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Demonstration of antibodies against *Mycobacterium leprae* both in immunoglobulin G and M in sera from pregnant and non-pregnant lepromatous leprosy patients

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Summary Antibodies against *M. leprae* antigen 7 have been shown to consist of both immunoglobulin G and M in a lepromatous leprosy serum pool and in individual sera from patients with active lepromatous leprosy. Various implications of the occurrence of anti-*M. leprae* antibodies in several immunoglobulin classes are discussed, particularly their use as an indicator of transfer of *M. leprae* antigens or of live leprosy bacilli to the foetus during pregnancy. With the present techniques, no IgM antibodies against *M. leprae* antigen 7 could be detected in several cord sera from babies born of mothers with active lepromatous leprosy.

Introduction

About 20 distinct antigenic components have so far been detected in *Mycobacterium leprae* by crossed immunoelectrophoresis (CIE) using rabbit antisera against purified armadillo-grown leprosy bacilli (Harbo *et al.*, 1977a;[1] 1977b;[2] Closs *et al.*, 1978).[3] One of these components, *M. leprae* antigen 7, has been purified and labelled with ^{125}I, and this preparation has been used to develop a radio-immuno-assay (RIA) for detection and quantitation of anti-*M. leprae* 7 antibodies (Melsom *et al.*, 1978;[4] Yoder *et al.*, 1979).[5]

Patients with lepromatous leprosy have on average the highest concentration of antibodies towards *M. leprae* antigen 7 in their sera with a steady decline towards the tuberculoid end of the spectrum. Within each group there

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is a large variation in concentration of anti-*M. leprae* antigen 7 in sera from individual patients (Yoder *et al.*, 1979).[5]

In lepromatous leprosy patients there is only a small decline in anti-*M. leprae* antigen 7 activity during one and a half years of treatment with DDS (Melsom *et al.*, 1978),[3] while in tuberculoid leprosy the decline appears to occur faster (Yoder *et al.*, 1979).[5]

The specificity of anti-mycobacterial antibodies in leprosy has been characterized by various gel precipitation techniques, particularly CIE with patient serum in the intermediate gel. The number of antibody specificities is lower in tuberculoid than lepromatous leprosy. However, even in most cases of lepromatous leprosy the antibodies are directed against only a limited number of antigenic components of bacillus (Axelsen *et al.*, 1974;[6] Myrvang *et al.*, 1974;[7] Kronvall *et al.*, 1975).[8]

Patients with active lepromatous leprosy have an increased concentration of IgG and possibly also of IgM and IgA in their sera (Gupta *et al.* 1978).[9] Antibodies towards *M. leprae* has been demonstrated but not quantitated in both IgG and IgM class (Abe *et al.*, 1972)[10] but antibodies of IgA class have not yet been demonstrated.

The present RIA is based upon using protein A on the surface of Cowan I strain of staphylococci (NCTC 85308) as solid phase to separate free antigen from antibody-bound antigen. It was previously believed that protein A could only bind IgG immunoglobulins of subclass IgG1, IgG2 and IgG4 (Kronvall and Williams, 1969).[11] It has later been shown that protein A also can bind IgA of subclass IgA2 and IgM of subclass IgM2 (Harboe and Følling, 1974;[12] Saltvedt and Harboe, 1976).[13] The assay is therefore now also expected to detect IgA2 and IgM2 antibodies.

The purpose of the present work was to use this sensitive RIA to establish if antibodies against *M. leprae* antigen 7 occurred both in IgG and IgM in leprosy.

Materials and methods

PATIENT SERA

A serum pool was made from 40 sera obtained from patients suffering from lepromatous leprosy (LL, LI and BL) who had been either newly diagnosed or had been treated with DDS for less than 6 months. Sera were also collected from 4 mothers suffering from active lepromatous leprosy (LL, LI and BL) (Ridley and Jopling, 1966;[14] Ridley and Waters, 1969;[15] Myrvang *et al.*, 1973)[16] and their babies, from one mother suffering from tuberculoid leprosy and her baby, and from one healthy mother and her baby. The samples were taken at delivery from the mother and from the cord of the newborn baby.

