Activation of Macrophages by Products of Lymphocytes from Normal and Syphilitic Rabbits

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Received 18 December 1981/Accepted 15 March 1982

The production of soluble macrophage-activating factors by lymphocytes from syphilitic and normal rabbits was examined. Culture supernatants of splenic lymphocytes cultured with Treponema pallidum antigens or concanavalin A were incubated with rabbit peritoneal macrophages in vitro. The macrophage monolayers were then washed and infected with log-phase Listeria monocytogenes. Activation of the macrophages by lymphocyte products was measured by the ability of the macrophages to resist intracellular multiplication of Listeria and thus survive infection. Macrophages incubated with supernatants of unstimulated lymphocytes or T. pallidum-stimulated lymphocytes from normal rabbits were unable to resist intracellular multiplication of Listeria. Specifically stimulated lymphocytes from syphilitic rabbits and mitogen-stimulated lymphocytes from both normal and syphilitic rabbits demonstrated a clear ability to produce soluble factors which conferred upon macrophages the ability to limit the intracellular growth of the bacteria. Antigen or mitogen alone was unable to activate the macrophages; the presence of lymphocyte products was required.

Primary infection with Treponema pallidum results in the appearance of a firm, indurated, crusted or ulcerative lesion (chancre) in humans and in intradermally infected rabbits. Rabbits infected intrathecally develop a firm orchitis (30). The bacteria, which multiply at the site of infection, are abundant in early lesions in the rabbit but are virtually cleared from the local site within several days after peak mononuclear cell infiltration (15). The lesions then heal without antibiotic therapy. Although the infiltrating cells, which have been identified as T lymphocytes and macrophages (15), are implicated in the destruction and clearance of the treponemes, the exact mechanism is not known. It has been proposed by several investigators (2, 10, 15-18) that macrophages, particularly activated macrophages, may be the ultimate effector cells in the clearance of treponemes from early lesions.

The central role of macrophages in immunity to infectious diseases such as listeriosis (13, 19), toxoplasmosis (12), and tuberculosis (23) is well described. In these infections, destruction of the infectious organism is mediated by macrophages that have been activated by secreted products of specifically sensitized T lymphocytes. The initial sensitization of lymphocytes and the subsequent release of macrophage-activating factors (MAF) are triggered by a specific antigen. Once macrophage activation is accomplished, phagocytosis and destruction of antigenically unrelated organisms can occur (12); the effector mechanism is nonspecific.

Macrophage activation has been assessed by a variety of physical, biochemical, and functional parameters (4, 5, 21), including increased spreading capacity and membrane ruffling, increased production of superoxide anion, ATP, secretory enzymes, and lysosomal constituents, and increased phagocytic, microbicidal and tumoricidal activity. With the exception of microbicidal and tumoricidal capacity, stimulant-induced (inflammatory) macrophages share the changes in the physical and biochemical parameters described above. Only macrophages which have been activated by a lymphokine-mediated event acquire a cytotoxic capacity.

Macrophages may be activated by lymphokines in vitro, with resultant microbicidal activity. This has been described for many experimental systems (4, 5, 12, 23). In preparation for an examination of the interaction of activated macrophages with T. pallidum, the production of MAF by antigen- and mitogen-stimulated lymphocytes from syphilitic and normal rabbits was examined. This report presents evidence that treponemal antigens will induce the production of soluble MAF by specifically sensitized lymphocytes. Concanavalin A (ConA) induces MAF production by lymphocytes from both normal and syphilitic rabbits.

MATERIALS AND METHODS

Rabbits. Adult male New Zealand white rabbits were examined upon receipt for clinical and serological evidence of Treponema paraluis-cuniculi infection.

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The animals were housed individually and given antibiotic-free food and water ad libitum; rabbits used as sources of treponemes were maintained at 18 to 20°C.

**Serological tests.** Before use, all rabbits were bled and tested by Venereal Disease Research Laboratory and fluorescent treponemal antibody absorption tests (31). Known negative and positive rabbit sera were employed as controls. Venereal Disease Research Laboratory reagents were obtained from Scientific Products Div., McGraw Park, Ill. The fluorescent treponemal antibody absorption test was modified for use with rabbit serum. Antigen and sorbent were obtained from Palomar Chemicals, Carlsbad, Calif. Fluorescein-labeled goat anti-rabbit immunoglobulin G (Cappel Laboratories, West Chester, Pa.) was used at a final dilution of 1:1,600.

