Environmental mycobacteria may induce recognition of auto-antigens. Circumstantial evidence using maternal and cord blood

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ABSTRACT

Background: It was assumed that the recognition of auto-antigens induced by environmental and infective micro-organisms may be involved in the clinical presentation of leprosy and a number of autoimmune diseases. Methods: Serum antibodies to epidermal antigens in the blood of 21 healthy mothers and in the cordblood of their new-born were compared using immunoblotting before and after absorption of the sera with mycobacteria (M. marinum, M. tuberculosis, M. kansasii). Results: Sera of the mothers contained a higher antibody titre than that of their respective babies. The pattern of bands for IgG was the same, for IgM the sera of 14 mothers showed more bands than that of their offspring. Absorption with the mycobacteria, especially M. tuberculosis resulted in the partial or total disappearance of some bands. Conclusion: The results of this study provide some supportive evidence for the above-mentioned assumption. The observation that M. tuberculosis was the most effective in the absorption experiment may be because of the BCG vaccination all the mothers had received in infancy.
**Key Words:** Leprosy, autoimmunity, environment, mycobacteria, cord blood.

**INTRODUCTION**

Similarity between antigenic determinants of *M. leprae* and the human host was suggested as a possible alternative explanation for the immune responses responsible for the clinical spectrum of leprosy and the reactional episodes (Naafs et al., 1990). It was observed that monoclonal antibodies (MoAbs) directed against *M. leprae* reacted with cryostat sections of normal human skin and nerve using an indirect immunoperoxidase technique. Later, the same technique and Western blotting with normal human skin extract provided further evidence for this similarity (Van Den Akker, 1992). It was hypothesized, that the recognition of self not only could account for the leprosy spectrum, but also for a number of "autoimmune diseases" such as sarcoidosis, Crohn's disease, diabetes, rheumatoid arthritis, biliary cirrhosis, psoriasis, palmoplantar pustulosis (M. Andrews-Barber) and chronic granulomatous disease (Holoshitz et al., 1986; Shoenfeld, 1988; Esaquy et al., 1991; Vilagut et al., 1994a; Vilagut et al., 1994b; Izaki et al., 1994; Yokota et al., 1993; Rook & Stanford, 1992).

In order to show that human beings develop recognition of auto-antigens during their lifetime that may have been induced by environmental microorganisms, it was decided to investigate the sera from healthy mothers for the presence of antibodies against human epidermis. The results before and after absorption with mycobacteria were compared with the results obtained with the sera derived from cord blood from their babies.

**MATERIAL AND METHODS**

Blood was obtained from 21 pregnant women in labour after informed consent. They all lived in the area of Bauru, Sao Paulo, Brazil, where leprosy and tuberculosis are known to be endemic. They were all healthy and stated that they had had no known contact with patients with mycobacterioses (specifically, they were questioned for leprosy, tuberculosis and persistent skin ulcers). Their age varied from 16 to 35 years. All of them had been vaccinated in infancy with BCG vaccine.

Neonates' blood was obtained from the umbilical cord immediately after delivery. Sera were prepared according standard procedures and stored at -20°C. and were transported to The Netherlands on dry ice and stored again at -20°C. upon arrival.
HUMAN EPIDERMAL EXTRACT

Skin obtained from healthy women who had undergone mammoplasty at the department of plastic surgery of the University Hospital Dijkzigt was shaved using a dermatome to harvest the epidermis. Briefly, the extract was prepared by homogenising the epidermis in 100ml of 0.25 M phosphate-buffered saline (PBS, pH 7.4) containing 1% sodium dodecyl sulphate. The homogenate was then centrifuged at 40,000 g for 60 minutes at 4°C, after which the pellet was discarded and the supernatant concentrated by ultra filtration. Thereafter, the supernatants of 3 different skin samples were combined and the protein concentration of the epidermal extract was determined to be 12 mg/ml using bovine albumin as a standard.

MYCOBACTERIA

Mycobacteria obtained from the Royal Tropical Institute (Kolk), M. marinum, M. kansasii and M. tuberculosis were used for the absorption experiments.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The human epidermal extract was electrophoresed using SDS-PAGE according to the Laemmli discontinuous buffer system (Laemmli, 1970) in a mini-slab cell, using 11% poly-acrylamide gel with 0.1% SDS. Samples of extracts containing 100mg/ml protein were treated with 5% mercapto-ethanol and 2% SDS at 100°C in a waterbath for 5 minutes.

The molecular weight standard marker used was a mixture of phosphorylase B (94,000 Da), BSA (67,000 Da) ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsine inhibitor (20,100 Da) and lactalbumin (14,400 Da).

