Identification of Lymphocyte Subsets in Leprosin A Positive Sites Following Vaccination

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Abstract

In Leprosy a skin test has been prepared by Rees from armadillo tissues carefully freed from armadillo antigen. This reagent is called "Leprosin A." Several healthy normals, contacts and leprosy patients negative to Leprosin A were vaccinated with a mixture of \textit{M. leprae} plus BCG. Forty four Leprosin A positive sites were biopsied following the vaccination. Immuno peroxidase technique with monoclonal antibodies has been used to identify T cells, T helper cells and T suppressor cells. Enumeration of the T Cell subsets shows that the large number infiltrating the positive skin test sites are T helper-induce subset. Multibacillary leprosy patients following the vaccination demonstrated large numbers of T helper-inducer lymphocytes. We speculate that the T helper cells are due to the immunostimulating properties of the \textit{M. leprae} plus BCG.

Introduction

Soluble skin tests in mycobacterial diseases have been helpful in understanding the mode of spread of the disease and the individuals susceptibility to infection. In leprosy, a skin test has been prepared by Rees from armadillo tissues carefully freed from armadillo antigen. This reagent is called "Leprosin A" and has been used extensively in leprosy patients, contacts and non-contacts in various parts of the World(1). There has been renewed interest in recent times to develop a vaccine against leprosy. Convit reported that injection of $6 \times 10^8$ \textit{M. leprae} with variable amounts of BCG has resulted in positive responses to soluble skin test antigens among leprosy patients and healthy contacts(2). Chemotherapy alone is not sufficient to cure multibacillary leprosy patients as they lack cell mediated immunity to lyse the bacteria which are lodged in the macrophages. In addition, a vaccine against leprosy would need to boost the protective immune response among contacts and non-contacts in a population who may develop the clinical disease.

We have vaccinated healthy persons, contacts and leprosy patients who were negative to Leprosin A soluble skin test antigen with killed \textit{M. leprae} $1 \times 10^7$, and live BCG $1 \times 10^6$(3). The Leprosin A positive sites and the vaccinated sites were subsequently biopsied. In this
communication we report the histological and lymphocyte subsets on tissue sections of Leprosin A positive sites.

**Materials and Methods**

Patients selected for vaccination had the details of the study explained to them. The personal particulars of each patient were recorded and they underwent a thorough clinical examination. Patients were classified on the leprosy spectrum using the Ridley-Jopling scale and the initial clinical classification was confirmed histologically. All the patients were on combined chemotherapy from Anandaban Leprosy Hospital.

The following multidrug regimens were used:

(i) Multibacillary patients. Skin smear BI>2+
   - Rifampicin 600mg 2 doses a month
   - Clofazimine 100mg or
   - Dapsone 100mg od

(ii) Paucibacillary patients. Smear BI<2+
   - Rifampicin 600mg 2 doses a month
   - Dapsone 100mg od.

The duration of treatment varied from 4 to 16 months.

**Contacts**: Residents in the same house as multibacillary patients were entered in the study. Clinical examination was done and those apparently free of clinical lesions were included in the study.

**Healthy persons**: Those with no history of contacts with leprosy or tuberculosis were entered in the study.

**Informed consent**: The details of the study was explained to all the subjects and their consent was obtained prior to the study.

**Skin test procedure**: Leprosin A was administered per person using Gillette Scimitar 1 ml disposable tuberculin syringe fitted with 26 gauge needle. Erythema and induration were measured at 48 hours. Biopsies were taken from macroscopically positive sites. 44 positive skin test biopsies were obtained from 5 healthy persons, 8 contacts, 10 borderline leprosy patients, 11 multibacillary patients (BL/LL) and 10 indeterminate patients.

**Preparation of leprosin A**: From batches of *M. leprae* prepared as per the protocol, the bacilli were broken open by sonic disruption, centrifuged and the soluble supernatant was standardised on the basis of protein content. 10μg/ml protein concentration was used in the skin test.

**Vaccines used in the study**:
1. BCG-Glaxo, freeze dried 10^6 live organisms
2. 10^7 killed *M. leprae*

**Skin biopsies**: Skin biopsies were taken under local anaesthesia. They were divided into two portions, one was fixed in formal-zenkar for histological examination and another snap frozen in liquid nitrogen until used for immunostaining.