PREPARATION AND LABELLING OF *M. LEPRAE* ANTIGEN 7

M. leprae purified from infected armadillo liver by Draper's technique (Draper, 1976)[17] were provided by R.J.W. Rees and P. Draper through the IMMLEP programme. ^{125}I-labelled *M. leprae* antigen 7 was made by electrolytic iodination and tested for purity by crossed immunoelectrophoresis and autoradiography. The properties of the presently used preparation corresponded closely to those of the preparation described in detail previously (Melsom *et al.*, 1978).[4]

RADIO-IMMUNO ASSAY (RIA)

The procedure described previously for assay of antibodies against *M. leprae* antigen 7 was used. In brief, the fractions after density gradient ultracentrifugation were diluted 1:50 in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA). 100 µl of these dilutions were added to 100 µl of labelled *M. leprae* antigen 7, and protein A containing staphylococci were used as solid phase to separate labelled antigen 7 bound to antibody from free labelled antigen 7 (Melsom *et al.*, 1978).[4]

DENSITY GRADIENT ULTRACENTRIFUGATION

The IgM fraction was separated from IgG in human serum by zonal ultracentrifugation on a 10–40% sucrose density gradient in Tris-NaCl buffer of pH 8.0. When the effect of the acid pH was tested, a similar sucrose gradient in 0.1 M/glycine-HCl buffer of pH 3.0 was used (Hannestad, 1967).[18] Ultracentrifugation was carried out in a Spinco L-50 preparative ultracentrifuge equipped with a SW 65 K rotor 18 hours at 4°C at 36,000 rev./min. Fractions of 4–5 drops each were collected from a pinhole in the bottom of each tube.

DETERMINATION OF IGM AND IGG CONCENTRATIONS

The IgM and IgG concentrations were determined in the fractions after density gradient ultracentrifugation by single radial diffusion method as previously described (Melsom *et al.*, 1979).[19]

REDUCTION WITH 2-MERCAPTOETHANOL

To abolish antibody activity in the IgM fraction, reduction with 2-mercaptoethanol was performed as follows: To 100 µl of serum, 100 µl of 0.9% NaCl and 7.5 µl of 2-mercaptoethanol were added. The mixture was incubated at 20°C for 2 minutes and layered over a sucrose gradient followed by immediate start of the centrifuge. This provided a concentration of 0.5 M.

2-mercaptoethanol in the sample for reduction and did not lead to solidification of serum which occurred when higher concentrations of 2-mercaptoethanol or undiluted serum were used (Solheim and Harboe 1972).[20]

Results

When the lepromatous leprosy serum pool was subjected to density gradient ultra-centrifugation and the fractions tested by RIA, antibody activity against *M. leprae* antigen 7 was found in two distinct peaks; denoted peak 1 and peak 2 in Fig. 1. The concentration of IgM and IgG as determined by single radial diffusion in the different fractions, is also indicated in the figure. IgM was clearly separated from IgG. As seen in Fig. 1, maximal IgM concentration corresponded to maximal antibody activity in peak 1 and the distribution of IgG concentration corresponded to antibody activity in peak 2.

