

Antimycobacterial Antibodies in Diffuse Lepromatous Leprosy Detected by Counterimmunoelectrophoresis¹

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Antimycobacterial antibodies have been widely demonstrated in the sera of patients suffering from lepromatous leprosy. This has usually been done by using the precipitin reaction in agar plates by the method described by Ouchterlony (7). By using this method, however, positive results are obtained only with sera containing rather large amounts of antibodies but not with small or trace amounts of circulating antibodies.

Sera of patients with lepromatous leprosy have been reported to give precipitin reactions when tested against *Mycobacterium lepraemurium* extracts (10, 12), or against purified polysaccharide(s) from *M. lepraemurium* (9). From this it is clear that *M. leprae* and *M. lepraemurium* share several antigens or that several antigens in both microorganisms are chemically related and therefore cross reactions are commonly observed.

This communication describes the findings when the counterimmunoelectrophoresis (CIE) technic is adapted for a quick, easy and sensitive demonstration of circulating antimycobacterial antibodies in the serum of patients having diffuse lepromatous leprosy (DLL). DLL, as well as the nodular type of lepromatous leprosy (NLL) are clinical forms of leprosy in which the presence of antimycobacterial antibodies has been reported to be a common finding. The role and significance of these antibodies in the lepromatous state is still unclear.

MATERIALS AND METHODS

Serum samples. Sera from 21 patients with lepromatous leprosy, clinically classified as "diffuse lepromatous," were obtained from the medical staff of the Centro Derma-

tológico Pascua at Mexico City. Control sera were obtained from this laboratory's personnel and from medical students at our department of immunology. Although the patient group was heterogeneous regarding age, sex, duration of disease and treatment, all of them had the disease in an active form and were adequate for the goals of the work. The control group was formed by 22 healthy individuals with no familial antecedents of mycobacterial disease (leprosy or tuberculosis).

Antigen (*Mycobacterium lepraemurium*). CFW mice bearing a four month infection with the Hawaiian strain of *Mycobacterium lepraemurium* were killed by exsanguination via the periorbital venous sinus and served as the source of infected tissue for the isolation of murine leprosy bacillus. The strain of *M. lepraemurium* was a gift from Dr. Y. T. Chang (National Arthritis and Metabolic Diseases, Bethesda, Maryland, U.S.A.) and has been maintained in our laboratory by serial infections of CFW mice. In order to facilitate the isolation of the bacilli we preferred to use the subcutaneous lepromata and the fatty tissue associated with genitalia, spleen and other organs, instead of the infected organs themselves.

The isolation and purification of bacilli from the infected tissue was done according to the procedure reported by Ueno *et al* (14). Briefly, a 2% trypsin solution (Difco 1:250, Detroit, Michigan, U.S.A.) is added to a 10% suspension of infected tissue in a final concentration of 0.2%, and the mixture is incubated in a water bath for 30 to 60 minutes at 37°C. After centrifugation at 1,000 rpm for three minutes (radius = 14.5 cm), the fluid supernatant is separated and recentrifuged at 3,000 rpm for 60 minutes. The resulting sediment is collected, resuspended in the original volume with distilled water and mixed with a sodium deoxycholate solution³

³ 1.0% sodium deoxycholate (Difco 0248-12) solution, diluted 1:10 with the bacillary suspension so that the final concentration was 0.1% (sodium deoxycholate is a protein-denaturing agent, but is used in a concentration which has no destructive effect on the infectivity of the bacilli).

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to a final concentration of 0.1%. Following incubation at 37°C for 30 minutes, the mixture is centrifuged at 1,000 rpm for three minutes and the resultant fluid supernatant is recentrifuged at 3,000 rpm for 60 more minutes. The sediment is collected, washed twice and resuspended with distilled water or saline solution as desired.

