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An ELISA-inhibition test using monoclonal antibody for the serology of leprosy

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SUMMARY

In this study a mouse monoclonal antibody (47–9) is described, which recognized an epitope on the 36 kD protein antigen of *M. leprae*. The monoclonal antibody showed specificity for *M. leprae*. An ELISA-inhibition test based on the competitive inhibition by antibodies from human test sera of the binding of the enzym labelled monoclonal antibody to *M. leprae* was developed. Seropositivity was found in 100% of the multibacillary leprosy patients group and in 91% of the paucibacillary patients. Only 5% of the 223 control sera were positive.

Because of the high seropositivity found in both multi- and paucibacillary patients, it is suggested that the epitope on the 36 kD antigen is immuno-dominant.

Therefore the ELISA-inhibition test described herein might well be a suitable tool for diagnosis of leprosy.

Keywords leprosy monoclonal antibody ELISA-inhibition test

INTRODUCTION

Despite the availability of anti-leprosy drugs since the 1940's the fight against leprosy has been only partly successful. Drug treatment is started when the diagnosis of leprosy is made on the appearance of clinical symptoms. However, long before the symptoms are manifest, the leprosy patients and in particular the lepromatous patients are highly infectious, transmitting the disease in the community. Early case finding, accompanied by highly effective drug treatment, would not only minimise the pathological effects of the infection but also reduce the infectious reservoir in the population (Sansarricq, 1981).

Serological tests for leprosy could detect subjects incubating the infection and assist in making decisions about the type and duration of treatment, monitoring the patient's response to treatment and determining the prognosis of the disease. Furthermore, at the community level, a serodiagnostic test could have great value as an epidemiological tool.

An advantage of methods based on antibody detection for diagnosis is that they exploit the amplification provided by the immune system. Various workers have developed serological tests for leprosy based on antibody detection. Among these are a fluorescent antibody absorbtion test (FLA-ABS), using whole *M. leprae* bacilli (Abe *et al.*, 1980), a radioimmunoassay (RIA) using antigen 7 of *M. leprae* (Harboe *et al.*, 1978), an enzyme linked immuno assay (ELISA) with the phenolic glycolipid of *M. leprae* (Cho *et al.*, 1983) and recently an antibody competition RIA using monoclonal antibody for the detection of antibodies to the MY2a determinant of *M. leprae* (Sinha *et al.*, 1983).

Since antigen-sharing across mycobacterial species in leprosy is extensive (Daniel & Janicki,

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1978; Goren, 1982), the specificity of a serodiagnostic test should be high. With the use of monoclonal antibodies, through their recognition of a single epitope, the specificity of a serodiagnostic test would be improved (Mitchell, 1981).

Previously we reported the identification of specific *M. leprae* protein antigens which were recognized by leprosy patient's sera (Klatser, van Rens & Eggelte, 1984). We now have a monoclonal antibody which recognizes one of these specific antigens, a 36 kD protein, and have used this monoclonal antibody to develop an ELISA-inhibition test. We present here results of the screening of various sera in this ELISA-inhibition test.

MATERIALS AND METHODS

Antigen preparation. M. Leprae was purified from livers of experimentally infected nine-banded armadillos (World Health Organization, 1980). Other mycobacteria were grown on Sauton medium. Ultrasonication was done as previously described (Klatser *et al.*, 1984).

The antigenic preparation used in the ELISA-inhibition assay was made by extracting a 10,000 g pellet fraction of an *M. leprae* sonicate with 2% sodium deoxycholate for 10 min at 100°C. The extract was centrifuged at 10,000 **g** for 5 min and diluted (1:1) with anhydrous glycerol. Thimerosal was added to a final concentration of 0·02% and the preparation was stored at −20°C.

Hybridoma technology. BALB/c mice were immunized intra-peritoneally with 0·2 mg of an alum precipitate of a 100,000 g *M. leprae* sonicate supernatant. An intraveneous booster with 0·25 mg sonicate was given 3 months later and the spleen was removed 4 days after the booster injection. The methods of hybridization, cloning, bulk culture of selected hybridomas and immunoglobulin class determination were done as previously described (Kolk *et al.*, 1984). SDS-polyacrylamide gel electrophoresis immunoperoxidase (SGIP) assay, ELISA and immunofluorescence (IF) were used to select hybridomas for cloning, as described before (Kolk *et al.*, 1984).