**Monoclonal antibodies**: For demonstration of T-cell subsets, the following antibodies
The Percentage of anti-Leu 3a positive or anti-Leu 2a positive T cells was calculated as follows:

\[
\frac{\text{Anti-Leu 3a positive cells}}{\text{Anti-Leu 3a positive cells + Anti-Leu 2a positive cells}} \times 100
\]

or

\[
\frac{\text{Anti-Leu 2a positive cells}}{\text{Anti-Leu 3a + Anti-Leu 2a positive cells}} \times 100
\]

were used from the commercially available murine monoclonal anti-T-cell antibodies: Leu series (Becton Dickinson, U. S. A). The properties of these antibodies are as follows: Anti-Leu-4 reacting with 100% of peripheral T-cells; anti-Leu-3a reacting with 50-60% of peripheral T-cells including helper inducer subsets. Anti-Leu 2a reacting with 20-30% of peripheral T-cells including suppressor/cytotoxic cells. Other antisera used in this study consisted of rabbit anti-mouse gammaglobulins (Cappel), goat anti-rabbit Ig (Fujizoki, Japan) and PAP (Daco, Denmark).

**Staining procedure:** The four step PAP method of Poppema et al for various T-cell subsets, was modified. In summary the 4μ frozen sections were air dried and fixed with acetone ethanol (3:1) for 30 seconds. Each section was treated with monoclonal anti-T with the optimal dilutions (1:200–1:400). After washing with PBS, each section was successively incubated with rabbit anti-mouse gammaglobuline serum (1:400), goat anti-rabbit Ig serum (1:400), and peroxidase rabbit anti-peroxidase complex (PAP, 1:100). Finally, the chromogen, 0.05% 3-3' diaminobenzidine in PBS with 0.03% hydrogen peroxide, was placed on each section for five minutes. Some of the sections were refixed with acetone for 1 minute after incubation with the first serum and washed with PBS, to prevent detachment of the sections. This procedure did not hinder subsequent immunohistochemical reactions. The control sections were incubated with mouse serum instead of first antibody. These control slides were treated similarly thereafter.

**Evaluation of the immunohistologic stain:** Cells whose cytoplasmic rims were stained brown were regarded as positive. Cells with dark brown granular staining was excluded, as these cells were observed in negative control slides as well, presumably indicating intrinsic peroxidase positive cells. Otherwise, the results were easy in differentiating positive and negative cells. To determine the ratio of anti-Leu-4 positive anti-leu 2a positive cells, the number
Legends for Figures

Fig. 1 Positive skin test response of healthy persons. (Cryostat section, HE Section ×120)

Fig. 2 Anti-Leu 4 positive cells in the Leprosin A positive reaction of healthy person. (Cryostat section, immunoperoxidase stain for Leu 4 ×240)

Fig. 3 Positive Leprosin A reaction of BL patient. (Cryostat section, HE section ×120)

Fig. 4 Anti-Leu 3a positive cells in Leprosin A positive reaction of BL patient. (Cryostat section, immunoperoxidase stain for Leu 3a ×240)

Fig. 5 Anti-Leu 2a positive cells are few in number in a positive skin test reaction of BL patient.

Fig. 6 Anti-Leu 3a positive cells in Leprosin A positive skin test of LL patient.
of positive cells in 200 mononuclear cells in the approximately same fields of two slides, one stained with anti-Leu 3a and the other with anti-Leu 2a were counted and calculated following the formula listed in Table 1. The individual ratios were averaged for each group of subjects.

Vaccination protocol: This is designed to observe and record conversion from negative to positive of Leprosin A skin tests in as many non-contacts, contacts and patients as possible after vaccinating them with *M. leprae* plus BCG. Each time the vaccine was freshly reconstituted and given in 0.1ml intradermally over the deltoids at intervals of 8 to 12 weeks(7).

Re-skin testing with leprosin A: In contacts and paucibacillary patients first repeat skin testing was performed after 4 vaccinations and subsequently prior to every re-vaccination. In multibacillary patients repeat skin testing was performed after every 4 vaccinations.

Results

Histological reactions: The histopathological changes observed were perivascular infiltration of mononuclear cells characteristic of delayed type hypersensitivity reactions. The extension of the cellular infiltration was variable. Nevertheless the cells were lymphocytes and monocytes. Eosinophils and basophils were not observed in the biopsies.

Healthy persons: The histology of the positive sites of skin tests in non-contacts was characterized by extensive lymphocytic infiltration around blood vessels and many of the capillaries and venules were packed with lymphocytes. The cellular infiltration extended into deep dermis.

Contacts: The histological picture was essentially uniform in all 8 biopsies and consisted of mononuclear cellular infiltration around the blood vessels and appendages.

Borderline tuberculoid patients: In all 10 biopsies the cellular infiltration was not similar. In 3 of these biopsies moderate mononuclear cells were observed perivascularly. Polymorphs were scanty in all the biopsies. Acid-fast bacilli were not detectable in 5 patients, rare in 1 patients and moderate number of bacilli were identified in the remaining patients.

