INFECTION AND IMMUNITY, May 1973, p. 743-746
Vol. 7, No. 5
Copyright © 1973 American Society for Microbiology
Printed in U.S.A.

Specificity of a BCG-Induced Pulmonary Granulomatous Response in Rabbits

VERNON L. MOORE[1], QUENTIN N. MYRVIK, AND EVA S. LEAKE

Department of Microbiology, The Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

Received for publication 29 January 1973

The specificity of Bacillus Calmette Guérin (BCG)-induced accelerated pulmonary granuloma formation has been evaluated in rabbits by cross sensitization-challenge experiments by using another granulomagenic organism, *Corynebacterium granulosum*. BCG-sensitized rabbits responded to challenge with homologous but not heterologous antigen, indicating that BCG-induced accelerated granuloma formation displays specificity characteristic of immunological reactions. These differences were also observed in local pulmonary delayed hypersensitivity, as determined by the migration inhibition test. The relationship between local pulmonary delayed hypersensitivity and the accelerated granulomatous response is discussed.

Pulmonary granulomas can be induced in rabbits by the intravenous (i.v.) injection of Bacillus Calmette Guérin (BCG) suspended in light mineral oil (9, 12, 13). The full expression of these granulomas requires 2 to 4 weeks; thus, the reaction has been termed a chronic pulmonary granulomatous response (CGR; references 9, 12). An accelerated pulmonary granulomatous response (AGR) can be elicited in sensitized rabbits (those undergoing the CGR) by an i.v. challenge with BCG suspended in saline. In contrast to the 2- to 4-week interval required for the full expression of the CGR, a maximal AGR develops within 4 days (9, 12, 13). Local pulmonary delayed hypersensitivity (DH) can be detected in both the CGR and the AGR by the migration inhibition test; however, dermal DH cannot be detected in animals exhibiting either of these pulmonary reactions (5; V. L. Moore and Q. N. Myrvik, unpublished data). The morphological features of these chronic inflammatory reactions in the lung have been described (9, 15).

The AGR resembles a dermal DH reaction in tempo and may represent the counterpart of cutaneous DH expressed in another organ.

The purpose of the present study was to assess the specificity of the BCG-induced accelerated pulmonary response by using another granulomagenic organism, *Corynebacterium granulosum* (2).

[1] Present address: Research Service, Veterans Administration Center, Wood, Wisconsin 53193.

This paper represents a portion of a Ph.D. dissertation submitted to the Graduate Faculty of the Bowman Gray School of Medicine, Department of Microbiology, Winston-Salem, North Carolina.

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 BCG strain of *Mycobacterium bovis*, the Battey bacillus, and the Bostrum strain of *Mycobacterium kansasii* were grown in Proskauer and Beck's broth. The organisms were harvested, heat-killed by autoclaving, washed in sterile water, and lyophilized. Killed *C. granulosum* was obtained from the Wellcome Research Laboratories, Beckenham, Kent, England. The organisms were washed in sterile water and lyophilized. Killed mycobacterial vaccines for sensitization were prepared by grinding the organisms in light mineral oil (Marcol 52, Humble Oil Co.) for at least 5 min with a mortar and pestle. The final concentration of the suspensions was adjusted to 1.0 mg of organisms per ml of oil. The killed *C. granulosum* vaccine used for sensitization was prepared similarly, except that the final concentration was adjusted to 10 mg of organisms per ml of oil. Suspensions of killed organisms for the elicitation of the accelerated granulomatous response were prepared by grinding the organisms with a mortar and pestle in 0.15 M NaCl. BCG, Battey bacilli, and *M. kansasii* were adjusted to a final concentration of 5.0 mg of organisms per ml, and *C. granulosum* was adjusted to a final concentration of 20 mg of orga-

nisms per ml. All vaccines were sonicated in a Cole-Parmer ultrasonic cleaner (Cole-Parmer Instrument and Equipment Co., Chicago, Ill.) for at least 15 min just prior to their inoculation into rabbits.