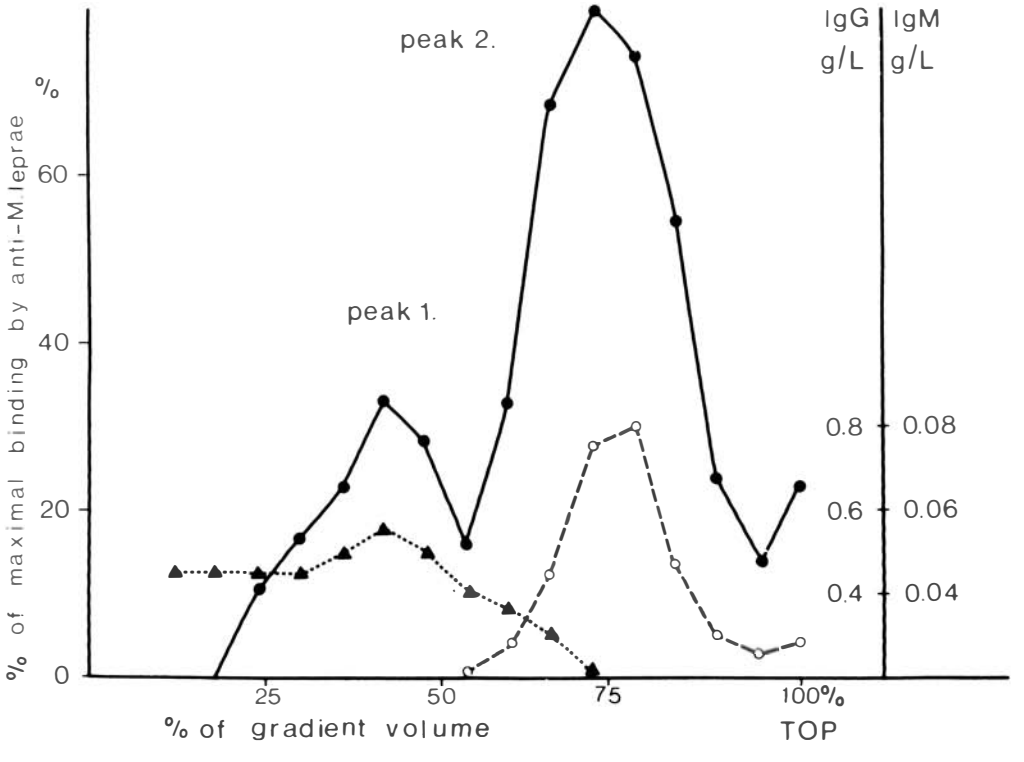

Figure 1. ●—● antibody activity against *M. leprae* antigen 7, ▲---▲ IgM concentration and ○---○ IgG concentration in different fractions after density gradient ultracentrifugation of LL serum pool.

The lepromatous leprosy serum pool was then tested in the same way after reduction of the serum with 2-mercaptoethanol. RIA with labelled *M. leprae* antigen 7 showed virtually no antibody activity corresponding to peak 1 after reduction of the serum with 2-mercaptoethanol. Nor could IgM be detected in the rapidly sedimenting fractions, while both peak 2 antibody activity and the IgG peak were found in the usual location (Fig. 2). The same results are seen in Fig. 3 where one individual serum has been treated with 2-mercaptoethanol. No antibody activity against *M. leprae* antigen 7 nor any IgM could be detected in the rapidly sedimenting fractions.

