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SDS-PAGE analysis of *M. leprae* protein antigens reacting with antibodies from sera from lepromatous patients and infected armadillos

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SUMMARY

Studies have been conducted to characterize *M. leprae* antigens from purified leprae bacilli derived from infected armadillos. First, the proteins of the mycobacterial extracts were fractionated by SDS-PAGE. Subsequently, the proteins in the gel were electrophoretically transferred on a strip of nitrocellulose paper by the technique of 'electrophoretic blotting'. The separated bacterial protein bands, thus immobilized on the nitrocellulose paper were made to react immunologically with sera from the lepromatous patients, infected armadillo sera and other experimental mycobacterial antisera. It was observed that a majority of *M. leprae* proteins contained antigenic determinants also present on proteins of BCG. In addition, only two specific antigen bands of 33KD and 12KD were conspicuously detected by the patients' sera and the infected armadillo sera. These substances were further identified as polysaccharides or glycoproteins since they could only be stained by Schiff's reagent or alcian blue. Only 12KD glycoprotein band reacted with concanavalin A, whereas wheat germ agglutinin (WGA) did not show any reaction with them. These 33KD and 12KD glycoprotein antigens were found to lose their antigenicity after pepsin treatment and can be considered as glycoproteins. Further, radiolabelling experiments showed that 12KD antigen underwent radioiodination under usual conditions, but 33KD glycoprotein failed to be similarly radiolabelled. It is suggested that these protein antigens have *M. leprae* specific determinants on a cross-reacting component.

INTRODUCTION

Lepromatous patients having high bacillary load are characterized by high level of antibodies towards *M. leprae*. Some authors have analysed antigens of *M. leprae* using double diffusion precipitin tests in gel (Stanford *et al.*, 1976; Caldwell, Kirchheimer & Buchanan, 1979). Some particular antigenic components of *M. leprae* have also been identified by crossed immunoelectrophoresis (CIE), using rabbit antisera against *M. leprae*, patients' sera and the sera from the armadillos having systemic mycobacterial infections after inoculation with *M. leprae* (Harboe *et al.*, 1977, 1978; Kronvall, Stanford & Walsh, 1976; Closs, Mshana & Harboe, 1979). Further indirect immunofluorescence studies have provided evidence concerning the presence of *M. leprae* specific antigens in pooled leprosy sera free from cross-reacting antibodies to common mycobacterial antigens (Abe *et al.*, 1980). Characterization of antigenic composition of the leprae bacillus is

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essential to the understanding of the immunological features of leprosy. All the above studies have provided some information about the antigenic characteristics of the leprosy bacillus, but further investigations are needed to determine the molecular identity of these antigenic components. In addition to the complex problem of mycobacterial cross-reactivity, resolution of a large number of protein antigens obtained from *M. leprae* has also posed a technical problem. In our present study, we have adapted a sensitive method for analysing and characterizing the antigenic materials of *M. leprae* with the help of a recent technique known as electrophoretic blotting (Towbin, Staehelin & Gordon, 1979). The proteins of *M. leprae* were first separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a slab gel on the basis of their molecular weights. Subsequently, these were transferred electrophoretically from the gel onto a strip of nitrocellulose paper. The separated proteins were electrophoretically blotted by the paper which retains an exact replica of the original gel pattern. Subsequently, the transferred proteins were made to react with appropriate antisera for immunochemical characterization, followed by reaction with radiolabelled protein A. After autoradiography, only the protein bands which reacted with the antisera were expressed. The method offers a great advantage of 'sorting out' the different protein components immobilized on nitrocellulose with the help of antigen–antibody reaction, followed by the ease of processing for autoradiography. The present preliminary communication describes the attempts made to analyse and identify the antigenic materials of *M. leprae* derived from systemically-infected armadillos, particularly with respect to the sera from infected individuals.

