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Influence of delayed immune reactions on human epidermal keratinocytes

(delayed-type hypersensitivity/tuberculin reaction/leprosy/leishmaniasis/Ia antigen)

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ABSTRACT **The epidermal changes that occur in human cutaneous immune responses have been investigated in the tuberculin reaction and in the lesions of tuberculoid and lepromatous leprosy and cutaneous leishmaniasis. In each situation, there was a dermal accumulation of monocytes and T cells, and the epidermis exhibited thickening. In the tuberculin response, the thickness of the epidermis sometimes doubled in 48–72 hr, and this was attributed to increases in both size and number of keratinocytes. In addition, the phenotype of the keratinocytes changed from Ia$^-$ to Ia$^+$. Similar changes in keratinocyte Ia-antigen expression occurred in the epidermis overlying untreated tuberculoid leprosy and cutaneous leishmaniasis lesions, but not in lepromatous leprosy. We suggest that one or more epidermal growth factors may be generated in the course of a delayed immune reaction in the dermis.**

The cutaneous lesions of lepromatous leprosy are characterized by a sparse lymphocytic infiltrate and foamy macrophages laden with *Mycobacteria leprae*. In contrast, tuberculoid leprosy lesions exhibit large numbers of lymphocytes, granuloma formation, and the absence of appreciable numbers of intracellular bacteria (1, 2). Monoclonal antibodies to human leukocytes and their subsets have been used to determine the phenotype of the cells in dermal infiltrates (3–6). These studies showed that in lepromatous lesions there is not only a marked reduction in the numbers of T cells (compared to tuberculoid lesions) but also a selective absence of T lymphocytes of the OKT4$^+$ subset.

We wanted to establish whether this represented a specific unresponsiveness to *M. leprae* antigens or a more general defect in the emigration and accumulation of OKT4$^+$ T cells. For this purpose we generated tuberculin reactions in the dermis of lepromatous patients by use of the purified protein derivative of tuberculin (PPD). During the course of these studies, we observed striking changes in the thickness and Ia-antigen expression of the epidermis overlying the PPD-induced delayed-type hypersensitivity (DTH) lesions. Similar changes also occur in the epidermis overlying the lesions of patients with tuberculoid leprosy and cutaneous leishmaniasis.

MATERIALS AND METHODS

The Generation of DTH Response to PPD. After informed consent was obtained, we evaluated the DTH response to 5 units of PPD in 90 Indian leprosy patients from New Delhi (a highly endemic area for tuberculosis). The study group included 25 lepromatous (LL) (see diagnosis below), 10 borderline lepromatous (BL), and 55 tuberculoid (BT and TT) patients and 50 non-leprosy control individuals. Sex and age distribution were as follows: BL and LL patients, 3 female and 32 male, ages 16–68 (median 30) years; BT and TT patients, 12 female and 43 male, ages 11–68 (median 30) years; non-leprosy controls, 15 female and 35 male, ages 15–65 (median 28) years. The leprosy patients were examined in collaboration with A. K. Sharma and R. S. Mishra (Department of Dermatology, Safdarjung Hospital, New Delhi). Antigen was injected intradermally into the leprosy lesions. Two 4-mm punch-biopsy samples, one from the PPD-injected leprosy lesion and one from an uninjected adjacent leprosy lesion, were taken at 68–92 hr from consenting LL and BL patients. The specimens were fixed as described below and transported to the United States for further processing.

Additional Patient Populations. *Leprosy.* Skin biopsy samples (taken after informed consent was obtained) from 16 patients from Brazil, 6 patients from Colombia, and 4 patients from the United States with various forms of leprosy were examined. All patients were untreated at the time of biopsy. The Brazilian patients were examined in collaboration with the Department of Dermatology and General Pathology, Hospital de Clinicas, Universidade do Rio de Janeiro. The Colombian patients were studied in collaboration with the Federico Lleras Acosta Hospital of Dermatology, Bogota. The U.S. patients were studied in collaboration with W. R. Levis (The Rockefeller University Hospital). Clinical diagnosis was accompanied by a histopathological diagnosis established by us (I.N. and E.N.S.) and C. K. Job (Public Health Service National Hansen's Disease Center, Carville, LA) according to the Ridley–Jopling classification (7).

