Effect of Antimacrophage Serum on Dermal Tuberculin Sensitivity and Allergic Pulmonary Granuloma Formation in Rabbits

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Anti-rabbit alveolar macrophage goat serum (AMS), adsorbed of hemolysins and hemagglutinins, exhibited complement-dependent cytotoxicity for the following rabbit target cells: alveolar macrophages, thymocytes, and kidney (RK-13) cells. The intravenous injection of AMS, sufficient to reduce markedly the dermal tuberculin reaction in BCG-sensitized rabbits, did not suppress the development of BCG-induced chronic and accelerated pulmonary granulomas. These observations are discussed in relation to delayed sensitivity, allergic granuloma formation, and cell-associated immunity.

Classical dermal tuberculin “sensitivity” (used here in preference to the more commonly used “hypersensitivity”) and acquired cellular immunity are apparently induced by different components of Mycobacterium tuberculosis because the two responses can be separated (18, 22). Granulomas are formed in diseases of chronic inflammation such as tuberculosis and leprosy, and are characterized by the focal accumulation of lymphocytes, macrophages, epithelioid cells, and giant cells; thus, there are histological similarities between local delayed sensitivity reactions and granuloma formation. Granulomas can be readily induced experimentally in the lungs of rabbits by the intravenous injection of killed BCG cells in oil (the chronic response; 8, 9). This response becomes accelerated in time and intensity when animals with chronic pulmonary granulomas are challenged intravenously with BCG cells in saline (the accelerated granulomatous response). A similar period of time is required for the elicitation of both dermal delayed sensitivity and the accelerated pulmonary granulomatous response. In this regard, it has been suggested that accelerated tubercle formation is dependent upon delayed sensitivity (4). Despite the similarities of the two responses, they can be dissociated under special experimental conditions (8). Accordingly, the immunological mechanisms or antigens (or both) mediating these responses must be different.

Antilymphocyte serum (ALS) seems selectively to depress cell-mediated immune responses by complement-dependent immune cytolysis (14), and probably exerts its maximal cytotoxic effect on thymus-derived small lymphocytes (13). Thus, ALS will reduce the tuberculin skin reaction (7), suppress contact allergy (21), and inhibit the lymphocyte transfer reaction (10). It has been reported that antimacrophage serum (AMS) also causes a reduction in delayed sensitivity reactions (12). To the authors’ knowledge, no information is available on the effects of either ALS or AMS on allergic granuloma formation in the lung.

The present studies indicate that AMS can suppress delayed sensitivity reactions but not the formation of allergic pulmonary granulomas.

MATERIALS AND METHODS

Animals. New Zealand white rabbits weighing 2 to 4 kg were used throughout these studies. Anti-rabbit alveolar macrophage serum was produced in a goat.

Cells. Alveolar macrophages were obtained by a method previously described (15). Rabbit thymocytes were collected by dicing the organ in medium 199 (Grand Island Biological Co.) and collecting the upper portion of cell suspensions that had been allowed to sediment for 4 min at 25 C. Rabbit kidney cells (RK-13), maintained in continuous tissue culture, were supplied by Jean Acton.
Microorganisms. The BCG strain of M. bovis was grown on Proskauer and Beck's broth. Cultures were autoclaved, harvested, washed in distilled water, and lyophilized. Vaccines were prepared by grinding organisms in a mortar in light mineral oil (Bayol F) or 0.15 M NaCl.

Production of AMS. A goat was immunized according to the schedule in Table 1. The proportion and viability of alveolar macrophages in each cell suspension was 95% or greater; the remaining cells resembled small lymphocytes. The goat was bled at 3- to 5-day intervals beginning 45 days after the initial injection. The antisera were pooled and adsorbed of detectable rabbit hemolysins and hemagglutinins.

Cytotoxicity testing. Target cells were suspended in medium 199 containing 10% normal inactivated rabbit serum. Fresh rabbit serum was used as a complement source to avoid the possible deleterious effects of xenogeneic serum (3). Viability was assessed by trypan blue exclusion. Cytotoxicity was expressed as TCLD_{50}, e.g., that dilution of AMS which kills 50% of the cells during 1 hr of incubation at 37 C in a shaking water bath (60 cycles/min). TCLD_{50} titers were calculated by the Reed-Muench method (5).

