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An *in Situ* Immunohistological Study of Mitsuda Reactions[1]

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Three types of skin test responses to lepromin have been described: a late (21–28 day) Mitsuda reaction, a 48-hour or Fernandez reaction, and an immediate or Medina reaction ([12, 13]). The Mitsuda reaction is the classical lepromin test and, although of little diagnostic value, is generally accepted as a useful tool in assessing the immunologic status and the prognosis of patients with leprosy. First described by Mitsuda in 1919, lepromin has been available for general use since 1940. The Mitsuda response is usually considered to be a delayed-type hypersensitivity (DTH) reaction, but this has never been proven and, therefore, must be regarded as an hypothesis.

The Mitsuda response is characterized initially by a diffuse cellular infiltrate which ultimately becomes more focal. At its peak it resembles a tuberculoid granuloma, consisting of some collection of epithelioid cells, lymphyocytes, and a variable number of giant cells ([1, 13]). The presence of epithelioid cells has been detected as early as 5–8 days in patients with borderline tuberculoid (BT) and polar tuberculoid (TT) types of leprosy ([1]).

In the present study, we have sought to further characterize Mitsuda reactions by the use of immunoperoxidase techniques and monoclonal antibodies. Our findings, when compared to tuberculin reactions, tuberculoid (BT) leprosy lesions, and reversal reactions, further support the hypothesis that the Mitsuda reaction is a DTH response.

MATERIALS AND METHODS

Patients were classified according to the criteria of Ridley ([14]). The six patients selected for lepromin testing were those designated as TT/BT or BT, incontrovertible TT being very rare in our patient population.

The three contacts selected for lepromin testing were first degree kindred of, and household contacts of recently diagnosed lepromatous patients.

Lepromin A (armadillo derived), 0.1 ml of 2×10^6 cobalt-irradiated bacilli per ml, was injected intradermally into the upper outer aspect of an arm. Reading was in mm of induration and was performed at 21 or 28 days. Two mm or less in diameter of induration was considered to be a negative reaction. All reactions sampled had 5 mm (diameter) of induration or more.

Tissue preparation. Punch or ellipsoidal skin biopsy specimens were embedded in optimal cutting temperature compound (OCT) (Ames Co., Elkhart, Indiana, U.S.A.), rapidly (snap) frozen in liquid nitrogen, and stored at −70°C until just prior to sectioning. Tissues were allowed to equilibrate to the cryostat temperature of −20°C to facilitate the cutting of thin sections. Six μm sections were cut, air dried (25°C) overnight, and fixed in fresh acetone (reagent grade) for 10 min at room temperature immediately prior to staining.

Monoclonal antibodies. Primary mouse monoclonal antibodies were at concentrations predetermined by checkerboard titrations. Each specimen was stained with a panel of monoclonal antibodies, including anti-interleukin-2 (IL-2) at 1:10 (Dr. Steven Gillis, Immunex Corp.) ([18]) and an antibody to IL-2 receptor, Tac, at 1:5000 (Dr. Thomas A. Waldmann, National Cancer Institute) ([5]). Previous studies in leprosy,

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including double-labeling experiments, have indicated that IL-2-positive cells may be producer cells ([7]). Also used were antibodies against T lymphyocyte subpopulations, including the pan-T cell marker Leu4 (1:60) (Becton, Dickinson & Co., Rutherford, New Jersey, U.S.A.), the helper/inducer T cell marker Leu3a (1:60), the suppressor/cytotoxic T cell marker Leu2a (1:60 or 1:100), the HLA-DR or Ia group specific antigen and the thymocyte marker OKT6 (1:50) (Ortho Pharmaceutical, Raritan, New Jersey, U.S.A.) which also is known to react with Langerhans' cells in the epidermis ([2]) and dermis ([10]).

One control was the use of mouse monoclonal antibody of irrelevant specificity at a concentration equivalent to the monoclonal antibody, in place of the primary antibody. In a second control, the primary antibody was omitted.

Immunoperoxidase staining. After fixation, frozen sections were washed with a modified phosphate-buffered saline (PBS) for 10 min. All stages were carried out at room temperature.

For Leu4, Leu2a, Leu3a, OKT6, and HLA-DR, a two-step indirect immunoperoxidase technique was used: slides were sequentially incubated for 15 min with normal goat serum, primary mouse monoclonal antibody and peroxidase conjugated goat anti-mouse at 1:10 (Tago Inc., Burlingame, California, U.S.A.). For anti-IL-2 and anti-Tac, the above technique was also used but incubation periods lasted 30 min (or the avidin-biotin-complex method) ([4]). Five-to-ten min washes with PBS were performed between all incubations. Amino-ethyl carbazole was the chromogenic substrate. Slides were counterstained with Mayer's hematoxylin for 1 min, washed, and mounted in glycerol jelly.