Cutaneous leishmaniasis. Patients with cutaneous leishmaniasis were studied in collaboration with W. M. Rojas (Corporacion para Investigiones Biologicas) and with M. I. Restrepo and F. M. Restrepo (The Regional Health Laboratory, Medellin, Colombia). After informed consent was obtained, 4-mm punch-biopsy specimens were taken from the periphery of the lesions of 5 patients.

Fixation and Processing of Cutaneous Biopsy Samples. Skin samples were fixed for 4 hr at 4°C in phosphate-buffered saline (PBS) containing 3% (wt/vol) paraformaldehyde, 75 mM lysine, and 10 mM sodium metaperiodate, as described by McLean and Nakane (8). This fixative preserves structural details without inhibiting the binding of monoclonal antibodies to their respective antigens (9). The biopsy samples were stabilized for freezing by overnight washing in PBS containing 10% (wt/vol) sucrose and 40 μM digitonin, followed by successive suspension in graded solutions of sucrose

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Abbreviations: DTH, delayed-type hypersensitivity; PPD, purified protein derivative of tuberculin.

(15–25%). The samples were stored in PBS with 25% (wt/vol) sucrose and 5% (vol/vol) glycerol until frozen.

Immunocytochemical Staining of Sections. The biopsy samples were embedded in OCT compound (Miles Scientific, Naperville, IL) and frozen at −20°C. Sections (6–8 μm) were cut on a cryostat and applied to gelatin-coated multiwell slides (Carlson Scientific, Peotore, IL). The sections were dried overnight at 37°C, rehydrated in PBS, and incubated with mouse monoclonal antibodies followed by biotinylated horse anti-mouse Ig and then avidin–biotin-peroxidase complexes (Vector Laboratories, Burlington, CA). The reaction product was developed with diaminobenzidine (0.4 mg/ml) in 0.02 M Tris Cl buffer (pH 7.6) containing 0.03% H_2O_2. Sections were counterstained with hematoxylin.

Monoclonal Antibodies. Mouse anti-human monoclonal antibodies were used for the identification of specific cell types. OKT6 (anti-thymocyte and Langerhans cells) was obtained from Ortho Diagnostics (10); 9.3F10 (anti-HLA class II) was produced in this laboratory (11); B8.11 (anti-HLA-DR) and PSV-L3 (anti-HLA-DQ) were obtained from R. deVries (Leiden, The Netherlands) (12, 13); VIC-Y1 (anti-human Ia invariant γ chain) was obtained from W. Knapp (Institute of Immunology, University of Vienna, Austria) (14).

Evaluation of Staining and Morphological Changes. Adjacent sections were evaluated for specific cell staining with a Zeiss light microscope. The thickness of the epidermis was measured following projection of the section image onto a television screen. At least 40 thickness determinations were made at successive 100-μm intervals on each section, and the mean and standard deviation of the mean were calculated. This procedure resulted in the selection of sites that were random with respect to the epidermal retia. In addition, the number of keratinocyte cell layers was determined by counting the number of cells between the basal and keratinized layers in at least 30 random evenly spaced sites of the section.

Table 1. PPD responsiveness in leprosy patients and in non-leprosy control subjects

Diagnosis	No. tested	No. of responders (percent)
LL	25	14 (56)
BL	10	8 (80)
BT/TT	55	44 (80)
Non-leprosy	50	34 (68)

DTH reaction to a single injection of 5 units of PPD administered intradermally to lepromatous leprosy (LL), borderline lepromatous leprosy (BL), and borderline and tuberculoid leprosy (BT/TT) patients and to non-leprosy control individuals was evaluated.