MATERIALS AND METHODS

Preparation of M. leprae *extracts. M. leprae* was provided by the World Health Organization, through its Immunology of Leprosy (IMMLP) programme, by Dr R.J.W. Rees as freeze-dried bacilli purified (protocol 1/79) from the liver tissues of infected armadillos. *M. leprae* (15 mg dry weight) was suspended in 2 ml of 0·01 M phosphate-buffered saline (PBS), pH 7·2 and sonicated on ice for 15 min using the MSE sonicater (N. Zyvy & Cie, Switzerland). After centrifugation at 10,000 *g* for 30 min at 4°C, the supernatant was collected and labelled as soluble *M. leprae* antigenic extract (SE). The pellet from the centrifugation was treated with 2% SDS and 2% mercaptoethanol for 20 min at 60°C, for solubilization. This was labelled as pellet extract (MSE). Both the preparations were stored at −20°C.

Patients' sera and the sera from the infected animals. Individual and pooled leprosy sera were obtained at Leprosy Home, Shahadra, Delhi and Safdarjung Hospital, New Delhi. The serum samples were kindly sent to our laboratory by air by Dr Kunal Saha of G. B. Pant Hospital, New Delhi. All the patients were on usual dapsone therapy and they were classified clinically and histopathologically as lepromatous patients (LL, BL). Some of the serum samples were also from the lepromatous patients with ENL. Lyophilized absorbed leprosy sera were kindly sent to us by Dr Abe of the National Institute for Leprosy Research, Tokyo, Japan. Cross-reacting antibodies to common mycobacterial antigens were removed from the serum pool by treating the same with BCG and *M. vaccae* suspensions and later with cardiolipin–lecithin solution. Sera from infected and non-infected normal nine-banded armadillos (*Dasypus novemcinctus*) were kindly provided by Dr W.F. Kirchheimer, U.S. Public Health Service Hospital, Carville, USA.

Antisera. Rabbit anti-BCG antibodies (code 124) was purchased from DAKO-Immunoglobulins a/s, Copenhagen, Denmark. Rabbit anti-*M. leprae* antibodies were a kind gift from Dr Harboe of the Institute for Experimental Medical Research, Ulleval Hospital, Norway. Rabbit anti-*M. leprae* antiserum was prepared in our laboratory as follows. Six milligrams of lyophilized *M. leprae* suspended in 1·5 ml of saline was emulsified in the cold with an equal volume of Freund's incomplete adjuvant with the help of a high speed homogenizer. The whole emulsions divided into three equal parts were kept at −20°C. For immunization, one healthy rabbit was injected intracutaneously at multiple sites in the neck and back region on day 0, day 7 and day 15 with the emulsions. The immunized rabbit was bled on day 34 and sera separated. The purified immunoglobulin fractions as obtained from the sera by $(NH_4)_2SO_4$ precipitation was stored with azide at 4°C.

Pepsin digestion. Whole leprae bacilli were suspended with pepsin (enzyme: bacterial proteins

1:40) in 0·001N HCl, pH = 3, at 37°C for 48 hr. Subsequently, the bacterial proteins were extracted with SDS-2ME.

Radiolabelling. The extracts of *M. leprae* were radioiodinated by the chloramin T method (McConahey & Dixon, 1966). Lectins Con A and WGA (wheatgerm agglutinin) and the extract of *M. leprae* after treatment with 2% SDS were radioiodinated by the iodogen method (Fraker & Speck, 1978). Protein A (Pharmacia, Sweden) was labelled with ^{125}I by the Bolton and Hunter reagent (Bolton & Hunter, 1973). Free iodine was removed either by extensive dialysis against PBS at 4°C or by gel filtration through G-25.

Discontinuous SDS-electrophoresis and staining of protein and glycoprotein bands. The extracts of *M. leprae* were analysed by SDS-PAGE according to the Laemmli discontinuous buffer system (King & Laemmli, 1971) in a slab gel electrophoresis using 6–20% gradient polyacrylamide gels with 0·1% SDS. For the staining of glycoproteins, the gels were first washed with 40% methanol and 7% acetic acid overnight to remove all SDS. They were then stained with the Schiff's reagent according to Glossman & Neville (1971). The gels were also stained for glycoproteins with alcian blue (Wardi & Michos, 1972).