Cells staining positively for IL-2 were few and were therefore enumerated in the entire section under standard light microscopy. The section area was measured and the percent occupied by inflammatory infiltrate was estimated with a calibrated reticule. Dividing the number of positive cells by the area of granuloma was performed to normalize to square millimeter of infiltrate. Tac positive cells were also few and counted in three random fields per section on a square millimeter grid and the average obtained. The percent of cells in the infiltrate staining with these antibodies was obtained by dividing the number of positive cells per square millimeter of the granuloma and multiplying by 100 ([7]). For all tissues studied, the total number of cells per square millimeter of infiltrate was 5200.

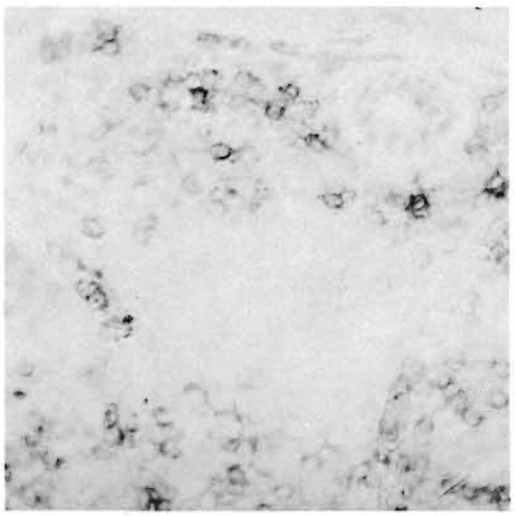

FIG. 1. Leu2a. A granulomatous response near an eccrine gland in a 21-day Mitsuda reaction. Cells bearing the Leu2a phenotype appear to be excluded from the epithelioid cell aggregate (×250).

Leu4, Leu3a, and Leu2a positive cells were numerous, i.e., 5% or greater. The percent of positive cells was directly estimated by two independent observers.

In normal epidermis, Langerhans' cells account for 3–5% of all epidermal cells, and evidently "connecting" or "touching" of dentriated adjacent cells is unusual, being seen in only 2 of 20. Epidermal Langerhans' cells were judged to be increased if frequent connections of dendritic extensions from neighboring cells were observed, i.e., in 5 in 20 or more.

RESULTS

The numerical results expressed as mean percent of cells staining positively for each antibody are summarized in The Table. Leu3a : Leu2a (helper/suppressor) ratios are also given.

The histologic appearance of all of the lepromin responses was granulomatous in

THE TABLE. *Percent of cells of dermal infiltrate staining positively for each specificity sought.*

Antibody	No. tested	Mean % positive	S.D.	Range
Leu4	9	61	11	45–75
Leu2a	9	19	4	15–25
Leu3a	9	47	12	30–65
(Leu3a/Leu2a)	9	2.4	0.6	1.7–3.8
IL-2	7	0.18	0.11	0.09–0.21
Tac	8	1.6	0.4	0.96–2.2

nature, i.e., some epithelioid cell aggregates in some areas of each specimen. Lymphocytes bearing the pan-T phenotype (Leu4) were found throughout the infiltrate, and comprised from 45–75% of the infiltrating cells. From 15–22% (mean 19%) of the lymphocytes expressed the suppressor/cytotoxic (Leu2a) phenotype; generally these cells were excluded from epithelioid cell aggregates but were present in the lymphocytic mantle surrounding the epithelioid tubercle (Fig. 1). Staining of serial tissue sections showed that cells with the helper/inducer (Leu3a) phenotype were from 30–60% (mean 47%) of the infiltrate; these cells were distributed throughout the infiltrate including the epithelioid cell aggregate. The helper/suppressor ratio ranged from 1.7 to 3.8.

Anti-IL-2 positive cells comprised a mean of 0.2% of the infiltrate. These cells were large and had cytoplasmic staining, indicating that such cells may be IL-2 producers, as previously hypothesized ([7]) (Fig. 2). Anti-Tac positive cells ranged from 1.0–2.2% (mean 1.6%) of the infiltrate; these were present throughout the reaction within both the epithelioid aggregates and the lymphocytic mantle.

