Dithiothreitol-Dependent Antilisterial Activity of Lysates from Normal Macrophages Exposed In Vitro to Culture Fluids from Spleen Cells of BCG-Immunized Mice

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The effect of culture fluids from mouse lymphocytes on the antilisterial activity of normal macrophages in vitro was determined. Titers of lysates of macrophages incubated with or without culture fluids from lymphocytes from control mice had titers of 10 to 270 with or without a reducing agent, dithiothreitol. Lysates of macrophages after incubation with culture fluids of spleen lymphocytes from BCG-immunized mice had antilisterial titers with and without dithiothreitol of 197,000 and 810, respectively. This may indicate a dithiothreitol-dependent antilisterial system inherent in activated macrophages of cell-mediated immunity. Lysates of incubated spleen cells and their culture fluids also had slight antilisterial activity.

Culture supernatant fluids from lymphocytes of immunized animals incubated in vitro with specific antigen reportedly affects the activity of macrophages in vitro. Nathan et al. (7) reported increased macrophage adherence, phagocytosis, and hexose monophosphate oxidation in macrophages from normal guinea pigs incubated with supernatants of lymphocytes from o-chlorobenzoyl bovine gamma globulin-immunized animals after the lymphocytes were incubated with the specific antigen. Patterson and Youmans (8) found a significant decrease in the number of virulent Mycobacterium tuberculosis H37Rv in normal macrophages when splenic lymphocytes from mice immunized with avirulent M. tuberculosis H37Ra were added. They also observed intracellular inhibition when culture fluids from immune spleen cells incubated in vitro with specific antigen were added to normal macrophages. Howard et al. (3) reported similar observations when lymphocytes from Histoplasma capsulatum-immunized mice were added to normal macrophages.

The increased macrophage antimicrobial activity is not specific for the immunizing organism. Culture supernatant fluids of lymphocytes from listeria-immunized mice incubated in vitro with listeria confer the ability to inhibit virulent mycobacteria on normal macrophages (5), and culture filtrates of BCG-immune lymphocytes incubated with BCG activate normal macrophages to greater killing of listeria (Sal-mon, B. J., J. I. Kreisberg, and G. M. Middleton, Fed. Proc. 32:1039, 1973).

We detected antilisterial activity in mouse peritoneal cell lysates that depends on the presence of a reducing agent, dithiothreitol (DTT) (6). This is a report of the detection of this activity in lysates of normal macrophages and an increased activity in lysates of macrophages incubated with culture fluids from BCG-immune lymphocytes.

MATERIALS AND METHODS

Mice. CBA/J female mice, approximately 9 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Me. They were housed five per cage and given water and food pellets ad libitum.

Bacterial preparations. Mycobacterium bovis BCG was grown at 37 C for 2 weeks in Dubos broth base without enrichment or Tween 80 (Difco Laboratories, Detroit, Mich.) with 0.5% dextrose, centrifuged, and homogenized with mortar and pestle. Cells were suspended in tissue culture media, the concentration estimated by centrifugation in a Hopkin's tube, and adjusted to approximately 10^8 organisms/ml.

Listeria monocytogenes was maintained on brain heart infusion agar (Difco). Cultures used for measuring antilisterial activity were grown for 18 h in brain heart infusion broth (Difco) at 37 C with shaking.

Immunizations. Mice were inoculated once intraperitoneally with approximately 2 mg (wet weight) of BCG in physiological saline in a total volume of 0.2 ml.

Lymphocyte cultures. Spleens from control mice and mice immunized 4 weeks previously with BCG were removed aseptically and placed in cold Hanks...
basal salt solution (HBSS) (Microbiological Associates, Bethesda, Md). Both groups were treated in the same manner. The spleens were punctured at various sites and injected with cold HBSS, and the cells were expressed with sterile forceps.

The cells were dispersed by forcing them first through a 20-gauge and then through a 27-gauge syringe needle. The cells were centrifuged at 5 C for 10 min at 250 x g and resuspended in HBSS with 10% fetal calf serum and supplemented with 1% each glutamic acid, sodium pyruvate, glutamine, vitamins, essential and nonessential amino acid pools, and 100 µg of streptomycin and 100 U of penicillin per ml.

Cell numbers were determined by hemocytometer. Differential cell counts were made in a solution of 2.1% citric acid-0.1% crystal violet. Viability counts were made by the trypan blue exclusion technique.

Spleen cell suspensions were adjusted to 10^6 viable lymphocytes per ml, and 15 ml was placed in plastic tissue culture dishes (100 by 15 cm). BCG was added to one-third of the spleen cell cultures for a final concentration of 10^6 organisms per ml. The cell cultures were incubated for 2 h at 37 C in an atmosphere of 95% air and 5% CO2. Nonadherent cells in the culture medium were transferred to new tissue culture dishes, incubated 3 days, and centrifuged. The culture fluids were decanted and set aside. The lymphocytes were washed once and resuspended to 10^7 cells/ml in 0.1 M phosphate buffer, pH 7. Cell suspensions after incubation were 93 to 99% lymphocytes.