Electrophoretic transfer of proteins. Proteins of the *M. leprae* extracts were first separated by SDS-PAGE in one dimensional gel slabs. They were electrophoretically blotted from the gel onto a strip of nitrocellulose paper, using the procedure as described by Towbin *et al.* (1979).

Immunological identification of proteins 'blotted' on nitrocellulose strips. The nitrocellulose strips containing the protein 'blots' from the gel were treated as follows: (i) they were incubated in 3% BSA solution for 1 hr at 40°C (3 g of BSA dissolved in 100 ml of saline/10 mM Tris-HCl, pH 7·4); (ii) they were then incubated in leprosy sera or anti-mycobacterial antisera appropriately diluted with 3% BSA solution for 3 hr at 4°C; (iii) they were washed with saline/Tris-HCl buffer, pH 7·4 for 3 hr with several changes of the washing buffer; (iv) they were further incubated with ^{125}I-Protein A (7×10^6 c.p.m./20 ml) dissolved in 3% BSA solution for 2 hr at room temperature; (v) they were washed extensively till the washings were free of radioactivity; (vi) they were then thoroughly dried and autoradiographed (Kodak BB 5 X-ray film) at −70°C for 1–5 days.

RESULTS

Identification of different proteins and glycoproteins in the bacterial extracts by SDS-PAGE

After coomassie brilliant blue staining, more than 20 thin distinct protein bands were observed in the reduced pellet fraction (Fig. 1). Molecular weights of the proteins ranged from 94KD to 14·4KD. The soluble fraction displayed only nine protein bands whose molecular weights were as follows: 75·8KD, 59KD, 40KD, 27·5KD, 20KD, 18KD, 16KD, 15·5KD, and 14·4KD. Of these, 20KD, 18KD and 14·4KD bands were the major components. The gel after being stained with Schiff's reagent revealed only three major diffuse bands (Fig. 1) in both fractions. Their molecular weights were calculated as 33KD, 20KD and 12KD. 12KD component appeared as a doublet. A similar picture containing these three bands was obtained by staining with alcian blue.

Reaction of patients' sera and infected armadillo sera with the bacterial proteins

Fig. 2 records the immunological reaction of a leprosy patient's sera with the separated proteins of the bacterial extracts. Only two major antigen bands corresponding to 33KD and 12KD of both the soluble and pellet fractions were shown to react with the sera. We have obtained consistent results in the experiments, using five different sera from lepromatous individuals, with or without ENL, and three pooled leprosy sera from several other patients. With absorbed leprosy sera, no antigenic proteins could be observed in the soluble extracts whereas two antigen bands in the 33–30KD region were detected only in the pellet fraction. Pooled normal human sera, diluted 1:6, did not react with the bacterial extracts; at 1:2 dilution a faint reaction was seen with the 12KD component. Four infected armadillo sera reacted with the 33KD and 12KD Schiff stained bands in the extracts, in addition to other components of 67KD, 50KD, 40KD (Fig. 3). One of these serum samples was also observed to react with 20KD band of the extracts. Normal armadillo sera only reacted with the 50KD band.