Preparation of the mycobacterial extract. Once purified, the bacillary suspension (about 12 ml) was subjected to disruption in the cold by using an electric sonifier S-125 (Branson Sonic Power, Denbury, Conn., U.S.A.) to maximal intensity (9.5 Amp, D.C.) for a total of 60 minutes. Then the suspension was centrifuged at 10,000 rpm for 15 minutes (Sorvall RC2-B, rotor SS-34), and the supernate was dialyzed against two changes (2,000 ml each) of saline-borate solution, pH 8.6 (H_3BO_3 : 0.3092 g, $Na_2B_4O_7 \cdot 10 H_2O$: 0.4768 g, NaCl: 8.30 g, distilled water to 1,000 ml) for 18 hours in the cold and stored frozen until used. The total amount of extractable protein in the mycobacterial preparation as determined by Lowry's technic⁽⁶⁾ was found to be 0.65 mg protein per ml (bovine serum albumin was used as a reference protein solution).

Counterimmunoelectrophoresis (CIE). From preliminary experiments, using the immunoelectrophoresis technic, it was observed that the mycobacterial preparation contained antigenic components which moved cathodally as well as components which moved anodally when tested against a pool of sera from leprosy patients which gave strong precipitin lines in the presence of a mycobacterial extract by Ouchterlony's double diffusion method. According to this, by the CIE technic, mycobacterial antigens which move anodally but not those which move cathodally are detected (antibodies moved cathodally at pH 8.6).

The CIE was performed in plastic plates (8 × 13 cm) containing 25 ml of 1% agarose gel (Sigma Chemical Co.) made in 0.05 M barbital buffer, pH 8.6, which fit on a Hyland electrophoresis unit. The sample wells of the agar plate were 2 mm in diameter, and the distance between antigen and antibody wells was 10 mm. The usual conditions for CIE comprised a running time of 70 minutes with a current of 30 mA and using the same 0.05 M barbital buffer, pH 8.6, as the electrode solution.

RESULTS

To establish the optimal protein concentration for CIE in the mycobacterial extract, 2 ml of the sample (0.65 mg protein per ml) were concentrated six times by putting the aqueous extract into a narrow dialysis tubing and pouring Sephadex G-200 powder (Pharmacia Fine Chemicals, Uppsala, Sweden) outside the dialysis tubing until the desired concentration was achieved. From this concentrated extract (now having 3.9 mg of protein per ml) twofold serial dilutions were prepared and each dilution was tested against two of the patients' sera. The small wells on the agarose plate were filled once and CIE was performed as indicated above. As can be seen in Figure 1, very clear cut precipitin bands between the patients' sera and the mycobacterial extract were observed with the higher concentrations of the extract. On this basis, we decided to use as a working extract, that having between 0.65 and 0.92 mg of protein per ml. The results obtained by testing 21 sera from patients with DLL against the extract were as follows: when tested by the classical Ouchterlony's method, 15 of 21 leprosy sera (71.4%) gave precipitin lines, while when tested by CIE, 18 of 21 sera (85.7%) reacted. It is important, however, to point out that in the Ouchterlony's method the precipitin lines were not as sharp and clear as those observed by CIE, and it took about 48 hours to reach the maximal development of the bands. Figure 2 shows the kind of results obtained by CIE where, in every case, it was possible to score the number of precipitin lines. This number was variable depending on the level and variety of antibodies in the sera tested, but never was greater than six. By using the CIE method, not one of 22 normal sera gave precipitin lines, while by Ouchterlony's method 3 of the same 22 normal sera gave doubtful results, probably due to the larger amount of serum required in the test.