Staining with the HLA-DR antibody was virtually universal in the dermal infiltrate. In the epidermis and hair follicles of all specimens, more than 90% of the nucleated keratinocytes bore the HLA-DR antigen, giving a pemphigus-like pattern (Fig. 3). In addition, dendritic cells staining with the OKT6 antibody showed a definite degree of hyperplasia comprising greater than 5% of nucleated cells, indicating an increase in the Langerhans' cell population (Fig. 4); also, 10 of 20 Langerhans' cells showed dendrites in continuity. OKT6 positive cells were also seen within the dermis in areas of granulomatous or lymphohistiocytic infiltration. A mild, focal, lymphocytic infiltrate into the epidermis was usually found (Fig. 5), consisting of pan-T, helper/inducer, and suppressor/cytotoxic phenotypes.

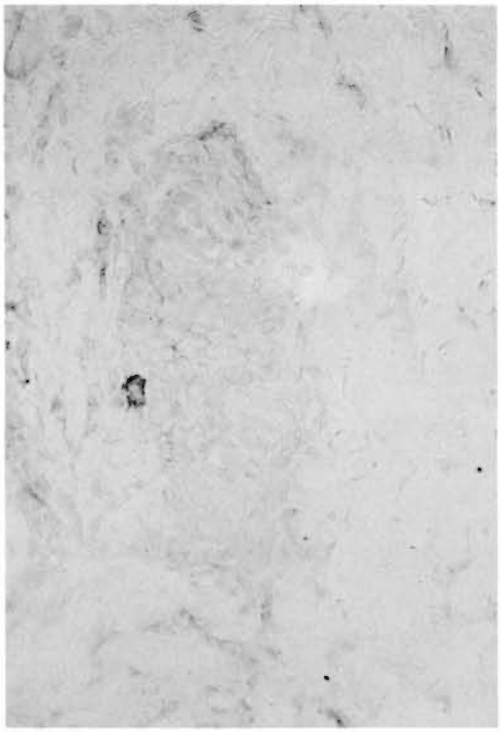

FIG. 2. IL-2. A small perivascular infiltrate in a 21-day Mitsuda reaction. A single anti-IL-2 positive cell is seen at the periphery of the infiltrate (×250).

DISCUSSION

Our *in situ* immunohistological findings in Mitsuda reactions are in part consistent with a DTH immunopathogenesis.

Comparing the dermal infiltrates of the Mitsuda reaction to those of tuberculin reactions, the benchmark DTH response, demonstrates many similarities ([3, 6, 11, 16]). For example, in nine 48-hour tuberculin reactions studied ([3]), the mean value for the pan-T phenotype was 47% of the infiltrate, not significantly different from the 61% mean value in the Mitsuda reactions. Also, the mean values for the Leu3a, Leu2a and the helper/suppressor ratio in Mitsuda reactions (47, 19, and 2.5, respectively) was

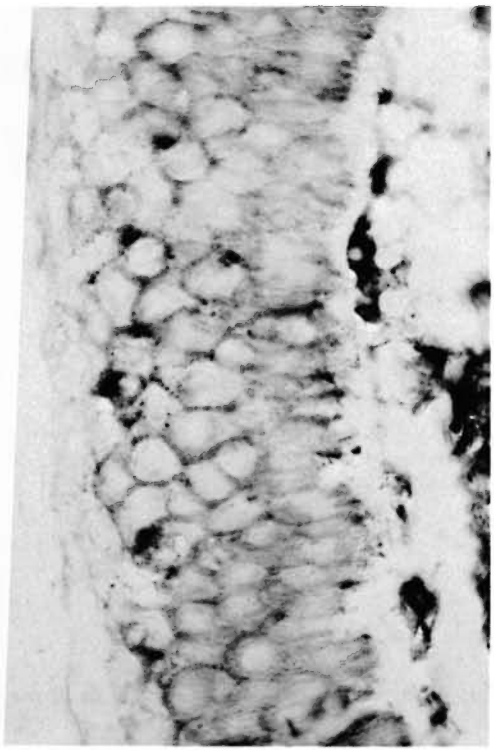

FIG. 3. HLA-DR. The epidermis in a 28-day Mitsuda reaction. Most nucleated epidermal cells appear to express the HLA-DR antigen, giving a pemphigus-like pattern. Cells in the dermis also express the antigen (×400).