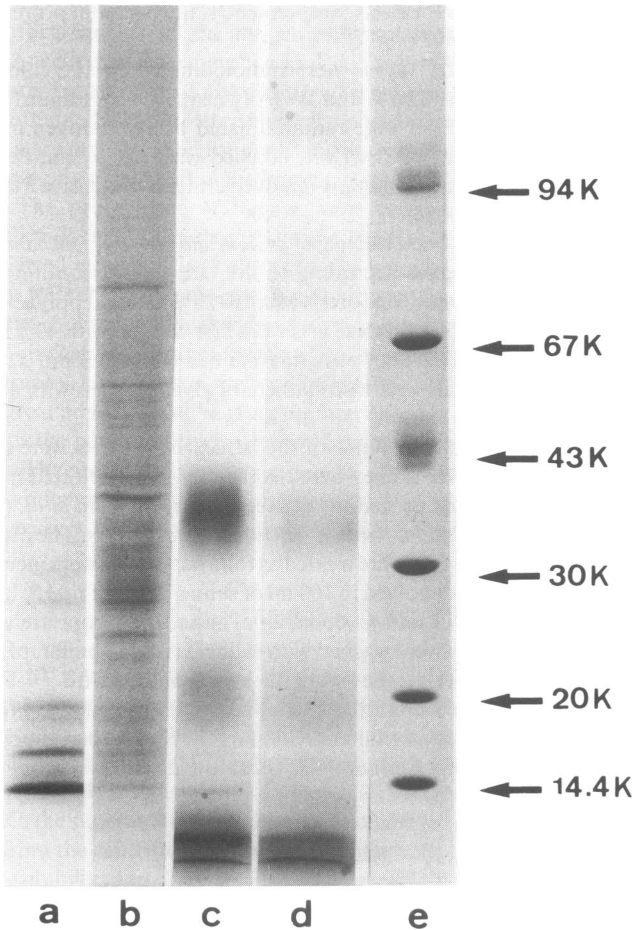

Fig. 1. SDS-electrophoresis on a 6–20% gradient PAA slab gel to analyse the protein extracts of *M. leprae*. Protein bands stained with coomassie brilliant blue. (a) A soluble sonic extract of *M. leprae* (SE); (b) pellet fraction treated with SDS-2ME after centrifugation of the sonic extract of *M. leprae* (MSE); (c) SE fraction stained with Schiff's reagent for carbohydrate as described in Materials and Methods; (d) MSE fraction stained for carbohydrate; (e) low molecular weight protein standards, phosphorylase b (94KD), albumin (67KD), ovalbumin (43KD), carbonic anhydrase (30KD), trypsin inhibitor (20KD) and lactalbumin (14·4KD).

Reaction of different experimental antisera with the bacterial extracts

Anti-BCG antibodies in 1:80 dilution reacted strongly with most of the bacterial proteins from 67KD region to 12KD region except the proteins near 65–60KD and 18–14KD regions (Fig. 4). However, anti-*M. leprae* antibodies in 1:40 dilution detected only 33KD and 12KD (doublet) antigen bands in the extracts under identical conditions (Fig. 4). Normal rabbit sera did not react with the protein fractions of the bacterial extracts.

Study of different properties of the bacterial antigens

The bacterial components, which primarily reacted with the patients' sera were shown to be susceptible to peptic cleavage, including the 33KD and 12KD Schiff reacting bands. Radiolabelled concanavalin A and wheatgerm agglutinin (WGA) were used as markers. The bacterial proteins did not show any reaction with wheatgerm agglutinin while only the 12KD Schiff reacting band reacted with concanavalin A (Fig. 5).

