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Detection of Mycobacterial Antigens in Leprosy Serum Immune Complex

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The antigens from immune complexes of sera from patients with mycobacterial diseases were released by sodium dodecyl sulfate. The antigenic activity of the released proteins was tested by agar gel diffusion and immunoelectrophoresis. This simple method provided direct evidence for the presence of mycobacterial antigens in the immune complexes of sera from patients with leprosy and tuberculosis.

The presence of circulating immune complexes (CICs) in the bloodstream and its clinical importance in the immunopathology of disease is well known (19). In leprosy patients also, CICs containing *Mycobacterium leprae* antigens have been demonstrated (1, 15). Since certain antigens of *M. leprae* and *M. tuberculosis* have been found to exert an immunoregulatory effect (14, 20), the characterization of antigens involved in the formation of CICs in leprosy and tuberculosis is likely to help in understanding the pathogenesis of the diseases (8). It would also be interesting to observe whether CICs formed along the spectrum of leprosy (16) or tuberculosis (10) differ in their antigenic constituents and to observe the effect of treatment on these constituents. Therefore, several attempts have been made to isolate and characterize antigens from CICs. However, the methods of dissociation of antigen and antibody by treatment with acids, alkalies, or high-molarity salt solutions (4, 9, 18), by isoelectric focusing (11, 12), or by ion-exchange chromatography (6) are lengthy and time consuming. We report here a simple and novel method for the demonstration of mycobacterial antigens in the CICs of patients suffering from mycobacterial infections.

Eighty-four serum samples were obtained from patients with various types of active leprosy who were attending the out-patient department of the Central JALMA Institute for Leprosy, Agra, India. Classification of the disease was made according to the Ridley-Jopling scale (16). Of these patients, 35 belonged to the tuberculoid/borderline tuberculoid (TT/BT) group, 11 belonged to the borderline (BB) group, and 38 belonged to the borderline lepromatous/lepromatous (BL/LL) group. The patients were either untreated or had been undergoing treatment (for up to 2 years). Twenty sera from patients with pulmonary tuberculosis were obtained from the T.B. Demonstration and Training Centre, Agra, India. Normal (control) sera were obtained from 20 healthy volunteers. All of the sera were preserved with 0.1% (wt/vol) sodium azide and stored at −20°C. *M. leprae* was purified from an autopsied liver of an LL patient by the World Health Organization protocol (3). Rabbit antibodies against the sonic extract of *M. leprae* (R-AMLAB) and against other mycobacteria were prepared by the method described previously (5). Pooled LL serum (PLLS) was also used as an antibody source, since it has been observed by us (17) and others (2) that the sera from LL patients contain high titers of circulating antibodies to *M. leprae* antigens.

The precipitation of CICs with 3.5% (wt/vol) polyethylene glycol 6000 was done by the method of Zubler et al. (21). The final pellets were reconstituted in normal saline to the original serum volume (2 ml).

A 10% sodium dodecyl sulfate (SDS) solution and CICs dissolved in normal saline were mixed in proportions of 1:4, and the mixture was incubated for 2 h at 37°C. For complete removal of SDS from CICs, the mixture was then dialyzed against phosphate-buffered saline (pH 7.2) for 48 h with four changes of phosphate-buffered saline. The samples thus prepared were used for the immunoassay. Ouchterlony immunodiffusion was performed in 1% (wt/vol) agarose (Sigma Chemical Co.) in 0.045 M barbitone buffer (pH 8.3). For the detection of antigen, 20 μl of R-AMLAB or PLLS was applied to the central well, and 10 μl (50 to 100 μg of protein) of test samples was applied to the surrounding wells. Immunoelectrophoresis was done on a 7.5-by-2.5-cm slide layered with 2.5 ml of 1% agarose (Sigma) in 0.045 M barbitone buffer (pH 8.3). Each sample to be tested for the presence of antigen was electrophoresed in the sample well. After electrophoresis, the precipitation reaction was developed with R-AMLAB. In an attempt to study the nature of constituent antigens, the CIC preparations were either kept in boiling water for 5 min (for determining the heat stability) or digested with pepsin (for finding out whether the antigenic

TABLE 1. Incidence of mycobacterial antigens in CICs

Disease (status) of patient	No. of patients (n = 124)	No. (%) antigen positive
BL/LL leprosy		
Untreated	15	11 (73)
Treated[a]	23	11 (48)
BT leprosy		
Untreated[b]		
Treated	11	3 (27)
TT/BT leprosy		
Untreated	15	7 (47)
Treated	20	8 (40)
Pulmonary tuberculosis		
Treated	20	18 (90)
Healthy	20	0 (0)

[a] The disease was still active in all of the treated patients.
[b] Only one patient was untreated.