*T. pallidum.* *T. pallidum,* Nichols strain (generously provided by James N. Miller), was maintained by rabbit testicular passage as described previously (17). For infection of experimental animals, infected testes were sliced longitudinally and extracted by rotation in 10% Venereal Disease Research Laboratory nonreactive rabbit serum in 0.14 M saline. The extraction medium, containing treponemes, was centrifuged at 280 × g for 10 min to pellet gross cellular debris; the motile organisms in the supernatant were counted by dark-field microscopy and adjusted to approximately 3 × 10⁷ *T. pallidum* cells per ml. Serologically nonreactive rabbits were infected intratesticularly with 1.0 ml of treponemal suspension per testis.

**Cell cultures.** The production of MAF by lymphocytes was determined by the protocol outlined in Fig. 1. Rabbits which had been infected with *T. pallidum* for 5 to 8 weeks were used as sources of lymphocytes for the production of culture supernatants containing putative MAF. Animals were sacrificed by injection of a lethal dose of T-61 euthanasia solution (Taylor Pharmaceutical Co., Decatur, Ill.). Spleens were removed aseptically and placed in cold, sterile balanced salt solution (BSS). Spleen cell suspensions were prepared for culture by gentle teasing and washing as previously described (17). The concentration of viable lymphocytes, determined by hemacytometer counts of cell suspensions in trypan blue, was adjusted to 10⁶ lymphocytes per ml in medium 199 (Flow Laboratories, Inglewood, Calif.) supplemented with 10% heated (56°C for 30 min) nonreactive rabbit serum. Cells were cultured (2 × 10⁶ lymphocytes per dish) in plastic tissue culture dishes (35 by 10 mm; Falcon Plastics, Oxnard, Calif.) with and without *T. pallidum* antigen or ConA (Miles Laboratories, Inc., Elkhart, Ind.) at 37°C for 4 days in an atmosphere of 5% CO₂ in air. Culture supernatants were obtained after the removal of cells by centrifugation and filtration through a 0.22-µm membrane filter. Control supernatants were obtained from cultures of splenic lymphocytes from serologically nonreactive, uninfected animals.

The proliferation of lymphocytes in culture was measured by determining the incorporation of [¹²⁵I]-iododeoxyuridine (17). Replicate lymphocyte cultures, prepared as described above, were pulsed with 0.1 µCi of [¹²⁵I]iododeoxyuridine 24 h before harvest. Cells

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**FIG. 1.** Experimental protocol. Abbreviations: M199, medium 199; NRS, nonreactive rabbit serum; PBS, phosphate-buffered saline; MΦ, Macrophage.
were harvested on filter papers with a multiple automatic sample harvester; the radioactivity incorporated by each culture was quantitated on a Packard gamma counter.

Peritoneal macrophages were obtained from proteose-peptone-injected normal rabbits and placed in culture dishes containing cover slips as previously described (18). After incubation at 37°C for 2 to 3 h and removal of nonadherent cells, the culture medium was replaced with fresh medium or lymphocyte culture supernatants; incubation was continued at 37°C for 36 h.

The macrophage monolayers were then washed three times in warm BSS and infected for 1 h at 37°C with 2 × 10⁶ to 5 × 10⁷ log-phase Listeria monocytogenes cells in medium 199–10% nonreactive rabbit serum. Extracellular bacteria were then removed from the culture dishes by vigorous extensive washing with warm BSS; fresh culture medium was added, and incubation was continued at 37°C.

At 0, 4, 12, and 24 h after infection of the macrophages with Listeria, the monolayers were washed twice with BSS, fixed with methanol, and stained with Wright-Giemsa stain. The cover slips were then inverted onto microscope slides. The resistance of the macrophages to the Listeria challenge was determined by counting the number of macrophages remaining in 10 random high-power microscope fields (12); counting was performed without knowledge of the experimental condition represented by each slide.

Antigens and mitogens. T. pallidum antigen was prepared by sonication of whole washed T. pallidum, Nichols strain, as described previously (17). The antigen concentration used in the lymphocyte cultures (equivalent to 4 × 10⁷ T. pallidum cells per ml) was determined by titration to be optimal. ConA was used at a concentration of 4 μg/ml.

L. monocytogenes. L. monocytogenes A1581 was generously provided by Patricia Totten, University of Washington, Seattle. The organisms were stored at −70°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 25% horse serum. For infection of macrophage monolayers, the bacteria were grown in tryptic soy broth for 18 h at 37°C, washed three times with BSS, and suspended in medium 199–10% nonreactive rabbit serum. For quantitation of the inocula, dilution plates were prepared on tryptic soy agar (Difco Laboratories).

Statistical analysis. The mean percentages of macrophages remaining at various times for each experimental condition were compared by Student’s t test. In all cases, differences between compared groups were considered to be significant when P < 0.05.

