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An Assay for Antibodies in Leprosy Sera Reacting with Ribonucleoprotein (RNP), a Mycobacterial Ribosomal Antigen, Using Crossed Immunoelectrophoresis with Intermediate Gel[1]

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The immune response to bacterial ribosomes has been a subject of increasing interest since the initial work of Youmans and Youmans [29] on ribosomal fractions of *Mycobacterium tuberculosis*. This is largely due to the fact that ribosomal preparations appear to provide effective, non-toxic vaccines against a variety of intra- and extracellular bacteria including, in addition to *M. tuberculosis*, *Staphylococcus aureus* [28], *Salmonella typhimurium* [7], *Salmonella typhi* [16], *Nocardia asteroides* [26], *Pseudomonas aeruginosa* [12] and *Bordetella pertussis* [4].

However, investigations of the immune response to particular ribosomal components following immunization or during the course of an infection have been hampered by the lack of a convenient quantitative antibody assay. Studies of the antibody response to ribosomal fractions have most frequently employed the qualitative techniques of double immunodiffusion and immunoelectrophoresis, sometimes with semiquantitative modifications [2, 9].

We describe here a quantitative method for determination of antibodies found in the sera of leprosy patients which react with the 16S ribosomal subunit of *M. smegmatis*. This ribonucleoprotein (RNP) antigen, which was originally termed the β precipitinogen by Navalkar, *et al.* [18], corresponds to antigen no. 1 in the *M. smegmatis* reference system of Kronvall, *et al.* [8]. The β precipitinogen is present in most mycobacteria and numerous nocardia species [21] and forms a major precipitin line with sera from lepromatous leprosy patients, both in double immunodiffusion [17] and in crossed immunoelectrophoresis [8]. The *M. leprae* antigen which corresponds to antigen no. 1 of *M. smegmatis* has been termed *M. leprae* antigen no. 5 [6].

Our method is based on the intermediate gel modification by Svendsen and Axelsen [27] of the Laurell crossed immunoelectrophoresis technique [10]. The method utilizes a lepromatous serum pool (LSP) both in the

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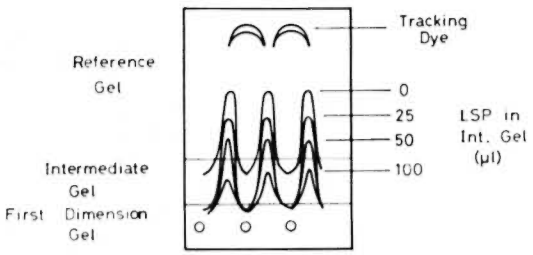

FIG. 1a. Precipitin lines obtained on four standard plates from a single run superimposed upon each other to show the effect of the addition of increasing amounts of lepromatous serum pool (LSP) in the intermediate gel. Total volume added was adjusted to 100 μl with 0.15 M NaCl. The position of the tracking dye is approximate and varies slightly from plate to plate.

reference gel and as standard in the intermediate gel. The antigen preparation consists of the supernatant fraction of an *M. smegmatis* sonicate. The method depends on a reduction in the rate of migration of the peak of the precipitin line formed in the reference gel by the incorporation of leprosy sera in the intermediate gel. The method should be applicable to other precipitating systems in which a major precipitin reacts with an antigen of high electrophoretic mobility.

MATERIALS AND METHODS

Patients' sera. Serum samples were obtained from 24 newly-diagnosed, untreated leprosy patients. The patients were classified according to the Ridley-Jopling system on the basis of clinical features, skin smears for acid-fast bacilli (AFB), and histopathologic criteria ([22]). Twelve of the patients had skin smears which were negative for AFB. Of these, one was classified as polar tuberculoid (TT), nine were classified as borderline tuberculoid (BT), and one was borderline (BB). One was a "neural" tuberculoid patient with no skin lesions, whom we have included in the TT group. The remaining 12 patients were positive for AFB in skin smears. Of these, six were classified as borderline lepromatous (BL) and six as polar lepromatous (LL). Blood was obtained by venipuncture and allowed to clot at room temperature. After standing overnight at 4°C, the serum was separated by centrifugation and stored at −20° or −70°C.