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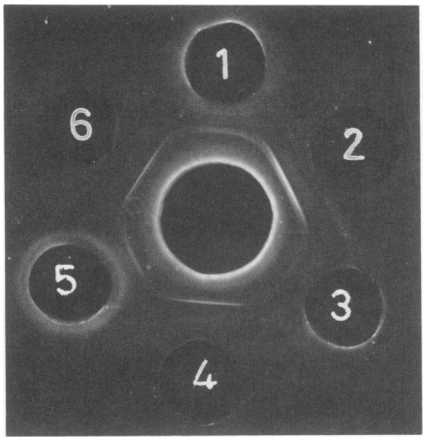

FIG. 1. Double diffusion (Ouchterlony) test. Wells 1, 3, and 5, SDS-treated CICs from LL sera; wells 2, 4, and 6, *M. leprae* sonic extracts; central well, pooled LL serum.

moieties were proteinaceous) by the method described previously (7).

Only the SDS-treated CICs showed precipitin reactions with R-AMLAB and PLLS by double diffusion. The sera, untreated CICs, or supernatants obtained after CIC precipitation did not react with antibodies. In the CIC test, 73% of untreated BL/LL patients and 48% of treated BL/LL patients showed antigen in the CICs, whereas in the TT/BT group, 47% of the untreated cases and 40% of the treated cases showed antigen (Table 1). Only 3 out of 11 cases in the BB group (one patient was untreated) showed antibody reactivity. Mycobacterial antigens could also be detected in the SDS-treated CICs in 90% of the sera from tuberculosis patients by use of rabbit anti-*M. tuberculosis* antibodies. None of the 20 healthy volunteers showed any precipitin reaction. The double diffusion results are represented in Fig. 1. Some of the antigens in SDS-treated CICs from leprosy and tuberculosis patients were found to be shared by other mycobacterial species also, as shown by reactions of identity in double diffusion tests using sonic extracts of *M. vaccae* and *M. bovis* BCG and corresponding rabbit antibodies.

By immunoelectrophoresis, it was noted that the antigens in most of the samples had a mobility between the α and β regions, whereas in a few others the mobility was in the γ region (Fig. 2 and 3). Even after boiling of the CIC preparations for 5 min, the antigenic components were detectable by immunodiffusion. However, after digestion with pepsin, the activity of antigens was completely lost, and no precipitin lines could be seen.

The present study has shown that the antigens can be released from the CICs by SDS treatment. This detergent, which seems to have antimicrobial activity, has the property

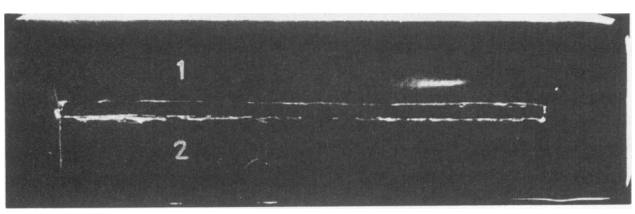

FIG. 2. Immunoelectrophoresis. Well 1, SDS-treated CIC of LL serum; well 2, SDS-treated CIC of normal serum; central trough, rabbit anti-*M. leprae* antibody.

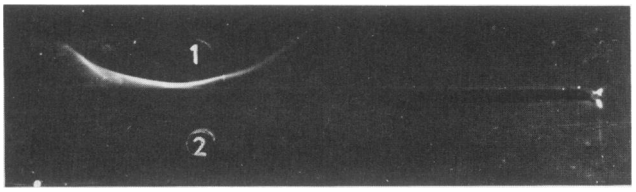

FIG. 3. Immunoelectrophoresis. Well 1, SDS-treated CIC of LL serum; well 2, SDS-treated CIC of normal serum; central trough, pooled LL serum.

of breaking down proteins into their constituent polypeptide chains (13). The affinity column-purified immunoglobulin G fraction, when electrophoresed by SDS-polyacrylamide gel electrophoresis, is known to dissociate into heavy and light chains. The present study has shown that this chemical also has the capacity to dissociate the antigen-antibody complex in such a way that after the dissociation the antigen and antibody cannot combine to form the complex again. On the other hand, the epitopes of certain antigens may not be affected by this treatment. This study also indicates that there may be an antibody-excess situation in leprosy sera, since the serum as such or the supernatant obtained after the precipitation of CICs did not react with either R-AMLAB or PLLS. Recently, three protein components of *M. leprae* origin have been described in the CICs of sera from patients with leprosy. These antigens were not *M. leprae* specific, as they reacted with anti-BCG antibody but not with anti-*M. leprae* antibody which was either raised in rabbits or found in LL serum (1). However, in our system, we observed precipitin reactions between the antigens of CICs and R-AMLAB and PLLS.