Multibacillary patients (BL/LL): In all 11 biopsies the cellular infiltration was minimal in comparison with the other groups. In 4 of the LL biopsies were devoid of acid fast bacilli and were superimposed with large numbers of lymphocytes.

In the remaining two, macrophages contained fragmented acid fast bacilli and superimposed with minimal numbers of mononuclear cells. The cellular infiltration was seen throughout the dermis.

Indeterminate patients: Histologically, a significant number of lymphocytes infiltrated the dermis around the blood vessels. Acid fast bacilli were not identified in 6 biopsies. In 4 acid fast bacilli were observed.

The intensity of mononuclear cellular infiltration was proportional to the size of the macroscopic reaction of the skin test.

T-lymphocyte subsets:

Healthy persons: Following immunostaining lymphocytes bearing the Pan T Cell antigen were the predominant cell type. Eighty five percent of the infiltrate were lymphocytes expressing T helper-inducer antigen and fifteen percent of lymphocytes expressed the T suppressor-
cytotoxic phenotypes. T helper cells are scattered in the center of the granulomas while the T suppressor cytotoxic phenotypes confined to the periphery of the granulomas.

**Contacts:** In eight contact specimens, the T cell was the dominant cell type. Eighty percent of the lymphocytes expressed T helper-inducer antigen and twenty percent of lymphocytes expressed the T suppressor-cytotoxic phenotypes. T helper and T suppressor cells distribution in the granuloma was similar to that observed in healthy persons.

**Borderline tuberculoid patients:** Lymphocytes staining for T helper inducer antigen were in large numbers. Seventy six percent; in contrast to the few T suppressor cells (twenty five percent). The distribution of the T cell phenotypes in the granuloma was less distinctive.

**Multibacillary patients (BL and LL):** In eleven specimens, cells expressing the T helper phenotypes were sixty seven percent while T suppressor phenotypes were thirty three percent. On enumeration T helper cells were in large numbers among BL patients than in LL patients. The helper and the suppressor phenotypes were diffusely distributed in both BL and LL patients.

**Indeterminate patients:** The predominant cell in all the nine biopsy specimens was bearing T helper phenotype. However there were distinctive differences as to the location of the T cell subsets in the granulomas. Six specimens show the typical location of suppresser cells at the periphery of the granuloma while the helper cells are found in the centre. On the other hand, in three specimens this pattern is not observed. Both cell types are distributed diffusely in the granulomas.

**Discussion**

The large quantities of *M. leprae* obtained from Armadillos has made possible a soluble skin test for use in leprosy. The basic assumptions of skin tests based on cell mediated hypersensitivity are that the antigen content of the skin tests are minimal and avoid sensitization of the test subjects and histologic findings are that of perivascular infiltration of mononuclear cells. The histological evaluation of Leprosin A has indicated that Leprosin A induced a characteristic delayed type hypersensitivity response in healthy persons, contacts and patients following vaccination with BCG plus *M. leprae*. The perivascular infiltration at the Leprosin A positive site is an indication of the state of tissue reactivity to antigens of *M. leprae* and is therefore an index of the resistance in the test subject. However, the relationship between delayed hypersensitivity and immunity is a problem with a long history(7).

The availability of specific monoclonal antibodies for all T cells has made it possible in the understanding of the immunopathological abnormalities of human diseases. In leprosy several workers have reported the T cells subsets in dermal lesions of leprosy and its significance in the disease spectrum and Erythema nodosum leprosum(8-10). The prime intention of this study is to enumerate the T cell phenotypes in the cellular infiltration of the Leprosin A positive sites. It is of interest to note that in all groups the predominant lymphocyte was T helper-inducer subset. Of considerable interest are the large numbers of T helper-inducer lymphocytes demonstrated in the Leprosin A sites of multibacillary patients, especially in lepromatous leprosy patients. This is in contrast to the T cell subsets observed in skin lesions in these patients where the T-suppressor-cytotoxic phenotype predominates(11). We speculate that the observed
T-helper-inducer phenotypes in large proportion is due to the immuno-stimulating properties of the *M. leprae* plus BCG. Therefore repeated vaccinations in LL patients will result in partial restoration of the cell-mediated immunity. It is hoped that the immuno-histological characterization of soluble skin test reaction will increase the value of the test in the protective evaluation of vaccines. As far as we know this is the first study to report the T cell phenotypes in soluble skin test sites following vaccination in leprosy patients and controls.

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**References**