DISCUSSION

The counterimmunoelectrophoresis technic (CIE) has been successfully used for diverse diagnostic purposes, among which are the detection of antitreponemal antibody⁽³⁾, anti-DNA antibody⁽²⁾, anti-*Entamoeba histolytica* antibody⁽¹³⁾, anti-RNA antibodies⁽¹⁾, Australian antigen^(5,8), bovine myco-

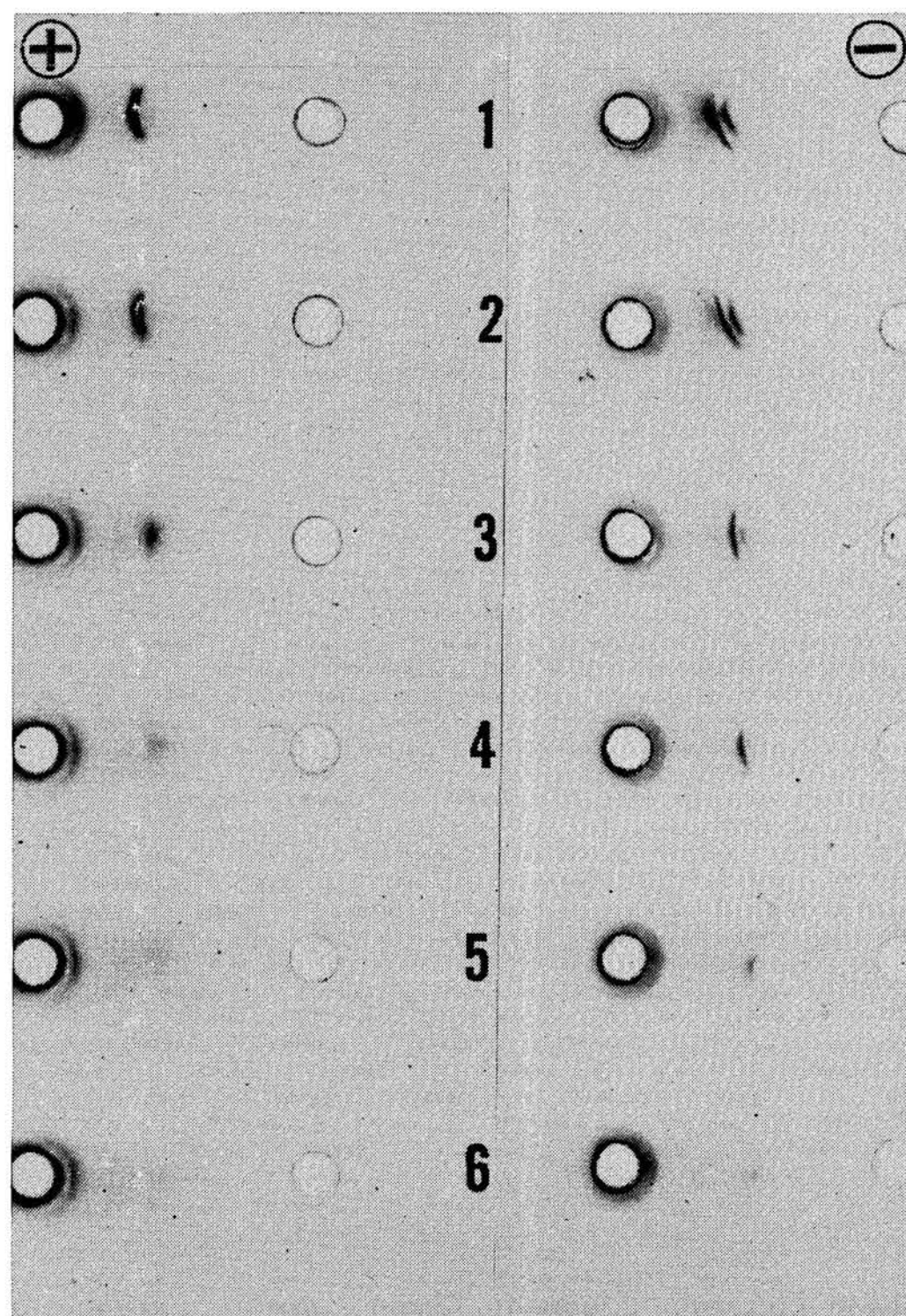


FIG. 1. Precipitin reaction between two sera from patients with diffuse lepromatous leprosy and several concentrations of a mycobacterial extract as observed by counterimmunoelectrophoresis. The sera in each pair of wells are in the left row and the cathode is at the right side. The extract concentrations, in mg of protein per ml, are 1) 1.95; 2) 0.92; 3) 0.41; 4) 0.20; 5) 0.10; and 6) 0.05.