RESULTS

Effect of infection with L. monocytogenes on in vitro survival of rabbit peritoneal macrophages. Proteose-peptone-induced rabbit peritoneal macrophages were harvested and placed in culture as described above. At 0, 4, 12, and 24 h after infection with L. monocytogenes, monolayers were washed, fixed, and stained; the number of macrophages remaining in 10 random high-power microscope fields was determined. Replicate monolayers which were not infected with Listeria were also counted. The mean percentages of macrophages remaining at each time point (compared with 0 h) from six separate experiments are shown in Fig. 2. Macrophages which are not infected with Listeria survive well in vitro: more than 70% remain in the monolayer for 24 h. On the other hand, infection with Listeria results in a significant decrease in macrophage survival as early as 4 h after infection. At 12 and 24 h, only 20 and 4% of the macrophages survived in the monolayers, respectively. Listeria could be observed by microscopic examination within surviving macrophages. Thus, L. monocytogenes can infect rabbit peritoneal macrophages and significantly decrease macrophage survival in vitro.

Effect of lymphocyte culture supernatants on activation of macrophages. Splenic lymphocytes from normal and syphilitic rabbits were placed in culture with and without T. pallidum antigen or ConA. Supernatants from these cultures were incubated with peritoneal macrophage monolayers, and the monolayers were then infected with Listeria. The effects of supernatants from normal lymphocytes are shown in Fig. 3. Although supernatants from unstimulated and T. pallidum-exposed normal lymphocytes afforded the macrophages no protection against infection with Listeria (21 and 18% survival at 12 h, respectively), supernatants from ConA-stimulated lymphocytes caused activation of the macrophages and consequent protection (83% survival).

As shown in Fig. 4, supernatants collected from lymphocytes from syphilitic rabbits were capable of activating macrophages after in vitro stimulation with ConA and thus were able to confer protection against Listeria infections (90% survival with stimulated lymphocyte supernatants versus 36% survival with unstimulat-

FIG. 2. Effect of infection with L. monocytogenes on in vitro survival of rabbit peritoneal macrophages. Each value represents the mean ± standard error of six separate experiments.
ed lymphocyte supernatants \( P < 0.001 \)). It is important to note that cultures of immune lymphocytes which were stimulated with \textit{T. pallidum} antigens also contained MAF: 75% of the macrophages remained at 12 h \( P < 0.05 \).

Macrophages which were incubated in culture medium containing \textit{T. pallidum} antigens or ConA alone (without lymphocytes) did not show increased resistance to the \textit{Listeria} challenge (8 and 10% survival, respectively, compared with 9% survival without antigen or mitogen). Culture medium from purified protein derivative-exposed syphilitic lymphocytes (unrelated antigen) did not contain MAF (data not shown).

Proliferative responses of splenic lymphocytes used for production of activating factors. The incorporation of \(^{125}\text{I}\)iododeoxyuridine by proliferating spleen cells was measured in cultures of normal and immune lymphocytes in the presence and absence of treponemal antigens and ConA. The mean proliferative responses of lymphocytes which were used for production of MAF are shown in Table 1. As anticipated, lymphocytes from normal rabbits responded well to the mitogen ConA but showed no response to treponemal antigens. On the other hand, lymphocytes from syphilitic rabbits showed significant proliferation in response to both \textit{T. pallidum} antigens and ConA.

**DISCUSSION**

A role for activated macrophages in resistance to early syphilitic infection has been proposed by several investigators (2, 15–18, 28). The infiltration of early lesions by T lymphocytes and macrophages and the resultant clearance of treponemes from those sites strongly suggest a local T cell-macrophage-\textit{T. pallidum} interaction (15). The cellular infiltration of syphilitic lesions is very similar to that seen in mycobacterial infections (tuberculosis and leprosy), in which activated macrophages have been demonstrated.

**TABLE 1. Responses of splenic lymphocytes to \textit{T. pallidum} antigens and ConA**

<table>
<thead>
<tr>
<th>Source of spleen cells</th>
<th>Lymphocyte proliferative responsea</th>
<th>T. pallidum antigens</th>
<th>ConA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.67 ± 0.046</td>
<td>2.65 ± 0.153</td>
<td>5.29 ± 0.066</td>
</tr>
<tr>
<td>Syphilitic</td>
<td>2.77 ± 0.065</td>
<td>3.72 ± 0.318</td>
<td>5.17 ± 0.107</td>
</tr>
</tbody>
</table>

\(^a\) Mean values ± standard error of log counts per minute of \(^{125}\text{I}\)iododeoxyuridine incorporated by \(2 \times 10^6\) lymphocytes; each mean represents data from three to four separate experiments.

\(^b\) Response to \textit{T. pallidum} stimulation of normal versus syphilitic spleen cells \( P < 0.05 \).