RESULTS

PPD Responsiveness in Leprosy Patients. Leprosy patients and non-leprosy control individuals were tested for their responsiveness to an intradermal injection of 5 units of PPD in 0.1 ml of diluent. Induration was measured 68–92 hr after injection and considered positive if >10 mm. The respon-

Table 2. PPD responses and the changes in epidermal keratinocytes

Patient	Diagnosis	Induration, mm	Epidermal thickness			Keratinocyte Ia	
			Uninj., μm	PPD, μm	Ratio	Uninj.	PPD
1	LL	30 × 38	42 ± 9	86 ± 13	2.0	−	+
2	LL	32 × 38	70 ± 13	76 ± 20	1.1	−	+
3	LL	33 × 35	30 ± 8	80 ± 31	2.7	−	+
4	BL	25 × 25	38 ± 10	49 ± 13	1.3	−	+
5	LL	22 × 22	48 ± 16	94 ± 31	2.0	±	+
6	LL	22 × 22	71 ± 27	107 ± 43	1.5	NT	+
7	LL	20 × 22	29 ± 8	70 ± 25	2.4	−	+
8	LL	20 × 20	48 ± 17	78 ± 23	1.6	−	+
9	LL	20 × 20	39 ± 7	68 ± 19	1.7	−	+
10	LL	19 × 21	52 ± 15	99 ± 26	1.9	−	+
11	LL	20 × 20	56 ± 25	86 ± 40	1.5	−	+
12	LL	18 × 20	59 ± 15	94 ± 16	1.6	−	+
13	BL	16 × 17	53 ± 18	101 ± 30	1.9	−	+
14	BL	16 × 16	64 ± 20	105 ± 31	1.6	−	+
15	LL	16 × 16	49 ± 15	118 ± 39	2.4	±	+
16	BL/LL	15 × 17	45 ± 17	56 ± 19	1.2	−	±
17	BL/LL	15 × 15	37 ± 16	85 ± 24	2.3	−	+
18	LL	12 × 12	44 ± 21	87 ± 26	2.0	−	+
19	LL (ENL)*	12 × 12	41 ± 11	70 ± 23	1.7	−	±
20	BL	11 × 12	42 ± 20	65 ± 27	1.5	−	+
21	LL	8 × 10	45 ± 21	82 ± 35	1.8	−	+
22	LL	8 × 12	47 ± 15	52 ± 18	1.1	−	+
23	LL	None	39 ± 17	56 ± 17	1.4	−	+
24	BL	2 × 2	NT	NT	NT	NT	−
25	LL	None	50 ± 20	63 ± 24	1.3	−	−
26	LL	None	41 ± 13	37 ± 10	0.9	+	±
27	LL	None	54 ± 16	44 ± 24	0.8	±	±
28	BL	None	69 ± 23	57 ± 18	0.8	−	−

Lepromatous (LL) and borderline lepromatous (BL) leprosy patients were tested for their response to a single injection of 5 units of PPD. The induration was measured in mm. Epidermal thickness in uninjected leprosy lesions (Uninj.) and PPD-injected lesions (PPD) was measured as described in *Materials and Methods*; results are expressed as mean thickness ± SD (in μm). Ratio = thickness of the PPD lesion divided by the thickness of the uninjected lesion. Keratinocyte Ia was evaluated and expressed as follows: +, all keratinocytes stained; ±, foci of stained keratinocytes; −, no keratinocyte stained. NT, not tested.

*ENL, erythema nodosum leprosum.

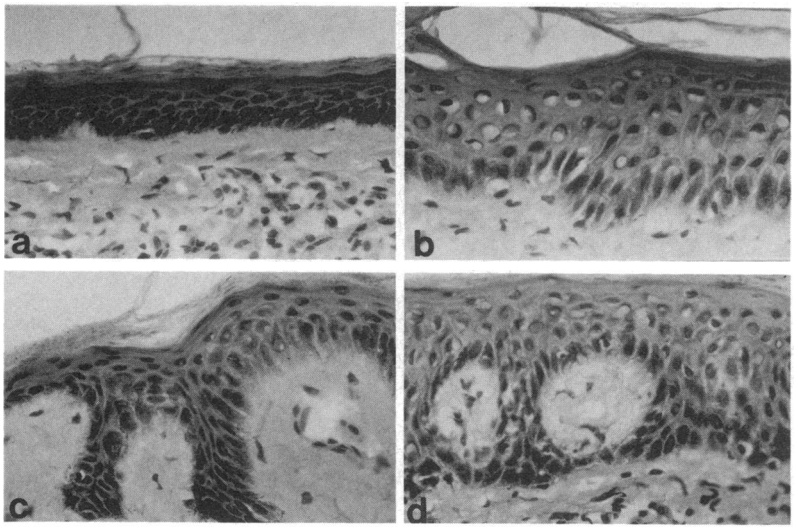

FIG. 1. Reaction of epidermal cells to a positive PPD reaction. Photomicrographs are shown of sections through the epidermis of uninjected leprosy lesions (*a* and *c*) and PPD-responsive injected lesions (*b* and *d*) of patients 3 (*a* and *b*) and 10 (*c* and *d*). In *b* and *d*, thickening of the epidermis, accompanied by enhanced numbers and enlargement of the keratinocytes, is observed. (Hematoxylin/eosin stain; ×250.)