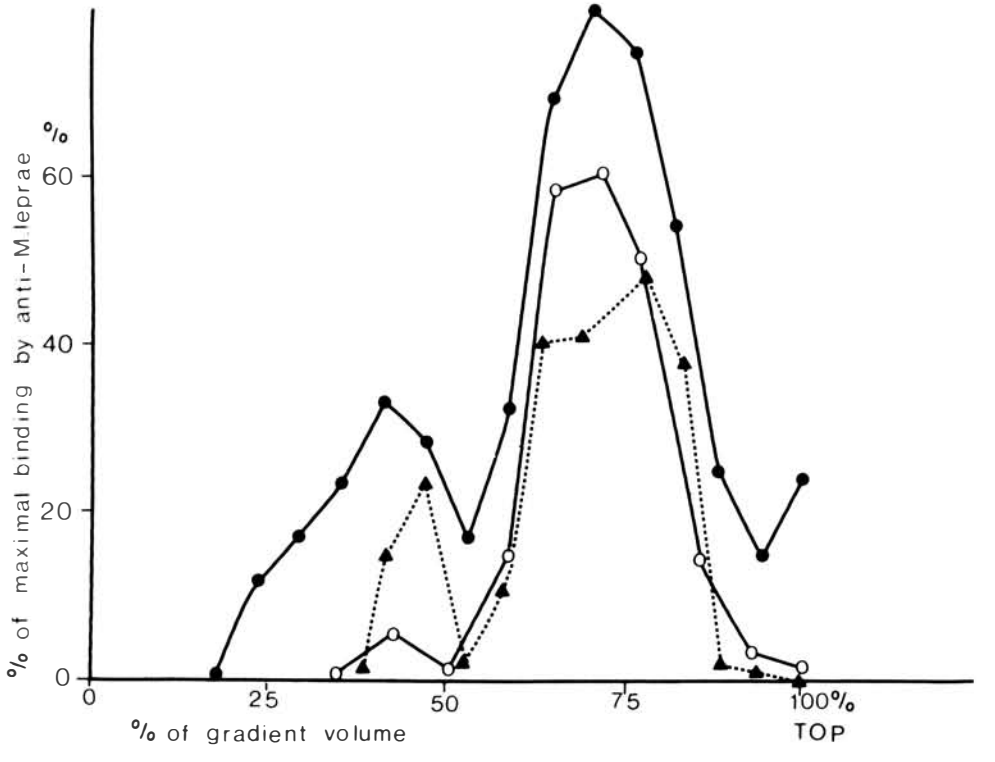

Figure 2. Antibody activity against *M. leprae* antigen 7 in fractions after density gradient ultracentrifugation of ●—● LL serum pool at pH 8.0, ○—○ LL serum pool at pH 3.0 and ▲---▲ LL serum pool after reduction with 2-mercaptoethanol.

Pretreatment with low pH would dissolve immune complexes consisting of labelled *M. leprae* antigen 7 and corresponding antibodies and other types of IgG complexes. At pH 8.0 such complexes would sediment faster than the bulk of monomeric IgG and together with other faster sedimenting components such as IgM. To exclude that the antibody activity in peak 1 consisted of IgG complexes, one individual serum from a mother suffering from lepromatous leprosy