\(^c\) Response of \textit{T. pallidum}-stimulated syphilitic spleen cells versus unstimulated syphilitic spleen cells \( P < 0.05 \).
to be important effector cells. This report provides direct evidence that lymphocytes from syphilitic rabbits produce soluble products (MAF) which can activate macrophages in vitro. The production of these factors by T. pallidum-stimulated T lymphocytes required prior sensitization of the lymphoid cells; lymphocytes from nonsyphilitic rabbits did not produce MAF in the presence of treponemal antigens. ConA, on the other hand, stimulated the production of MAF by lymphocytes from both normal and syphilitic rabbits.

Earlier evidence for the existence of activated macrophages during syphilitic infection has been indirect. Schell and Musher (25) and Schell et al. (26) demonstrated that syphilitic rabbits show increased resistance to infection with L. monocytogenes; rechallenge of the infected animals with virulent T. pallidum can prolong the period of macrophage activation. Although the activated macrophages are capable of resisting infection by an antigenically unrelated intracellular pathogen such as L. monocytogenes, their ability to destroy virulent T. pallidum has been examined only indirectly. Histological studies have revealed the presence of treponemal fragments within macrophages in healing lesions (2, 16). Electron microscopic examinations of tissue from a primary human chancre (1, 29) and from experimental rabbit orbititis (14) have resulted in the identification of treponemes within macrophages and other nonphagocytic cells. Treponemes in various stages of degradation were seen, however, only within known phagocytic cells. Ovcinnikov and Delektorskij (22) conducted a careful study of treponeme-phagocyte interactions in early and resolving rabbit dermal lesions: treponemes were observed within macrophages in typical phagocytic vacuoles in association with lysosomal structures. Similarly, Sell et al. demonstrated the presence of T. pallidum in phagocytic vacuoles in macrophages in tissue from resolving testicular infection in rabbits; evidence for bacterial destruction within vacuoles was also provided (27a). Despite the fact that treponemes in various stages of degradation may be seen within phagocytes, it is not yet clear whether these organisms were actually killed by the macrophages or whether the phagocytes function simply as scavenger cells which ingest organisms that have been killed by another mechanism.

Attempts to protect rabbits against treponemal challenge by systemic or local activation of macrophages have yielded conflicting results. Several investigators have failed to demonstrate protection against treponemal infection after systemic activation of macrophages with Mycobacterium bovis BCG (8, 9, 27) or Propionibacterium acnes (3). Harris et al. (11), on the other hand, stated in an early study that the nonspecific in vivo activation of macrophages in rabbits by lecithin or trypan blue causes the early resolution of progressing syphilitic testicular lesions. Similarly, Hardy et al. (10) described rapid enlargement and early onset of healing of primary lesions after inoculation of mixed BCG and T. pallidum in BCG-sensitized rabbits. This healing corresponded temporally with the peak infiltration of macrophages to the site, and treponemes were identified by silver stain in phagocytic vacuoles of macrophages in those healing lesions. Harris and Thoen also demonstrated that the intradermal injection of BCG close to a site of T. pallidum challenge inhibits the development of treponemal lesions in BCG-sensitized rabbits, suggesting the early destruction of the treponemes by cells infiltrating the site in response to BCG antigens (D. L. Harris and C. O. Thoen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E32, p. 86). In contrast, the intradermal injection of heat-killed P. acnes mixed with the treponemal challenge organisms failed to alter the course of treponemal infection in P. acnes-sensitized recipients (3).

A number of investigators have examined the ability of lymphocytes from syphilitic humans or rabbits to produce macrophage or leukocyte migration-inhibitory factors. The results are somewhat contradictory. Although two studies (20, 24) clearly demonstrated migration-inhibitory factor production by lymphocytes from syphilitic rabbits, other investigators were unable to demonstrate antigen-induced inhibition of leukocyte migration in patients with various stages of syphilis (6). Two additional studies revealed enhanced macrophage migration in early human (7) or rabbit (32) syphilis, with migration inhibition appearing only later in the course of infection.

Clearly, the role of macrophages in resistance to syphilitic infection remains unresolved. Although the in vitro phagocytosis of T. pallidum by proteose-peptone-induced rabbit peritoneal macrophages has been described by Lukehart and Miller (18), the direct interactions of lymphokine-activated macrophages with virulent T. pallidum have not been examined. The demonstration that rabbit macrophages can be activated in vitro by products of T. pallidum-sensitized lymphocytes provides a useful experimental system for the investigation of these interactions. Such an examination may provide a key to the further definition of host-parasite relationships in syphilitic infection.

ACKNOWLEDGMENTS

I thank Sharon A. Baker-Zander and King K. Holmes for helpful comments, Cathy Critchlow for statistical analyses, and Carol Taylor for manuscript preparation.

S.A.L. is the recipient of a Venereal Diseases Research Fund Postdoctoral Fellowship from the American Social
Health Association. This work was supported by Public Health Service grant AI 12192 from the National Institutes of Health.

LITERATURE CITED