A lepromatous serum pool (LSP) was prepared from 15 BL and LL donors. Some of these were treated patients, but all had been positive for AFB in their skin smears within the past three years.

Antigen. An *M. smegmatis* supernatant fraction following ultrasonic disruption of the bacilli was kindly provided by Dr. Göran Kronvall, Department of Medical Microbiology, University of Lund, Sweden ([8]).

Quantitative intermediate gel crossed immunoelectrophoresis. The method is a modification of that described in detail by Axelsen ([1]). Gels were prepared from 1% agarose (Litex, Glostrup, Denmark) in barbital buffer (pH 8.6, ionic strength = 0.02).

(a) First dimension gel. The first dimension gel was cast between glass plates (11 cm × 20 cm) using a U-shaped plastic spacer 1.1 mm thick. Antigen wells of 4 mm diameter were cut 1.5 cm apart in three rows also spaced 1.5 cm apart. Wells were filled with 10 μl of the *M. smegmatis* antigen preparation, except the end wells in each row, which contained 10 μl of tracking dye (0.1% bromphenol blue).

Electrophoresis was performed in a Behringwerke AG apparatus (Marburg-Lahn) at 100 volts (10 V/cm) until the tracking dye had advanced 1.5 cm (approximately 7 min). This procedure places the RNP antigen midway between the holes and results in the formation of three precipitin lines per plate during the second dimension electrophoresis. The first dimension gel was cut in strips 1.5 cm wide and 5.5 cm long and placed on an agarose precoated glass plate (5.5 cm × 8.0 cm) in such a way that the first hole was located 0.5 cm from the left side of the plate (Fig. 1a).

(b) Intermediate gel. The intermediate gel was poured in a space 1.5 cm wide formed by the first dimension gel and the edge of a second glass plate 1.1 mm thick. The intermediate gel contained 1.0 ml 1% agarose in buffer and 100 μl of the patient's serum (12 μl/cm²) or 100 μl of the standards.

(c) Reference gel and second dimension electrophoresis. The reference gel contained 70 μl LSP (2.5 μl/cm²) in 3.0 ml agarose solution in buffer. Immediately before the second dimension electrophoresis, 10 μl of the tracking dye was added to the second and third wells in the first dimension

gel. The second dimension electrophoresis was performed at 12 volts (1.5 V/cm) until the leading edge of the tracking dye was about 0.5 cm from the top edge of the plate (approximately seven to nine hr). During the electrophoresis the gels were cooled by water circulating at a temperature of 15°C.

(d) Washing, drying, and staining. At the end of the run the position of the leading edge of each spot of tracking dye was recorded (to the nearest 0.5 mm), measuring from the lower edge of the intermediate gel. Plates were then developed in a moist box at room temperature overnight, then washed by immersion in 0.1 M NaCl for an additional 24 hr. Following pressing, rinsing for 15 min in distilled water, and re-pressing, the plates were dried at 37°C. Staining with Coomassie Blue and destaining were performed as described ([1]).

(e) Quantitation. The height of each precipitin peak above lower edge of the intermediate gel was measured to the nearest 0.1 mm. The ratio of the height of each peak to the distance migrated by the leading edge of the nearest tracking dye was calculated to give the relative migration. For the middle precipitin peak the peak height was divided by the average distance migrated by the two tracking dye spots in order to obtain the relative migration.

(f) Assay of unknown samples. For determinations of antibody activity eight unknown samples were run simultaneously with four standards. For each plate the values of the relative migration of the three peaks were averaged, and the average was used to calculate the anti-RNP activity as % LSP from the standard curve (Fig. 1b). Each unknown sample was assayed in four separate runs on four different days. The data (Fig. 3) are expressed as the mean of the four determinations.

Statistical analysis was done using the Mann-Whitney U Test ([23]).

RESULTS

Sketches of the precipitin lines obtained from a set of four standard plates are shown superimposed upon each other (Fig. 1a). (Minor precipitins reacting with antigens of lower electrophoretic mobility are not shown.) The height of the peak is progressively reduced by the incorporation of increasing amounts of LSP into the intermediate gel. When the average relative migration for the lines of each standard plate is calculated, the standard curve shown in Fig. 1b is obtained. The curve is not linear due to the discontinuity between the intermediate and reference gels.

