Immunohistological Analysis of Skin Reaction to My1 Derived from *Mycobacterium leprae*[1]

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Leprosy is a chronic infectious disease caused by *Mycobacterium leprae.* Various groups of workers have carried out the antigenic analysis of *M. leprae* ([1, 4, 21]). Recently, three protein antigens (My1, My2 and 68K) carrying distinct antigenic determinants which are expressed by *M. leprae* but not by several other species of mycobacteria have been identified by monoclonal antibodies ([4]). Two of the monoclonal antibodies, MLO4 and MLO6, recognize mycobacterial antigens My2, and My1 of *M. leprae,* respectively. Both of these antigens are localized in the cytoplasm of the mycobacteria ([2]). It has been observed that My1 evoked only an early, delayed-type hypersensitivity skin reaction (24–48 hours) in tuberculoid patients but not in lepromatous patients and was similar to that seen with standard Dharmendra lepromin. The infiltrates of this skin reaction are composed of lymphocytes and polymorphonuclear leukocytes. The immunological characteristics of the cells in these infiltrates are not clear. Monoclonal antibodies against cell surface antigens have been used to delineate the immunopathological mechanisms involved in the granulomas of leprosy lesions ([6–12, 15–20]), sarcoidosis ([5, 8]), the mechanism of induction of the lepromin reaction ([13]), and other skin reactions ([16, 18]).

The present study was aimed at understanding the elicitation of the early skin reaction to My1 by characterizing the infiltrating cells using monoclonal antibodies against T-cell subsets, Ia-like antigens, Langerhans' cells, and soluble antigens of *M. leprae.*

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MATERIALS AND METHODS

Antisera. Monoclonal antibodies OKT11 (pan T cells), OKT8 (suppressor/cytotoxic T cells), OKT6 (subset of T cells, Langerhans' cells), and OKIa (activated T cells, macrophages, B cells) were obtained from Ortho Pharmaceutical Corporation, Raritan, New Jersey, U.S.A.; Leu3a (inducer/helper T cells) was obtained from Becton, Dickinson Monoclonal Center, Inc., Mountain View, California, U.S.A.; fluorescein conjugated rabbit anti-human IgM (Dakopatts A/S, Denmark); FITC-conjugated sheep anti-mouse Ig $F(ab)_2$ (New England Nuclear, Boston, Massachusetts, U.S.A.); purified My1 and MLO6 monoclonal antibody against soluble antigen (My1) of *M. leprae,* as ascites fluid, were kindly provided by Dr. J. Ivanyi, MRC Unit, Hammersmith Hospital, London. My1 was purified by monoclonal (MLO6) antibody based affinity chromatography ([3]).

A solid-phase radioimmunoassay (RIA) analysis showed that the My1 protein was considerably enriched in the eluate fraction and was completely depleted from the filtrate of the affinity column.

Skin biopsies. The leprosy patients were selected from the outpatient clinic of the Central JALMA Institute for Leprosy, Agra, India, and were classified according to the criteria of Ridley and Jopling ([17]). My1, 1 μg in 100 μl, was injected intradermally into the interscapular area on the back and, after 24 hr, a biopsy from the site of the reaction was collected in isopentane and frozen at −20°C for cryostat sections.

Immunofluorescence. Cryostat sections 4–5 μm thick were cut and fixed in a cold acetone-chloroform mixture (1:1 ratio) for 10 min. The sections were then dried, and incubated with 1:20 dilutions of monoclonal antibodies at room temperature for 45 min. Sections layered with phosphate buffered saline (PBS) served as controls. In addition, sections were also incubated with 25

μl of neat (approximate concentration 1 mg/ml) stock solution of MLO6 for 45 min. As a control for the MLO6 treatment, sections were layered with normal mouse serum, diluted to a concentration of 1 mg/ml similar to MLO6. Subsequently, all of the sections were washed for 20 min in 0.85% w/v saline. They were then incubated with 25 μl of a 1:80 dilution of FITC-conjugated sheep anti-mouse Ig $F(ab)_2$ mixed with pontochrome violet (1%) for 30 min at room temperature and then washed in saline for 45 min. For the definition of B cells, direct immunofluorescence was done using a 1:120 dilution of FITC-conjugated rabbit anti-human IgM. The sections were mounted in 90% PBS-glycerol and viewed by epi-illumination using an HBO 50 mercury lamp and a Leitz inverted microscope, with incident light excitation filter block no. 12 and transmitted light excitation filter block no. 4 (513604).