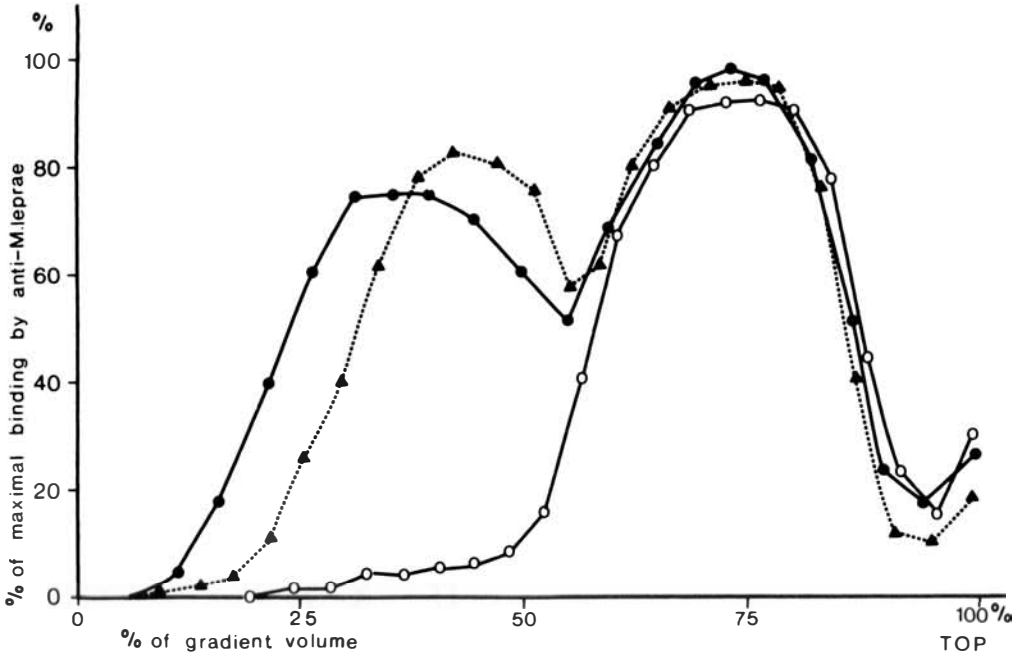

Fig 3. As for Figure 2 but with serum 242/76 from a patient with active lepromatous leprosy.

was fractionated by density gradient ultracentrifugation at pH 3.0. IgM and IgG were still demonstrated in fast and slow sedimenting fractions respectively. The *M. leprae* antigen 7 antibody activity was still present in the fractions containing IgM and IgG, again forming two distinct peaks, peak 1 and peak 2, as seen in Fig. 3. The position and the amount of *M. leprae* antigen 7 antibody activity was almost identical with the pattern seen after density gradient ultracentrifugation at pH 8.0. Similar results were obtained after density gradient ultracentrifugation of the LL serum pool. These findings indicated that the *M. leprae* antigen 7 antibody activity demonstrated in the fast sedimenting fractions after density gradient ultracentrifugation (denoted peak 1 in Fig. 1) did not consist of immune complexes.

Cord sera were fractionated by density gradient ultracentrifugation and the fast sedimenting fractions were tested in the RIA for possible *M. leprae* antigen 7 antibodies of IgM class. Fig. 4 shows a typical pattern of antibody activity against *M. leprae* antigen 7 in different fractions from both maternal and cord serum at delivery. After separation by density gradient ultracentrifugation, no antibody activity against *M. leprae* antigen 7 could be demonstrated in the cord serum sample in the rapidly sedimenting fractions, while the maternal serum had strong antibody activity in the fractions corresponding to IgM. Five additional cord-mother pairs were examined, and IgM antibodies against

M. leprae antigen 7 could not be dected in any of these cord sera. IgM does not cross the placenta, while IgG does. Therefore, the demonstration of *no* antibodies in rapidly sedimenting fractions of cord serum support the conclusion that the activity in these fractions of maternal sera was due to IgM antibodies towards *M. leprae* antigen 7.

