

Two-Dimensional Electrophoretic Analysis of Humoral Responses to Culture Filtrate of *Mycobacterium bovis* BCG in Patients with Leprosy and Tuberculosis¹

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Leprosy still remains a major chronic infectious disease affecting more than 1,260,000 patients. There are two polar types of the diseases. One is lepromatous leprosy (LL), which represents no specific cellular immune response to *Mycobacterium leprae* and has a large number of bacilli within phagocytes in the macrophage-rich granuloma. The other is tuberculoid leprosy (TT), which is characterized by a vigorous cellular immune response and can result in irreversible nerve destruction in a high proportion of cases. Precise early diagnosis is important for the initiation of efficient chemotherapy. A variety of techniques to detect an infection with *M. leprae* have been offered. In prognosis and treatment, there are differences between the LL and TT types of the disease. Many studies on the diagnosis of leprosy also have been reported (^{4, 12}).

The antigen 85 (Ag85) complex, Ag85-A = MPB44 (⁹), Ag85-B = MPB59 = α antigen (⁶) and Ag85-C = MPB45 (⁹), is secreted by mycobacteria. These antigens are presented both on the cell surface and in the cytosol of *M. leprae* within the infected lesions of LL patients (¹³). The humoral responses to Ag85 complex have been reported by different groups (^{5, 17}). However, so far no comparisons of the reactions with whole secreted proteins have been presented.

The genes for the culture filtrate (CF) proteins, α antigen (⁶), MPB51 (¹¹), MPB64 (¹⁸), MPB70 (¹⁵), MPB83 (⁷), and MPB57 (¹⁹) were first cloned by us from *M. bovis* bacillus Calmette-Guerin (BCG). On the basis of this background, comparative analyses were carried out on humoral responses of patients against whole CF proteins in the hope that these proteins might contain antigens useful in predicting the prognosis of the disease. Two-dimensional electrophoresis (2-DE) has great resolving power to separate individual proteins. The CF of BCG was analyzed by 2-DE. Ag85 complex and MPB51 were immunodominant antigens as previously reported (¹²). In addition, we identified three new antigens that were recognized by leprosy sera.

MATERIALS AND METHODS

Serum. Patients were classified clinically and histopathologically. Serum samples were collected from 3 LL, 3 borderline lepromatous (BL), 3 mid-borderline (BB), 3 borderline tuberculoid (BT), 2 TT and 4 tuberculosis (TB) patients (The Table). All patients were studied before chemotherapy. The healthy control sera were collected from three persons who had no prior history of exposure to leprosy.

CF. BCG substrain Tokyo was used in this study. It was cultured on the surface of Sauton liquid medium for 3 weeks. The culture was passed through a membrane filter with a pore size of 0.45 μ m to remove BCG cells. The proteins in the filtrate were concentrated by solid ammonium sulfate at 80% saturation, as described previously (⁸), and the concentrate was dialyzed against TMNSH buffer (10 mM Tris-HCl buffer, pH 7.8, 10 mM Mg acetate, 60 mM NH₄Cl and 6 mM 2-mercaptoethanol).

2-DE. The proteins in the CF were analyzed by 2-DE according to the method of

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THE TABLE. *Classification and clinical history of untreated patients^a.*

Patient no.	Clinical/histopathological diagnosis	Clinical history
1	BL	Recurrence
2	BT	Recurrence
3	LL	Recurrence
4	TT	Primary infection
5	BL	Primary infection
6	LL	Primary infection
7	LL	Primary infection
8	TT	Primary infection
9	BT	Primary infection
10	BB	Primary infection
11	BL	Primary infection
12	BB	Primary infection
13	BB	Primary infection
14	BT	Primary infection
15	TB	Primary infection
16	TB	Primary infection
17	TB	Primary infection
18	TB	Primary infection

^a Sera were obtained from Kyoto University Hospital attached to the School of Medicine, Kyoto (patients 1–12); Kitano Hospital, Osaka (patient 13); Kitasato University Hospital attached to the School of Medicine, Kanagawa (patient 14); Johoku Hospital, Aichi (patient 15) and Tokyo Hospital of National Sanatorium, Tokyo (patients 16–18).

