Relationship of BCG-Induced Pulmonary Delayed Hypersensitivity to Accelerated Granuloma Formation in Rabbit Lungs: Effect of Cortisone Acetate

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The role of local pulmonary delayed hypersensitivity in accelerated pulmonary granuloma formation was investigated using cortisone acetate, an immunosuppressive drug that appears to preferentially eliminate committed lymphocytes at appropriate doses. Data are presented showing that cortisone acetate suppressed local pulmonary delayed hypersensitivity at the time of and subsequent to challenge with BCG. Furthermore, cortisone damage appeared to involve primarily committed lymphocyte populations since the defect was repairable with sensitized spleen cells, an unlikely source of macrophage precursors.

An accelerated pulmonary granulomatous response (AGR) can be elicited in the lungs of BCG-sensitized rabbits by an intravenous (i.v.) challenge with BCG suspended in saline (8, 17, 18). Initial studies in this laboratory revealed that there is no correlation between dermal delayed hypersensitivity (DH) and the capacity of an animal to undergo an AGR, because sensitized rabbits desensitized with intraperitoneal doses of old tuberculin, until they were skin-test negative, were still capable of developing an AGR when challenged i.v. with BCG in saline (8). This study also suggested that the AGR was independent of a DH mechanism. Later studies revealed that cells recovered from the lungs of sensitized (rabbits displaying a chronic pulmonary granulomatous response [CGR]) and sensitized-challenged (the AGR) rabbits exhibited DH when evaluated by the migration inhibition test (4, 5). Furthermore, i.v. sensitization and challenge resulted in the induction of local pulmonary DH in the absence of detectable dermal DH, indicating that dermal DH is not necessary for the development of the AGR.

Since it is conceivable that the AGR is dependent upon local DH existing in the lung and perhaps other sites such as the spleen and bone marrow, we investigated the effect of cortisone acetate on pulmonary DH and accelerated pulmonary granuloma formation. Cortisone acetate, at appropriate doses, appears to preferentially eliminate committed lymphocytes, the cells which mediate DH (21, 22). If accelerated pulmonary granuloma formation is dependent upon local DH, an immunosuppressant capable of damaging the functional capacity of or eliminating committed lymphocytes should also produce depression of the accelerated granulomatous response.

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MATERIALS AND METHODS

Animals. White New Zealand, outbred rabbits of either sex weighing 1.5 to 2.5 kg were used throughout the experiments. They were maintained in the laboratory animal facility of the Bowman Gray School of Medicine.

Microorganisms. The Bacillus Calmette Guérin (BCG) strain of Myobacterium bovis was grown on Proskauer and Beck broth. The organisms were harvested, heat-killed by autoclaving, washed in sterile water, and lyophilized. Killed BCG vaccine for sensitization was prepared by grinding the organisms in a mortar with a pestle for at least 5 min in light mineral oil (Marcol 52, Humble Oil Company). The final concentration of the suspension was adjusted to 1.0 mg of organisms per ml of oil. Vaccine for challenge was prepared similarly except that the

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organisms were ground on 0.15 M NaCl and adjusted to a final concentration of 5.0 mg of organisms per ml of saline. Both vaccines were sonically treated in a Cole-Parmer ultrasonic cleaner (Cole-Parmer Instrument and Equipment Company, Chicago, Ill.) for approximately 15 min just prior to inoculation.

Induction, elicitation, and quantification of pulmonary granulomas and pulmonary delayed hypersensitivity. Rabbits were sensitized by the injection (marginal ear vein) of 100 μg of killed BCG suspended in 0.1 ml of light mineral oil (Marcol 52). Such animals develop chronic pulmonary granulomas in 2 to 3 weeks and local pulmonary DH can be demonstrated by the migration inhibition test (5). The AGR was elicited by challenging sensitized rabbits i.v. with 5.0 mg of BCG in 1.0 ml of 0.15 M NaCl. It was assessed 4 days later. These procedures have been described previously (17, 18).