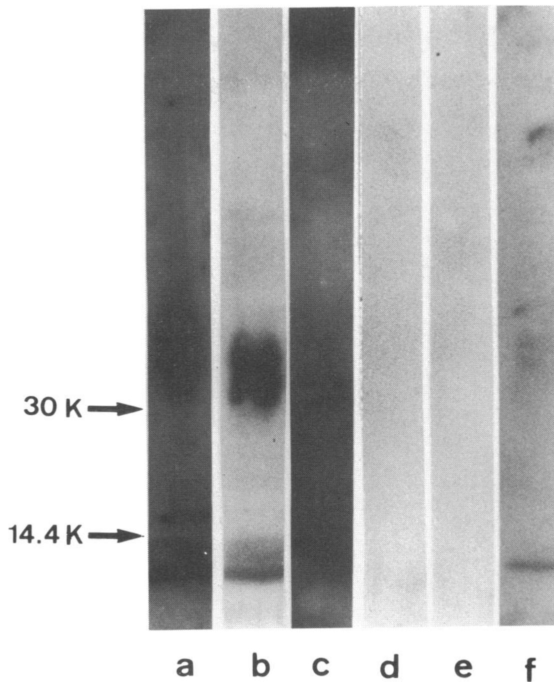

Fig. 2. Detection of antigenic components in *M. leprae* by reacting with the sera from leprosy patients. Protein bands as obtained from SE and MSE fractions on the slab gel by SDS-PAGE were electrophoretically blotted on the strips of nitrocellulose paper. The latter were incubated with diluted patient's sera, washed, treated with ^{125}I-Protein A and subsequently autoradiographed. (i) Reaction with the sera from lepromatous patients (1:6 dilution): (a) SE fraction; (b) MSE fraction; (ii) reaction with the pooled absorbed leprosy serum (1:3 dilution): (c) MSE fraction; (iii) reaction with normal human sera (1:6 dilution): (d) SE fraction; (e) MSE fraction; (iv) reaction with normal sera (1:2 dilution): (f) MSE fraction.

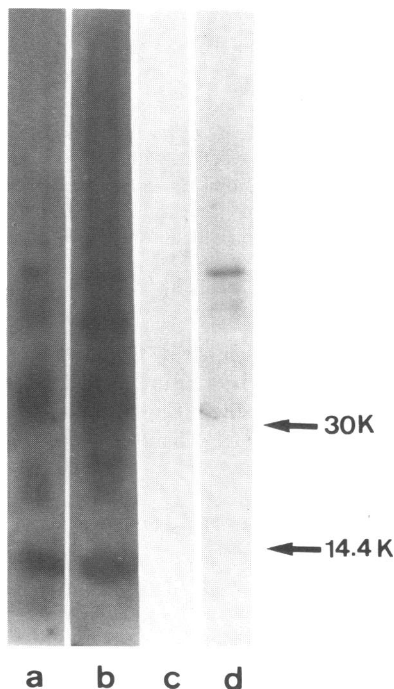

Fig. 3. Detection of antigenic components in *M. leprae* by reacting with the sera from armadillo. Technical procedure is the same as described before. (i) reaction with the sera from armadillo with systemic infection with *M. leprae* (1:6 dilution): (a) SE fraction; (b) MSE fraction; (ii) reaction with normal uninfected armadillo sera (1:6 dilution): (c) SE fraction; (d) MSE fraction.