Macrophage collection and culture. A modification of the method of Klun and Youmans (4) was used. Peritoneal cells of normal, unstimulated mice were collected as described elsewhere (6). The cells were pooled, centrifuged 10 min at 5 C at 250 x g, and resuspended in the above medium with 10% horse serum added. Differential cell counts and viability were determined as above, and cell numbers were adjusted to 10^6 macrophages per ml. A 15-ml amount was placed in plastic tissue culture dishes and incubated at 37 C in 95% air and 5% CO2. After 2 days, the medium and unattached cells were removed, fresh medium was added, and the macrophages were incubated for an additional 24 h.

After 3 days of incubation, the medium was removed and replaced with 10 ml of fresh medium and 5 ml of lymphocyte culture fluid. The cell cultures were incubated for 24 h. The medium was decanted and the adherent cells were rinsed quickly and gently with 1:1 trypsin-ethylenediamineetraacetic acid at room temperature. The cells were covered with cold trypsin-ethylenediamineetraacetic acid and removed with a rubber policeman. The macrophages were washed, counted, and resuspended to 2 x 10^5 macrophages per ml in 0.1 M phosphate buffer, pH 7.0. The cell suspensions were 99% macrophages.

Preparation of cell lysates and lymphocyte culture fluids for assay. Macrophage and lymphocyte suspensions were lysed with staphylococcal delta-hemolysin as described elsewhere (6). When microscopic examination determined that greater than 95% lysis had occurred, portions were removed and assayed immediately for antilisterial activity.

 Supernatant fluids from cultured lymphocytes were dialyzed in distilled water that had been degassed and flushed with argon. The dialyzed culture fluids were assayed immediately.

Samples of cell lysates and dialyzed lymphocyte culture fluids were stored at 4 C and reassayed after 3 weeks.

Antilisterial assay. Antilisterial activity was measured by a modification of the assay described in a previous paper (6). Serial threefold dilutions of the material to be tested were made in two diluents, 0.1 M phosphate buffer (pH 7) containing 0.01% bovine serum albumin with and without DTT at a concentration of 10 mM. The controls were tubes containing 1.0 ml of the diluent. An 18-h broth culture of L. monocytogenes was centrifuged, resuspended in 0.85% saline, and diluted to about 2 x 10^6 colony-forming units per ml. A 0.1-ml amount was added to each dilution and control tubes. The tubes were incubated for 2 h at 37 C. The suspensions were diluted, and 10 µl of each dilution were added to 15 ml of warm, melted brain heart infusion agar. After mixing, the contents were poured into petri dishes, allowed to solidify, and incubated overnight at 37 C. The number of colonies was counted, and the titer was taken as the highest dilution which inactivated 50% of listeria when compared to the buffer control.

RESULTS

Antilisterial activity of lymphocyte culture fluids. In the absence of DTT, no detectable antilisterial activity was found in fresh lymphocyte culture fluids except in those from BCG-stimulated lymphocytes of BCG-immunized mice (Table 1). Without DTT, no antilisterial

| TABLE 1. Antilisterial activity with or without DTT of culture fluids from mouse spleen lymphocytes, incubated in vitro with or without BCG, from normal and BCG-immunized mice |
|---|---|---|---|
| Source of lymphocytes* | Added to lymphocytes for incubation in vitro | Included in assayed solutions | Antilisterial titer* when assayed immediately | Antilisterial titer of supernatants after storage |
| | | | | |
| Unimmunized | 0 | DTT* | <10 | <10 |
| | 0 | DTT | <10 | <10 |
| BCG | 0 | DTT | <10 | 270 |
| BCG-immunized | 0 | DTT | >270 | 810 |
| | 0 | DTT | >270 | <10 |
| | 0 | DTT | 90 | 270 |

* Lymphocyte concentration in vitro culture = 10^6 cells/ml.
* Titer = highest dilution which inactivated 50% of listeria.
* DTT concentration in assay solutions = 10 mM.
* BCG concentration in vitro culture = 10^6 cells/ml.
activity in culture fluids was detected after 3 weeks of storage. Slight activity was detected in the presence of DTT culture fluids from control lymphocytes which had been incubated with BCG, and from BCG-immune lymphocytes incubated with or without BCG.

**Antilisterial activity of lymphocyte lysates.** There was antilisterial activity in lysates from lymphocytes from all groups, with and without DTT (Table 2). Titers of stored lysates were similar to initial titters.

**Antilisterial activity of macrophage lysates.** End points were not reached in several of the initial titrations (Table 3). When reassayed, the greatest activity was found in lysates of macrophages incubated with culture fluids from BCG-immune lymphocytes incubated with BCG. In the presence of DTT, lysates in this group had titers of 197,430. Without DTT, the titer was 810. Macrophage lysates from cells incubated with culture fluids of BCG-immune lymphocytes, unstimulated in vitro, had similar titers. End points reached with macrophage lysates in other groups were not greater than 270.