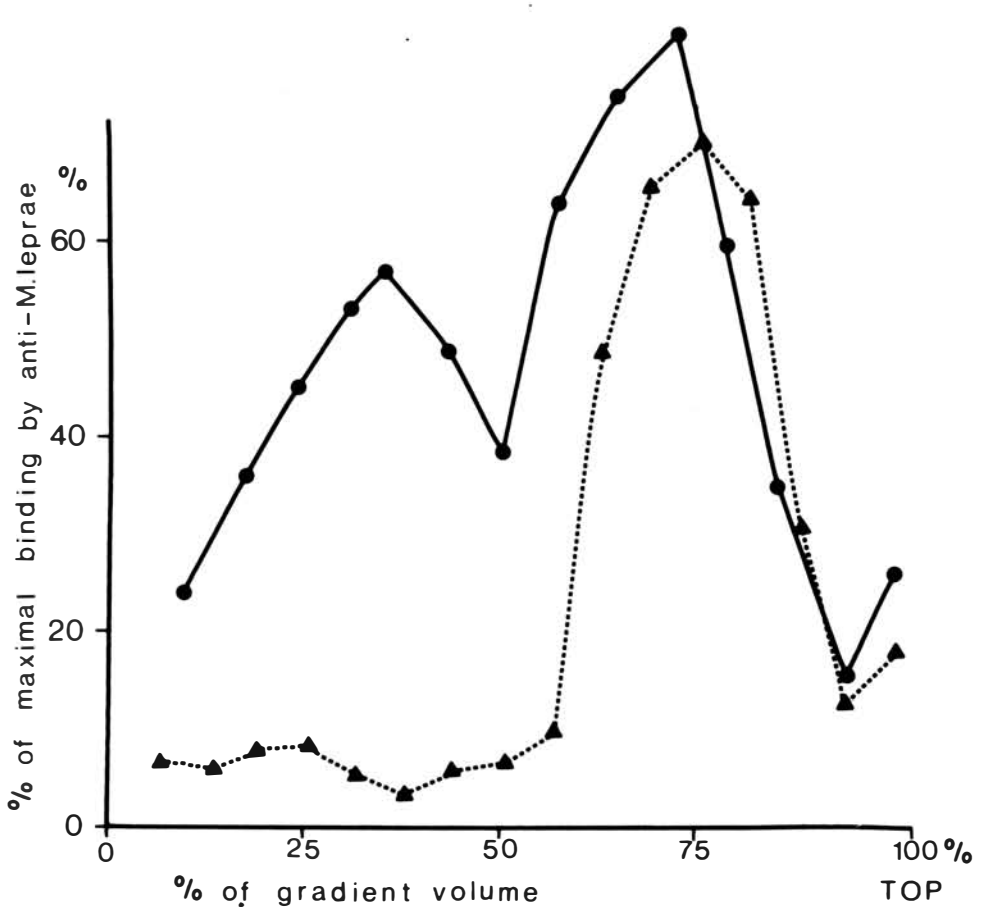

Figure 4. Antibody activity against *M. leprae* antigen 7 in fractions after density gradient ultracentrifugation of ▲---▲ baby's cord serum and ●——● the corresponding maternal serum taken at delivery.

Fig. 5a and 5b illustrate that different amounts of antibodies against *M. leprae* antigen 7 occurred in the IgM and the IgG class in four mothers with active lepromatous leprosy (Fig. 5a), in one mother with tuberculoid leprosy and in one mother who was a normal, non-leprosy control (Fig. 5b). These two figures showed that the sera from three out of four pateints with active leprosy contained antibodies of both the IgM and the IgG class against *M. leprae*

