BCG Antibody Profiles in Tuberculoid and Lepromatous Leprosy

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In sera from 12 patients with polar tuberculoid leprosy, 12 with subpolar tuberculoid leprosy, and 16 with lepromatous leprosy were demonstrated a total number of 125 anti-BCG precipitins by means of crossed immunoelectrophoresis with intermediate gel. Up to 14 different precipitins were found in individual sera, and the complexity in antibody response was higher than previously realized. The specificity of 69% of the antibodies was defined, and these antibodies were titrated in three arbitrary titer units. A highly significant difference (P < 0.002) was found in antibody response between the tuberculoid and the lepromatous group. Due to simplicity, sensitivity, and high resolution, the method used is a promising tool for providing exact data to be used as guidelines for purification of important individual mycobacterial antigens. The need for reference antisera is emphasized.

A considerable body of evidence suggests that the expression of immunity to intracellular bacteria such as Mycobacterium leprae depends on cell-mediated immune reactions. Humoral antibody responses to M. leprae, on the other hand, appear to be involved in immunological complications of leprosy such as erythema nodosum leprosum (8, 18) and may also be involved in the specific defect of cell-mediated immunity (CMI) in lepromatous leprosy, although they do not appear to operate through the mechanism of immunological enhancement (5, 6). To throw light on these and other immunological phenomena in leprosy, the study of circulating antibodies to M. leprae and their corresponding antigens appears to be of considerable interest. The main hindrances for exact studies of this kind have been the lack of appropriate and enough material from M. leprae for antigen production and lack of sensitive methods with high resolution for complex antigen-antibody systems. In the past these problems have been partly solved by utilizing antigen from mycobacteria related to M. leprae and diffusion-in-gel ad modum Ouchterlony. Rees et al. (11) observed that lepromatous in contrast to tuberculoid sera contained strong antibodies against M. tuberculosis, and that the amount of antibody decreased upon treatment. These observations suggested that the antibody response is proportional to the antigenic load, i.e., the amount of bacilli in the tissues. Norlin et al. (12) studied the reactivity of leprosy sera with different cross-reacting mycobacteria and found that M. smegmatis, M. kansasii and M. phlei gave most precipitin bands and that many lepromatous sera contained antibodies to the beta and delta antigens which were common to several mycobacterial species. Navalkar (10) extended these studies and showed that M. leprae in addition to the beta and delta contained three antigens which were specific for this species. In the first antibody study (B. Myrvang, C. M. Feek, and T. Godal, manuscript in preparation) using the Ridley-Jopling classification of leprosy (13, 14), it was demonstrated that the proportion of leprosy sera with precipitins increased gradually from the tuberculoid towards the lepromatous end of the spectrum. The precipitin formation is thus inversely related to the CMI response to M. leprae (9; Myrvang et al., manuscript in preparation).

The recent development of the sensitive and highly resolving quantitative immunoelectrophoretic methods (4) makes more precise studies of even very complex antigen-antibody systems possible. In the present report we introduce the crossed immunoelectrophoresis with intermediate gel (1) for investigation of antibodies in leprosy.

MATERIALS AND METHODS

Leprosy patients and sera. Sera were obtained from selected leprosy patients at the All Africa Leprosy and Rehabilitation Training Centre
BCG antibody profiles in leprosy

(ALERT). The great majority were untreated. Diagnosis was in each case based on clinical and histopathological examination, and classification was made according to the criteria of Ridley and Jopling (13) and Ridley and Waters (14). Criteria for distinction between polar tuberculoid (TT) and subpolar tuberculoid (TT/BT) cases have been described elsewhere (9). Sera from 12 TT, 12 TT/BT, and 16 patients with lepromatous leprosy (LL) were examined in the present study. One of the LL patients suffered from erythema nodosum leprosum. Venous blood samples were obtained, and the sera were stored at −25 °C for periods of up to 3 years before examination.