siveness of this patient population is shown in Tables 1 and 2. The frequency of responders among patients with LL was not significantly different from that among non-leprosy control subjects (56 and 68%, respectively). A higher percentage (80%) of BT/TT patients were PPD-reactive. Responders from all three patient groups (LL, BL, BT/TT) and from the non-leprosy control group showed no differences in the extent of induration.

All patients responding to PPD had dermal infiltrates containing large numbers of monocytes, $OKT4^+$ and $OKT8^+$ T cells (ratio about 2:1), and other cellular components, similar histologically to the DTH response to PPD reported in normal tuberculin responders (15–18). The composition of these dermal infiltrates will be reported in more detail elsewhere.

PPD Responsiveness and the Reaction of Epidermal Keratinocytes. The delayed dermal response of the tuberculin reaction was accompanied by marked changes in the epidermis. The epidermis overlying the indurated PPD site thickened relative to the epidermis overlying the uninjected site (Fig. 1). Epidermal thickening was sometimes >2-fold in responsive patients (Table 2). A limited correlation between the extent of induration and epidermal thickening was observed (correlation coefficient 0.526). The biopsy samples taken from patients with negative PPD reactions (<10 mm) failed to show epidermal thickening relative to uninjected control sites. The change in epidermal thickening was associated with increases in both the size and the number of keratinocytes (Figs. 1 and 2). Quantitation indicated that the epidermis of uninjected lesions contained an average of

