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Serological study of leprosy employing ELISA with arabinogalactan of *Mycobacterium smegmatis* as antigen

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Summary An ELISA has been developed for detecting circulating antibodies in leprosy sera using arabinogalactan, a cell-wall polysaccharide of *Mycobacterium smegmatis* as the antigen. In normal sera, arabinogalactan specific IgM is higher than IgG, whereas in untreated leprosy sera anti-arabinogalactan (AG) IgG is more than the corresponding IgM. With long-term treatment of the disease, IgM level goes up compared to IgG.

Introduction

Infection with mycobacteria is usually associated with the induction of a non-protective humoral immune response against its various antigens. The search for a specific antigen, which might be useful for early detection of leprosy or for screening of at-risk populations, has been intensive. *Mycobacterium leprae* contain antigenic components which are specific as well as highly cross-reactive with other mycobacteria. In the latter class, belong 2 immunogenic polysaccharides, arabinomannan (AM) and arabinogalactan (AG).[1, 2] One study observes[3] that pretreatment antibody level of arabinomannan was directly proportional to the quantity of *M. leprae* present, and patients with low antibody titres represent a paucibacillary state. Moreover in paucibacillary states the titre against AM goes down with treatment, but in multibacillary states such a correspondence cannot be established. A further study[4] thoroughly investigated the AM, developed an ELISA and showed high specific antibody in untreated cases, low antibody response in the treated cases and almost negligible antibody to the control. This antigen, although very specific for LL cases, fails to discriminate TT/BT and household contacts from normal populations. One study[5] showed that *M. smegmatis* was most reactive against lepromatous sera using the ELISA technique. The other polysaccharide component, arabinogalactan (AG), has

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been shown to be more seropositive than AM.[6] We, therefore, decided to investigate the feasibility of AG as a screening antigen.

We have isolated arabinogalactan from cell-wall surface of *M. smegmatis* and have tested it for reactivity with serum antibodies in individual patients. A specific ELISA has been developed for the demonstration and quantification of specific IgG and IgM antibodies against arabinogalactan. Antibodies against this component occur frequently in leprosy patients as well as in normal individuals. With a highly-purified preparation of AG we have observed that in normal individuals antigen specific IgM level in sera is higher than that of IgG. In untreated leprosy patients specific IgG level is higher than that of IgM level, while with treatment of the disease the specific IgM level goes up. These findings, though surprising, might be due to the fact that our population is exposed to a plethora of atypical mycobacteria and are mostly BCG vaccinated, and hence maintain a high level of antibody against this cross-reactive antigen. We show here that a comparison of IgG and IgM levels against this antigen, in other words the ratio, IgM:IgG, provides us with an interesting discriminatory point to confirm the disease status. Our objective in the present study was to investigate whether AG specific IgM, IgG antibody levels in normal and infected individuals have any significant correlation with the development of the disease.

Materials and methods

Arabinogalactan was obtained from *M. smegmatis* by the published procedure[2, 6] of fractional precipitation with ethanol, 80% alcoholic precipitated fraction was utilized as the major source of antigen. The fraction was dissolved in a minimum quantity of distilled water and reprecipitated 3 times with absolute ethanol, then it was triturated with ether and acetone. It was finally purified by passing through Sephacryl 1200 (Pharm. Fine. Chem., Sweden) giving a single fraction, which was used for ELISA.

CHEMICAL PROPERTIES OF PURIFIED POLYSACCHARIDE

Purified polysaccharide fraction was positive for the phenolsulphuric acid reaction.[8] Galactose and arabinose were detected and estimated by paper chromatography and also by gas liquid chromatography (Hewlette Packard, column SE-30). Arabinogalactan peptidoglycan comprised 24% carbohydrate, 3% protein[9] and approximately 65% of fatty acid.[10]

ENZYME-LINKED IMMUNOSORBENT ASSAY

Linbro plates were coated with arabinogalactan solution (100 μl/well) in bicarbonate buffers (pH 9·6). Wells were then washed with phosphate buffered

saline (PBS), incubated with 100 μl of PBS containing 5% BSA at 37°C for 2 h. The contents were aspirated and 100 μl of human serum, diluted with PBS containing 20% normal bovine serum was added and incubated overnight in a moist chamber. After 3 washes with PBS, rabbit antihuman IgM, IgG-HRPO conjugate (Dakopatts, Denmark, diluted in 1:4000, PBS-1% BSA) was added, incubated and washed with PBS. One hundred microlitres of H_2O_2-O-phenylene-diamine substrate dye reagent in citrate phosphate buffer was then added and kept for 15 min. Reactions were terminated with 50 μl of 5N H_2SO_4 and the absorption was read at 492 nm in titre Tek Multiskan (Flow Laboratories, Inc. USA) ELISA reader.