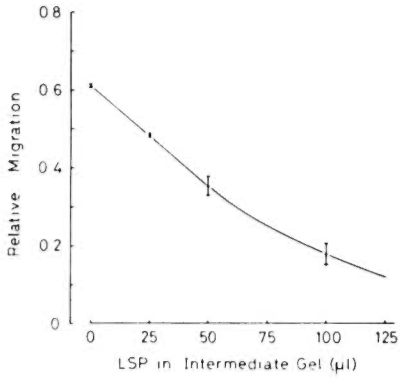

FIG. 1b. Standard curve obtained from the plates in Fig. 1a. Relative migration calculated with respect to the position of the tracking dye as described in Materials and Methods. Data represent the mean ± S.D. for the three precipitin peaks on each plate.

The results from a set of nine standard plates run in duplicate on the same day are shown in Fig. 2. There is good agreement between duplicate plates. Again, curvature in the region above 50 µl LSP is evident. This occurs at about relative migration 0.25, which is the point at which the precipitin peak emerges from the intermediate gel. The standard curve is again linear at higher serum concentrations up to at least 150 µl LSP (data not shown). It is evident from Fig. 2 that the method is extremely sensitive. As little as 5 µl LSP in the intermediate gel can give a significant reduction in the migration of the reference precipitin peak.

The results obtained from 24 leprosy sera are illustrated in Fig. 3. The AFB-negative group is clearly differentiated from the AFB-positive group by their anti-RNP activity. The AFB-negative group has an activity of 22 ± 17% LSP (mean ± S.D.). The AFB-positive group has an activity of 60 ± 24% LSP. This difference is highly significant ($p < 0.001$). Within the AFB-positive lepromatous group in which the bacillary index (BI) of the skin smears ranged from 1.2 to 5.3, the level of anti-RNP activity of

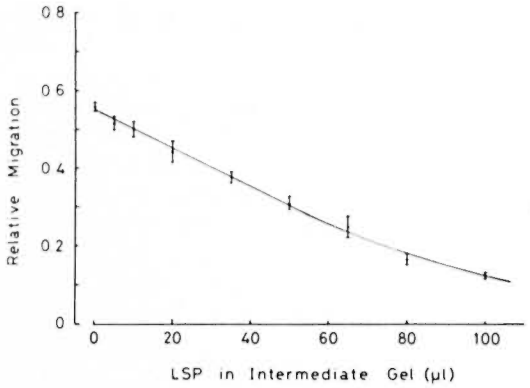

FIG. 2. Standard curve obtained from duplicate plates. Each point represents the mean ± S.D. for the six precipitin peaks (three per plate).

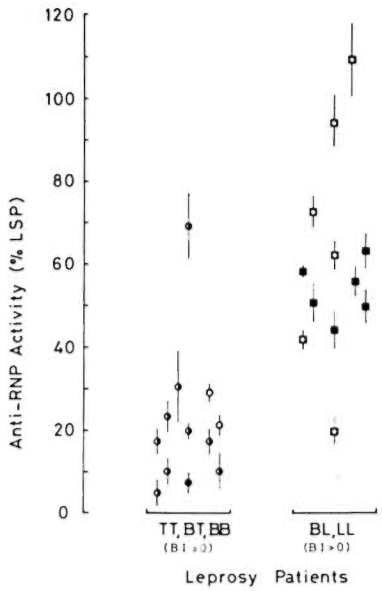

FIG. 3. Anti-RNP activity in the sera of untreated leprosy patients. Left: TT, open circles; BT, half-filled circles; BB, closed circle. Right: BL, open squares; LL, filled squares. The results represent the mean ± S.E.M. of four separate determinations.

the sera showed no correlation with the BI of the patient (data not shown). Nor did the anti-RNP activity of the six BL sera (66 ± 33% LSP) differ significantly from that of the six LL sera (54 ± 6.8% LSP). In this series of BL and LL patients, the scatter in the BL group was much greater than in the LL group.