In an attempt to further simplify the method of SDS-mediated antigen release, we discovered that prior precipitation of CICs with polyethylene glycol is not necessary. The direct addition of a 0.25 volume of 10% SDS to serum can release the antigens.

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LITERATURE CITED

1. **Chakraborty, A. K., M. Maire, K. Saha, and P. H. Lambert.** 1983. Identification of components of IC purified human sera. Clin. Exp. Immunol. **51**:225–231.
2. **Cho, S. N., D. L. Yanagihara, S. W. Hunter, R. H. Gelber, and P. J. Brennan.** 1983. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. Infect. Immun. **41**:1077–1083.
3. **Draper, P.** 1979. Report of the enlarged steering committee meeting for research on immunology of leprosy (IMMLEP). World Health Organization, Geneva.
4. **Givol, D., S. Fuchs, and M. Sela.** 1962. Isolation of antibodies to antigens of low molecular weight. Biochim. Biophys. Acta **63**:222–229.
5. **Harboe, N., and A. Ingild.** 1973. Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immunol. **2**(Suppl. 1):161–164.
6. **Hoffken, K., F. Bosse, U. Steih, and C. G. Schmidt.** 1982. Dissociation and isolation of antigen and antibody from immune complexes. J. Immunol. Methods **53**:51–59.
7. **Hudson, L., and F. C. Hay (ed.).** 1980. Practical immunology, p. 196–202. Blackwell Scientific Publications, Ltd., Oxford.
8. **Jones, V. E., and E. Orlans.** 1981. Isolation of immune com-

plexes and characterisation of their constituent antigens and antibodies in some human diseases. A review. J. Immunol. Methods **44**:249–270.
9. **Kabat, E. A.** 1961. Experimental immunochemistry. Charles C Thomas, Publisher, Springfield, Ill.
10. **Lenzini, L., P. Rottoli, and L. Rottoli.** 1977. The spectrum of human tuberculosis. Clin. Exp. Immunol. **27**:230–239.
11. **Maidment, B. W., L. D. Papsidero, and T. M. Chu.** 1980. Isoelectric focusing—a new approach to the study of immune complexes. J. Immunol. Methods **35**:297–306.
12. **Maidment, B. W., L. D. Papsidero, T. Nemoto, and T. M. Chu.** 1981. Recovery of immunologically reactive antibodies and antigens from breast cancer immune complexes by preparative isoelectric focusing. Cancer Res. **41**:795–800.
13. **Maizel, J. V.** 1969. Acrylamide gel electrophoresis of proteins and nucleic acids, p. 334–362. *In* K. K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press, Inc., New York.
14. **Mehra, V., P. J. Brennan, E. Rada, J. Convit, and B. R. Bloom.** 1984. Lymphocyte suppression induced by unique *M. leprae* glycolipid. Nature (London) **308**:194–196.
15. **Ramanathan, V. D., O. Parkash, G. Ramu, D. Parkar, J. Curtis, U. Sengupta, and J. L. Turk.** 1984. Isolation and analysis of circulating immune complexes in leprosy. Clin. Immunol. Immunopathol. **32**:261–268.
16. **Ridley, D. S., and W. H. Jopling.** 1966. Classification of leprosy according to immunity. A five group system. Int. J. Lepr. **34**:255–273.
17. **Sinha, S., U. Sengupta, G. Rámu, and J. Ivanyi.** 1985. Serological survey of leprosy and control subjects by a monoclonal antibody-based immunoassay. Int. J. Lepr. **53**:33–38.
18. **Slobin, L. I., and M. Sela.** 1965. Use of urea in purification of antibodies. Biochim. Biophys. Acta **107**:593–604.
19. **Theofilopoulos, A. N., and F. J. Dixon.** 1979. The biology and detection of immune complexes. Adv. Immunol. **28**:89–220.
20. **Wadee, A. A., D. Mendelsohn, and A. R. Rabson.** 1983. Characterisation of a suppressor cell activating factor (SCAF) released by adherent cells. J. Immunol. **130**:2266–2270.
21. **Zubler, R. H., L. H. Perrin, W. D. Creighton, and P. H. Lambert.** 1977. The use of polyethylene glycol (PEG) to concentrate immune complexes from serum or plasma samples. Ann. Rheum. Dis. **36**(Suppl.):23–25.