In Vitro Induction of Nonspecific Resistance in Macrophages by Specifically Sensitized Lymphocytes

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Experiments were carried out to determine whether macrophages can be activated in vitro to resist challenge with heterologous microorganisms. Sensitized spleen cells from guinea pigs chronically infected with Toxoplasma gondii were cultured with normal guinea pig peritoneal macrophages in the presence and absence of Toxoplasma antigen. Macrophage monolayers incubated with sensitized spleen cells and antigen were markedly resistant to challenge from Listeria monocytogenes. Resistance was manifested by prolonged survival of the monolayers and rapid intracellular killing of the bacteria. Macrophages incubated with sensitized spleen cells but in the absence of antigen, as well as macrophages cultured with normal spleen cells, in the presence or absence of antigen, were rapidly destroyed. Sensitized spleen cells responded to the presence of Toxoplasma antigen by increased uptake of tritium-labeled thymidine. Supernatant fluid medium obtained from cultures of macrophages, sensitized spleen cells, and antigen contained a macrophage migration inhibitory factor(s). In addition, these supernatant fluids were capable of inducing increased resistance to Listeria in normal macrophages.

Resistance to a variety of facultative or obligate intracellular pathogens (e.g., Listeria monocytogenes, Brucella abortus, Salmonella typhimurium, Mycobacterium tuberculosis) appears to be effected by “activated” macrophages which possess an enhanced microbicidal capacity. The work of Mackaness and his colleagues, largely through in vivo studies, has revealed an important antigen-specific role which is played by the immunologically committed lymphocyte in inducing macrophage resistance to homologous as well as heterologous organisms (12). Little is known, however, of the actual mechanisms whereby lymphocytes activate macrophages. The recent results reported by Patterson and Youmans (18) and Mooney and Waksman (17), using a mouse and rabbit system, respectively, suggest that previously sensitized lymphocytes exposed to the specific sensitizing antigen will cause activation of normal macrophages in vitro.

In the mouse tubercle bacillus model employed by Patterson and Youmans, both induction of immunity and elicitation of resistance were performed by using homologous antigen. Since infection with Toxoplasma gondii confers in vivo resistance to heterologous challenge [e.g., against mengovirus (23), Listeria, Salmonella (26), etc.], an attempt was made to determine whether normal guinea pig macrophages could be activated in vitro to resist challenge with a heterologous organism (Listeria) when an unrelated intracellular parasite (T. gondii) is employed as the original immunizing organism.

MATERIALS AND METHODS

Cell cultures. Cells for all experiments were obtained from inbred strain XIII guinea pigs. Sensitized lymphocytes were obtained from the spleens of guinea pigs that had been infected for approximately 6 months with the C56 strain of T. gondii by methods similar to those previously described (11). The macrophage composition of these sensitized spleen cell suspensions as well as those from normal spleen cells never exceeded 10% when examined morphologically or by studies utilizing phagocytosis of Candida albicans. Unstimulated peritoneal cells were washed from the peritoneal cavities of several normal guinea pigs.
by using heparinized Hanks balanced salt solution (HBSS; 10 units of heparin/ml). These cell suspensions, consisting of 70 to 75% macrophages, were pooled and washed twice with HBSS at 150 × g. They were then resuspended in medium 199 (Grand Island Biological Co., Grand Island, N.Y.) containing 20% heat-inactivated fetal calf serum (199–20% FCS), 100 μg of streptomycin, and 100 units of penicillin per ml and counted with a hemocytometer. Leighton tubes were inoculated with 1 ml of medium containing 2 × 10⁸ peritoneal cells and allowed to incubate for 2 hr at 37 C in an atmosphere of 5% CO₂, 95% air. Thereafter, nonadherent cells were removed by four washes with 2.0 ml of medium. Two million spleen cells from normal or Toxoplasma-infected guinea pigs were then added to each monolayer in 2.0 ml of medium alone (199–20% FCS plus antibiotics) or in 2.0 ml of medium containing 10 μg of Toxoplasma antigen per ml. The Leighton tubes were then reincubated for 72 hr. At this time, the supernatant fluid medium was decanted and frozen at −20 C, and the monolayers were washed four times with 2.0 ml of antibiotic-free medium. A 1.0-ml suspension of Listeria was then added to a series of Leighton tubes from each group. After reincubation for 1 hr, the extracellular bacteria were removed by washing four times with antibiotic-free medium. Finally, 1.0 ml of antibiotic-free medium was added, and the tubes were reincubated. At various times after Listeria infection, cover slips from each group were fixed in methanol and stained with Giemsa stain.