Pulmonary DH was evaluated by the direct migration inhibition test (5). Lung cells were removed by means of a lavage technique with Hanks balanced salt solution (19). The cells were washed once and packed cells were suspended in 10 volumes of medium 199 (Grand Island Biological Company) containing 20% fetal bovine serum (Flow Laboratories), 100 units of penicillin G per ml, 100 μg of streptomycin sulfate per ml, 0.2 mM L-glutamine, and sufficient 8.0% NaHCO₃ to attain a pH of 7.0. Either no antigen (migration control) or 10 μg of PPD per ml (test) was added to separate fractions of the cell suspensions. Fractions were inoculated into 1.0- to 1.2-mm capillary tubes and packed by centrifugation (50 x g for 5 min). The capillary tubes were severed at the cell-supernatant fluid interface and loaded into separate Mackaness-type chambers. Test chambers were filled with TC-199 plus 10 μg of PPD per ml, and control chambers were filled with TC-199 without antigen. The cells were allowed to migrate onto glass surfaces at 37 C for 30 h; they were magnified approximately 10 times with a Bower 35-mm slide projector. The results are expressed in relative migration units; one unit is equal to a 5-mm² area.

Pulmonary granulomas were quantified, as previously described (17, 18). In addition, lung cells were collected and the packed cell volumes were estimated as an approximation of the increase in cellularity due to BCG-induced chronic inflammation.

Treatment of rabbits with cortisone acetate. Cortisone acetate (Cortone, Merck, Sharp and Dohme) was obtained as a saline suspension at a concentration of 50 mg/ml. At each injection interval, each rabbit received 100 mg of cortisone acetate contained in 2.0 ml of 0.15 M NaCl intraperitoneally. No indications of toxicity were noted throughout the experiments.

Repair of cortisone damage with spleen cells from sensitized rabbits. Donor animals were sensitized i.v. 3 weeks previously with 100 μg of killed BCG in 0.1 ml of light mineral oil (Marcol 52). Spleens were removed and teased apart with a syringe, needle, and forceps in minimal essential medium (Grand Island Biological Company) containing 5% normal rabbit serum, 0.2 mM L-glutamine, 100 units of penicillin G per ml, 100 μg of streptomycin sulfate per ml, and sufficient 8.0% NaHCO₃ to attain a pH of 7.0. The free cells were passed through a 200-mesh wire screen prior to injection into recipients. The recipients were injected i.v. with 500 units of heparin, approximately 30 min prior to the injection of sensitized spleen cells, in an attempt to prevent the infused cell population from clumping. For injection into recipients, spleen cells were suspended in 15.0 ml of the minimal essential medium just described and administered slowly i.v. Each recipient received one donor equivalent of spleen cells. No signs of toxicity were detected during the 4-day interval that sensitized spleen cells remained in the recipients.

RESULTS

Granuloma indices and delayed hypersensitivity. Typical values for BCG-induced pulmonary granuloma formation in rabbits are shown in Fig. 1. By definition, the baseline granuloma index of normal rabbits is 1.0 (17, 18). The granuloma index of rabbits sensitized i.v. 3 weeks previously with 100 μg of killed BCG in oil is approximately 2.2, and those sensitized and challenged is about 4.4. These differences are also reflected in the cell yields from the lungs of these animals. The morphological features of lungs of rabbits with chronic and accelerated pulmonary granulomas have been described in previous publications from this laboratory (11, 20). It has been established that the granuloma index is a function of an increase in cellularity in the organ. Accordingly, an increase in the granuloma index from 1.0 (normal lung) to 2.0 indicates that the organ has approximately doubled in weight.