Monoclonal antibody, selected for its specificity for *M. leprae*, was produced in ascites fluid in BALB/c mice.

IgG was isolated from the 50% ammonium sulphate precipitate of ascites fluid by DEAE-Affigel Blue (Bio-Rad, Richmond, CA, USA) as has been described (Bruck *et al.*, 1982) and conjugation of the monoclonal antibody with peroxidase was done according to the method of Nakane & Kowaoi (1974).

Sera. Individual leprosy sera was kindly provided by Dr D. L. Leiker (Department of Dermatology, Academical Medical Centre, Amsterdam, The Netherlands). All patients were on multidrug therapy. Patients were classified clinically and histopathologically according to the Ridley-Joplin scale (Ridely & Jopling, 1966).

Sera from tuberculosis patients was kindly provided by Dr D. G. Groothuis of the National Institute of Public Health, Bilthoven, The Netherlands and Dr. W. F. M. Strankinga (Academical Hospital of the Free University, Amsterdam, The Netherlands). Control sera from healthy Vietnamese subjects was kindly provided by Dr Pham Van Than (St Paul's Hospital Hanoi, Vietnam).

Control sera from patients with various diseases other than leprosy included those with leishmaniasis, toxoplasmosis, leptospirosis, schistosomiasis, syphilis, auto-immune disease and cancer.

ELISA-inhibition test. Polystyrene ELISA microtitre plates (Dynatech, Alexandria, VA, USA) were coated with 100 μl per well of the soluble *M. leprae* preparation (0·5 μg/ml) in 0·05 M sodium bicarbonate buffer, pH 9·6, for 3 h at 37°C. The plates were washed three times with phosphate buffered saline (PBS) containing 0·05% Tween 20. To each well was added at the same time 20 μl of serum and 80 μl of peroxidase-labelled monoclonal antibody diluted 1:1000 in PBS containing 0·06% Tween 20 and 0·6% BSA and the plates were incubated for 3 h at 37°C. Sera were tested in duplicate. After washing with PBS containing 0·05% Tween 20, wells were incubated with 100 μl substrate solution (1 mg/ml 5-aminosalicylic acid in phosphate/EDTA buffer, pH 6·25, with 0·003% hydrogen peroxide).

Plates were incubated for 2 h at room temperature and overnight at 4°C. Optical densities (O.D.)

were then measured in a Titertech Multiscan (Dynatech, Alexandria, VA, USA) using a 492 nm filter.

Expression of results. The mean value of the optical density of a group of 40 Dutch control sera was taken as negative inhibition value (O.D. neg. = 0·37, s.d. = 0·05). The percentage inhibition of the test sera was found using the formula:

$$\text{Inhibition (\%)} = \left[1 - \frac{\text{O.D. sample}}{\text{O.D neg.}}\right] \times 100\%.$$

Sera which gave more than 41·8% inhibition (O.D. neg. − 3·09 × s.d., $P = 0{\cdot}001$) were scored as positive.

RESULTS

Monoclonal antibody

Hybridoma supernatants were first screened in ELISA on *M. leprae* sonicate; 20% (125/620) were positive. When the same supernatants were tested in an immunofluorescence assay on intact *M. leprae* bacilli, only 8% were positive. In addition 49 (including weak positive in ELISA) were selected for further screening in the SGIP assay, using *M. leprae* sonicate; 92% were positive.

Clones were established from positive cultures and their antibodies characterized by testing as many as 20 different species of mycobacteria in ELISA, IF and SGIP (*M. avium, Armadillo Derived Mycobacteria* 1, 2, 3 and 4, *M. bovis BCG, M. duvalii, M. flavescens, M. fortuitum, M. gadium, M. gastrii, M. gordonae, M. kansasii, M. lepraemurium, M. nonchromagenicum, M. scrofulaceum, M. smegmatis, M. terrae, M. tuberculosis* & *M. vaccae*). On the basis of its antigen specificity, one clone (F47-9), an IgG1, was selected to be used in an ELISA-inhibition test; other interesting clones will be described elsewhere.