After running (30 mA, 70 min, 0.05 M barbital buffer, pH 8.6), the agarose plate was dialyzed in the cold for 48 hours against three changes (2,000 ml each) of 0.85% saline solution and overnight against 1.0% glycerol in water. After that, the gel was dried at 37°C, stained for ten minutes with 0.25% amido black 10B (Sigma Chemical Co.) in methanol (5): water (5): glacial acetic acid (1), and destained as needed with the same mixture without dye.

plasma antigen (4), influenza antigens (11), and others.

In this work we applied the principles of the CIE, taking advantage of the fact that at least some of the antigen components of *M. lepraemurium* which cross react with human antimycobacterial antibodies move anodally at pH 8.6. When these components are tested against sera from patients having antimycobacterial antibodies (which move cathodally

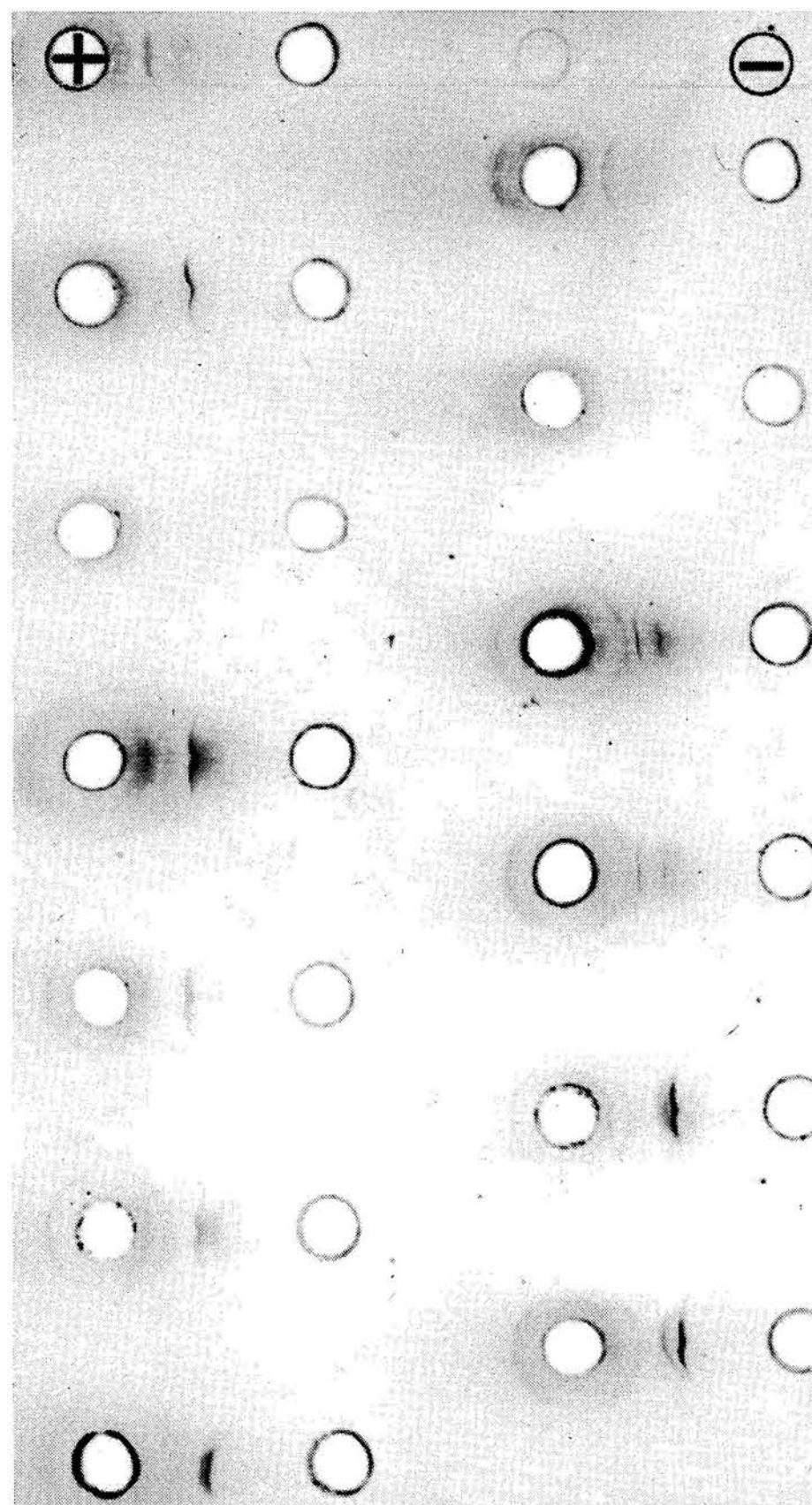


FIG. 2. Thirteen sera samples from patients with diffuse lepromatous leprosy were reacted (counterimmunoelectrophoresis) against a mycobacterial extract having 0.92 mg protein per ml. Very clear precipitin lines can be observed with at least 11 sera (84.6%). Even though photography does not show clear-cut details, with some sera multiple precipitin bands could be easily observed. Technic as for Figure 1.

under the influence of an electric field at pH 8.6), very clear precipitin lines are obtained within relatively short periods of time (40 to 70 minutes at 30 mA). Besides the short time required, the technic is very easy to perform since any immunoelectrophoresis unit can be used. The results are highly reproducible and very small or trace amounts of antibody can be detected. This, together with the simple method for isolation of the bacilli from infected tissue (14), makes the use of CIE very attractive when antimycobacterial antibodies need to be detected.

We believe that studies such as these are