O'Farrell⁽¹⁰⁾ with a few modifications as described previously⁽⁹⁾. In each 2-DE gel, 20 µg of CF protein was loaded. The first dimension was isoelectric focusing in 8 M urea with a 2% ampholyte mixture (Bio-Lyte; Bio-Rad mixture of 3/10:3/5:5/7 = 4:1:1) (Bio-Rad Laboratories, Hercules, California, U.S.A.). The second dimension was sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% polyacrylamide gel. The gels were processed for Western blotting or proteins were visualized by silver staining.

Immunoblotting. Immunoblotting was carried out according to the method of Towbin, *et al.*⁽¹⁶⁾. The blots were probed with 1:100 diluted serum antibodies, and detected with 1:1000 diluted peroxidase-conjugated rabbit antihuman immunoglobulin G (Dako A/S, Glostrup, Denmark).

RESULTS

2-DE. 2-DE of the CF derived from BCG is presented in Figure 1. The silver-stained gel showed the well-separated proteins. For example, Ag85-A, α antigen,

Ag85-C, MPB51, MPB64, MPB70, the 65-kDa protein (groEL/hsp60 analog)⁽¹⁴⁾, MPB57 (groES/hsp10 analog), and BCG 45/47-kDa complex⁽⁹⁾ were defined as clear spots.

Humoral responses of LL and BL patients to CF proteins. Represented in Figure 2A, all sera showed the strongest humoral reactions to Ag85 complex and MPB51 among the CF proteins. A majority of the sera reacted to BCG 45/47-kDa complex (5/6 = 83%). Only a smaller number of sera recognized the MPB57 and 65-kDa protein spots (MPB57: 1/6 = 16.7%, 65-kDa: 3/6 = 50%). These responses were weaker than those to the Ag85 complex and BCG 45/47-kDa complex. There were also three small seroreactive spots, the 29-, 24- and 23-kDa spots, that had not as yet been characterized (arrows in Fig. 2). All sera reacted weakly to the 29- and 24-kDa spots. Concerning the 23-kDa spot, half of the sera gave a positive reaction.

Humoral responses of BB, BT and TT patients to CF proteins. Proteins were recognized relatively less intensely or not at all when probed with BB, BT and TT sera (Fig. 2B). All of these sera showed weak reactions against Ag85 complex and MPB51, but only 1 out of 3 BB and 1 out of 3 BT sera showed strong reactions. Other spots were recognized with only a few sera (MPB57: 2/8 = 25%; 65-kDa: 2/8 = 25%; BCG 45/47-kDa complex: 1/8 = 12.5%; 29-kDa: 3/8 = 37.5%; 24-kDa: 2/8 = 25%; 23-kDa: 1/8 = 12.5%). There were no spots that were recognized by only the BT and TT patients.

Humoral responses of TB patients to CF proteins. TB patients showed reactions similar to the TT and BT patients. Ag85 complex, MPB51 and 29-kDa spots were weakly recognized by these sera (Fig. 2C). Other spots were recognized with some sera (65-kDa: 3/4 = 75%; 24-kDa: 1/4 = 25%; 23-kDa: 1/4 = 25%). MPB57 showed no reaction with any sera. The humoral responses of healthy control sera showed no reactions to any of the CF proteins (data not shown).

DISCUSSION

One of the main limitations on the successful control of leprosy as a public health problem is the lack of methods for its early