WESTERN BLOT

Electrophoretic transfer of the separated protein from polyacrylamide gels to nitro-cellulose membranes was done using a Bio-Rad transblot cell (Bio-Rad laboratories). After blotting the membranes were blocked with PBS containing 0.03% SDS for 30 minutes and cut into strips.

ABSORPTION

A part of each mother's serum was divided into 3 aliquots. The aliquots were absorbed with sonicates of M. marinum, M. tuberculosis or M. kansasii, as described previously (Klatser et. al., 1984). After absorption the final dilution of the sera used was 1:50 for maternal sera and
1:12.5 for the neonatal sera, the optimal dilutions chosen after dilution studies. No additional activity was detected when lower dilutions were used. For each serum studied, the same procedures were followed without absorption with the mycobacterial sonicate. These unabsorbed sera served as controls.

**INCUBATION AND IMMUNOGOLD SILVER STAINING**

The blotted nitro-cellulose strips were incubated with the absorbed or unabsorbed sera for one hour. After incubation the strips were washed in PBS with 0.005% SDS and then incubated with the second antibody. For the detection of the IgM antibodies from the sera that had bound to the strips, they were incubated with a mouse-anti human IgM Moab (Southers Biotechnology Associated Inc. USA) for two hours at room temperature, and then washed with PBS containing 0.5% SDS. The strips were then incubated with gold-labelled goat anti-mouse IgG (Auro probe B1 plus, Amersham UK), according to the manufacturer's instructions. For the colour reaction the immuno-gold silver staining (Intense TM 2, Amersham UK) was according to the instructions by the manufacturer.

An extra control consisted of IgG. The IgG antibodies were detected by incubating the strips with gold-labelled goat anti-human IgG Moab (Auro probe BL plus, Amersham UK). A negative control was always included.

**RESULTS**

The results showed that both the sera from the mothers’ blood and the cord blood (neonatal sera) contained IgM and IgG antibodies to epidermal proteins of molecular weight ranging from 17 to 90 kDa. Most of the proteins were in the 42-65 kDa range. The reactivity pattern was similar for all sera tested. However each serum showed an individual reaction pattern. When the mother’s serum was compared with that of her baby (neonatal serum) it was observed that the bands were more intensely stained after blotting with the mother's serum, indicating a higher level of antibody in the mother's serum. The patterns for IgG antibody except for the staining intensity were exactly the same for both mother and her baby. However, for IgM sera of 14 of the 21 mothers showed more bands than their babies did. These bands are shown in TABLE 1. The most frequently encountered bands are 80 (3x), 36 (4x), 26 (3x), 21 (5x), 18 (4x) and 16 kDa (5x). When the reactivity pattern of these 14 mother's sera was compared before and after absorption there was a either a total or a partial disappearance of the reactivity to some of these epidermal protein bands after absorption with sonicate of *M. marinum*, *M. kansasii* and *M. tuberculosis* in 7 mothers. Reactivity with a band of approximately 80 kDa diminished in one serum after absorption with each of the 3 strains of mycobacteria in one serum only.
after absorption with *M. tuberculosis*. Two sera had a diminished reactivity with a band of 43 kDa, one after absorption with each of the three strains of mycobacteria, one after absorption with both *M. kansasii* and *M. tuberculosis*. One serum showed lower reactivity with a band of 67 kDa after absorption with each of the three strains and another with the 16 kDa band. After absorption with *M. marinum*, one serum showed a diminished reactivity with a band of 28 kDa, another with a band of 21 kDa after absorption with *M. kansasii* and *M. tuberculosis*. In one serum the reactivity with an 18 kDa band diminished after absorption with *M. marinum* and *M. kansasii*. One serum lost activity in a 28 kDa band after absorption with *M. marinum*. In the range between 43 to 67 kDa where no distinct bands were present, a diminished reactivity was observed in 9 of the 21 sera. Two of the sera showed lower reactivity after absorption with each of the three mycobacteria, one with *M. kansasii* and *M. marinum*, two with *M. kansasii* only and four with *M. marinum* only (results not shown). The results are summarised in TABLE I and II and shown in Figures 1-5.

**TABLE 1** - Additional bands observed after reaction with the mothers’ sera in relation to their neonates’ sera, according to the weight in kDa.