HUMAN SERA

Sera from 15 normal healthy laboratory workers (without any known mycobacterial diseases), from All India Institute of Medical Sciences, New Delhi, and also sera from 30 apparently healthy Indians (with a record of BCG vaccination), comprised our normal control population.

The second test group of sera was from 44 untreated LL leprosy patients, whose ages ranged from 15 to 60 years, with a wide spectrum of leprosy, and was donated by Dr S N Choudhury, School of Tropical Medicine, Calcutta.

The third group of sera was from treated leprosy patients, and was donated by Dr Narayanan, TRC, Madras, India. Except for 6 BT cases the rest of the sera were from LL patients; the bacillary index in the group ranged from 0 to 5. All the patients were on therapy at the time serum was obtained. The duration of therapy ranged from 2 to 25 years. Sera were preserved by the addition of 0·1% sodium azide and were stored at -70°C until used.

Serum was assayed in triplicate and the arithmetic mean of the OD_{492} readings was used in all further analysis. The mean and standard deviations for each of the 3 study populations were calculated.

Results

Results from Figure 1 illustrate a standard serum dilution curve for 5 untreated LL pooled sera, with different antigen concentrations. From this curve, the optimum level of 50 μg/well of antigen and sera dilution of (1:100) was chosen and used in all subsequent ELISA experiments.

Figure 2 shows the ELISA results for 44 individual cases each of normal, treated and untreated leprosy sera using Dakopatts Rab antihuman IgM, IgG, IgA—HRPO conjugate. The mean value ($0{\cdot}625 \pm 0{\cdot}164$) for normal cases was significantly higher than that observed in untreated patients ($0{\cdot}332 \pm 0{\cdot}125$). Interestingly, the treatment of lepromatous leprosy patients for 2 or more years resulted in an elevation of the level ($0{\cdot}516 \pm 0{\cdot}210$.

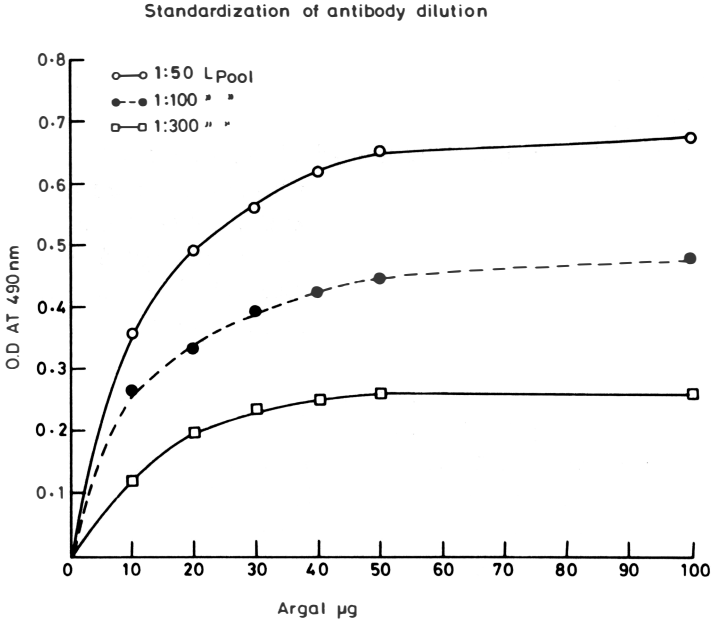

Figure 1. Standardization of arabinogalactan by ELISA technique. An antigen concentration range of 10–100 μg was used and titrated against 3 different dilutions of sera pooled from 5 individual untreated lepromatous leprosy patients.