Tuberculin sensitivity. Animals were vaccinated subcutaneously at the base of the ear with 150 µg of killed BCG cells in 0.1 ml of oil. To elicit dermal reactivity, animals were challenged intracutaneously with 10 µg of purified protein derivative (PPD), in 0.1 ml of 0.15 M NaCl. Skin reactions (24 hr) are expressed as erythema (mean of the greatest and smallest "diameter") and induration (increase in skin thickness as measured with a Schnelltaster, Kroplin).

Pulmonary granulomas. Pulmonary granulomatous responses were induced as previously described (8). Chronic pulmonary granulomas were induced by vaccinating animals intravenously (iv) with 100 µg of BCG cells in 0.1 ml of oil. Two weeks later, the animals were weighed and killed; their lungs were removed and weighed. Alveolar cells were collected and a granuloma index was calculated. [Granuloma index = (lung weight/body weight of test animal)/ (lung weight/body weight of normal animal). Assuming that lung weight/body weight of normal animal = 4 X 10^{-3}, then the granuloma index = (lung weight/body weight of test animal) X (2.5 X 10^9).] Accelerated pulmonary granulomas were elicited by challenging animals undergoing a chronic pulmonary granulomatous response with 5 mg of BCG cells in 1.0 ml 0.15 M NaCl given iv. At 4 to 5 days after challenge, animals were sacrificed and processed. Packed-cell volumes were determined to estimate the increase in cellularity of granulomatous lungs. It is known that rabbits displaying increased ratios of lung weight to body weight after BCG vaccination by these methods are undergoing pulmonary granulomatous responses as evaluated histologically (8, 9).

RESULTS

In vitro activity of AMS. Table 2 shows the results obtained when AMS was incubated with various rabbit target cells. AMS had a higher TCLD_{50} titer against rabbit thymocytes than against alveolar macrophages; dilutions of 1:6 and 1:12 were cytolytic for thymocytes but not for alveolar macrophages. Since the cell suspensions used for antibody production contained small percentages of lymphocytes, low levels of antilymphocyte and antithymocyte activity were expected. Adsorption with thymocytes removed all detectable cytotoxic activity against both thymocytes and alveolar macrophages. Furthermore, adsorption with thymocytes and alveolar macrophages calculated to possess equivalent surface areas revealed that thymocytes were more efficient than alveolar macrophages in removing cytotoxic activity against alveolar macrophages (Table 2). The minimal diameter of the alveolar macrophages was estimated at 15 µm, and the maximal diameter of the thymocyte was estimated at 10 µm; accordingly, the surface area of 2.0 X 10^4 alveolar macrophages is minimally equivalent to the sur-

### Table 1. Protocol for production of goat anti-rabbit alveolar macrophage serum

<table>
<thead>
<tr>
<th>Day</th>
<th>Route</th>
<th>No. viable alveolar cells injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intravenous</td>
<td>2.1 X 10^6</td>
</tr>
<tr>
<td>4</td>
<td>Intravenous</td>
<td>9.0 X 10^6</td>
</tr>
<tr>
<td>8</td>
<td>Intravenous</td>
<td>1.7 X 10^6</td>
</tr>
<tr>
<td>13</td>
<td>Intravenous</td>
<td>3.9 X 10^6</td>
</tr>
<tr>
<td>40</td>
<td>Subcutaneous</td>
<td>5.0 X 10^6</td>
</tr>
<tr>
<td>60</td>
<td>Intravenous</td>
<td>7.5 X 10^6</td>
</tr>
<tr>
<td>68</td>
<td>Intravenous</td>
<td>2.3 X 10^6</td>
</tr>
</tbody>
</table>