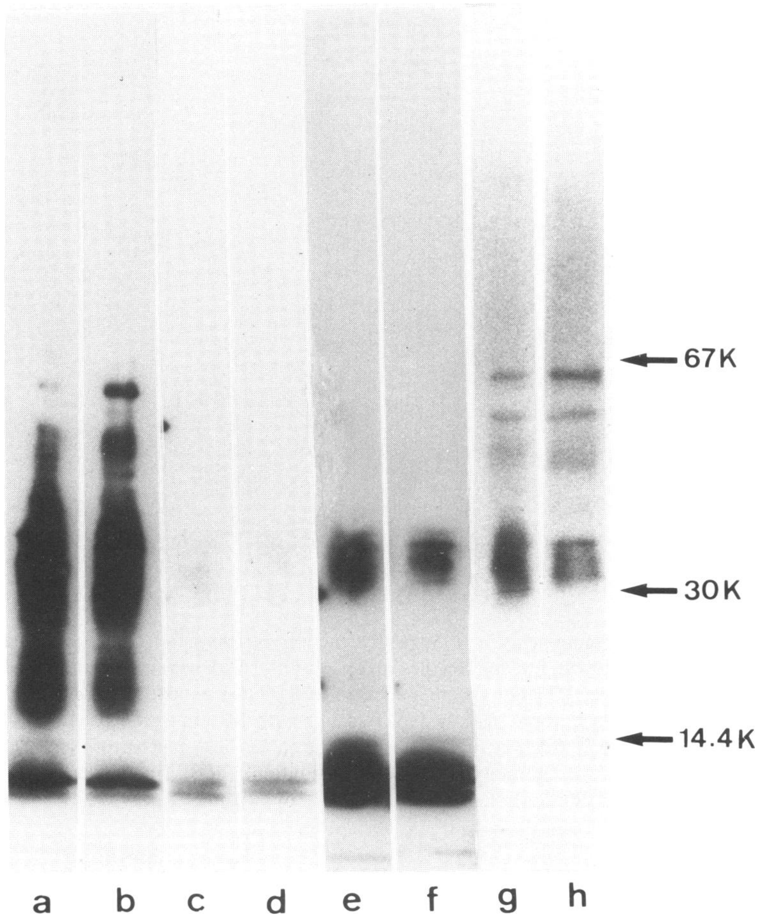

Fig. 4. Characterization of antigenic content of *M. leprae* by reacting with different antisera. Technical procedure is the same as described in Fig. 2. (i) reaction with anti-BCG antibodies (1:80 dilution): (a) SE fraction; (b) MSE fraction; (ii) reaction with anti-*M. leprae* antibodies (1:40 dilution—Dr Harboe's preparation): (c) SE fraction; (d) MSE fraction; (iii) same as (ii), but autoradiographed for a longer period (5 days); (e) SE fraction; (f) MSE fraction; (iv) reaction with anti-*M. leprae* in 1:20 dilution (our preparation): (g) SE fraction; (h) MSE fraction.

Radioiodination of the bacterial proteins either by the chloramin T method or by the iodogen method, showed most of the bacterial proteins to be insensitive to radioiodination. Proteins above 14·4KD band could only be faintly radiolabelled (Fig. 6). Both the methods were found efficient enough to radioiodinate both 14·4KD and 12KD band of the soluble fraction of *M. leprae*.

DISCUSSION

This study describes the identification of antigenic components of *M. leprae* derived from infected armadillos. In our present approach, the fractionated protein components of the leprosy bacillus, after immobilization on a nitrocellulose paper strip, are allowed to immunologically react with patients' sera and other experimental anti-mycobacterial antisera. Only the protein antigens that have reacted are identified.

The protein extracts obtained from the sonicated *M. leprae* materials were analysed by

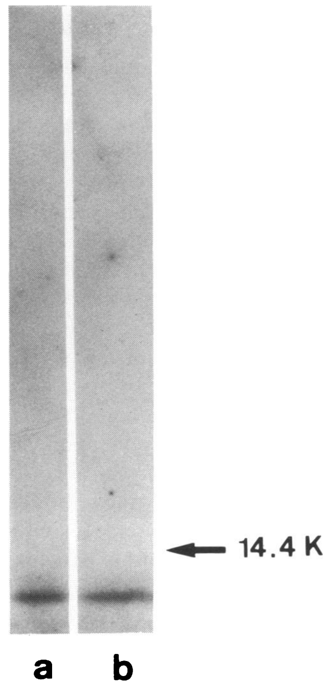

Fig. 5. Concanavalin A binding activity of the proteins of *M. leprae* extract. Con A (a) SE fraction; (b) MSE fraction. WGA treatment did not show any band after autoradiography.

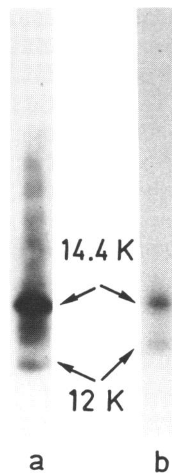

Fig. 6. SDS-electrophoresis of *M. leprae* extracts labelled with radioiodine. After electrophoresis, either the dried gel was directly autoradiographed, or the electrophoretically blotted nitrocellulose strip was put for autoradiography. The bacillus extracts were radiolabelled by the different ways. (i) the chloramin T method: (a) SE fraction only; (ii) the iodogen method: (b) SE fraction only.