Quantitation of resistance to Listeria. Methods of quantitating resistance to Listeria were modified from those previously described (25). The integrity of each monolayer was checked microscopically by counting the number of cells in 10 randomly chosen high-power fields. To enumerate the growth of Listeria, the number of intracellular bacteria was counted in 100 cells.

Antigen preparation. Soluble T. gondii antigen was prepared from purified (19) and osmotically lysed trophozoites of the RH strain as previously described (Krahnenhuhl, Ruskin, and Remington, submitted for publication). Protein determination was performed by using the method of Folin-Ciocalteu (8) with bovine serum albumin (fraction V, Mann Research Laboratory, New York) as a standard.

Listeria monocytogenes. An 18 hr-old culture of L. monocytogenes type 4a (tryptic soy broth, Difco) was washed three times in HBSS at 3,000 × g and restored to the original volume of the culture with antibiotic-free 199–20% FCS. A challenge dose of bacteria was then prepared by using a 1:25 dilution of this suspension. Dilution plates were immediately prepared on tryptic soy agar (Difco) to quantitate the inoculum.

Migration inhibitory factor. The supernatant fluid from the different groups of Leighton tubes were centrifuged at 10,000 × g to remove cellular debris and tested for their ability to cause inhibition of migration of normal guinea pig peritoneal exudate cells by using methods described previously (10).

Lymphocyte transformation. Suspensions of spleen cells in screw-capped plastic culture tubes (3033, Falcon Plastics, Los Angeles, Calif.) were prepared to contain 4 × 10⁶ cells per ml of medium (199–20% FCS plus antibiotics). Quadruplicate sets of normal or sensitized spleen cell cultures were then prepared in the presence or absence of Toxoplasma antigen by adding either 1.0 ml of medium alone or 1.0 ml of medium containing 20 μg of Toxoplasma antigen. After incubation for 4 days, 2.0 μCi of tritium-labeled thymidine (3H-TdR, specific activity 6.0 μCi/m mole, New England Nuclear Corp., Boston, Mass.) was added to each tube for 16 hours. The cells were then washed three times with saline at 150 × g, frozen and thawed three times, and extracted as follows. Each pellet of lysed cells was resuspended in 6.0 ml of cold 5% trichloroacetic acid and centrifuged at 400 × g. The precipitate was dissolved in 2.0 ml of 1 M KOH and incubated at 37 C for 1 hr. This alkaline solution was neutralized with an equivalent amount of 1 M HCl and 4.0 ml of 10% trichloroacetic acid was added. Precipitation was carried out at 4 C for 1 hr at which time the precipitate in each tube was collected on individual membrane filters (0.25 μm, Millipore Corp., Bedford, Mass.) by repeatedly rinsing the tubes with cold 5% trichloroacetic acid. After placing the filters in vials, scintillation fluid was added (Omnifluor, New England Nuclear Corp.), and the samples were counted in a Nuclear-Chicago scintillation counter. The amount of 3H-TdR incorporation is presented as the ratio A/C where A is the mean counts per minute in cultures incubated in the presence of antigen and C is the mean counts per minute in cultures incubated in the absence of antigen (Krahnenhuhl et al., submitted for publication).

RESULTS

Effect of sensitized spleen cells and antigen on activation of normal macrophages. Monolayers of normal macrophages were exposed for 72 hr to 2 × 10⁶ normal or sensitized spleen cells in the presence or absence of Toxoplasma antigen. Cultures of normal macrophages, without added lymphocytes, were also prepared and incubated with and without antigen. The results of two representative experiments are shown in Fig. 1. After infection with Listeria (10⁴), the number of cells in the monolayers which had been exposed to sensitized lymphocytes in the absence of antigen and normal lymphocytes in the presence or absence of antigen were rapidly destroyed (Fig. 1-I, II). Antigen alone afforded no resistance to macrophages cultured without added lymphocytes (Fig. 1-I). In addition, the number of bacteria observed in the cells from these groups had greatly increased by the fifth hour after infection, and by 12 hr the intracellular Listeria cells in the few remaining macrophages were too numerous to count (Fig. 1-III, IV). In marked contrast were the results observed in monolayers exposed to sensitized lymphocytes in the presence of Toxoplasma antigen. Although some destruction of the macrophages did occur after challenge with Listeria cells, the decrease
in cell numbers closely paralleled that of uninfected control monolayers (Fig. 1-I). Intracellular growth of *Listeria* was barely perceptible and had increased only slightly as late as 12 and 16 hr after infection (Fig. 1-III, IV). Figure 2 depicts the obvious differences in integrity of representative cell monolayers challenged with *Listeria* after exposure to sensitized lymphocytes in the presence or absence of antigen.