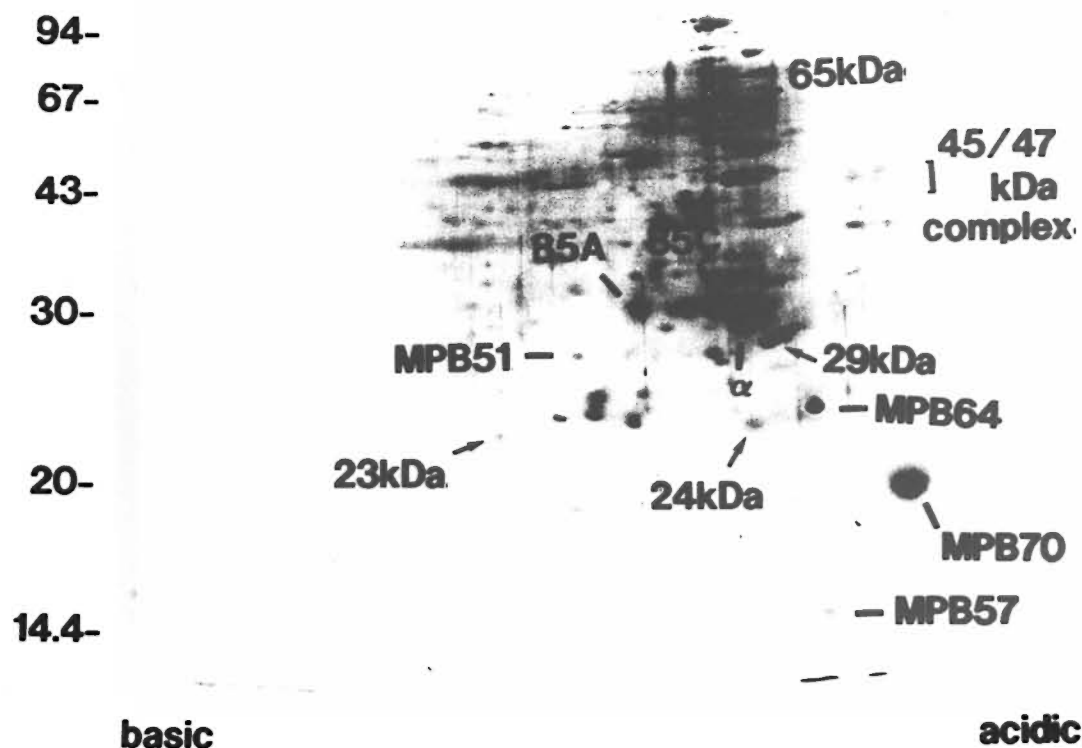


FIG. 1. 2-DE of CF proteins derived from BCG. The gel was visualized by silver staining. Apparent sizes of molecular weight standards are indicated in kDa along the left side. 85A = Ag85-A; α = α antigen; 85C = Ag85-C; 65 kDa = 65-kDa protein (groEL analog); MPB = major protein secreted from BCG.

diagnosis in subclinical cases. Because of the long incubation period of the disease, the liberation and spread of *M. leprae* into the environment during that stage constitute the main sources of infection. We searched for antigens that could be applied to the prediction of leprosy infection in subclinical cases.

Separation of proteins in one-dimensional electrophoresis is generally not sufficient to resolve a large number of constituents. 2-DE as described by O'Farrell⁽¹⁰⁾ is very powerful since two different principles of protein separation are applied in the first and second dimensions, permitting a precise definition of individual antigens of samples. Proteins secreted by mycobacteria have been suggested as major immune targets in the early phase of infection⁽¹⁾. The CF of BCG was utilized to examine reactivities with sera from leprosy patients because cultivation of *M. leprae* has not yet succeeded *in vitro*. A study of

crossreactivities with these proteins that contain well-characterized antigens may have potential value for finding new immunogenic antigens. The early cultures were used to exclude the possible contaminants with degraded products of bacteria.

In this study, we have defined three new antigens that are recognized by sera from leprosy and tuberculosis patients, the 29-, 24- and 23-kDa antigens (Fig. 2). The 29- and 24-kDa antigens were recognized very often in lepromatous patients but rarely in tuberculoid or TB patients. The reactions to these antigens can be good candidates for prediction of the infection, especially in lepromatous patients. Further study of these antigens may provide a better understanding of the pathogenesis.