Induction, elicitation, and quantification of pulmonary granulomas and pulmonary delayed hypersensitivity. For sensitization with BCG, Battey, or *M. kansasii*, rabbits were injected in the marginal ear vein with 100 μg of the organisms suspended in 0.1 ml of oil (9, 12, 13). In the case of sensitization with *C. granulosum*, rabbits were given 1.0 mg of the organisms suspended in 0.1 ml of oil in the marginal ear vein and 25 mg of organisms suspended in 2.5 ml of oil into five subcutaneous sites: two inguinal areas, two axillary areas, and the nuchal area of the neck. Accelerated granulomatous responses were elicited by challenging sensitized rabbits i.v. with 5.0 mg of BCG, Battey, and *M. kansasii* or 20 mg of *C. granulosum*, all suspended in 1.0 ml of 0.15 M NaCl. CGR were evaluated 3 weeks after sensitization, and AGR were evaluated 4 to 6 days subsequent to challenge.

Pulmonary DH was evaluated by the direct migration inhibition test. Lung cells were removed by means of a lavage technique with Hanks' balanced salt solution (14). The cells were washed once and suspended in medium 199 (Grand Island Biological Co.) containing 20% fetal bovine serum (Flow Laboratories), 100 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml, 0.2 mM L-glutamine, and sufficient 8% $NaHCO_3$ to attain a pH of 7.0. Either no antigen (migration control in medium 199), 10 μg of BCG per ml in the medium 199 described above, or 20 μg of *C. granulosum* per ml in medium 199 was added to separate Mackaness-type chambers. (These particulate antigens were dispersed in suspension by grinding with a mortar and pestle.) The cells were allowed to incubate at 37 C for 30 h. The migration patterns were magnified approximately 10 times in a Bower 35 mm slide projector. The results are expressed in relative migration units. A migration unit is equal to a 5 mm^2 area.

Pulmonary granulomas were quantified, as previously described (12, 13). In addition, lung cells were collected, and the packed cell volumes were estimated.

RESULTS

BCG- and Corynebacterium-induced pulmonary granulomas. The pulmonary granulomatous response of rabbits to killed *C. granulosum* and to killed BCG is shown in Fig. 1. In contrast to the CGR produced by BCG vaccination, sensitization with *C. granulosum* did not result in a detectable increase in lung weight or cellularity. Nevertheless, sensitization did occur because challenge with 20 mg of *C. granulosum* resulted in a substantial pulmonary granulomatous response when evaluated 6 days later. Normal rabbits challenged with 20 mg of *C. granulosum* exhibited no detectable pulmonary granulomatous response within 6 days, indicating that sensitization is necessary for an accelerated response. Another difference between *C. granulosum* and BCG-induced pulmonary granulomas is the magnitude of the response. Even though greater quantities of *C. granulosum* were used in sensitization (26 mg) and challenge (20 mg), the BCG-induced accelerated response is about twofold greater. Despite these differences, the values obtained indicated that cross sensitization-challenge experiments could be used to assess the specificity of the BCG-induced accelerated pulmonary granulomatous response.

Lung sections from rabbits displaying *C. granulosum*-induced accelerated granulomatous responses were indistinguishable histologically from those of BCG-induced chronic or accelerated responses (15).

Cross sensitization-challenge experiments. The results of the cross sensitization-challenge experiments indicate that rabbits sensitized with BCG do not undergo an accelerated granulomatous response when challenged with 20 mg of *C. granulosum*, even though homologous sensitization and challenge with *C. granulosum* resulted in a significant increase in the granuloma index (Fig. 2). In fact, the granuloma indices of BCG-sensitized, *C. granulosum*-challenged rabbits were not significantly increased over that of animals with BCG-induced chronic granulomas.