### Table 2. Titration of in vitro cytotoxicity of antimagrophone serum (AMS)

<table>
<thead>
<tr>
<th>AMS prep(a)</th>
<th>Target cell</th>
<th>TCLD_{50} titer(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadsorbed</td>
<td>Alveolar macrophage</td>
<td>1:20</td>
</tr>
<tr>
<td>Adsorbed with 4.5 X 10^6 thymocytes/ml of AMS</td>
<td>Thymocyte</td>
<td>1:47</td>
</tr>
<tr>
<td>Adsorbed with 2.0 X 10^6 alveolar macrophages/ml of AMS</td>
<td>Rabbit kidney cells(c)</td>
<td>1:19</td>
</tr>
<tr>
<td>Adsorbed with 2.0 X 10^6 alveolar macrophages/ml of AMS</td>
<td>Alveolar macrophage</td>
<td>&lt;1:6</td>
</tr>
</tbody>
</table>

\(a\) All preparations of AMS were depleted of detectable rabbit hemolysins and hemagglutinins by adsorption.

\(b\) Values were calculated from the mean of duplicate flasks.

\(c\) Maintained in continuous tissue culture.
face area of $4.5 \times 10^6$ thymocytes. From these results, we tentatively conclude that alveolar macrophages and thymocytes share surface antigens which are present in greater concentrations on thymocytes than on macrophages and, moreover, that the AMS did not contain cytotoxic antibodies specific for macrophages. Although we are designating our material AMS, functionally it is an "anti-rabbit cell serum."

**Effect of AMS on the tuberculin skin reaction.** After the animals became reactive to a challenge dose of PPD, test animals were treated with 1.0 ml of AMS iv for 5 days. One day after the cessation of AMS injections, test and nontreated control animals were skin-tested again with 10 µg of PPD in 0.1 ml of 0.15 M NaCl (Fig. 1). AMS treatment greatly reduced the dermal tuberculin reaction. Since control animals exhibited strong reactivity to the second skin challenge of PPD (Fig. 1), the marked inhibition of skin reactions by AMS in test animals could not have been due to desensitization with the primary dose of PPD. These results were expected, because antithymocyte serum has been shown to prolong allograft survival (16) and to inhibit the lymphocyte transfer reaction (11), and our antiserum exhibited antithymocyte activity. AMS adsorbed with thymocytes had no effect on the tuberculin skin reaction. In addition, equivalent doses of normal goat serum did not suppress tuberculin sensitivity.

![Fig. 1. Effect of antimacrophage serum (AMS) on the tuberculin skin reaction in rabbits.](image1)

![Fig. 2. Effect of antimacrophage serum (AMS) on pulmonary granuloma formation in rabbits. (1) Granuloma indices of 10 normal rabbits. (2) Granuloma indices of rabbits undergoing chronic granuloma formation; seven treated with AMS and five untreated controls. (3) Granuloma indices of rabbits undergoing accelerated granuloma formation; 10 treated with AMS and 7 untreated controls.](image2)
Effect of AMS on the formation of chronic pulmonary granulomas. Daily iv injections with 1.0 ml of AMS were started 2 days after the initial vaccination with BCG cells in oil and were continued until the day before the animals were killed; i.e., animals received 12 ml of AMS over a 2-week period. Figure 2 shows clearly that AMS did not significantly reduce the formation of chronic pulmonary granulomas. Figure 2 also demonstrates that the granuloma index is a reliable indicator of the degree of granuloma formation, because the lung weight to body weight ratio of normal rabbits is remarkably constant. The yield of packed cells from the lungs of rabbits undergoing the chronic granulomatous response usually ranges from 1.5 to 2.0 ml. Treatment with AMS did not appreciably reduce the alveolar cell yields.

Effect of AMS on the accelerated pulmonary granulomatous response. Animals were treated with five daily iv doses of AMS beginning 2 days prior to the challenge dose of BCG cells in saline. Figure 2 shows that AMS did not reduce accelerated granuloma formation; in fact, there was a small significant increase ($P < 0.01$). The yield of packed cells from the lungs of either AMS-treated or nontreated animals undergoing the accelerated response commonly ranged from 3.0 to 5.0 ml.