BCG antigen. BCG bacilli, strain Bergen, were supplied by the BCG Laboratory, Bergen, Norway, as lyophilized vaccine containing 20 mg (wet weight) of bacilli per ampoule. The contents of 10 ampoules were suspended in 0.15 M NaCl and sonically treated in a Branson sonifier (model B-12) for 30 min at an output effect of 100 W. The sonic extract was centrifuged at 10,000 × g for 20 min and the supernatant was collected. The protein concentration of the supernatant determined by a modified Folin-Ciocalteau method (7) was 0.8 mg/ml, using human immunoglobulin G as the standard. The supernatant was stored at −20 °C.

Rabbit anti-BCG sera. Four rabbits were immunized according to the following protocol. Each injection was divided into three doses, two given intramuscularly in each hind leg and one given subcutaneously in the neck. Initially 2 ml of a suspension of BCG, strain Bergen, in incomplete Freund adjuvant, containing 4 mg (wet weight) of bacilli per ml, was injected into each rabbit. After 3.5 weeks, 1 ml of sonically treated BCG containing 12 mg (wet weight) of bacilli per ml was given. This injection was repeated at 8 and 13 weeks after the first injection. Ten days after the fourth injection the animals were bled from the ear. Sera were stored at −20 °C.

Crossed immunoelectrophoresis. For testing the rabbit anti-BCG sera, crossed immunoelectrophoresis was carried out in a micro-modification using glass plates measuring 5 by 5 cm as described by Weeke (17). The electrophoreses were performed using the DL immunoelectrophoresis equipment (Dansk Laboratoriumsbyr A, Copenhagen, Denmark) and 1% (wt/vol) agarose gel (batch AGS 091 A. Litex, Glostrup, Denmark) in barbital buffer (pH 8.6), ionic strength 0.02. The temperature of the cooling water was 12 C, and the gel thickness was 1 mm. The amount of BCG-antigen applied was 10 μl, and the separation (first-dimension electrophoresis) was carried out with a potential gradient of 10 V cm⁻¹ for 25 min. The second-dimension electrophoresis was performed with a potential gradient of 2 V cm⁻¹ overnight; the second dimension gel contained 12.5 μlitters of rabbit serum per ml.

After electrophoresis the plates were pressed under filter paper and soft blotting paper (20 min), dried under a hair drier (15 min), stained with Coomassie brilliant blue R (Michrome no. 1137, Edward Gurr Ltd., London) (5 min) destained (5 min, three times), and finally dried again (10 min).

Crossed immunoelectrophoresis with intermediate gel. In this modification of crossed immunoelectrophoresis a gel with patient serum is interposed between the first-dimension gel and the gel containing the rabbit antibodies (reference gel) against the antigens. This allows a direct comparison of rabbit and patient antibodies. The theoretical and technical details of this method have been recently reviewed (1).

In the present study the immunoelectrophoreses were carried out in a micro-modification using glass plates (5 by 5 cm) as described above. The dimensions of the gels appear in Fig. 2A. In the test plates the intermediate gels contained 16.7 μlitters of patient serum per cm²; in the control plates the same amount of control serum obtained from a healthy Danish person who had never suffered from leprosy or tuberculosis was used (she was BCG vaccinated as a child and had a positive Mantoux reaction). The reference gel contained 16.7 μlitters of rabbit anti-BCG serum per cm². In our set-up (1), eight first-dimension electrophoreses were run simultaneously in the same apparatus: one was used for a control plate, the rest were used for test plates. First- and second-dimension electrophoreses and the pressing, washing, and staining of the plates were carried out as described above.

To minimize bias, the sera were randomized and coded prior to the investigation: the code was broken after the evaluation of the plates. Examination of the precipitate patterns was performed by looking for differences between the test plate and the corresponding control plate; the differences were interpreted by the method of Axelsen (1). The identified human precipitins were titrated semi-quantitatively by comparing each precipitate of the test plates with corresponding precipitates of plates in which the reference anti-BCG serum was incorporated in the intermediate gel. The human titers were expressed in arbitrary units referring to two concentrations of the reference rabbit anti-BCG in the intermediate gel: titer 3 ≥ 8.3 μlitters/cm² > titer 2 ≥ 0.83 μlitters/cm² > titer 1 > titer 0 = 0 μlitters/cm² (control plate).