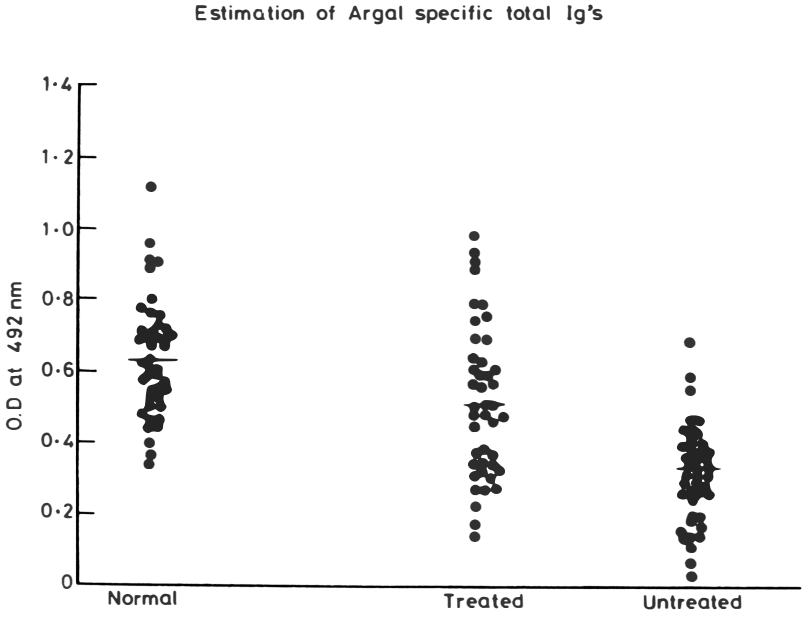

Figure 2. ELISA with Argal antigen. Each dot represents total antibody level against arabinogalactan, 44 individual cases each of normal, treated LL and untreated LL sera were used.

To determine whether the observed differences involved a class specific antibody response to AG, we estimated specific IgG and IgM levels in individual normal, LL and TT as well as in normal and leprosy-pooled sera using rabbit-antihuman-HRPO conjugates. The results are given in Figure 3. While there is no dramatic difference in total immunoglobulin level against AG among these categories, specific IgG response is considerably more compared to IgM response in pooled and individual leprosy sera than in normal controls. The same trend is confirmed in the results given in Figure 4, where groups of individual normal, treated and untreated LL patients are compared. The picture as presented is significantly different in untreated LL patients from that of normal controls, while interestingly the treated patients show a mixed picture. In Figure 5, we compared anti-AG, -IgG and-IgM levels in a number of short-term treated LL and BT patients' sera; again IgG, IgM levels in these cases and consequently the ratio, IgM:IgG is less than unity.

Discussion

Results presented above indicate an interesting phenomenon that basal anti-arabinogalactan IgM levels in normal healthy individuals are found to be greater than specific IgG levels. With *M. leprae* infection, however, the relative levels are modulated, and in LL cases IgG titres are higher than IgM titres. We do not know if this trend is universal or specific to the Indian population we tested. Because the population in tropical countries, like India, is continually exposed to many

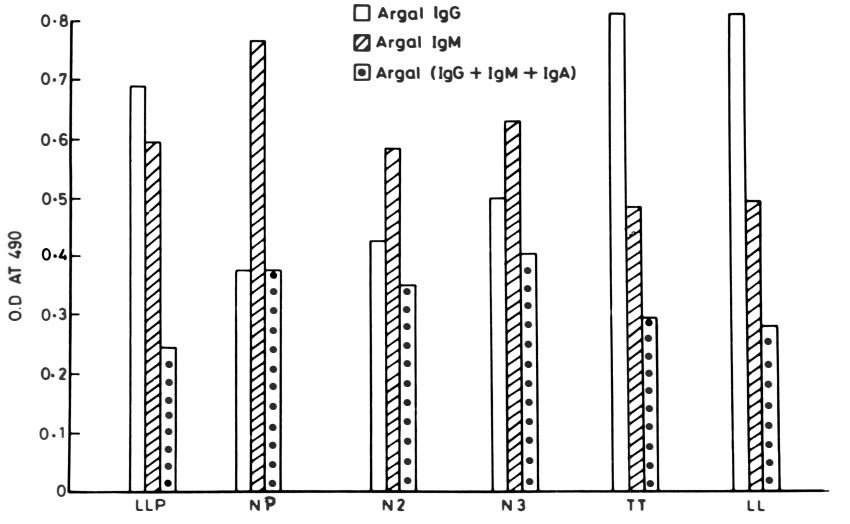

Figure 3. Comparison of antibody levels against arabinogalactan (AG) in normal pooled, leprosy pooled and individual control as well as individual untreated TT and LL patients.

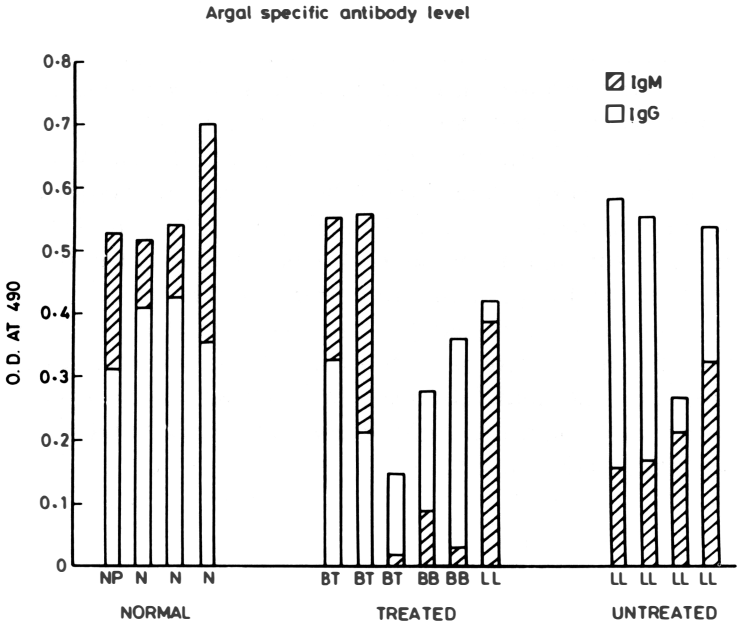

Figure 4. Comparison of specific antibody levels against Argal. The level of IgM is more in normal than in untreated patients' sera.