**DISCUSSION**

The lymphocyte culture fluids and lysates of spleen cells inactivated listeria to some extent. Whether the activity was due to a material produced and secreted by lymphocytes or whether this base-line activity was a result of the small number of macrophages present could not be determined.

Low levels of antilisterial activity were detected in lysates of macrophages with or without culture fluids from unsensitized lymphocytes. This was interpreted to represent the normal antibacterial factors of macrophages, although an effect by pinocytosed antibiotics cannot be eliminated. Titers with or without DTT did not differ markedly.

In the presence of DTT, lysates of macrophages incubated with culture fluids of BCG-stimulated lymphocytes from BCG-immunized mice had greater antilisterial activity than the above controls. Unpublished data indicates this is a qualitative difference, not quantitative. Culture fluid from BCG-immune spleen cells cultured in vitro were also able to stimulate macrophages, and titers were obtained with lysates in this group when DTT was included in the assay system. Spleen cells had been collected from mice 1 month after injection with BCG and, therefore, the cells may still have been near the peak of their immune response. Blanden et al. (1) found significant resistance to *L. monocytogenes* in mice infected with BCG 4 weeks earlier.

The nature of the stimulus affecting macrophages is unknown. The macrophages may be pinocytosing and concentrating an active lymphocyte product or receiving a signal to produce and/or activate a macrophage antilisterial product(s). The latter is more probable, assuming complete uptake of an active factor(s) from culture fluid and the action additive to normal macrophage antibacterial systems. There was not sufficient total activity in the volume of culture fluid added to macrophages to account for the increased activity, even using least favorable data. It is possible that a DTT-dependent antibacterial factor produced by lymphocytes is taken up and is acting synergistically with macrophage systems. Lysates of macrophages incubated with culture fluids of BCG-sensitized lymphocytes, incubated in vitro with or without BCG, had titers of 197,430 and 65,610, respectively. In this same test group, titers were 270 with or without DTT when BCG was added to culture fluid at the time of transfer to macrophages. This suggests the removal of an active factor(s) by the BCG.

We have reported a DTT-requiring antilisterial factor(s) from mouse peritoneal cell lysates. Slightly greater activities were detected in cell lysates from BCG-immunized and pre-

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**Table 2. Antilisterial activity with or without DTT of lysates of mouse spleen lymphocytes, incubated in vitro with or without BCG, from normal and BCG-immunized mice**

<table>
<thead>
<tr>
<th>Source of lymphocytes*</th>
<th>Added to lymphocytes for incubation in vitro</th>
<th>Included in assayed solutions</th>
<th>Antilisterial titer when assayed immediately</th>
<th>Antilisterial titer of supernatants after storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unimmunized BCG*</td>
<td>0 DTT&lt;sup&gt;v&lt;/sup&gt; 0</td>
<td>30</td>
<td>30</td>
<td>270</td>
</tr>
<tr>
<td>BCG-immunized</td>
<td>0 DTT&lt;sup&gt;v&lt;/sup&gt; 0</td>
<td>&gt;270</td>
<td>270</td>
<td>90</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Lymphocyte concentration in in vitro culture = 10<sup>9</sup> cells/ml.

*<sup>b</sup> Titer = highest dilution which inactivated 50% of listeria.

*<sup>v</sup> DTT concentration in assay solutions = 10 mM.

*<sup>+</sup> BCG concentration in in vitro culture = 10<sup>4</sup> cells/ml.
stimulated mice (6). The dependency of the antilisterial factor on a reducing agent for activity may explain why it has not been detected by previous investigators. The elevated antilisterial titer, with DTT, of lysates of macrophages exposed to culture fluid of BCG-immune lymphocytes suggests that the DTT-dependent factor(s) may be important in antibacterial cell-mediated immunity.

Other authors have reported an inhibitory influence of culture supernatant fluids from immune, stimulated lymphocytes on intracellular parasites in normal, infected macrophages (2-5, 8, 9). Pearsall et al. (9) reported a direct effect on yeast cells by lymphokine-containing lymphocyte supernatants. Klun and Youmans (4) and Fowles et al. (2) found no direct effect on extracellular mycobacteria or listeria by lymphocyte culture fluids but suggested that these results may not be definitive. Fowles et al. (2) found that the addition of sensitive lymphocytes had a greater effect on macrophage bacteriostasis than did supernatant fluids and suggested that the mediators produced by lymphocytes may be labile. In their experiments, the effect of lymphocytes on macrophages was detected by determining the bacterial inactivation in intact macrophages. Our system offers the advantage of measuring bacterial inactivation in a cell-free system and examining the process at a subcellular level.

**LITERATURE CITED**