The optimal dilutions of the antibodies and checking of monoclonal antibodies was done on cryostat sections from lymph nodes, normal skin, and skin lesions of leprosy patients and smears of Ficoll-Hypaque purified mononuclear cells from peripheral blood of normal individuals.

Quantitation (approximate percentage) of subsets of cells in the infiltrates was done by enumeration of positive cells with green fluorescence and negative cells staining red with pontochrome violet. In addition to the cells in the infiltrates, OKT6+ Langerhans' cells per high power field in the epidermis were also estimated. All of the sections were read blind.

RESULTS

Preliminary experiments were carried out on the cryostat sections of leprosy lesions to determine the optimal dilutions of antibodies required for staining. The antibodies were also evaluated by indirect immunofluorescence on peripheral blood mononuclear cells. OKT11, Leu3a, and OKT8 antibodies stained 70–80%, 40–50%, and 15–20% of peripheral blood mononuclear cells, respectively.

Ten patients each of tuberculoid (BT/TT) and lepromatous (BL/LL) leprosy classified on the scale of Ridley and Jopling ([17]) were included in the study. The skin reaction to My1 was strongly positive in the tuberculoid patients with a peak response at 24 hr. However, in the lepromatous patients it did not evoke any reaction. Only 24-hr skin reactions in the ten tuberculoid patients were analyzed.

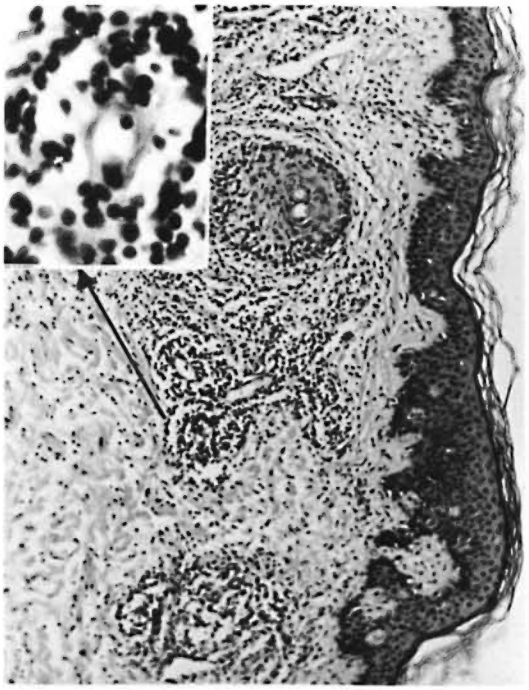

FIG. 1. Dermal infiltrate of 24-hour skin reaction induced by My1, showing predominantly lymphocytes with a few polymorphonuclear leukocytes (×60). Inset shows a high-power view of the lymphocytic infiltration (H&E ×200).

Dermal infiltrate characteristics in 24-hr skin reactions induced by My1

Lymphocytes. The infiltrates in this reaction consisted of lymphocytes and polymorphonuclear leukocytes (Fig. 1). The intensity of staining was maximal with Leu3a, OKT8, OKIa, and OKT6 antibodies, and moderate with OKT11 antibodies. A high proportion of cells in the infiltrates expressed OKT11, Leu3a, and OKT8 antigens (Fig. 2). Leu3a and OKT8+ cells were also seen in the epidermis. The ratio of Leu3a+ :OKT8+ cells in the infiltrates was 1.2 ± 0.50 (The Table). Ia-like antigens were expressed by a large proportion of these cells, but were not discernible on the polymorphonuclear leukocytes. No staining was observed in the infiltrates with OKT6 antibodies or with fluorescein conjugated rabbit