Effect of various numbers of sensitized spleen cells on activation of normal macrophages. To emphasize further the importance of the sensitized spleen cells in this experimental system, various numbers of such cells (10⁸, 5 x 10⁸, 10⁹) were incubated with normal macrophages in the presence or absence of antigen. After 72 hr, *Listeria* challenge (10⁹) was rapidly followed by destruction of macrophages and growth of the bacteria in all groups except those originally containing 5 x 10⁸ or 10⁹ sensitized lymphocytes plus *Toxoplasma* antigen (Fig. 3). With the former spleen cell concentration, resistance apparent 8 hr after challenge was overwhelmed by 16 hr. One million sensitized spleen cells plus antigen induced a state of resistance to *Listeria* growth which was still apparent at 16 hr, the latest period sampled (Fig. 3).

Activation of normal macrophages and demon-
stration of migration inhibitory factor(s) by supernatant fluids from sensitized spleen cells and macrophages cultured in the presence of antigen. Supernatant fluids were collected at 72 hr from experiments utilizing macrophage monolayers and normal or sensitized spleen cells with and without Toxoplasma antigen. Groups of normal monolayers were incubated in the presence of these four supernatant fluids for 72 hr and challenged with Listeria. The results, indicating that resistance was induced in macrophages exposed to the supernatant fluid obtained from previous incubation of sensitized spleen cells with Toxoplasma antigen, revealed a slower growth rate of intracellular bacteria after challenge with Listeria (Fig. 4).

Separate determinations for migration inhibitory factor activity in samples of these supernatant fluids obtained from two different experiments were performed. In the first determination, the per cent inhibition of macrophage migration caused by normal spleen cell supernatant fluids was 2%, indicating that no migration inhibitory factor had been produced. In contrast, the supernatant fluid from sensitized spleen cells incubated in the presence of antigen caused a 33% inhibition of migration. In the second migration inhibitory factor determination with different samples of similar supernatant fluids, macrophage migration was inhibited by 34% in the case of sensitized spleen cell supernatant fluids as compared to -2% inhibition for normal spleen cell culture supernatant fluids.

Demonstration of increased 3H-TdR uptake by spleen cells used to activate normal macrophages. Portions of the normal and sensitized spleen cells used in the two experiments described above (Fig. 1) were cultured in the presence and absence of antigen and tested for their ability to incorporate 3H-TdR. Cultures prepared in this manner

Fig. 2. Representative experiment showing the effects of challenge with Listeria in normal macrophages previously cultured with sensitized spleen cells in the presence and absence of Toxoplasma antigen. (A) Spleen cells plus antigen 8 hr after challenge, (B) spleen cells plus antigen 16 hr after challenge, (C) spleen cells without antigen 8 hr after challenge, (D) spleen cells without antigen 16 hr after challenge.
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from the normal spleen cells used in two of the above experiments did not reveal increased levels of uptake of \(^{3}H\)Tdr as evidenced by A/C ratios of 0.96 and 1.03, respectively (Table 1). However, spleen cells from *Toxoplasma*-infected guinea pigs used in these experiments responded with A/C ratios of 4.30 and 6.92, respectively.

**DISCUSSION**

The results described above reveal that sensitized spleen cells in vitro, in the presence of specific antigen, have the capacity to activate or enhance the microbicidal properties of normal peritoneal macrophages. Although the system we employed is different, we consider that our results confirm those of Patterson and Youmans (18), who described enhanced resistance to *M. tuberculosis*.

**Table 1. Lymphocyte transformation in spleen cells obtained from guinea pigs infected with Toxoplasma gondii**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Source of spleen cellsa</th>
<th>+ or – antigenb</th>
<th>Mean counts/min</th>
<th>A/Cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>–</td>
<td>751 (5)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>+</td>
<td>721 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toxoplasma-infected</td>
<td>–</td>
<td>767 (5)</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td>Toxoplasma-infected</td>
<td>+</td>
<td>3303 (5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>–</td>
<td>189 (5)</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>+</td>
<td>195 (5)</td>
<td></td>
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<tr>
<td></td>
<td>Toxoplasma-infected</td>
<td>–</td>
<td>279 (4)</td>
<td>6.92</td>
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<tr>
<td></td>
<td>Toxoplasma-infected</td>
<td>+</td>
<td>1932 (4)</td>
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</tr>
</tbody>
</table>

a Each culture contained \(4 \times 10^4\) cells in 2.0 ml of medium 199–20% fetal calf serum.
b *Toxoplasma* lysate (10 \(\mu\)g/ml).
c A/C = mean counts per minute of cells cultured with antigen (A)/mean counts per minute of cell culture without antigen (C).
d Values in parentheses indicate number of cultures.
losis in mouse macrophages cultured in the presence of this organism and spleen lymphocytes from M. tuberculosis-immunized donors. Our studies served also to extend their observations in that we noted nonspecific activation of macrophages in vitro; enhancement of killing of Listeria was observed in macrophages exposed to Toxoplasma antigen and lymphocytes from Toxoplasma-infected guinea pigs. This activation was evidenced by prolonged survival of macrophage monolayers and rapid killing of intracellular bacteria. No such activation was observed when antigen alone or spleen cells alone (either normal or sensitized) were used.