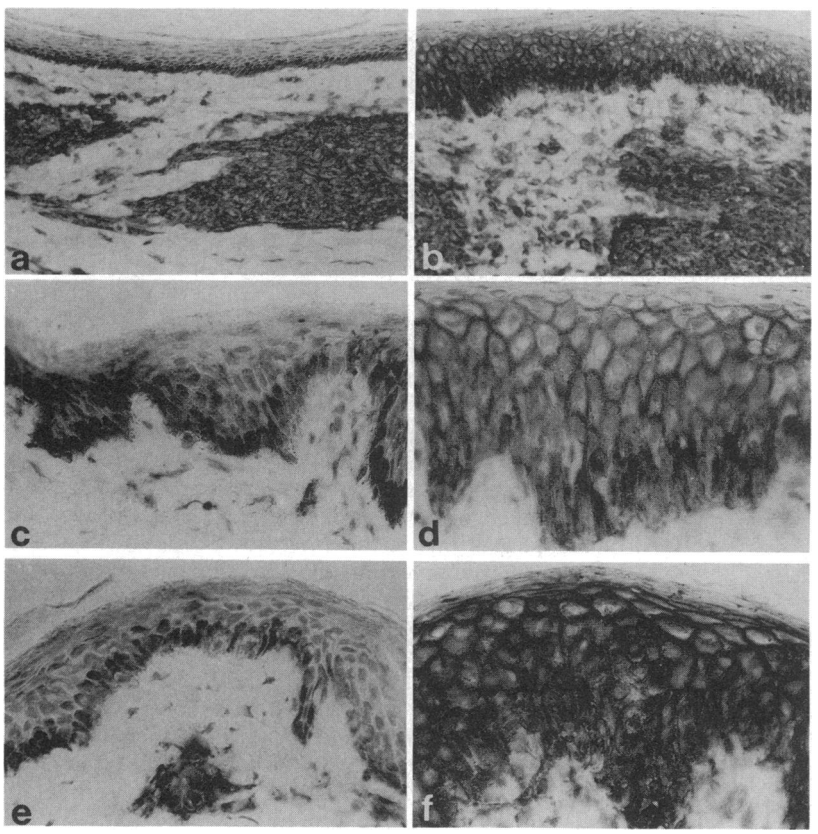

FIG. 2. Expression of keratinocyte Ia in response to intradermal tuberculin reactions. Photomicrographs of anti-Ia (9.3F10) monoclonal antibody staining of the epidermal cells of uninjected leprosy lesions (*a*, *c*, and *e*) and tuberculin-responsive injected lesions (*b*, *d*, and *f*) of patients 3 (*a* and *b*), 7 (*c* and *d*), and 28 (*e* and *f*). Keratinocyte Ia staining is observed only in the lesion from the PPD response sites. The dark areas in the basal layer of the uninjected sites are due to melanin. (*a* and *b*, ×100; *c*–*f*, ×250.)

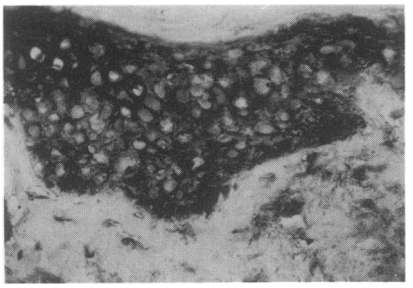

FIG. 3. Expression of keratinocyte Ia in an untreated tuberculoid leprosy lesion, shown by anti-Ia staining of the epidermal cells. Epidermal thickening and Ia staining of the keratinocytes are observed. (×250.)

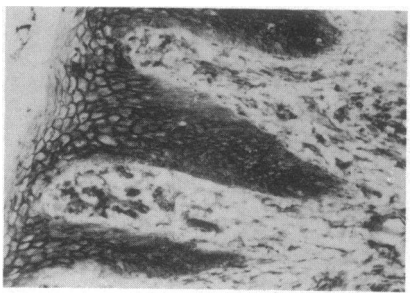

FIG. 4. Expression of keratinocyte Ia in a cutaneous leishmaniasis lesion, shown by anti-Ia staining of the epidermal cells. Extensive epidermal thickening and Ia staining of keratinocytes are observed. (×100.)

3.5–5.9 (mean 4.6 ± 0.7) epidermal cell layers, whereas that of PPD reactions was 5.1–8.5 (mean 7.1 ± 0.9) layers. Keratinocytes in the epidermis of uninjected lesions were flatter than those observed in PPD-positive lesions.

In addition to the thickening of the epidermis, the Ia expression of the keratinocytes changed as a result of the tuberculin reaction in the dermis. Most if not all the keratinocytes of 20/22 patients became Ia^+ after the induction of the DTH reaction (Fig. 2). The keratinocytes were HLA-DR^+, HLA-DQ^- and showed a weak cytoplasmic staining for the invariant γ chain (Inv). As a control, we noted that both dermal macrophages and epidermal Langerhans cells were DR^+, DQ^+, Inv^+. In contrast, the keratinocytes of 5/6 non- or low responders remained Ia^-. The keratinocyte changes were unrelated to the number and size of $OKT6^+$, Ia^+ Langerhans cells.

Expression of Keratinocyte Ia Antigen in Lesions of Untreated Leprosy Patients. The epidermal modifications associated with the accumulation of T cells and monocytes in the tuberculin reaction suggested that similar epidermal changes might occur in the lesions of tuberculoid leprosy. Lesions of untreated polar and borderline forms of the disease were biopsied. A representative example of a tuberculoid lesion is shown in Fig. 3. All of the tuberculoid patients and 5/6 borderline tuberculoid patients exhibited Ia^+ keratinocytes (Table 3), whereas none of the biopsies from polar lepromatous patients showed reactive cells. Again, epidermal Ia expression could not be attributed to staining of $OKT6^+$, Ia^+ Langerhans cells. Thickening of the epidermis overlaying the lesions was observed in many of the tuberculoid leprosy lesions but could not be evaluated more accurately because no control biopsy samples of normal skin were available for these patients.

Cutaneous Lesions of Leishmaniasis. A large accumulation of mononuclear cells occurs at the edge of the lesions of cutaneous leishmaniasis. Examination of biopsy specimens from five Colombian patients revealed extensive epidermal thickening over the area of dermal infiltrate (Fig. 4). Each of the five patients showed strongly positive Ia staining outlining the enlarged spherical keratinocytes.