SDS-PAGE. The soluble and the reduced fractions of the mycobacterial extracts presented different profiles of separate protein bands on the gel after coomassie brilliant blue staining. We observed a large number of protein bands as reported earlier (Closs *et al.*, 1979; Harboe *et al.*, 1977). Of nine bands present in the soluble extracts, the major ones identified are 20KD, 18KD and 14·4KD, the latter being the predominant one. In addition we have characterized three polysaccharide

containing bands of 33KD, 20KD and 12KD, which can only be stained by Schiff's reagent or by alcian blue. Two of these components (33KD and 12KD) were found sensitive to pepsin treatment and are probably glycoproteins. The 12KD glycoprotein is observed as a doublet and reacts with concanavalin A.

It is of interest that the sera from the lepromatous patients exhibited a significant immunological reaction only with the 33KD and 12KD glycoproteins. However, one cannot exclude that SDS and ME would alter the antigenic reactivity of some of the *M. leprae* antigens. It is not clear why the sonic extraction failed to release all the soluble antigenic material and it is likely that some antigens are linked with larger insoluble components via disulphide bridges. Further, it is difficult to verify from the autoradiographs whether the 33KD glycoprotein characterized as one of *M. leprae* protein antigens is a single substance or a mixture of two or more substances with similar molecular weights. Several authors have investigated the antigenic constitution of *M. leprae* using different immunological techniques. By double diffusion tests in gel, Stanford *et al.* (1975) identified four antigens 'specific to the leprosy bacillus'. From studies based on crossed immunoelectrophoresis in gel, Closs *et al.* (1979) identified 20 different antigenic components of *M. leprae*, whereas Kronvall *et al.* (1976) described a unique antigenic determinant present only on one *M. leprae* antigen. The sera from armadillos having systemic infection with *M. leprae* are also shown to react significantly with 33KD and 12KD glycoproteins of the bacterial extracts. In addition, three more minor protein antigens are also detected by the infected armadillo sera. It seems that the major *M. leprae* specific antibody activities in sera from both the lepromatous patients and the infected armadillos are mainly directed against antigens (33KD and 12KD) which are also the antigens recognized by rabbit anti-*M. leprae* immunoglobulins.

In this study, there has been no attempt to do an extensive analysis of the leprae antigens that cross react with other bacteria. However, in the present preliminary investigations, it was seen that most of the *M. leprae* proteins appearing on the electrophoregram react with anti-BCG antibodies. Other authors have also reported that *M. leprae* and BCG have common antigens (Brown, Brown & Sljivic, 1981; Webb, Mims & Turk, 1980; Harboe *et al.*, 1979). It is likely that the antigenicity of most *M. leprae* proteins is too low to induce a good immune response either in man or in rabbit, unlike those from other cultivable mycobacteria (Harboe *et al.*, 1979).

Reaction of the *M. leprae* extracts with pooled leprosy sera, depleted of anti-BCG and anti-*M. vaccae* antibodies has also been studied. The present results indicate that the *M. leprae* antigens which are associated with antibody formation in lepromatous patients also contain a significant number of cross-reacting components. Perhaps a very minute fraction of the antigenic materials may define the specific antigenicity of the bacillus.

The present investigations provide some information on the antigenic constitution of *M. leprae* and on some characteristics of these *M. leprae* antigens. Antigen 33KD does not react with normal human sera and cannot be radioiodinated under usual conditions, whereas 12KD is shown to react very faintly with normal human sera and can be radioiodinated easily. Both substances do have antigenic determinants common to BCG and are susceptible to peptic digestion.

Recently, it has been shown that of all the antigenic proteins of *M. leprae* only antigen 7 can easily be radioiodinated and it is being used to monitor the antibody activity in sera from leprosy patients or from individuals exposed to leprosy infection (Harboe *et al.*, 1977; Melsom *et al.*, 1980; Yoder *et al.*, 1979). It is difficult to compare *M. leprae* antigens identified using different reference systems. It is suggested that antigen 7 and antigen 12KD may be related to each other.

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