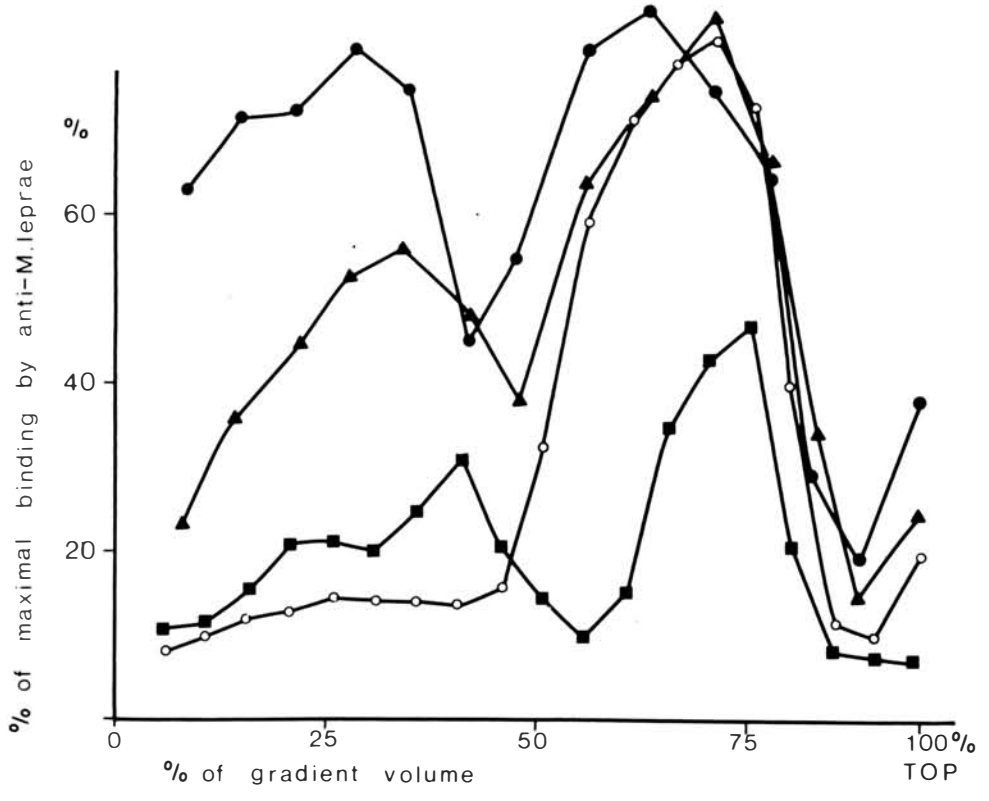

Figure 5a and b. Antibody activity against *M. leprae* antigen 7 in fractions after density gradient ultracentrifugation of: (a) 4 mothers suffering from active lepromatous leprosy, (b) 1 healthy mother ▲—▲, and 1 mother with tuberculoid leprosy ●—●.

antigen 7, while sera from the normal control and the patient with tuberculoid leprosy did not contain antibodies of this class against *M. leprae* antigen 7 detectable by the present technique.

Discussion

Patients with active lepromatous leprosy have more antibodies (both in concentration and specificity) than patients with tuberculoid leprosy or non-leprosy normal controls (Axelsen *et al.*, 1974;[6] Yoder *et al.*, 1979).[5] These antibodies have been considered mostly to be of the IgG class. Besides this no information is available regarding specific antibodies towards *M. leprae* in the different immunoglobulin classes.

IgM can be separated from IgG based on its faster sedimentation rate during density gradient ultracentrifugation of LL serum pool. Quantitation of IgM and

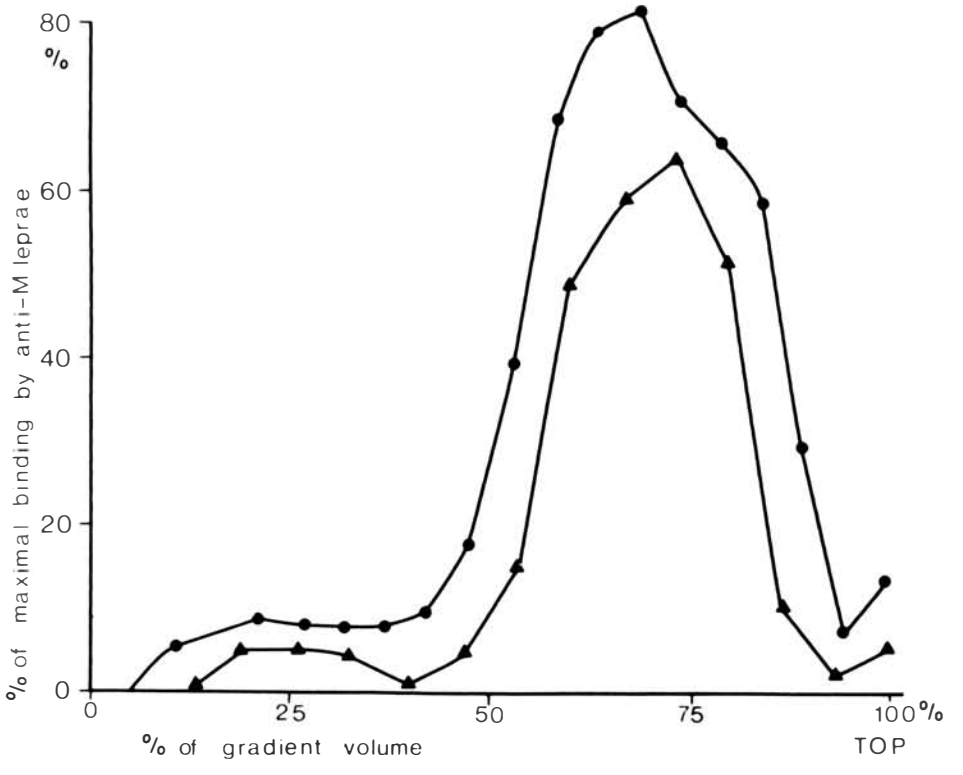

Figure 5a and b. Antibody activity against *M. leprae* antigen 7 in fractions after density gradient ultracentrifugation of: (a) 4 mothers suffering from active lepromatous leprosy, (b) 1 healthy mother ▲—▲, and 1 mother with tuberculoid leprosy ●—●.