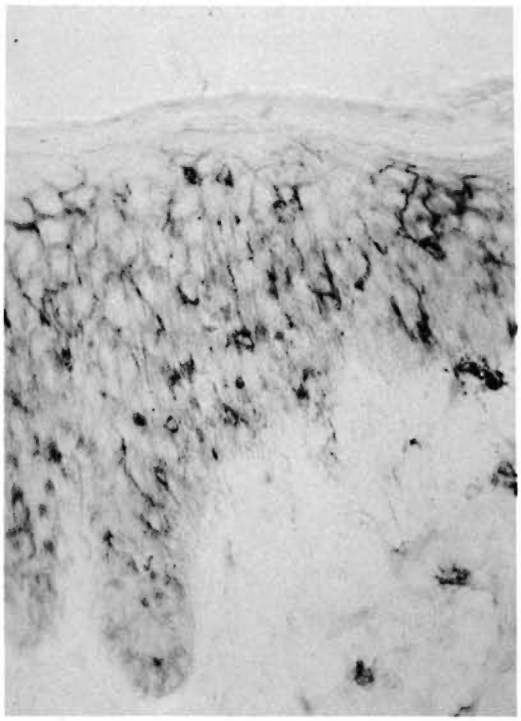

FIG. 4. OKT6. The epidermis in a 21-day Mitsuda reaction. Cell bodies and dendritic processes bear the OKT6 phenotype. A few positive cells are present in the dermis (×250).

not dissimilar to those found in tuberculin reactions (52, 17, and 3.3). In addition, OKT6 positive cells in the dermis were found in both groups, and Ia positivity was virtually universal in each reaction. Also, the mean percentage of anti-Tac positive cells in lepromin reactions, 1.5, did not differ significantly from those in our tuberculin reactions, 1.0.

The percentage of anti-IL-2 positive cells in Mitsuda reactions, 0.18 ± 0.11, was less than that found in tuberculin reactions, 0.6 ± 0.3; this difference was statistically significant, $p < 0.01$. Because the prevalence of anti-IL-2 positive cells in tuberculoid skin lesions, 0.48, was intermediary between the lepromin and tuberculin values, we think it likely that the numerical differences do not indicate a difference in the underlying mechanism but rather differences in the kinetics of 28-day and 2-day reactions.

Comparisons of epidermal changes also show many similarities. Both have Langerhans' cell hyperplasia and lymphocytes infiltrating the epidermis ([3]). The keratinocytes in lepromin reactions are Ia positive; in tuberculin reactions Ia positivity is also observed, not at 24 or 48 hours but at 96 hours ([3, 17]). Another immunohistological difference between Mitsuda and tuberculin reactions appears to be a consequence of granuloma formation in the former, i.e., sequestration of the Leu2a phenotype to the lymphocytic mantle about the epithelioid tubercle. These differences, therefore, are not indications that a Mitsuda reaction is not a DTH response.

Comparison of the Mitsuda reaction to tuberculoid (BT) or reversal reaction lesions, two circumstances where DTH is thought to be important in lesion pathogenesis, also shows many similarities ([6, 8]). In the dermis, all three have similar percentages of cells bearing the Leu4, Leu3a, Leu2, OKT6, and Ia phenotypes. In the epi-

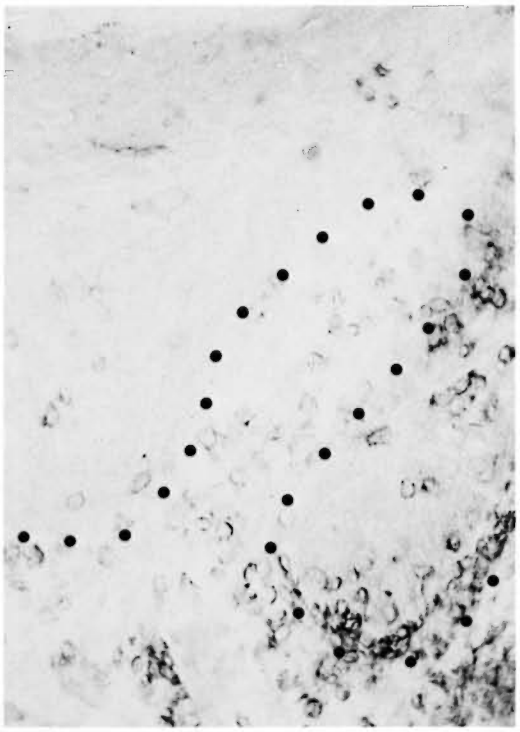

FIG. 5. Leu3a. A 21-day Mitsuda reaction. The dermal epidermal junction is indicated by dots. Cells bearing the Leu4 phenotype are present in both the dermis and epidermis but are more numerous in the dermis (×250).

dermis, all three display lymphocytic infiltration, Langerhans' cells hyperplasia and Ia antigen upon keratinocytes.