Statistical analysis. The significance of the difference, in number of precipitins per serum, between the three groups of patients was established by using the Mann-Whitney U test. P, the probability of difference being due to chance, was obtained from tables for U (16).

RESULTS

Crossed immunoelectrophoresis of the BCG antigen against sera from each of four rabbits immunized with this antigen showed that one of the rabbits had a far stronger precipitin response than the other animals, i.e., more precipitins and higher titers. It was therefore decided to use this antisemum as reference for the study of human antibodies.

Figure 1A shows the crossed immunoelectrophoresis with control serum in the intermediate gel and rabbit anti-BCG in the reference gel. It appears (Fig. 2A) that 19 precipitates could be seen in the control plate although some were very faint. Precipitates numbers 11, 16, and 17
were "fuzzy" and antigen number 16 was composed of at least two antigenically related components since a spur was observed at the anodic "leg" of the precipitate.

Serum from a patient with lepromatous leprosy was included in the intermediate gel of the test plate of Fig. 1B. Two strong precipitins were identified as anti-17 and anti-19 (Fig. 2). Weak antibodies with specificities for antigen numbers 5, 6, 12, and 18 were demonstrated as deflections (1) of the reference precipitates. The three distinct precipitates marked with dots represent human antibodies with no counterpart in the reference antiserum and therefore are "unidentified" in terms of the rabbit reference antibody specificities. The unidentified precipitins were not analyzed in detail but some gave very characteristic precipitates, e.g., the most anodic one of Fig. 1B which was present in several sera as judged from form and position.

A total number of 125 antibodies were found in the 40 sera from the patients: 6 in the 12 TT sera, 17 in the 12 TT/BT sera, and 102 in the 16 LL sera. Figure 3A shows the distribution of patients according to the total number of antibodies found in each serum (criterium A): the "total" number of antibodies includes regular precipitins seen in the intermediate gel as well as low-titer antibodies (titer 1) which were only demonstrated by deflections of the reference precipitates.

The specificity of 86 of the 125 human antibodies (69%) could be defined in terms of the 19 rabbit precipitins: 5 in the TT sera, 13 in the TT/BT sera, and 68 in the LL sera. Figure 3B shows the distribution of patients by the number of these identified antibodies per serum.

The remaining 39 human antibodies could not be identified in terms of rabbit antibody specificities, but according to position and morphology of the precipitates it was estimated that they belonged to about six different specificities. Figure 3C shows the distribution of patients by the number of unidentified antibodies in each serum.

The highly significant differences ($P < 0.002$) between the LL and the TT/BT groups and the LL and the TT groups are shown in Table 1. No statistically significant differences were found between the TT/BT and the TT groups. The LL group had a relatively small overlapping to the other groups (25%), using the criteria A and B (Table 1) (95% confidence limits: 7.3% to 52.3%).

The specificities of the 86 identified antibodies were confined to 11 of the 19 antigens enumerated in Fig. 2. The distribution of the

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**FIG. 1.** Crossed immunoelectrophoresis with intermediate gel using BCG as antigen and rabbit anti-BCG as reference antiserum. First-dimension electrophoresis with anode at the right and second-dimension electrophoresis with anode at the top. (A) Control plate with serum from a control person in the intermediate gel. (B) Test plate with serum from a patient with lepromatous leprosy. For details see the text and Fig. 2.
patients by the specificity and titer of the antibodies is shown in Fig. 4. It appears that the LL patients, with one exception, accounted for all antibodies with titers 2 and 3. Although the LL group accounted for all antibodies with specificity for antigens numbers 4, 8, 11, 12, 13, 18, and 19, there were 4 LL patients who had no antibodies against any of these 7 antigens. The dominating antigens, as regards antibody response in this material, were numbers 5, 6, 17, 18, and 19.

Although "free" antigens in serum, or immuno-complexes in antigen excess, might have been demonstrated with this method (3), none was found.

DISCUSSION

Crossed immunoelectrophoresis with intermediate gel (1) is especially suited for studies of human antibodies in infectious diseases since it is possible by this method to obtain information on the number, specificities, and titers of precipitins in terms of a reference system. Also, free antigens in serum can be demonstrated (3), and the sensitivity and resolving power of the method are higher than obtained with the conventional diffusion-in-gel methods.