Figure 2 also indicates that rabbits sensitized with BCG develop a small, but significant, accelerated granulomatous response when challenged with 5.0 mg of the Battey bacillus or the Bostrum strain of *M. kansasii*. However, this

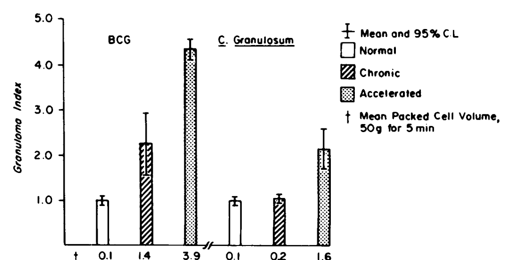

FIG. 1. *Characterization of BCG- and C. granulosum-induced pulmonary granulomas in rabbits. Animals were sensitized with 26 mg of C. granulosoum or 100 μg of BCG, and the chronic granulomatous response was evaluated 3 weeks later. Accelerated responses were elicited by challenging animals at 3 weeks with 20 mg of C. granulosum or 5 mg of BCG. The accelerated granulomatous response to BCG was assessed 4 days after challenge, and response to C. granulosum was assessed 6 days after challenge. Granuloma index = (lung wt/body wt test animal) (2.5 × 10²); (references 12, 13).*

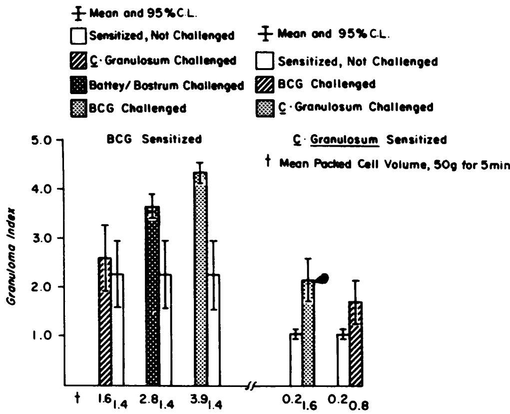

FIG. 2. *Specificity of the BCG- and C. granulosum-induced pulmonary granulomatous responses in rabbits. BCG sensitization was followed 3 weeks later by challenge with 20 mg of C. granulosum* (▨), *5 mg of Battey bacillus or the Bostrum strain of M. kansasii* (■), *or 5 mg of BCG* (▩). *C. granulosum sensitization was followed 3 weeks later by challenge with BCG* (▨) *or C. granulosum* (▩). *Accelerated granuloma formation was evaluated 6 days after challenge. For explanation of granuloma index, see Fig. 1.*

response is not as great as homologous sensitization and challenge with BCG. Presumably, this is because some, but not all, antigenic determinants on tuberculoproteins are shared by these different species of mycobacteria.

The results were not as definitive when *C. granulosum*-sensitized rabbits were challenged with 5.0 mg of BCG. In this case, there was a slight increase in the granuloma indices which is comparable to the response of normal rabbits challenged with 5.0 mg of BCG (V. L. Moore and Q. N. Myrvik, unpublished data). Furthermore, the granuloma indices of *C. granulosum*-sensitized, BCG-challenged rabbits are not as great as homologous sensitization and challenge with *C. granulosum* ($P < 0.05$).

Since BCG-sensitized rabbits did not undergo an accelerated granulomatous response upon challenge with *C. granulosum*, it seems reasonable to assume that the two organisms do not share an adequate number of antigenic determinants to permit sensitization and cross elicitation of allergic pulmonary granulomas. These differences should be reflected in pulmonary DH; i.e., cells from BCG-sensitized rabbits should not display DH to an in vitro challenge with *C. granulosum*. The results of these experiments show that pulmonary cells from BCG-sensitized rabbits were not inhibited from migrating in the presence of *C. granulosum* but were inhibited in the presence of BCG (Fig. 3). It is also obvious that pulmonary cells from *C. granulosum*-sensitized rabbits migrated well in the presence of BCG or *C. granulosum*. The most probable reason for failure to detect DH in *Corynebacterium*-sensitized rabbits is that sensitization alone did not cause a significant inflammatory response in the lung (Fig. 1).