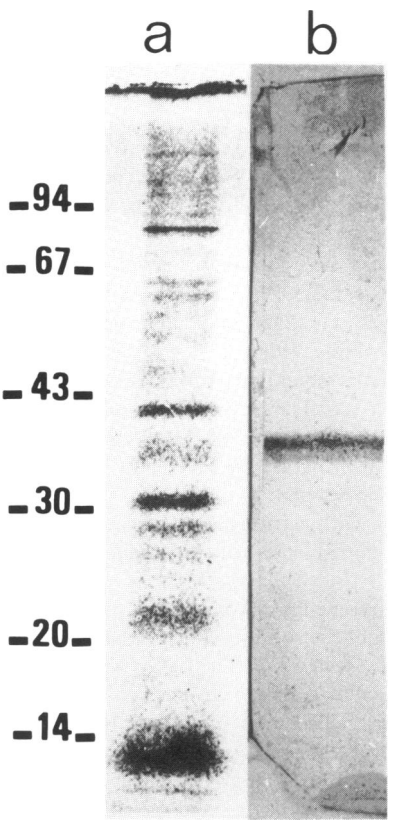

Fig. 1. Application of SGIP on slices from a SDS-gel with *M. leprae* sonicate. Reaction with a lepromatous leprosy serum (a) and monoclonal antibody F47-9 (b).

Table 1. Number of seropositive sera and mean inhibition values using an ELISA-inhibition test in groups and leprosy patients and controls

Diagnosis	No. positives/total	Mean inhibition (%)
Healthy subjects: Dutch	0/40	0 (5·9)*
Vietnamese	3/92	15·0 (9·5)
Pulmonary tuberculosis	3/34	24·7 (13·9)
Other diseases	5/57	14·6 (15·4)
Leprosy: Paucibacillary	79/87	66·1 (16·4)
Multibacillary	77/77	77·0 (12·7)

* Standard deviation

In the immunofluorescence assay, clone F47-9 was negative; in the ELISA it was weakly positive but reacted only with *M. leprae* and not with any of the 20 other mycobacteria tested, nor with armadillo tissue. In the SGIP assay, this monoclonal antibody recognized the 36 kD antigen in the pellet fraction of an *M. leprae* sonicate (Fig. 1); this antigen in heat and SDS stable but sensitive to trypsin treatment.

It was then found that the reaction of this monoclonal antibody in ELISA was greatly improved when the antigen was extracted from the pellet with sodium deoxycholate. This antigenic preparation was therefore used in the development of the ELISA-inhibition test.

ELISA-inhibition test

The number of seropositives and the levels of inhibition in the ELISA-inhibition test are presented in Table 1. All of the multibacillary patients were seropositive compared to 79 out of the 87 paucibacillary patients. The mean percentage inhibition was significant ($P < 0{\cdot}05$) higher in the multibacillary group (77·0%) than in the paucibacillary group (66·1%).

All of the control sera from healthy Dutch persons were scored negative, whereas of the 92 Vietnamese control sera three were positive.

Of the 34 from patients with tuberculosis three were positive and of the 57 sera from patients with other diseases, five were positive.

DISCUSSION

The ELISA-inhibition test described here makes use of the specificity of a monoclonal antibody (F47-9) recognizing a single antigenic determinant on a 36 kD protein antigen of *M. leprae*. This protein was first described using the SGIP technique, in which this same protein was shown to be recognized by leprosy patients' sera (Klatser *et al.*, 1984).

The 36 kD protein was mainly present in the insoluble pellet fraction of an *M. leprae* sonicate and could be solubilized by extraction with detergent (deoxycholate). These results suggest that the 36 kD protein is an integral part of the cell membrane of *M. leprae*.

In this monoclonal-based ELISA-inhibition test all of the multibacillary patients tested were seropositive. Of the paucibacillary patients, 91% were positive. All patients had been on multidrug treatment for variable periods of time when sera were collected and during chemotherapy the quantity of circulating antibodies antibodies may decrease (Yoder, *et al.*, 1979). Moreover, in tuberculoid patients, with their low bacillary load, the antibody level may be too low to be detectable with our test. Of the eight seronegative paucibacillary patients, five were classified as polar tuberculoid.

The epitope on the 36 kD protein antigen of *M. leprae* might well be immunodominant, since not only all multibacillary patients were seropositive, but also a majority of the paucibacillary patients, with high mean inhibition percentages. In contrast, several other authors (Abe, *et al.*, 1980; Harboe, *et al.*, 1978; Sinha, *et al.*, 1983; Young & Buchanan, 1983) have reported much less seropositivity,

based on measuring antibody levels, in patients at the tuberculoid pole of the disease, compared to those at the lepromatous pole. However, in the assay described here, only one serum dilution was used and therefore quantitive differences between antibody concentrations might possibly have been masked.