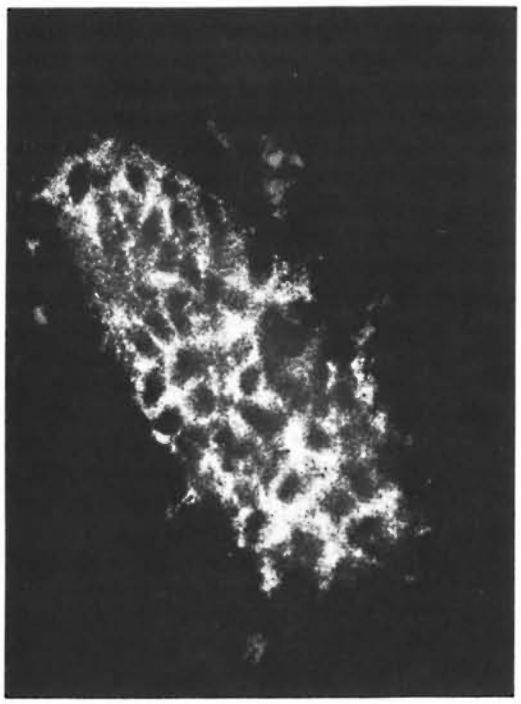

FIG. 2. Dermal infiltrate of the 24-hour skin reaction induced by My1, showing the Leu3a+ cells stained with the monoclonal antibody by indirect immunofluorescence (cryostat section counterstained with pontochrome violet ×320).

THE TABLE. In situ *characteristics of dermal infiltrates in 24-hour skin reactions induced by My1 in 10 tuberculoid (BT/TT) leprosy patients.*

	Mean ± S.D. percent positive cells in the infiltrates
Monoclonal antibodies	
OKT11	36.0 ± 17.9
Leu3a	60.0 ± 16.5
OKT8	56.0 ± 19.1
OKIa	81.0 ± 7.0
OKT6	Not detectable
B cells[a]	Not detectable
MLO6	Positive cells seen
Ratio Leu3a+ : OKT8+ cells	1.2 ± 0.50
OKT6+ epidermal Langerhans' cells	
Normal skin (5 subjects)	14.0 ± 1.8
My1 reaction	10.0 ± 3.4

[a] Heterologous (rabbit) anti-human IgM antibody.

anti-human IgM. Thus, the infiltrating cells appear to be predominantly activated T lymphocytes.

Mycobacterial antigens. The presence of My1 antigen on the infiltrating cells was observed using the MLO6 monoclonal antibody. In some of the cells, membranous staining was seen. The polymorphonuclear leukocytes also showed staining with this antibody. As a control, normal mouse serum diluted to the same concentration as that of the monoclonal antibody was used, and only occasional positive cells were seen.

Epidermal Langerhans' cells. No difference was observed in the numbers of epidermal Langerhans' cells in the skin of the My1 reactions in comparison to normal skin (The Table).

DISCUSSION

The lepromin reaction has been used as one of the parameters to assess the *in vivo* immune status of a leprosy patient or a contact, and to test the efficacy of an immunoprophylactic agent. It can also be considered as an experimental system to study the immunological mechanism involved in the elicitation of a delayed-hypersensitivity skin reaction. The method of preparation of lepromin is an important factor in determining the type of reaction. It has been observed that soluble components induce an early reaction, while killed intact organisms produce predominantly a late reaction ([19]). In this study, purified mycobacterial antigen My1 from *M. leprae* has been used. This elicits only an early skin reaction which is strongly positive in tuberculoid patients, negative in lepromatous patients, and is similar to that seen with Dharmendra lepromin. The infiltrates in this skin reaction are composed of lymphocytes and polymorphonuclear leukocytes, and we report here the immunological characteristics of these infiltrates as defined by monoclonal antibodies.

A high proportion of the cells in these infiltrates were activated T lymphocytes expressing OKT11, Leu3a, OKT8, and Ia-like antigens. Ia-like antigens were not discernible on polymorphonuclear leukocytes. The percentage of Leu3a+ and OKT8+ cells was similar but more than the OKT11+ cells. One of the possible explanations for this could be that Leu3a and OKT8 are better antibodies than OKT11. As described ear-

lier, the intensity of staining with OKT11 antibodies in the tissues was moderate. The ratio of Leu3a+ : OKT8+ cells was 1.2 ± 0.50. Similar observations of the presence of activated T cells expressing OKT11 and Ia-like antigens and a similar ratio of Leu3a+ : OKT8+ cells have been recorded in delayed-hypersensitivity reactions to PPD in humans ([16]), and recently in 24-hour skin reactions induced by Dharmendra lepromin and armadillo-derived leprosin in humans ([13]).