As BCG is antigenically related to M. leprae and commonly available, we decided to use this antigen in the present study. Our demonstration of 19 antigens in BCG contrasts to the finding of Roberts et al. (15) of 48 antigens in this species. The reason for this discrepancy is not obvious since it may be attributed both to differences in antigen preparation, immunization procedure, and antibody response of individual rabbits. Reference reagents, above all reference antisera, are therefore highly needed in such complex systems (2, 15) to make studies in different laboratories comparable. Such reference antisera should define certain antigens, but need not necessarily contain antibodies against all known antigenic components.

Since BCG antibodies in this study were demonstrated in all lepromatous sera, the method seems promising as a tool for further studies in this field. In previous studies the maximal number of precipitins to BCG was 5 in a single serum (Myrvang et al., in press), whereas we found that 75% of the sera in this study (15%) had more than 5, and that up to 14 different antibody specificities were demonstrable in individual sera. The previously demonstrated difference in antibody response in tuberculoid and lepromatous leprosy (11, 12; Myrvang et al., in press) was strongly supported by our findings (Table 1).

The specificity of the antibodies varied considerably in individual sera, and the complexity in antibody response was higher than expected. On the whole, however, antibodies against antigen numbers 5, 6, 17, 18, and 19 were most frequently found.

The finding of human BCG antibodies which could not be identified in the reference system are of interest since they may represent a human-specific or perhaps a leprosy-specific antibody response; the majority of unidentified antibodies occurred in lepromatous sera (Fig. 3C).

![Figure 3](http://iai.asm.org/) Distribution of 40 leprosy patients by three criteria. (A) Total number of antibodies in each serum (identified + unidentified); (B) number of identified antibodies per serum; (C) number of unidentified antibodies per serum. Symbols: 12 TT patients; 12 TT/BT patients; 16 LL patients.

**Table 1. Statistical analysis of the differences between the three groups of patients according to number of antibodies per serum**

<table>
<thead>
<tr>
<th>Criterium</th>
<th>Comparison</th>
<th>Result*</th>
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<tbody>
<tr>
<td>A</td>
<td>LL-TT/BT</td>
<td>P &lt; 0.002</td>
</tr>
<tr>
<td></td>
<td>LL-TT</td>
<td>P &lt; 0.002</td>
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<tr>
<td></td>
<td>TT/BT-TT</td>
<td>NS*</td>
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<tr>
<td>B</td>
<td>LL-TT/BT</td>
<td>P &lt; 0.002</td>
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<td></td>
<td>LL-TT</td>
<td>P &lt; 0.002</td>
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<td></td>
<td>TT/BT-TT</td>
<td>NS</td>
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<tr>
<td>C</td>
<td>LL-TT/BT</td>
<td>P &lt; 0.002</td>
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<td></td>
<td>LL-TT</td>
<td>P &lt; 0.002</td>
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<td></td>
<td>TT/BT-TT</td>
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* Mann-Whitney U test.

* NS, Not significant.
Strong CMI against *M. leprae* in patients with tuberculous leprosy and the continuous decrease in CMI towards the lepromatous end of the spectrum has been demonstrated in vitro and in vivo using whole *M. leprae* bacilli as antigen (9). The studies of the overall antibody response showing the inverse picture of increasing response towards the lepromatous end of the spectrum is based on studies on extracts of mycobacteria containing a multitude of soluble antigens (12; Myrvang et al., in press). Further information on the aberration of the immune response in leprosy requires study of CMI and antibody response against the same individual mycobacterial antigens. The method introduced in the present study and other refined immunoelectrophoretic methods (4) can provide exact data to be used as guidelines for purification of those mycobacterial antigens that are prone to induce formation of precipitating antibodies in leprosy patients. After purification, these antigens should then be studied with regard to CMI in different patients to see if the inverse relationship between CMI and antibody production is a feature which also is true on the level of individual mycobacterial antigen molecules.

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**LITERATURE CITED**


