

that the hypopigmented skin macule is an indicator of a hypersensitivity reaction to sensory nerve myelin. McDougall⁽⁶⁾ has put forward an attractive theory on the mechanism of hypopigmentation in leprosy. He relates the hypopigmentation as a consequence of free radical formation during the cell-mediated immune response. We may assume that a hypopigmented skin lesion is, therefore, an early indicator of the immunopathological mechanism affecting the nerve trunk.

The above-mentioned hypothesis allows us to raise questions on the possible modalities of preventive therapy to be established for patients without clinically detectable loss of nerve function but classified on the basis of skin lesion.

How important is the close association between the subclinical neuropathy and the skin lesion? Can we consider that in leprosy the earliest skin lesion may be considered a forerunner of clinical neuropathy?

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Detection of Antibodies Toward Secreted Mycobacterial Antigen 85 in Untreated Leprosy Patients' Sera

TO THE EDITOR:

Leprosy is an insidious disease that affects two million persons worldwide (1993) and continues to present a public health problem in various parts of the world. The efforts carried out by the World Health Organization (WHO) to eliminate leprosy by the year 2000 have been based mainly on monitored multidrug treatment⁽³⁾, which includes the use of new diagnostic, prevention and disease classification methods.

Mycobacterium leprae is one of the first

human pathogens to have been described, but the impossibility of its cultivation *in vitro* has impeded the isolation and characterization of its various antigenic components. It would be extremely important to determine the role these antigens may play in the immunopathology of the disease, both in humoral and cellular responses.

The chemical structure of *M. leprae* is complex. Considering the high degree of homology between this bacterium and *M. bovis*⁽¹⁾, in this study we analyze the humoral response to *M. bovis* secreted anti-

THE TABLE. Levels of IgG antibodies directed to *Mycobacterium bovis* excreted proteins and total *Mycobacterium leprae* proteins.

Serum 1:200	No.	Mycobacterial proteins	
		<i>M. bovis</i> excreted	Total <i>M. leprae</i>
Multibacillary	17	0.468 ± 0.2 (65%) ^a	1.168 (88%)
Paucibacillary	15	0.06 ± 0.04 (40%)	0.281
Control	10	0.025 ± 0.022	0.000

^a OD ± standard deviation (percent of positive sera, OD >0.2).

gens in patients with Hansen's disease and in normal persons who are contacts of leprosy patients. We used mycobacterial proteins, among them complex 85, actively secreted by this mycobacterium in culture media (⁴).

We studied sera from 17 multibacillary (LL, BL, BB) patients, 15 paucibacillary (TT, BT, LI) patients, and 10 Mitsuda-positive contacts and nonrelated persons, all adults, to detect antibodies toward *M. bovis* secreted and *M. leprae* cytoplasmic proteins by an immunoenzymatic assay.

The antigen used was *M. bovis* excreted protein. Danish BCG strain 1331 was cultured in Sauton medium (⁷). Ten freeze-dried BCG ampules (0.5 mg each) were diluted in 200 ml Sauton and seeded in Roux vials for a total final volume of four liters of medium, which was incubated at 37°C for 6 weeks. The medium was then filtered through Whatman No. 1 filter paper, centrifuged at 10,000 rpm (Sorvall RC-5B, rotor SS-34) at 4°C for 15 min and finally filtered through a 0.45-µm membrane. Ammonium sulfate (85% saturated) was added to each liter of medium with gentle shaking for 30 min. After centrifugation, the precipitate was resuspended in 100 mM TRIS-HCL, pH 7.4, and dialyzed for 48 hr against the same buffer. The material was concentrated using 10× polyethylene glycol. Protein concentration was determined by the BCA method (⁸).

The immunoenzymatic assay was performed as described previously (⁵). Circulating IgG values against excreted proteins were expressed in optical density (OD). Briefly, microtiter plates were sensitized with 2 µg protein/ml, 50 µl per well. All test sera were used at a 1:200 dilution. The secondary serum (peroxidase-labeled anti-human IgG) was used at a 1:5000 dilution.

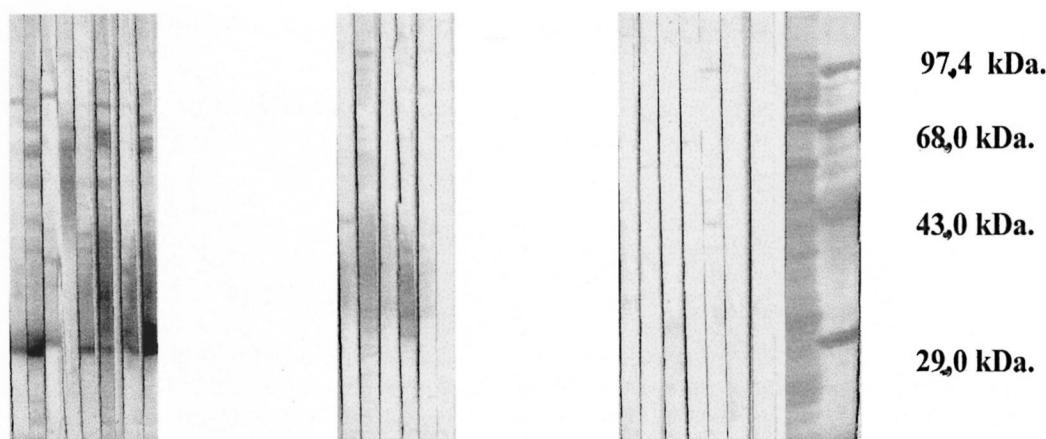
The substrate was OPD-H₂O₂ and the reaction was stopped with 2.5 N H₂SO₄. Readings were done at 490 nm.

For immunoblotting, the BCG excreted proteins were separated by electrophoresis in polyacrylamide gels containing 10% SDS (⁶). Samples (500 µg protein) were diluted in sample buffer pH 6.8 (62 mM TRIS-HCL, 2% SDS, 50 mM 2-mercaptoethanol and 10% glycerol), boiled for 3 min and applied to 11-cm gels. Running conditions were 25 mA (constant current) in the stacking gel and 35 mA in the separation gel until bromphenol blue reached the lowest part of the gel. The gel was stained with 0.25% Coomassie brilliant blue. A Sigma standard molecular weight mixture was used.

Proteins in the preparative gel were electrophoretically transferred to 0.45-µm nitrocellulose paper using a pH 8.3 buffer (25 mM TRIS base, 192 mM glycine and 20% methanol) at a constant current of 100 mA for 16 hr (⁹). Nitrocellulose membranes were cut into 4-mm thick strips and confronted with patient and control sera as follows: 2 hr incubation with test sera (1:400), three 10-min washes with PBS-Tween, 2 hr incubation with secondary peroxidase-labeled anti-IgG serum (1:5000), and three washes as above. 1-4 Chloronaphthol-H₂O₂ was added and the reaction was stopped with water.

The results showed greater intensity of the IgG antibody response directed toward a protein complex in the excreted antigens (The Table), with a relative mobility of approximately 30 kDa, in multibacillary as compared to paucibacillary patients (The Figure).

Other investigators have found low levels of antibodies directed toward secreted proteins (28.5% in multibacillary and 32.3% in paucibacillary untreated patients)



THE FIGURE. Immunoblotting of BCG excreted proteins in the presence of sera from Hansen's disease patients.

(²), and they suggest that there might be immune complexes associated to free antigen. This study does not correlate with the bacterial index or ELISA positivity.

Since *M. bovis* excreted proteins possess high homology with *M. leprae*, these results could be measuring bacterial viability; they suggest that these proteins could be candidates for serological follow up of multidrug therapy in leprosy.

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