DISCUSSION

The semi-quantitative method originally developed by Svendsen and Axelsen ([27]) utilized measurements of the area under the precipitin curve on plates run to near equilibrium (3 V/cm for 22 hr). Their method was designed to titer simultaneously numerous precipitins present in complex sera. Our method is a velocity adaptation, which permits quantitation of the major precipitin present in leprosy sera which interacts with a ribosomal component of *M. smegmatis*. In this adaptation the short electrophoresis time (1.5 V/cm for seven to nine hr) permits measurement of the rate of migration of the precipitin peak relative to the rate of migration of the tracking dye. This innovation serves two important purposes: First, use of the tracking dye compensates for the inevitable variations in the flow of electric current through the different plates and thus permits accurate quantitation of the rate of peak migration. Secondly, the short electrophoresis time with reduced current permits the use of concentrations of antibody in the reference gel which are so low that the top of the reference precipitin peak would migrate off the top of the plate if the electrophoresis were prolonged. The use of low concentrations of antibody in the reference gel maximizes the sensitivity of the system. Clearly, the greater the rate of migration in the reference gel in the absence of antibody in the intermediate gel, the greater is the sensitivity of the system to the addition of precipitins in the intermediate gel.

The *M. smegmatis* antigen which we call RNP was termed antigen no. 1 by Kronvall, *et al.* ([8]). This antigen is identical to the precipitinogen termed β by Navalkar, *et al.* ([18]) and Ridell, *et al.* ([20]). This immunogen has been found to consistently give rise to precipitins in the sera of lepromatous leprosy patients. Navalkar ([17]), using double immunodiffusion, found β precipitins in 23 of 26 lepromatous leprosy sera, in ten of 17 dimorphous (borderline) leprosy sera, and in only three of 56 tuberculoid leprosy sera. Using the more sensitive technique of crossed immunoelectrophoresis with intermediate gel, Kronvall, *et al.* ([8]) found precipitins to *M. smegmatis* antigen no. 1 in all lepromatous leprosy (BL and LL) patients (47/47). They also detected precipi-

tins in 22 of 44 tuberculoid leprosy sera. However, estimation of the titers showed that while 41 of the 47 lepromatous sera had titers of 3+ or 4+, only two of the 44 tuberculoid sera had titers that high. Our findings are in good agreement with these earlier qualitative and semi-quantitative results. We also found that the anti-RNP activity reflects the antigenic load of the patient: 11 of 12 patients who were skin-smear negative for AFB had activities of less than 35% LSP whereas 11 of 12 patients who were skin-smear positive for AFB had activities above 35% LSP.

It is clear that high levels of anti-RNP antibodies characterize the lepromatous group of patients. Measurement of the anti-RNP activity in the sera of leprosy patients could be a useful addition to the methods available for the classification of leprosy patients. An assay for antibodies to RNP might also have application in the early serodiagnosis of multi-bacillary leprosy.

It remains to be determined whether the increased anti-RNP activity in lepromatous leprosy sera merely reflects the increased antigenic load in the multi-bacillary lepromatous patient or, alternatively, whether anti-RNP antibody may directly or indirectly play an active role in the suppression of cell-mediated immunity which accompanies progressive leprosy infections ([25]). At the same time, it is known that ribosomal fractions ([24, 26]), including those of mycobacteria ([13, 19]), are capable of inducing delayed-type hypersensitivity as well as antibody responses, and it is conceivable that *M. leprae* ribosomes play a part in the induction of cell-mediated immunity following subclinical leprosy infection ([5]). The availability of purified *M. leprae* ribosomes would permit assessment of delayed-type hypersensitivity to *M. leprae* antigen no. 5 in leprosy patients and its relation, if any, to antibody formation to the same antigen.

Some workers have emphasized the possible role of contaminating extrinsic factors in the immune response to ribosomes ([2, 3, 11, 15]). However, Ridell, *et al.* ([20]) have localized the β precipitinogen to the 16S core particle of the mycobacterial ribosome, and it seems unlikely that it represents an extrinisic factor. Nevertheless, it does show an unusually high heat stability ([20]), and the chemical nature of the immunodominant group deserves further investigation.

This assay for antibodies to antigen no. 5 of *M. leprae* is the second described for antibodies to a defined *M. leprae* antigen. Melsom, *et al.* ([14]) have purified antigen no. 7 of *M. leprae* and developed a radioimmunoassay for antibodies to antigen no. 7. The availability of these and the development of additional assays for defined antigens will help to elucidate the role of particular antigens and their antibodies in the pathogenesis of leprosy.