The argument that the Mitsuda reaction represents a DTH reaction rests upon the communality of findings of Mitsuda reactions with tuberculin reactions and the lesions of BT or reversal reactions. It is further strengthened by comparison with lepromatous lesions, where DTH is absent (or inhibited or greatly reduced). In lepromatous lesions anti-IL-2 positive cells are scant ([7]), epidermal changes are nonexistent, dermal OKT6 positive cells are rare and the suppressor/cytotoxic phenotype predominates ([8]). Thus, four kinds of changes are candidates for being immunohistological correlates of DTH reactions. The reported experience with primary irritant and allergic contact dermatitis and with disseminated and localized cutaneous leishmaniasis indicates that the helper/suppressor ratio is not a correlate of DTH ([9, 15]). Anti-Tac positivity was similarly prevalent in all tissues studied and, therefore, although necessary for a DTH reaction to proceed, is not an *in situ* marker for a DTH response. IL-2 positivity and the epidermal triad of lymphocytic infiltration, Langerhans' cells hyperplasia, and Ia positive keratinocytes would appear to be consistent correlates of a DTH response.

SUMMARY

In an attempt to further define their immunopathogenesis, the cellular infiltrates of Mitsuda reactions were studied *in situ* using immunoperoxidase techniques and monoclonal antibodies. Lepromin A-elicited Mitsuda reactions from six patients with borderline tuberculoid leprosy (TT/BT or BT) and three healthy kindred and contacts of lepromatous patients were examined. In the dermis, cells bearing the Leu4 phenotype comprised a mean of 61% of the infiltrate; the Leu3a, 47%; the Leu2a, 17%; anti-IL-2, 0.2%; anti-Tac, 1.5%; and cells bearing the Ia phenotype were virtually universal; OKT6 positive cells were present. The Leu2 phenotype was sequestered to the periphery of epithelioid tubercules. In the epidermis, there were mild, focal lymphocytic infiltrates, hyperplasia of epidermal Langerhans' cells, and well-developed expression of Ia upon nucleated keratinocytes. These findings, when compared with those of a better-defined reaction, tuberculin, are further evidence that the Mitsuda response may be a delayed-type hypersensitivity phenomenon.

RESUMEN

Usando técnicas de inmunoperoxidasa y anticuerpos monoclonales, se estudiaron los infiltrados celulares de las reacciones de Mitsuda con el fin de entender mejor su inmunopatogénesis.

Se examinaron las reacciones de Mitsuda inducidas con lepromina-A en 6 pacientes con lepra tuberculoide-intermedia (TT/BT o BT) y en 3 contactos sanos de pacientes lepromatosos.

En la dermis, las células Leu4 constituyeron el 61% del infiltrado, las células Leu3a, el 47%; las células Leu2a, el 17%; las células anti IL-2, el 0.2%, y las anti-Tac, el 1.5%. Las células Ia fueron virtualmente universales y también hubieron células OKT6+.

Las células Leu2 estuvieron localizadas en la periféria de los tubérculos epitelioides.

En la epidermis se encontraron infiltrados linfocíticos focales de tamaño moderado, hiperplasia de las

células de Langerhans, y una buena expresión de Ia en los queratinocitos nucleados. Estos resultados, comparados con los encontrados en la reacción a la tuberculina, constituyen una evidencia adicional de que la reacción de Mitsuda representa un fenómeno de hipersensibilidad de tipo tardío.

RÉSUMÉ

Afin de tenter de mieux cerner leur immunopathogénèse, les infiltrats cellulaires caractéristiques de la réaction de Mitsuda ont été étudiés *in situ* au moyen de techniques à base d'immunoperoxydase et d'anticorps monoclonaux. On a examiné des réactions de Mitsuda provoquées par la Lepromine-A, chez 6 malades atteints de lèpre tuberculoïde dimorphe (TT/BT ou BT) et chez 3 individus sains, membres de la famille et contact de malades lépromateux. Au niveau du derme, les cellules porteuses du phénotype Leu4 constituaient en moyenne 61% de l'infiltrat; les cellules porteuses du phénotype Leu3a en représentaient 47%. En ce qui concerne les autres phénotypes, la répartition était la suivante: Leu3a, 47%; Leu2a, 17%; anti-IL2, 0,2%; anti-Tac, 1, 5%. Quant aux cellules porteuses du phénotype Ia, elles étaient pratiquement universelles. On notait également la présence de cellules positives pour OKT6. Le phénotype Leu2 était strictement limité à la périphérie des granulomes épithélioïdes. Dans l'épiderme, on notait des infiltrats lymphocytaires peu prononcés et en foyer, de même qu'une hyperplasie des cellules épidermiques de Langerhans, et une expression bien marquée du phénotype Ia sur les karétinocytes nuclées. Lorsqu'on compare ces observations avec celles relevées au niveau d'une réaction bien définie, celle de la tuberculine, on en déduit que la réponse de Mitsuda peut être un phénomène d'hypersensibilité de type retardé.

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