It is interesting that the infiltrating cells showed the presence of the My1 antigen using the MLO6 monoclonal antibody. MLO6 monoclonal antibody has been shown to recognize the mycobacterial antigen My1 of *M. leprae* by radioimmunoassay and immunoblotting techniques ([4]). It could be argued that the staining of infiltrates with MLO6 monoclonal antibody could be due to the nonspecific effect of mouse gamma globulin present in the antibody. This is unlikely, since we have used normal mouse serum (at a concentration similar to that of the monoclonal antibody) as a control, and only occasional positive cells were observed. However, it is difficult to define the precise nature of the positive cell from the present study. This result supports our recent findings using the same monoclonal antibodies where the presence of My1 antigen was noticed in the granulomas of leprosy lesions. However, it was not detectable in the lymph node granulomas of patients with tuberculosis or in the normal skin or in the skin lesions of patients with psoriasis ([14]). In addition, the cells in the leprosy granulomas did not show any staining with monoclonal antibodies to *M. tuberculosis* (upublished observations). No difference was observed in the number of epidermal Langerhans' cells in the My1 reactions in comparison to normal skin. These findings are similar to those observed in the skin lesions of leprosy ([10]) and in lepromin reactions ([13]).

Furthermore, the observations in the present study indicate that the immunological characteristics and the distribution of cells in the infiltrates of the 24-hour reaction induced by My1 appear to be similar to those seen in the 24-hour lepromin reactions ([13]) and other skin reactions ([16]).

In summary, it would appear that activated T lymphocytes are present in the infiltrates of early reactions induced by My1. Also, some of the cells seem to be expressing the My1 antigen. These T lymphocytes may interact with the antigen, thus causing a cell-mediated immune reaction which gives rise to delayed hypersensitivity and, eventually, the features of the skin reaction.

SUMMARY

A study was made on the *in situ* characteristics of dermal infiltrates in a 24-hr skin reaction using monoclonal antibodies defining T-cell subsets, Ia-like antigens, Langerhans' cells, My1, and indirect immunofluorescence. The skin reaction was induced by the mycobacterial antigen My1 derived from *Mycobacterium leprae.* In all, 10 biopsies were studied. The infiltrates were composed of lymphocytes and polymorphonuclear leukocytes. The predominant lymphocytes in the infiltrates were activated T lymphocytes expressing OKT11, Leu3a, OKT8, and Ia-like antigens. The ratio of Leu3a+ : OKT8+ cells was 1.2 ± 0.50. Some of the cells in the infiltrates showed the presence of My1, as seen by the staining with MLO6 monoclonal antibody. No difference was observed in the numbers of OKT6+ epidermal Langerhans' cells in the skin of My1 reaction biopsies and those of normal individuals.

RESUMEN

Se hizo un estudio por inmunofluorescencia sobre las características *in situ* de los infiltrados dérmicos en una reacción en piel de 24 horas inducida con el antígeno My1 del *Mycobacterium leprae.* Se usaron anticuerpos monoclonales contra subpoblaciones de células T, contra antígenos Ia, contra células de Langerhans y contra antígeno My1. Se estudiaron 10 biopsias. Los infiltrados estuvieron compuestos por linfocitos y polimorfonucleares. Los linfocitos predominantes en los infiltrados fueron Lc T activados que expresaron los antígenos OKT11, Leu 3a, OKT8 e Ia. La proporción de células Leu 3a+:OKT8+ fue de 1.2 ± 0.50. Algunas de las células en los infiltrados mostraron la presencia de My1 porque reaccionaron con el anticuerpo monoclonal MLO6. No se observaron diferencias en los números de las células epidérmicas tipo Langerhans OKT6+ entre las biopsias de piel de las reacciones inducidas con My1 y las biopsias de individuos normales.

RÉSUMÉ

Concernant les caractéristiques *in situ* des infiltrats du derme au cours d'une réaction cutanée s'étendant sur 24 heures, cette étude a fait appel aux anticorps monoclonaux définissant les sous-groupes de lymphocytes-T, les antigènes du type Ia, les cellules de Langerhans, My1, et l'immunofluorescence indirecte. La réaction cutanée était par l'antigène anti-bactérien My1 dérivé de *Mycobacterium leprae*. Au total, on a étudié 10 biopsies. Les infiltrats étaient composé de lymphocytes et de leucocytes polymorphonucléaires. Les lymphocytes qui prédominaient dans les infiltrats étaient activés par des lymphocytes-T exprimant OKT11, Leu3a, OKT8, et des antigènes du type Ia. Le ratio des cellules Leu3a+ aux cellules OKT8+ était 1,2 ± 0.50. Certaines cellules des infiltrats révélaient la présence de My1, ainsi que pouvait en témoigner la coloration avec l'anticorps monoclonal MLO6. Aucune différence n'a été observée dans les nombres de cellules épidermiques de Langerhans OKT6+, dans la peau des biopsies prélevées au niveau de réaction My1, par rapport aux valeurs observées chez des individus normaux.

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