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Efforts in diagnosing early leprosy using serological techniques

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Abstract. Skin scrapings obtained from the lesions of leprosy patients of all types showed 96 % positivity to the serum antibody competition test using monoclonal antibody (ML04)to 35 kDa antigen of *Mycobacterium leprae.* Further, *in vitro* culture of full thickness skin biopsies from lepromatous patients were noted to release IgG antibodies to *M. leprae* with a peak antibody response at 48 h. The significance of this local antibody response to *M. leprae* in skin has been discussed for its possible use in diagnosing early leprosy.

Keywords. Leprosy; *Mycobacterium leprae* 35 kDa antigen; skin scrapings; skin biopsies.

1. Introduction

Although recently multidrug therapy (MDT) has brought down significantly the number of leprosy cases in the world from 12 million (WHO 1988) to 2·4 million (Noordeen 1995), there has not been any change in the incidence of the disease indicating that an active transmission is still going on in the population. Similarly, in India, after MDT the number of cases has come down to 0·62 million in October, 1995 from 3 million cases in 1981 (D.G.H.S. 1995). In spite of such a significant reduction in the number of cases, the incidence has not shown any downward trend in any region in India. Hence, there is a need for a specific serological test for diagnosing leprosy at a very early stage of the disease for an early treatment and control.

During the last two decades several specific serological assays have been established (Sengupta 1990). Out of these, serum antibody competition test (SACT) (Sinha *et al.* 1985) and phenolic glycolipid-I (PGL-I) based ELISA (Brett *et al* 1983; Young and Bachanan 1983) have been found to be very specific. However, these tests also failed to identify a large population of early cases of tuberculoid (TT) and borderline tuberculoid (BT) leprosy. In one of our studies, it was further noted that 30 to 40% of patients did not show any elevation in the antibody levels against 35 kDa and PGL-1 antigens (Om Parkash *et al* 1995) and also against whole *Mycobacterium leprae* antigens (unpublished data). These observations clearly indicated that in TT/BT patients with negative antibody response the B-cell response was not at that level which could ultimately result in an elevated antibody response against the background level of antibody already present in the population. The present study was undertaken to find out the local antibody response, if any, in the skin lesions of leprosy and to explore it for it's use in the diagnosis of leprosy patients.

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2. Materials and methods

2.1 *Skin scrapings*

Skin scrapings (4 strokes each) from skin lesions of 47 leprosy patients (borderline tuberculoid leprosy = 22; borderline lepromatous leprosy and lepromatous leprosy = 22; mid borderline leprosy = 3) and from 20 non lesions of leprosy patients were collected in 0·5 ml normal saline (NS) using a standard method (Rees 1985) with minor modifications. Patients included in the study were active and were at various stages of treatment. Scrapings were also taken from lesions of 34 non-leprosy patients (sufering from other skin diseases like Vitiligo, fungal infection, etc.,) and 10 normal individuals.

2.2 *Full thickness skin biopsy culture*

Full thickness skin biopsies were obtained from borderline lepromatous (BL) and lepromatous (LL) patients after classifying them on the Ridley-Jopling scale (Ridley and Jopling 1966). The skin biopsies were cultured using a standard method (Le poole *et al* 1991). Briefly, 4 mm full thickness biopsies were positioned (epidermal side up) on a nitrocellulose paper (NCP) (1·2 μm pore size, Millipore Inc., USA). The NCP with the biopsy was placed on a stainless steel grid inside a sterile Petri dish. Enough medium (DME with 10% AB serum) was added just to wet the NCP without covering the skin tissue. Medium was collected at every 24 h with replenishment of culture with fresh media.

2.3 *Antigens*

Cell free soluble extract (CFE) of *M. leprae* was obtained from the IMMLEP WHO Bank at the National Institute of Medical Research, Mill Hill, London and was supplied by Dr R J W Rees.

2.4 *Antibodies*

Monoclonal antibody against 35 kDa (MLO4) raised by us at the Wellcome Laboratories, UK (Ivanyi *et al* 1983) was obtained from Dr J Ivanyi, MRC Tuberculosis and Mycobacterial Infections, Hammersmith Hospital, UK.

2.5 *SACT*

The test was carried out following the method described earlier (Chaturvedi *et al* 1991; Om Parkash *et al* 1995). Briefly, wells of ELISA plates (Nunc, round bottomed) were coated with M. *leprae* antigen (2.5 μg/well). After blocking the non-specific binding sites with 3% bovine serum albumin (BSA, Sigma Chemical Co., UK) in phosphate bufered saline (PBS) the wells were treated for 20 min with serum/skin scraping samples followed by addition of peroxidase labelled monoclonal (MLO4) antibody. After developing the colour in the wells, the enzymatic reaction was stopped by adding 7% H_2SO_4 to the wells. The readings were noted in at 492 nm in an ELISA reader (Titertek, Multiscan Plus, Flow Laboratories, UK) and the results were analysed.