The minimum number of sensitized spleen cells in the presence of antigen found necessary to activate sufficiently normal macrophages, such that enhanced microbial killing could be clearly demonstrated, was approximately one spleen cell per two to four macrophages. Since macrophages from Toxoplasma-infected animals have been shown to have enhanced microbicidal activity against intracellular bacteria (26), the question arises as to whether contamination of our sensitized spleen cell preparations with macrophages could have accounted for the results. If this were the case, one would expect monolayers treated with spleen cells of sensitized animals to be resistant to Listeria challenge, regardless of the presence or absence of specific antigen. Since activation occurred only in the presence of sensitized lymphocytes and specific antigen, it appears probable that the active cells in suspensions of sensitized spleen cells were immunologically committed lymphocytes.

Resistance to Listeria has been shown to be affected at the cellular level by macrophages possessing an enhanced microbicidal capacity (14). Although little is known about the mechanisms involved in macrophage activation in vivo, it appears to be at least a two-cell process involving an interaction between immunologically committed small lymphocytes and macrophages (12). Fortuitously, the activated macrophages resulting from this interaction are capable of killing not only the homologous infecting organism but also a wide range of unrelated intracellular pathogens (14, 27). An element of specificity does appear to exist, however (12). This specificity involves lymphocyte recognition of the antigen to which the host had previously been exposed; such recognition appears to be necessary before the macrophage-activation process occurs. Nonspecific cellular resistance appears to diminish with the disappearance of the antigenic stimulus from the host but can be rapidly recalled by reintroduction of the specific antigen (12). The Toxoplasma model used in our studies should afford an ideal situation since, unlike the Listeria or Mycobacterium model in which resistance either results in the complete elimination of the organism from the tissues (5, 14) or effectively sequesters it and renders it antigenically ineffective (6), a host infected with Toxoplasma remains infected for life, and there is ample evidence for continued antigenic stimulation throughout the life of the host (16, 20–22). Delayed hypersensitivity is acquired in guinea pigs within 1 week after Toxoplasma infection as measured by in vivo and in vitro techniques (10).

At least two hypotheses have been presented to explain the activation of macrophages (12, 13). The first involves the production by lymphocytes of a cell-bound antibody which allows the macrophages to become sensitive to antigen. Such an antibody would have to be extremely avid for these cells and thus be absent in an effective configuration or concentration from the serum, since immune serum has been shown to be ineffective in the passive transfer of resistance to such organisms as Listeria (15). The second hypothesis is based on the existence of soluble mediators that are released from lymphocytes after specific interaction with antigen [e.g., migration inhibitory factor (4, 7), lymphokinetin (9, 24), chemotactic factor (28), skin reactive factor (1), etc.]. These factors are defined functionally by in vitro techniques (except for skin reactive factor which is elicited in vivo) and appear to be quite uniform in their physicochemical characteristic (3). It is plausible that one or several of these mediators or an as yet unidentified factor is released specifically from sensitized lymphocytes and leads to the activation of macrophages.

The cytophilic antibody hypothesis as well as the mediator hypothesis suffers from the lack of direct in vivo evidence for its role in macrophage activation. However, the present in vitro studies, as well as those of Patterson and Youmans (18), tend to support the hypothesis that increased macrophage resistance may be caused by specifically released mediators. In their work and ours, supernatant fluid media obtained from cultures containing macrophages, sensitized spleen cells, and antigen were capable of inducing enhanced microbicidal properties in normal macrophages. Additional supporting evidence for these results has been presented by Mooney and Waksman (17), who demonstrated that supernatant fluids from sensitized rabbit lymph node cells cultured with specific antigen stimulated the spreading and motility of normal rabbit macrophages, two properties which are characteristic of the activated macrophage (2). That the sensitized lymphocytes used in our experiments were indeed responding to Toxoplasma antigen was shown by their increased
ability to incorporate $^3$H-TdR when cultured in the presence of antigen. In addition, supernatant fluids capable of activating normal macrophages were shown to contain at least one of the factors known to be released upon interaction of antigen and lymphocyte, migration inhibitory factor.

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**LITERATURE CITED**