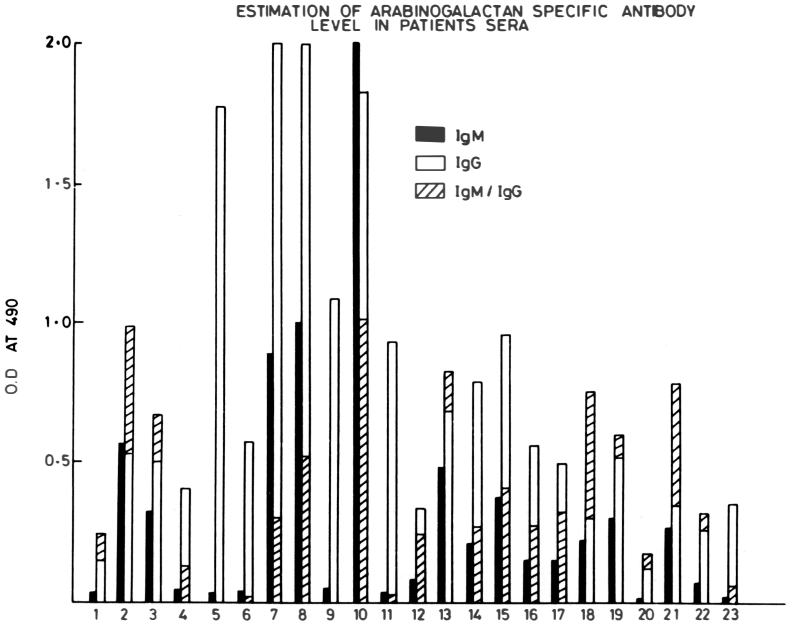

Figure 5. Profile of anti-AG-IgG, IgM and IgM : IgG ratio in 23 treated LL patients' sera. IgM is low compared to IgG, IgM : IgG ratio is below unity.

atypical and soil mycobacteria it is possible that normal individuals already maintain a sustained IgM response against very common, AG-antigen. Also, a vast number of the population in India have already been BCG vaccinated.[13] It has been demonstrated in lepromatous leprosy that anti-*M. leprae* IgG is about twice as high as IgM[14,15] and that the IgM response is mostly against specific phenolic glycolipids, which somehow lack an IgM to IgG switch.[16] More common polysaccharide antigens (AG and AM), after *M. leprae* infection, normally switch over to IgG response, so that the IgG level goes up, at the expense of IgM, giving rise to an inverse relationship with corresponding levels in the normal population.

The observed trend, however, is contrary to what has been observed with other antigens.[17, 18, 19] Usually increasing antibody activity is observed throughout the leprosy spectrum, lowest activity in household contacts, higher median-activity in sera from indeterminate leprosy patients (only of IgM response) and highest median activity in sera from untreated leprosy patients. Here with AG, the total specific immunoglobulin level shows a slightly reversed trend. The reason for this trend is not clear to us, and we feel from the wide range of scatter (Figure 3), that the difference in total immunoglobulin may not be as much if it is averaged over a very large number of samples. Instead of total immunoglobulin, if one compares specific IgG and IgM levels, the ratio of IgM:IgG $> 1{\cdot}0$ in the case of normal and $< 1{\cdot}0$ for persistent infection, and possibly increased again with treatment. This is a significant and consistent finding and provided us with an interesting point in terms of a cut-off value (1·0) to determine the status of the disease. In Figure 5, results are shown with sera from a number of individual lepromatous leprosy patients with a wide spectrum of severity of the disease, in all cases, except nos. 2 and 10, the ratio is much less than unity.

Chemotherapy of leprosy has been associated with decreasing antibody titres, whereas relapsed tuberculoid patients generate a higher antibody level.[10] Monitoring therapeutic improvement remains a difficult task as no clear correlation of antibody level including antiphenolic glycolipid-1 antibodies, with bacillary index has been established. IgM assay with specific phenolic glycolipid has been promising but is not foolproof, anti-AG, -IgM:IgG ratio offers an alternative method, and in combination with phenolic glycolipid-1 assay may be of better predictive value.

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