<table>
<thead>
<tr>
<th>SAMPLE NUMBER</th>
<th>ADDITIONAL BANDS IN THE MOTHER’S SERA (kDa)</th>
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<tbody>
<tr>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>18, 21, 26</td>
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<tr>
<td>3</td>
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<td>4</td>
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<tr>
<td>5</td>
<td>43, 36, 16</td>
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<tr>
<td>6</td>
<td>80, 67, 21</td>
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<tr>
<td>7</td>
<td>80, 36, 28</td>
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<tr>
<td>8</td>
<td>18, 16</td>
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<tr>
<td>9</td>
<td>30, 26, 18, 16</td>
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<td>10</td>
<td>18, 16</td>
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<td>11</td>
<td>38, 36, 21, 16</td>
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<tr>
<td>12</td>
<td>36</td>
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<tr>
<td>13</td>
<td>40, 34, 28, 26</td>
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<td>14</td>
<td>40, 34</td>
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</table>
TABLE 2 - Bands that showed less reactivity after incubation with the serum of the mother absorbed with *M. marinum*, *M. kansasiti* and *M. tuberculosis*, according to the weight in kDa.

<table>
<thead>
<tr>
<th>SAMPLE NUMBER</th>
<th>BAND WITH LESS REACTIVITY AFTER ABSORPTION WITH <em>M. MARINUM</em> (in kDa)</th>
<th>BAND WITH LESS REACTIVITY AFTER ABSORPTION WITH <em>M. KANSA SITI</em> (in kDa)</th>
<th>BAND WITH LESS REACTIVITY AFTER ABSORPTION WITH <em>M. TUBERCULOSIS</em> (in kDa)</th>
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<tr>
<td>1</td>
<td>80</td>
<td>80</td>
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<tr>
<td>2</td>
<td>18</td>
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<td>6</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>36, 28</td>
<td></td>
<td>80</td>
</tr>
</tbody>
</table>

Fig. 1 - Strips immunostained for human IgG of nine pairs of mother and child. The first strip on the left molecular weight standard and last one on the right is the negative control. In each group of two, the one on the left was reacted with the mother’s serum and the one on the right, with the newborn’s serum. There are individual differences among the bands and the pattern of each baby is similar to the one from its mother, but less heavily stained.

For FIGURES n° 2, 3 and 4 the results from two pairs of mother and child are shown in each figure. The two strips in the middle are controls for the immunostaining and the two next to them are controls for the absorption. From right to left in each group of 5 strips are seen: the one reacted with the newborn’s serum with the mother’s serum, with the mother’s serum absorbed with *M. marinun*, *M. kansasii* and *M. tuberculosis*, respectively.

Fig. 2 - Group 1 - band of approximately 24 kD is seen only on the strip reacted with the mother’s serum and disappears after absorption with the three strains of mycobacteria. Band of approximately 45 kD, only seen on the strip reacted with the mother’s serum disappears after absorption with *M. kansasii*. Group 2 - bands in the region of 26, 28 and 34 kD only seen on the strip reacted with the mother’s serum partially disappears after absorption with the three strains of mycobacteria.
Fig. 3 - Group 3 - band in the region of 43 kD, seen only on the strip reacted with the mother’s serum totally disappears after absorption with *M. kansasii* and *M. tuberculosis*. Group 4 - band of approximately 60 kD seen on the strip reacted with the mother’s serum disappears totally after absorption with *M. marinum* and partially after absorption with *M. kansasii*.
Fig. 4 - Group 5 - three bands from the region from 60 to 65 kD seen only on the strip reacted with the mother’s serum partially disappeared after absorption with *M. marinum* and *M. tuberculosis*. Group 6 - band of approximately 42 kD visible only on the strip reacted with serum from the mother can no longer be seen after absorption with the three strains of mycobacteria.

Fig. 5 - Both groups 7 and 8, from right to left, show strips reacted with the newborn’s serum mother’s serum, mother’s serum absorbed with *M. marinum*, with *M. kansasii*, with *M. tuberculosis*, control for absorption and control for immunostaining. Group 7 - band of approximately 16 kD, only seen on the strip reacted with the mother’s serum, partially disappears after absorption with *M. marinum* and *M. kansasii*. Group 8 - The same can be seen with bands in the region of 16, 21 and 40 kD after absorption with *M. kansasii*.

DISCUSSION

The results of a previous study using MoAbs against *M. leprae* antigenic determinants in cryostat sections of normal human skin strongly suggested a similarity between some of the *M. leprae* antigenic determinants and those of the human host (Naafs et al., 1990). This was supported by further studies using sera of leprosy patients and healthy human controls in Western blot against a human epidermal extract (Van den Akker et al., 1992). Subsequent absorption studies also supported this suggestion (Id, ibi).

It was theorised that such a reported similarity on one hand could induce a state of immunologic tolerance and on the other hand could induce auto-immunity (Naafs et al., 1990; Van den Akker et al., 1992).