Based on these data, it is concluded that the BCG-induced pulmonary granulomatous response in rabbits exhibits specificity.

DISCUSSION

In cross sensitization-challenge experiments, rabbits sensitized i.v. with 100 μg of killed BCG in oil failed to develop an accelerated pulmonary granulomatous response when challenged with *C. granulosum* (2). Notably, homologous sensitization and challenge with *C. granulosum* resulted in the production of an accelerated pulmonary response, albeit of less intensity than the BCG-induced pulmonary response. Furthermore, pulmonary cells from BCG-sensitized animals were not inhibited from migrating when challenged in vitro with *C. granulosum*, although challenge with homologous antigen resulted in inhibition of migration. Therefore, BCG-induced pulmonary granulomas exhibit specificity, the hallmark of immunological reactions.

The finding that BCG-sensitized rabbits undergo a small, but significant, accelerated granulomatous response when challenged with different species of mycobacteria is consistent with the concept of cross-reacting antigens within various species of a given genus. It is well recognized that human subjects injected with atypical mycobacteria develop small tuberculin reactions when challenged with PPD derived from *Mycobacterium tuberculosis*. These data also suggest that an immunological response to tuberculoprotein is essential for the production of allergic pulmonary granulomas.

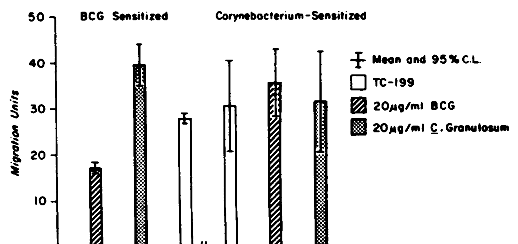

FIG. 3. *Evaluation of pulmonary delayed hypersensitivity in BCG- and C. granulosum-induced chronic granulomas in rabbits. Animals were sensitized with killed BCG or C. granulosum. Three weeks later they were evaluated for pulmonary DH in vitro by challenging pulmonary cells with 20 µg of BCG* (▨) *or C. granulosum* (▩) *per ml.*

Previous studies from this laboratory have indicated that dermal DH is not essential for the expression of the accelerated pulmonary granulomatous response (6, 12). However, Galindo and Myrvik (5) showed that cells from BCG-induced chronic and accelerated pulmonary granulomas display DH when evaluated by the migration inhibition test. Furthermore, local pulmonary DH can exist in the absence of detectable dermal DH, again demonstrating that dermal DH is not essential for the production of an accelerated pulmonary granulomatous response (5). Since DH reactions can exist in discrete anatomical foci (5, 10, 16, 18), probably because committed lymphocytes (the cellular mediators of DH) do not recirculate extensively from vascular to lymph compartments (1, 7, 8, 11), it is now understandable why dermal DH is not necessary for the development of allergic pulmonary granulomas. Since the i.v. route of sensitization induces a marked chronic inflammatory response in the lungs (the CGR), committed lymphocyte populations are undoubtedly sequestered at this site of inflammation; thus, these cells are not available in the peripheral circulation to mediate dermal DH. This is consistent with the concept that committed lymphocytes home to sites of inflammation (7, 8, 11).

Presumably, the mechanism of accelerated pulmonary granuloma formation involves the interaction of antigen with putative antibody-like receptors on the surface of committed lymphocytes, resulting in the synthesis and elaboration of molecular mediators of DH such as a chemotactic factor, mitogenic factor, and migration inhibitory factor. These mediators could be responsible for the accumulation and localization of bone marrow-derived, blood-borne macrophages at the site of chronic allergic inflammation in the lung.

Schistosoma mansoni eggs induce accelerated pulmonary granulomas in mice that are specific for the inducing antigen (17). Furthermore, the evidence is convincing that the production of these granulomata is dependent upon DH (3, 4, 17). The data presented here, together with other data from this laboratory, support the concept that BCG-induced pulmonary granulomas are allergic in nature and dependent upon the induction of local DH reactions in the lung and possibly other sites.