Typical values for pulmonary delayed hypersensitivity are depicted in Fig. 1. As expected, pulmonary cells from sensitized rabbits (the CGR) exhibited typical migratory inhibition patterns. However, cells from the lungs of sensitized-challenged rabbits (the AGR) exhibited migration inhibition in the absence of PPD; the addition of PPD did not produce additional migration inhibition. The inhibition of cells from lungs undergoing a granulomatous response in the absence of antigen has been termed the autoinhibition phenomenon (4, 5). Since these animals had received a relatively large challenge dose of BCG just 4 days prior to the evaluation of DH, it was conceivable that components of BCG equivalent to 10 μg of PPD per ml persisted for this short period of time and were recovered along with the cells. If this assumption is correct, the autoinhibition phenomenon in sensitized-challenged animals should disappear at some future interval when most of the BCG in the lung had been catabolized. Figure 2 shows the kinetics of DH
in the lung following sensitization and challenge. When DH was evaluated 6 or more days after challenge, migration patterns resembled those seen in the chronic pulmonary response (3-week postsensitization) in accordance with the above prediction. The data are consistent with the hypothesis that sufficient tuberculoprotein to mediate DH was recovered from the lungs 2 to 4 days following challenge with 5 mg of BCG.
Suppression of pulmonary delayed hypersensitivity and the accelerated pulmonary granulomatous response with cortisone acetate. Figure 3 shows that 200 mg (approximately 100 mg of body weight per kg) of cortisone acetate will markedly suppress pulmonary DH in sensitized (CGR) rabbits. Furthermore, 200 mg of the drug prior to challenge and 100 mg after challenge significantly inhibited DH in sensitized-challenged (AGR) rabbits. It is also obvious that these doses of cortisone did not detectably alter the migrating capacity of macrophages. Thus, cortisone acetate altered the functional capacity of or eliminated committed lymphocytes, but not the migrating capacity of sensitized-challenged (AGR) rabbits. It is also possible because pulmonary DH was suppressed at and subsequent to the time of challenge with BCG. However, it is also possible that cortisone acetate interfered with replicating macrophage precursors disrupting the traffic flow of mature macrophages into the lung.

Repair of cortisone damage with spleen cells from sensitized rabbits. Since the accelerated granulomatous response could have been suppressed because of cortisone elimination of committed lymphocytes or damage to macrophage precursors, we attempted to repair cortisone damage with sensitized spleen cells, an unlikely source of macrophage precursors and presumably a source of committed lymphocytes following i.v. sensitization (21, 22). Figure 4 demonstrates that it is possible to repair cortisone damage with sensitized spleen cells. In this case, the animals were not treated with cortisone acetate following challenge with BCG to avoid damage to the new infused population of sensitized spleen cells. However, appropriate controls (Fig. 4) demonstrated that 200 mg of cortisone acetate added prior to challenge with BCG significantly inhibited the development of the accelerated response. Figure 4 also shows that sensitized spleen cells restored the capacity of cortisone-treated animals to display DH in vitro 4 days after challenge with BCG. These data suggest that local pulmonary DH is an essential component for the development of an accelerated pulmonary granulomatous response in rabbits.

DISCUSSION

The autonihilation phenomenon was first described by Galindo and Myrvik (4, 5) who proposed that it was due to residual antigen. This observation has been confirmed, and addi-

Fig. 3. The effect of cortisone acetate on BCG-induced pulmonary delayed hypersensitivity and granuloma formation in rabbits. Animals were sensitized with 100 μg of killed BCG followed by inoculation of 100 mg of cortisone acetate 18 and 20 days later. Some animals were evaluated for pulmonary DH and the chronic granulomatous response on day 21 (Dashed) and others were challenged with 5 mg of BCG. The challenged group was given 100 mg of cortisone acetate 2 days later; pulmonary DH and the accelerated granulomatous response were evaluated 4 days after challenge with BCG (Dotted). Control rabbits were sensitized (Black) and challenged (Gray) with BCG but received no cortisone acetate. For description of granuloma index, see Fig. 1.
Fig. 4. Repair of cortisone damage with spleen cells from sensitized rabbits. Animals were sensitized with 100 μg of killed BCG and divided into three groups: (■) One group was given 100 mg of cortisone acetate 18 and 20 days after sensitization followed by challenge with 5 mg of BCG and replacement therapy with $5 \times 10^2$ to $10^4$ spleen cells from sensitized rabbits on day 21. (□) Another group was sensitized and challenged 21 days later (controls) and (▲) a third group was sensitized, given cortisone acetate 18 and 20 days later, and challenged with BCG on day 21. Pulmonary DH and the accelerated granulomatous response were evaluated 4 days after challenge with BCG. For description of granuloma indices, see Fig. 1.

tional data are presented consistent with their initial hypothesis.