Immunoblot analysis gave some patterns of reactivities with individual patient sera. There was a clear trend. Humoral response intensities against Ag85 complex and MPB51 were well correlated with the types

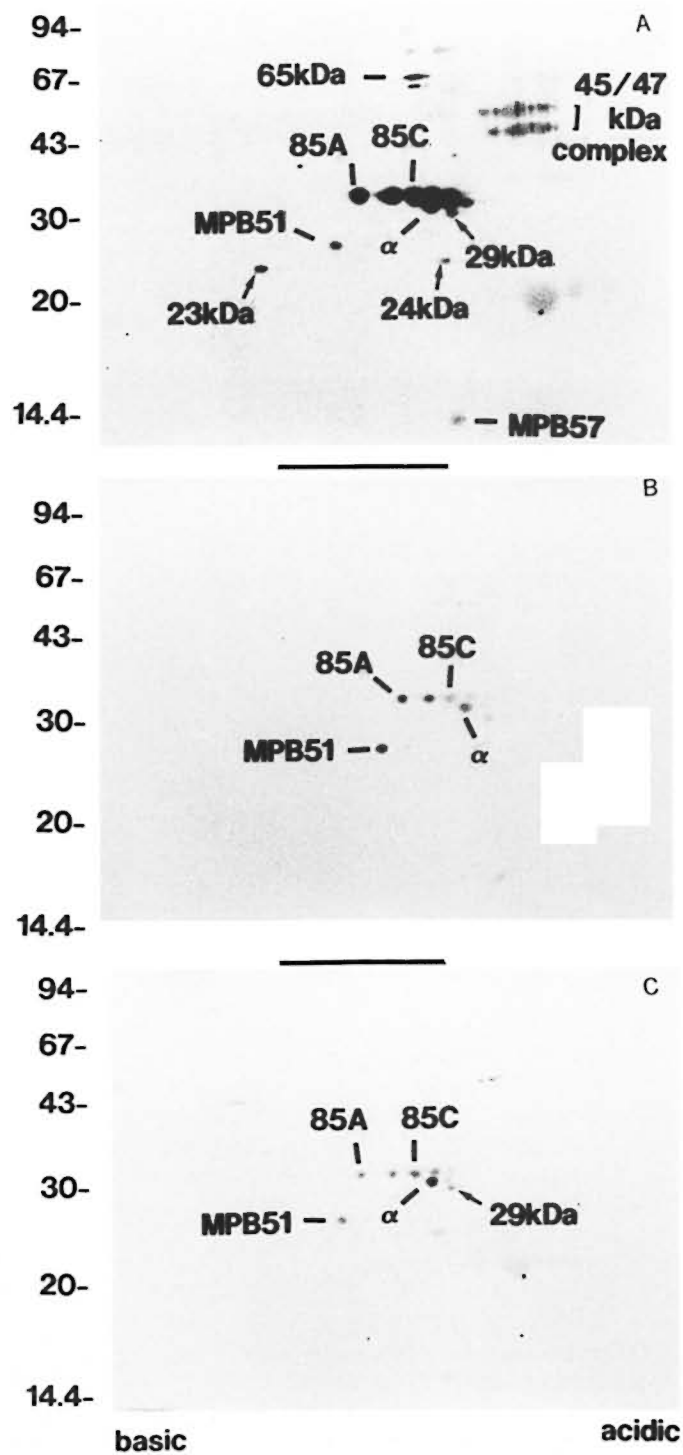


FIG. 2. Patterns of antibody responses to CF proteins separated by 2-DE of three individual patients: A = LL, B = TT and C = TB. \rightarrow = spots with molecular weights of 29-, 24-, and 23-kDa. Apparent sizes of molecular weight standards are indicated in kDa along the left side; other spots are as indicated in Figure 1.

of disease. The reactions with sera from lepromatous patients were stronger than those from tuberculoid and TB patients. Focusing on Ag85-B, an ELISA analysis was performed and yielded a similar conclusion (data not shown). Ag85 complex has recently been shown to be a mycolyl transferase that is one of the important enzymes for unique mycobacterial cell-wall synthesis (²). The Ag85 complex may play important roles as the first row antigens secreted by *M. leprae* after infection. The antigens are suggested to be the most principal ones for determining the clinical prognosis after infection.