All of the normal human sera from The Netherlands were negative. From the Vietnamese control sera, 3% were scored positive, but these sera came from an endemic area and the donors were not screened for leprosy, thus we cannot exclude the possibility that these had been infected with *M. leprae*.

Of both groups of sera from tuberculosis patients and patients with other diseases 9% were seropositive. Of the latter, one patient had leptospirosis, one schistosomiasis and the other three were cancer patients on BCG-immunotherapy. These false positive results could be attributed to steric hindrance by antibodies binding to sites in the vicinity of the epitope recognized by the monoclonal antibody.

There is a need for a quick, sensitive and specific test for early diagnosis of leprosy. Because the ELISA-inhibition test described herein detected both multi- and paucibacillary patients, it might well be a suitable tool for diagnosis. Its value should be assessed in large-scale screening of both patients and their contacts in endemic areas.

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REFERENCES

ABE, M., MINAGAWA, F., YOSHINO, Y., OZAWA, T., SAIKAWA, K. & SAITO, T. (1980) Fluorescent leprosy antibody absorbtion (FLA-ABS) test for detecting subclinical infection *Mycobacterium leprae*. *Int. J. Lepr.* **48,** 109.

BRUCK, C., PORTETELLE, D., GLINEUR, C. & BOLLEN, A. (1982) One-step puification of mouse monoclonal antibodies from ascites fluid by DEAE Affi-gel Blue chromatography. *J. Immunol. Methods*, **53,** 313.

CHO, S.N., YANAGIHARA, D.L., HUNTER, S.W., GELBER, R.H. & BRENNAN, P.J. (1983) Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. *Infect. Immun.* **41,** 1077.

DANIEL, T.M. & JANICKI, W. (1978) Mycobacterial antigens: a review of their isolation, chemistry and immunological properties. *Microbiol. Rev.* **42,** 84.

GOREN, M.B. (1982) Immunoreactive substances of mycobacteria. *Am. Rev. Resp. Dis.* **125,** 50.

HARBOE, M., CLOSS, O., BJUNE, G., KRONVALL, G. & AXELSEN, N.H. (1978) *Mycobacterium leprae* specific anibodies detected by radioimmunoassay. *Scand. J. Immunol.* **7,** 111.

KLATSER, P.R., RENS, M.M. VAN & EGGELTE, T.A. (1984) Immunochemical characterization of *Mycobacterium leprae* antigens by the SDS-polyacrylamide gel electrophoresis immunoperoxidase technique (SGIP) using patients' sera. *Clin. exp. Immunol.* **56,** 537.

KOLK, A.H.J., HO M.L., KLATSER, P.R., EGGELTE, T.A., KUIJPER, S., DE JONGE, S. & VAN LEEUWEN, J. (1984) Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis, M. bovis* (BCG) and *M. leprae*. *Clin. exp. Immunol.* **58,** 511.

MITCHELL, G.F. (1981) Hybridoma antibodies in immunodiagnosis of parasitic infection. *Immunol. Today*, **2,** 140.

NAKANE, P.K. & KAWAOI, A. (1984) Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* **22,** 1084.

RIDLEY, D.S. & JOPLING, W.H. (1966) Classification of leprosy according to immunity, a five-group system. *Int. J. Lepr.* **34,** 255.

SANSARRICQ, H. (1980) Recent changes in leprosy control. *Lepr. Rev.* **52 (suppl. 1),** 15.

SINHA, S., SENGUPTA, U., RAMU, G. & IVANYI, J. (1983) A serological test for leprosy based on competitive inhibition of monoclonal antibody binding the MY2a determinant of *Mycobacterium leprae*. *Trans. Royal Soc. trop. med Hyg.* **77,** 869.

WORLD HEALTH ORGANIZATION (1980) Purification of *M. leprae*. Report of the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy (IMMLEP), Geneva 25–26th June 1980. *WHO report* **no.TDR/IMMLEP-SWG(5)/80-3,** World Health Organization Geneva.

YODER, L., NAAFS, B., HARBOE, M. & BJUNE, G. (1979) Antibody activity against *Mycobacterium leprae* antigen 7 in leprosy: studies on variation in antibody content throughout the spectrum and on the effect of DDS treatment and relapse in BT leprosy. *Lepr. Rev.* **50,** 113.

YOUNG, D.B. & BUCHANAN, T.M. (1983) A serological test for leprosy with a glycolipid specific for *Mycobacterium leprae*. *Science*, **221,** 1057.