ACKNOWLEDGMENTS

This research was supported, in part, by a grant from The North Carolina Tuberculosis and Respiratory Disease Association and by Public Health Training grant AI0268 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Coe, J. E., J. D. Feldman, and S. Lee. 1966. Immunologic competence of thoracic duct cells. I. Delayed hypersensitivity. J. Exp. Med. **123**:267-281.
2. Collet, A. J. 1971. Experimental stimulation of alveolar macrophage production by *Corynebacterium anaerobium* and its quantitative evaluation. J. Reticuloendothel. Soc. **9**:424-446.
3. Domingo, E. O., and K. S. Warren. 1967. The inhibition of granuloma formation around *Schistosoma mansoni* eggs. II. Thymectomy. Amer. J. Pathol. **51**:757-768.
4. Domingo, E. O., and K. S. Warren. 1968. The inhibition of granuloma formation around *Schistosoma mansoni* eggs. III. Heterologous antilymphocyte serum. Amer. J. Pathol. **52**:613-632.
5. Galindo, B., and Q. N. Myrvik. 1970. Migration inhibition response of granulomatous alveolar cells from BCG-sensitized rabbits. J. Immunol. **105**:227-237.
6. Kawata, H., Q. N. Myrvik, and E. S. Leake. 1964. Dissociation of tuberculin hypersensitivity as mediator for an accelerated pulmonary granulomatous response in rabbits. J. Immunol. **93**:433-438.
7. Koster, F. T., and D. D. McGregor. 1971. The mediator of cellular immunity. III. Lymphocyte traffic from the blood into the inflamed peritoneal cavity. J. Exp. Med. **133**:864-876.
8. Koster, F. T., D. D. McGregor, and G. B. Mackaness. 1971. The mediator of cellular immunity. II. Migration of immunologically committed lymphocytes into inflammatory exudates. J. Exp. Med. **133**:400-409.
9. Leake, E. S., and Q. N. Myrvik. 1968. Changes in morphology and in lysozyme content of free alveolar cells after the intravenous injection of killed BCG in oil. J. Reticuloendothel. Soc. **5**:33-53.
10. Mackaness, G. B. 1971. Delayed hypersensitivity and the mechanism of cellular resistance to infection, p. 413-424. *In* B. Amos (ed.), Progress in immunology, Academic Press Inc., New York.
11. McGregor, D. D., F. T. Koster, and G. B. Mackaness. 1971. The mediator of cellular immunity. I. The life-span and circulation dynamics of the immunologically committed lymphocyte. J. Exp. Med. **133**:389-399.
12. Moore, V. L., and Q. N. Myrvik. 1970. Effect of antimacrophage serum on dermal tuberculin sensitivity and allergic pulmonary granuloma formation in rabbits. Infect. Immunity **2**:810-814.
13. Moore, V. L., Q. N. Myrvik, and M. Kato. 1972. Role of cord factor (trehalose-6,6′-dimycolate) in allergic granuloma formation in rabbits. Infect. Immunity **6**:5-8.
14. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. J. Immunol. **86**:128-132.
15. Myrvik, Q. N., E. S. Leake, and S. Oshima. 1962. A study of macrophages and epithelioid-like cells from granulomatous (BCG-induced) lungs of rabbits. J. Immunol. **89**:745-751.
16. Schlossman, S. F., H. A. Levin, R. E. Rocklin, and J. R. David. 1971. The compartmentalization of antigen-reactive lymphocytes in desensitized guinea pigs. J. Exp. Med. **134**:741-750.
17. Warren, K. S., E. O. Domingo, and R. B. T. Cowan. 1967. Granuloma formation around Schistosome eggs as a manifestation of delayed hypersensitivity. Amer. J. Pathol. **51**:735-756.
18. Yamamoto, K., and R. L. Anacker. 1970. Macrophage migration inhibition studies with cells from mice vaccinated with cell walls of *Mycobacterium bovis* BCG: characterization of the experimental system. Infect. Immunity **1**:587-594.