2.6 *Estimation of anti M. leprae-IgG antibody by ELISA*

The presence of IgG antibody to *M. leprae* sonicated antigen in the culture fluid produced by the skin biopsy was measured by ELISA. The wells of the ELISA plates (Nunc, round bottomed) were coated with 50 µl of *M. leprae* sonicated antigens at a concentration of 10 µg/ml in 0·05 M carbonate buffer pH 9·6 at 37°C for 4 h followed by incubating at 4°C overnight. Plates were washed with PBS twice and then blocked with 1% BS A (Sigma Chemical Co., USA) for 1 h. Plates were washed with PBS-Tween 20 (0·1%, 3X) followed by the addition of 50 µl of culture fluid at 1:10-fold dilution. Each dilution was tested in duplicate. Further, plates were incubated at 37°C for 2h and then washed thrice with 0·1% PBS-Tween. After washing 50 µl of peroxidase conjugated rabbit anti-human IgG (Sigma Chemical Co., UK) (1:1000) was added to each well. Plates were then incubated at 37°C for 90 min. After washing extensively with PBS-Tween, 50 µl of OPD substrate (O-phenylene diamine dihydrochloride containing H_2O_2) in citrate buffer (pH 5·0) was added to develop colour. The reaction was stopped after 30 min with 7% H_2 SO_4 and was read at 492 nm in an ELISA reader (Titertek Multiscan Plus, Flow Laboratories, UK.)

3. Results

3.1 *Experiments with serum samples and skin scrapings*

In the first experiment, serum samples were obtained from 28 leprosy patients of all types, 30 non leprosy patients suffering from other skin diseases and 10 normal individuals. SACT analysis of the sera samples revealed that while 68% of the patients showed positivity only 3·3% of non leprosy patients were positive to the test. None of the normal individuals were found to be positive by the assay (table 1).

In the second experiment the skin scrapings were collected from 47 leprosy patients from lesional areas, 20 leprosy patients from non-lesional areas, 34 non-leprosy patients from lesional areas and from normal skin from 10 healthy individuals. On screening these samples it was noted that in leprosy patients SACT positivity was 96% in samples from lesions, while in 95 % of the leprosy patients the test was positive even in samples obtained from non-lesional areas of the skin. Further, samples from non-leprosy patients showed 47·1 % positivity whereas all the normal individuals were

Table 1. Findings using SACT for sera from leprosy patients, non leprosy patients and normal individuals.

		SACT		
Types of cases	Total cases	+	–	Positivity (%)
Leprosy patients	28	19	9	68·0
Non-leprosy patients	30	1	29	3·3
Normal individuals	10	0	10	0

Borderline tuberculoid leprosy = 6/15; borderline lepromatous leprosy and lepromatous leprosy = 10/10 and mid borderline leprosy = 3/3.

again found to be negative for the assay (table 2). Thus the SACT positivity using skin suspension from non leprosy patients was 43·8 % more when compared to the per cent positivity with serum of non leprosy patients.

Table 2. Findings using SACT for skin suspensions from leprosy patients and non leprosy individuals.

		SACT		
Skin suspension from	Total	+	−	Positivity (%)
Lesions (leprosy patients)	47	45	2	96·0
Non-lesions (leprosy patients)	20	19	1	95·0
Lesions of non leprosy patients	34	16	18	47·1
Normal persons	10	0	10	0

Borderline tuberculoid leprosy = 21/22, borderline lepromatous leprosy/lepromatous leprosy = 22/22 and mid borderline leprosy = 2/3.

3.2 *Experiment with full thickness skin biopsies*

After obtaining the above informations with the skin scrapings we were tempted to carry out the *in vitro* skin culture study to understand the basic aspects related to the local synthesis of antibody in the skin lesions. Further, this approach may be helpful in establishing that the source of antibody is localized in the lesion.

In vitro IgG antibody response to *M. leprae* peaked at 48 h of culture and by 96 h there was a considerable fall in the yield of antibody (figure 1).

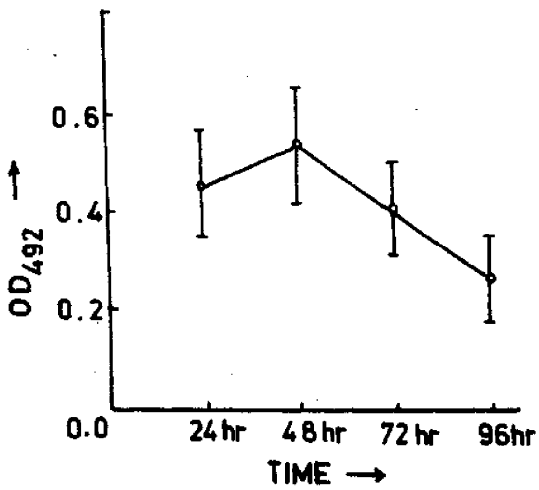

Figure 1. Kinetics of antibody production by skin biopsy *in vitro* culture.

4. Discussion

It is clear from the present study that there is an antibody response locally in the skin lesions of leprosy patients and a larger number of patients (96%) could be identified than that by using SACT or PGL-1 assays in serum. Thus, when the sensitivity of the assay was improved considerably the specificity of the assay declined because many of the non-leprosy patients afected with vitiligo and fungal infections were also positive in the assay. Since SACT assay is known to be specific for detection of *M. leprae* infection (Sinha *et al* 1985), it is possible that some of the non-leprosy patients found to be positive in the present study might be having concurrent infection (may be subclinical) with *M. leprae.* However, further studies to rule out the non specificity of the assay and its reason thereof would be worth attempting.

Like previous report (Lai A Fat *et al* 1980), in the present study also while standardizing the full thickness skin culture from BL/LL patients it was noted that antibody production to *M. leprae* could be measured *in vitro* which was released in the media. In addition, in the present study it was possible to measure the kinetics of antibody production in a continuous culture with a peak antibody response at 48 h which declined later (figure 1).

Though preliminary in nature, the present study has provided evidence for the presence and probably production of local antibody in leprosy lesions. These informations may prove to be helpful in designing some specific laboratory test which might help in diagnosing early leprosy. The studies on this direction are worth while attempting.

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