Table 3. Keratinocyte Ia in lesions of leprosy patients

Diagnosis*	No. of patients	Incidence of keratinocyte Ia^+		
		+	±	−
LL	9	0	0	9
BL	6	1	0	5
BT	6	3	2	1
TT	4	2	2	0

All patients tested were untreated at the time of biopsy.
*See legend to Table 1 for abbreviations.
†Scored as follows: +, all keratinocytes stained; ±, foci of stained cells; −, no keratinocytes stained.

DISCUSSION

The common denominator in each of the epidermal responses reported here is the accumulation of T lymphocytes and monocytes in the dermis. These infiltrates, induced by the local administration of antigen into sensitized hosts, represent the classic DTH response (15–18). In the case of the intradermal tuberculin reaction, these cells were present for no longer than 3 days. In tuberculoid leprosy and cutaneous leishmaniasis, the lesions had existed for many months, although the longevity of individual T cells and mononuclear phagocytes in the infiltrates is unknown. In each case, however, epidermal thickening and the expression of keratinocyte Ia antigen on the cell surface had taken place. Epidermal thickening is not a well-described feature of DTH, although it was noted by Turk (15) in the guinea pig tuberculin reaction. Gut epithelial hyperplasia has been described in the mucosal alterations during graft-vs.-host disease (19). Enhanced expression of epidermal and epithelial Ia has also been described in other cell-mediated immune responses (20, 21). Increased Ia expression could not be accounted for by an increase in the numbers or size of Ia^+ Langerhans cells. Using the OKT6 monoclonal antibody to identify Langerhans cells, we found that the numbers of $OKT6^+$ cells in the epidermis was often reduced during a DTH response. These results will be reported in more detail elsewhere.

In a clinical trial we are now conducting at the Hospital of The Rockefeller University, recombinant interferon γ (Genentech, South San Francisco) has been administered intradermally (Medajet gun) into the lesions of patients with lepromatous leprosy. Our findings, which will be published elsewhere, include a rapid (6-day) increase in epidermal thickness and the marked expression of Ia antigen on the surface of the keratinocytes. Therefore, interferon γ may, either directly or through secondary reactants, be a significant stimulator for the keratinocyte changes in DTH. The situation may be more complex if keratinocyte growth and Ia expression are generated by separate stimuli. Ia expression can be induced in a number of cell types by interferon γ, and in macrophages this is unrelated to cell division (22–25). Assuming interferon γ is involved in inducing the epidermal changes, our observations suggest that the infiltrating T cells of the dermal lesions of tuberculoid leprosy release interferon γ locally, leading to keratinocyte Ia expression and epidermal thickening, whereas the infiltrating cells of the lepromatous lesions do not release interferon γ. This model is consistent with our *in vitro* observations that peripheral blood lymphocytes from patients with tuberculoid leprosy release interferon γ in response to *M. leprae*, whereas cells from lepromatous leprosy patients release little or none (33).

The thickening of the epidermis, when coupled with changes in keratinocyte shape, larger numbers of cell layers, and greater numbers of mitotic figures, suggests that keratinocytes are undergoing more rapid growth, although modified keratinocyte differentiation must also be considered. We favor the idea that one or more epidermal growth stimulants are generated by the dermal cell populations or by the epidermis in response to cell-mediated immunity. Two agents that induce keratinocyte replication have been noted. (*i*) Stimulation of adenylate cyclase activity by cholera toxin promotes replication (reviewed in ref. 26). (*ii*) Epidermal growth factor(s) from a variety of cell types may be responsible for promoting replication (27). Establishing whether these mechanisms are operative in delayed-type reactions will require the use of keratinocyte cultures. The complex milieu of the DTH reaction contains many cell types and secreted cellular products that may be the source of the epidermal stimulant. These include T cells and their secreted lymphokines (28, 29), macrophages and their extensive secretory repertoire (30), fibroblasts (31), and even keratinocytes (32) themselves.

Additional questions remain concerning the rate of appearance and persistence of the epidermal changes, the role of other inflammatory cells, and the responsiveness of the skin of the normal control subjects. The generation of a number of soluble factors during an immune response, including interleukin 2, interferon γ, and epidermal growth factor(s), may promote the healing and closure of wounds.

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