Cortisone acetate has been reported to preferentially eliminate committed lymphocytes in mice at doses similar to those used in the present study (on a weight basis) (21, 22). North observed that the drug suppressed the development of cell-mediated immunity to Listeria monocytogenes because macrophages failed to accumulate at sites of infection, presumably indirectly as a result of the selective elimination of committed lymphocytes (21). Furthermore, committed lymphocytes appeared to be selectively eliminated from the red pulp of the spleen of Listeria-infected mice because cortisone acetate substantially reduced the capacity of spleen cells from infected mice to transfer anti-Listeria immunity to normal recipients (22). In addition, cellular proliferation in the red pulp of the spleen was directly correlated with the intensity of delayed hypersensitivity (22), the immunological response largely responsible for anti-Listeria immunity (14). Because cortisone damage was repairable with sensitized spleen cells, it appeared that the macrophage component of anti-Listeria immunity remained intact following cortisone treatment (21, 22). The evidence is convincing that cortisone, at appropriate doses, preferentially eliminates committed lymphocytes in mice.

The present studies have demonstrated that cortisone acetate can markedly suppress pulmonary DH and accelerated granuloma formation in rabbits. The data indicate that cells removed from the lungs of cortisone-treated rabbits are not inhibited from migration in the presence of antigen; therefore, the migrating capacity of macrophages does not appear to be impaired. The drug lesion seems to be in the committed lymphocyte population, perhaps by the elimination of these cells from the lung. This is even more likely since cortisone damage was repairable with sensitized spleen cells, e.g., spleen cells from BCG-sensitized rabbits restored the capacity of cortisone-treated rabbits to develop an accelerated pulmonary response and to display delayed hypersensitivity. Since the lesion seems to be in the committed lymphocyte population, the accelerated granulomatous response is probably dependent upon a cell-mediated hypersensitivity reaction.

The immunological reaction in the lung probably generates lymphokines, among them a chemotactic factor and migration inhibition factor which are responsible for the accumulation and localization of blood-borne macrophages whose initial source is bone marrow (12, 13). The reason that the accelerated pulmonary granulomatous response in rabbits is dependent upon local pulmonary DH, but independent of dermal DH, is more understandable in view of the present knowledge about delayed hypersensitivity reactions. These reactions can be restricted to certain anatomical locations (4, 5, 6, 15, 23, 25). It seems certain that the i.v. route of sensitization is largely responsible for the compartmentalization of DH observed in these studies. Sensitization results in the induction of an intense pulmonary granulomatous response, in-
dindicating that antigen has been deposited in the lung. It is probable that this chronic inflammatory response is responsible for the sequestration of a considerable proportion of committed lymphocytes that are generated. These cells appear to have a predilection for inflammatory foci and do not appear to recirculate extensively from blood to lymph compartments (1, 9, 10, 16).

Katz et al. (7) have reported that graft-versus-host reactions in guinea pigs nonspecifically induced an expanded population of carrier-specific cells in a hapten-carrier system, so it is possible that a graft-versus-host response in the experiments reported here could have expanded the population of residual host-committed lymphocytes which were not eliminated by cortisone treatment. A more likely explanation would be that a host-versus-graft reaction destroyed a portion of the infused committed lymphocyte population. However, delayed hypersensitivity in lung cells from cortisone-treated, allogeneic spleen cell-repaired rabbits was detectable 4 days subsequent to adoptive transfer, indicating that sufficient committed lymphocytes were functional to inhibit the migration of macrophages (Fig. 4).

Pulmonary granulomas induced by the eggs of Schistosoma mansoni in mice also seem to be dependent upon a delayed hypersensitivity mechanism (2, 3, 24). The present study suggests that BCG-induced pulmonary granulomas are another manifestation of delayed hypersensitivity; therefore, they differ from dermal DH primarily in nomenclature but both seem to represent chronic immunological inflammation.

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