This method requires neither purified antigen nor a monospecific antiserum. It should be widely applicable to the assay of precipitating antibodies in any system in which an antigen of high electrophoretic mobility forms a major precipitin in crossed immunoelectrophoresis.

SUMMARY

The technique of crossed immunoelectrophoresis with intermediate gel has been adapted to provide a quantitative assay for antibodies to a mycobacterial ribosomal antigen termed ribonucleoprotein (RNP) antigen. This antigen is no. 1 in the *M. smegmatis* reference system and corresponds to antigen no. 5 of *M. leprae*. The assay method measures changes in the rate of migration of the reference precipitin peak caused by the addition of serum in the intermediate gel and utilizes a lepromatous serum pool (LSP) both in the reference gel and as a standard in the intermediate gel. The anti-RNP activity in 24 leprosy sera differentiated the pauci-bacillary tuberculoid group (11 of 12 <35% LSP) from the multi-bacillary lepromatous group (11 of 12 >35% LSP). These findings confirm the work of others which indicates that assay of anti-RNP activity may have applications in the classification of leprosy patients and in the serodiagnosis of lepromatous leprosy infections. This method should also be applicable in other systems in which an antigen of high electrophoretic mobility forms a major precipitin in crossed immunoelectrophoresis.

RESUMEN

Se adaptó la técnica de la inmunoelectroforésis cruzada con gel intermedio para la determinación cuantitativa de los anticuerpos contra un antígeno ribo-

somal micobacteriano denominado "antígeno ribonucleoproteína" (RNP). Este antígeno es el No. 1 en el sistema de referencia del *M. smegmatis* y corresponde al antígeno No. 5 del *M. leprae*. El método de ensayo mide los cambios en la velocidad de migración del pico de precipitación de referencia causado por la adición de suero en el gel intermedio y utiliza una mezcla de sueros lepromatosos (MSL) tanto en el gel de referencia como en el gel intermedio (en este caso como estandard). La actividad de anti-RNP en 24 sueros de pacientes con lepra, permitió diferenciar al grupo tuberculoide escaso en bacilos (11 de 12 <35% MSL) del grupo lepromatoso multibacilar (11 de 12 >35% MSL). Estos hallazgos confirman el trabajo de otros que indica que el ensayo de la actividad de anti-RNP puede tener aplicaciones en la clasificación de los pacientes con lepra y en el serodiagnóstico de la lepra lepromatosa. Este método podría ser aplicable también a otros sistemas en los cuales un antígeno de alta movilidad electroforética reaccione con su anticuerpo y forme un precipitado en la inmunoelectroforésis cruzada.

RÉSUMÉ

La technique de l'immunoélectrophorèse croisée sur gel intermédiaire a été adaptée en vue de fournir une méthode quantitative pour doser les anticorps à un antigène ribosomique mycobactérien appelé l'antigène ribonucléoprotéinique (RNP). Cet antigène porte le n°1 dans le système de référence de *M. smegmatis*, et correspond à l'antigène n°5 de *M. leprae*. Cette méthode d'évaluation mesure les changements intervenus dans le taux de migration du pic de la précipitine de référence, après addition de serum au gel intermédiaire. On utilise un pool de serum lépromateux (LSP), à la fois pour le gel de référence, et comme standard dans le gel intermédiaire. L'activité anti-RNP dans 24 échantillons de serum lépromateux a permis de distinguer le groupe tuberculoïde pauci-bacillaire (11 des 12 échantillons en-dessous de 35% de LSP) du groupe lépromateux multi-bacillaire (11 des 12 échantillons au-dessus de 35% de LSP). Ces observations confirment celles qui ont été faites par d'autres auteurs et qui indiquent que l'évaluation de l'activité anti-RNP peut être appliquée à la classification des malades lépromateux, ainsi que pour le sérodiagnostic des infections lépromateuses. Cette méthode devrait également être appliquée à d'autres systèmes, dans lesquels un antigène présentant une mobilité électrophorétique élevée constitue une précipitine d'importance dans les réactions d'immunoélectrophorèse croisée.

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