DISCUSSION

In the present studies, we have demonstrated that doses of AMS which markedly reduce dermal tuberculin sensitivity do not reduce the intensity of chronic or accelerated pulmonary granuloma formation in rabbits. In subsequent studies, we have demonstrated that AMS will reduce dermal tuberculin sensitivity but not chronic granuloma formation in rabbits vaccinated by methods known to induce both types of reactivity (Moore and Myrvik, unpublished data). Kawata et al. (8) demonstrated that there is no correlation between accelerated pulmonary granuloma formation and the level of dermal tuberculin sensitivity. Furthermore, they observed that rabbits desensitized with tuberculin are still capable of developing strong accelerated granulomatous responses. Conversely, they were able to induce high levels of tuberculin skin reactivity in animals by repeated intradermal injections of killed BCG suspended in saline; these animals failed to develop accelerated pulmonary granulomatous responses. These observations support the concept that the BCG-induced accelerated pulmonary granulomatous response is not mediated solely by circulating sensitive small lymphocytes comparable to those which can mediate dermal tuberculin reactions.

The AMS used in the present study probably reduced tuberculin sensitivity by its toxic effects on thymus-derived small lymphocytes (14, 20; Table 2). In this regard, why does AMS fail to reduce the magnitude of allergic pulmonary granulomatous responses? It is conceivable that sensitive alveolar macrophages armed with cytotoxic phagocytic antibody (17) proliferate in response to specific antigen so that granulomas could be self-sustaining, provided that appropriate antibody-producing lymphocytes were present and were resistant to or protected from AMS.

The fact that cells from allergic granulomas are inhibited from migrating in the presence of antigen (6) suggests that these cells possess a delayed sensitivity response. If tuberculin sensitivity is an important mediator of allergic granuloma formation, the lymphocytes involved would have to be protected from the cytotoxic effects of AMS. An intravenous injection of BCG cells in oil causes the formation of many small granulomata distributed in several organs, such as the lungs, spleen, bone marrow, and liver. In this situation, it is possible that AMS does not effectively gain access to target cells because they are sheltered within these small nonvascular granulomatous foci. After challenge to produce the accelerated granulomatous response, such primary granulomata could shed large numbers of cells into the blood stream, and these cells would be rapidly filtered out by the lungs and arranged in new foci before damage by AMS could take place. Accordingly, cells of primary granulomata proliferating in the lungs, as well as in extra-pulmonary sites, could be the progenitors of the accelerated granulomatous reaction which takes place in the lungs after a challenge dose of BCG cells.

Different antigenic components of the tubercle bacillus may be responsible for the induction of tuberculin sensitivity and granulomatous responses. Bekierkunst et al. (1, 2) showed that cord factor, but not Wax D, from mycobacteria could induce granuloma formation in mice; these animals also exhibited increased resistance to challenge with the H37Rv strain of \textit{M. tuberculosis}. Raffel (18) demonstrated that Wax D and tuberculin protein components could induce delayed sensitivity in the absence of immunity. Youmans and Youmans (22) produced pulmonary granulomas in mice with the H37Ra strain of \textit{M. tuberculosis} and showed that increased immunity to the H37Rv strain was a function of the magnitude of the granulomatous response.

It is possible that allergic granulomas are
mediated by classical delayed sensitivity involving an antigenic component of the tubercle bacillus other than tuberculoprotein. However, if this were the case, AMS treatment should have suppressed the allergic granulomatous response, since it was highly effective against tuberculin sensitivity. In this regard, Reid and Mackay (19) observed a correlation between the extent of mycobacterium-induced granulomas and the intensity of the dermal tuberculin reaction in man. This relationship would be expected, because patients infected with *M. tuberculosis* are undoubtedly sensitive to antigenic components responsible for the induction of both dermal tuberculin sensitivity and granuloma formation.

Collectively, these data provide additional evidence in support of the concept that dermal tuberculin sensitivity and accelerated pulmonary granulomatous reactions involve different immunological mechanisms.

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**LITERATURE CITED**