMMARY

Mice treated with sea star factor (SSF), a protein extracted from sea star coelomocytes, became highly susceptible to infection with a normally sublethal dose of *Listeria monocytogenes*. This was in contrast to the expected result of increased resistance originally postulated because of the macrophage-activating properties of SSF. Enhanced susceptibility was seen when SSF was given from 96 h before to 48 h after infection with *Listeria*. Mice pretreated with SSF failed to develop immunity to *Listeria* when given a dose of organisms capable of immunizing nontreated mice. Treatment of immune mice with

SSF did not alter their immune status. In addition, incubation of immune lymphocytes with SSF *in vitro* did not alter their ability to adoptively transfer immunity to normal recipients. Immune lymphocytes treated with SSF and then incubated with anti-SSF and C did, however, lose the ability to transfer immunity. These results suggest that SSF enhances infection by binding to T lymphocytes, inhibiting their replication upon contact with *Listeria* antigen and thus preventing the generation of a population of sensitized lymphocytes capable of effecting anti-*Listeria* immunity.

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