The present study was designed to show that during their lifetime humans may develop an enhanced immunologic recognition of their own antigenic determinants which is not present at birth and that such a recognition of these determinants may be induced by mycobacteria. The study focused on IgM antibodies because IgG antibodies are known to cross the placenta (Avrech et al., 1994), as also shown in this study. The reactivity patterns of IgG antibodies in mother's and her neonate's sera were identical, as also reported by others (Nagao et al., 1998), though the titre of the antibodies was different, being lower in the neonate. Generally, IgM antibodies are considered not to pass from mother to fetus and the presence of IgM antibody in the neonate suggests an actual contact of the fetal immune system with the specific antigenic determinant (Avrech et al., 1994; Nagao et al., 1998). It was observed that 14 out of the 21 mother's sera reacted with more antigenic determinants present in the epidermal extract than their infant's sera. The absorption studies showed that in the sera of 7 mothers out of these 14, some of these antigenic determinants were identical to those also present on the mycobacteria used for the experiment.

Total absence of reactivity to a protein band in the blot after absorption with a mycobacterial sonicate indicates that all antibodies directed against that specific protein, which were present in the serum, had also bound to the mycobacterial antigenic determinants in the pellet and were thus absorbed out of the serum. Partial lowering of the reactivity can be explained by assuming that the sera also contained antibodies directed against antigenic determinants in that particular protein band that were not cross-reacting with the antigens in the mycobacterial sonicate used for the absorption study. Other less likely possibilities include the amount of antibodies in the serum exceeding the available antigenic determinants in the sonicate and a possible low affinity of the cross-reacting antibodies to the mycobacterial antigens.

Earlier studies showed that some antigens are common to all mycobacteria, most nocardia and some corynebacteria and other related genera (Stanford & Wong, 1974; Stanford et al., 1975). Such common antigenic determinants may be responsible for the observation that there
was no reactivity to some of the protein bands after absorption with two or all three strains of the mycobacterial sonicates. Disappearance of the reactivity with only one of the three strains may be due to an antigenic determinant that is more specific for that mycobacterium.

The observation that after absorption, some bands are still visible in one mother but not in another signifies a difference in the individual response of the mothers to the epidermal and/or mycobacterial antigens, reflecting the differences in their genetic make-up and their micro-bacterial history in the context of infecting and environmental micro-organisms.

The occurrence of autoantibodies against epidermal antigens in the sera of healthy individuals has well been recognised by several investigators (Serre et al., 1987; Abel Bystryn, 1976; Paluch & Bloch, 1982; Ackermann-Schopf et al.; Iwatsuki et al, 1986; Hintner et al, 1983; Holland et al, 1985). Studies comparing sera from healthy controls with that of patients with different diseases showed that anti-epidermal antibodies are frequently directed against keratin polypeptides with a molecular weight varying from 17 to 50 kDa (Sun et al., 1983). The antibodies observed in this study reacted mostly against epidermal proteins of 62-64 kDa and 16-35 kDa. It is not unlikely that some of these antibodies are directed against Heat Shock Proteins (HSPs) which are present in the epidermis together with the keratins (Rambukkana et al., 1992).

This study shows that the mothers sera in general recognised more auto-antigens than those of their neonates, suggesting that humans develop such recognition during their lifetime. Since some of the antigens studied in this study seem to share antigenic determinants with the mycobacteria studied, it is tempting to suggest that the recognition of self can be induced and/or maintained by exposure to infective and/or environmental microorganisms. Though other mechanisms like trauma to the epidermis with consequent exposure to antigenic determinants cannot be excluded. It was observed that \textit{M. tuberculosis} seemed to be the most effective mycobacterium in the absorption experiments. This could be due to the fact that all the mothers had been vaccinated with BCG in infancy.

Mycobacteria have a widespread distribution in nature and different species may vary in prevalence from one region to another (Collins et al., 1984; Lyons & Naafs, 1987) inducing different responses in the individuals living in such areas (Rook and Stanford, 1992; Lyons and Naafs, 1987). Besides the etiologic role of these microorganisms in tuberculosis, leprosy and some other less common infections, accumulating evidence indicates a possible participation of mycobacteria in a number of autoimmune diseases like diabetes, biliary cirrhosis, Crohn's disease, rheumatoid arthritis and systemic lupus erythematoses (SLE) (Avioach et al., 1990; Ehrenstein & Isenberg, 1991; Cohen & Young, 1991; Van Eden et al, 1987; Elias et al, 1990).

The study reported here provides further supportive evidence that infective and environmental microorganisms may induce recognition of auto-antigens.
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LITERATURE


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