IgG in the fractions showed peak of IgM concentration corresponding to the fractions denoted peak 1 in Fig. 1, while the peak of IgG concentration occurred in the slower sedimenting fractions corresponding to peak 2. The concentration of IgA was also quantitated in the fractions, and the distribution of IgA corresponded closely to that of IgG with maximum concentration in the same fraction as for IgG (data not shown).

M. leprae antigen 7 is one antigenic component of about 20 distinct antigenic components so far detected in *Mycobacterium leprae* by crossed immunoelectrophoresis. This preparation was labelled with ^{125}I, and was tested by crossed immunoelectrophoresis, and autoradiography and gel filtration. By these three techniques we could demonstrate that more than 95% of the radioactivity was localized to *M. leprae* antigen 7 (Melsom *et al.*, 1978).[4] We therefore used an isolated, defined labelled cell wall antigen which cross-reacts with several other mycobacteria.

RIA was carried out with labelled *M. leprae* antigen 7 on fractions obtained after density gradient ultracentrifugation of LL serum pool. Antibody activity

was found in the fractions with the highest concentration of IgM, peak 1, and in the fractions with IgG, peak 2. If any antibodies of IgA2 subclass against *M. leprae* antigen 7 are present in LL serum pool, these would be present in the same fractions as IgG, i.e. as part of peak 2.

After reduction and inactivation of IgM with 2-mercaptoethanol, almost no antibody activity towards *M. leprae* antigen 7 was found in the fraction corresponding to peak 1, while antibody activity towards *M. leprae* antigen 7 could be demonstrated in the fast sedimenting fractions after density gradient ultracentrifugation at pH 3.0, a procedure used to split IgG complexes whereas antibody activity due to IgM is not abolished.

We have demonstrated here antibodies towards *M. leprae* antigen 7 in both the IgM and the IgG classs. Thus several criteria used to distinguish IgM from IgG antibodies, i.e. presence in fast sedimenting fractions after density gradient ultracentrifugation, loss of activity after reduction, resistance to exposure to low pH, and lack of activity in cord blood have all been met in the present investigation.

It is still an open question if *M. leprae* bacilli or *M. leprae* antigens can cross the placenta. If the antibodies of the IgM class against *M. leprae* antigen(s) could be demonstrated in cord blood, this would strongly indicate that leprosy bacilli or antigen(s) from the bacilli cross the placenta. The present system used to demonstrate antibodies towards *M. leprae* antigen 7 in the IgM class, was therefore used to test if small amounts of IgM antibodies against this antigen are present in cord sera from babies of mothers with active lepromatous leprosy. But so far we have not been able to demonstrate IgM antibodies towards *M. leprae* antigen 7 in any of the cord sera we have tested. This can be due to our system being too insensitive. The protein A used as solid phase in this RIA system will only bind about 30% of the IgM present in adult sera (Harboe and Følling, 1974).[12] But these results could also indicate that *M. leprae* or *M. leprae* antigen 7 do not cross the placentae. A third possibility is that the foetus is unable to produce antibodies towards *M. leprae* antigen 7. To obtain more information on whether *M. leprae* or *M. leprae* antigens cross the placenta and affect the immune system of the foetus, we are presently developing other sensitive solid phase radio-immuno-assays for specific demonstration of anti-*M. leprae* antibodies in the IgG, IgA and IgM classes in cord serum.

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