important in the detection of antibodies which could be mediators of damage, both in natural and experimental infections. Data regarding the presence of antimycobacterial antibodies (and others which were not studied in this work) as well as their level in the blood circulation, could be used to complement clinical findings and they could help to explain certain pathologic situations usually present in patients suffering from lepromatous leprosy, such as kidney damage, skin lesions, and other alterations. Finally, the technic can be further simplified by using an antigenic extract prepared from *M. tuberculosis* or BCG cultures (unpublished observations).

SUMMARY

By using the counterimmunoelectrophoresis (CIE) technic it was possible to demonstrate the presence of antimycobacterial antibodies in the sera of patients suffering from lepromatous leprosy. The antigen was prepared from isolated *Mycobacterium lepraemurium*.

The technic was more reliable and quicker than Ouchterlony's classical double diffusion in gel. Clinical application of the method is therefore recommended.

RESUMEN

Por medio de la técnica de la contrainmuno-electroforésis (CIE) se demostró la presencia de anticuerpos contra antígenos micobacterianos en el suero de pacientes con lepra lepromatosa. El antígeno usado se preparó a partir de *Mycobacterium lepraemurium* purificado.

Como ya se ha demostrado por varios autores, la técnica de la CIE resultó más confiable y rápida que el método clásico de la doble difusión en gel diseñado por Ouchterlony. En base a lo anterior se recomienda la aplicación en la clínica del método de la CIE.

RÉSUMÉ

En utilisant des techniques de contreimmunoelectrophorèse, on a pu mettre en évidence la présence d'anticorps antimycobactériens dans le serum de malades souffrant de lèpre lepromateuse. L'antigène a été préparé à partir d'un isolat de *Mycobacterium lepraemurium*.

La technique était fidèle et plus rapide que la méthode classique de double diffusion sur gel d'Ouchterlony. On recommande dès lors l'application clinique de cette méthode.

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