Although relapse of lepromatous leprosy is a serious problem, this clinical change cannot be detected clearly in an early stage. Recently, multidrug-resistant *M. leprae* has been isolated from relapsing leprosy (³). Drug therapy for a long period to avoid relapse of the disease raises the possibility of selecting the drug-resistant *M. leprae*. The antibody responses to spots defined in this study might provide successful prediction for controlling the disease and for efficient therapy. Our findings may also contribute to the understanding of the pathogenesis of leprosy and the mechanism of the infection.

SUMMARY

Sera from 3 lepromatous (LL), 3 borderline lepromatous (BL), 3 mid-borderline (BB), 3 borderline tuberculoid (BT), 2 tuberculoid (TT) and 4 tuberculosis (TB) patients and 3 healthy individuals were examined for their reactivities against the proteins in the culture filtrate of BCG separated by two-dimensional electrophoresis (2-DE). The sera were obtained from patients who were untreated. Sera from LL and BL patients reacted strongly with the antigen 85 (Ag85) complex and MPB51. Sera from LL and BL patients also weakly reacted with the newly identified 29-, 24- and 23-kDa spots. Sera from the other patients reacted similarly, but the levels of reaction were remarkably lower than those from LL and BL patients. *Mycobacterium leprae* antigens that are analogous to BCG Ag85 and MPB51 are suggested as the main targets for the humoral immunity of untreated patients. The reactivities of sera with newly

identified antigens may provide the potential for predicting the severity and prognosis of diseases.

RESUMEN

Se probaron los sueros de 3 pacientes lepromatosos (LL), 3 lepromatosos subpolares (BL), 3 pacientes intermedios (BB), 3 tuberculoideos subpolares (BT), 2 tuberculoideos (TT), 4 pacientes con tuberculosis (TB), y 3 individuos sanos, para establecer su reactividad contra las proteínas secretadas por BCG fraccionadas por electroforesis bidimensional (2-DE). Todos los individuos fueron casos no tratados. Los sueros de los pacientes LL y BL reaccionaron fuertemente con el complejo antigénico 85 (Ag 85) y con MPB51. Los sueros de los pacientes LL y BL también reaccionaron débilmente con moléculas nuevas de 29-, 24- y 23 kDa. Los sueros de los otros pacientes reaccionaron de manera similar pero los niveles de reacción fueron marcadamente menores que los de los pacientes LL y BL. Se sugiere que los antígenos de *Mycobacterium leprae* que son análogos a los antígenos Ag 85 y MPB51 de BCG sean los blancos principales de la inmunidad humoral en los pacientes no tratados. Las reactividades de los sueros con los nuevos antígenos identificados podrían ser de utilidad potencial para predecir la severidad y el pronóstico de la enfermedad.

RÉSUMÉ

Les sérums collectés à partir de patients hanséniens incluant 3 lépromateux (LL), 3 lépromateux borderlines (BL), 3 borderlines (BB), 2 tuberculoïdes (TT), ainsi que 4 patients tuberculeux (TB) et 3 individus en bonne santé furent analysés pour leur réactivité contre les protéines obtenues à partir de filtrats de cultures de BCG, protéines séparées par électrophorèse à 2 dimensions (2-DE). Les sérums furent obtenus à partir de patients non traités. Les sérums des patients LL et BL ont fortement réagis contre le complexe antigénique 85 (Ag 85) et MPB51. Les sérums des patients LL et BL ont aussi faiblement réagis avec des spots nouvellement identifiés de 29, 24 et 23 kilodaltons (kDa). Les sérums des autres patients ont réagis de façon similaire, avec toutefois des niveaux de réaction très nettement inférieurs à ceux observés chez les patients LL et BL.

Il est supposé que les antigènes de *M. leprae* qui sont analogues à ceux des antigènes Ag85 et MPB51 du BCG sont les cibles principales de l'immunité à médiation humorale des patients non traités. La présence d'une réaction de ces sérums avec des antigènes nouvellement identifiés pourrait potentiellement permettre de prédire la sévérité et le pronostic de maladies.

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