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Microscopic Findings of Delayed Reactions Elicited by the Skin Test Reagent Leprosin A Derived from *M. leprae*[1]

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Cell-mediated hypersensitivity has generally been regarded as a homogenous group of reactions characterized by perivascular infiltrates on mononuclear cells ([4, 19, 20]). However, recently it has been established that at least two histologically discrete forms of cellular immune response exist in experimental animals ([7]). These are classical delayed hypersensitivity and cutaneous basophil hypersensitivity characterized by a basophil-rich infiltrate. Subsequently, basophils were observed in Jones-Mote reactions as well as in tuberculin reactions ([1]).

Additionally, and in no sense correlating with these histological differences, two distinct forms of delayed response to soluble reagents have been observed in mice challenged with *Mycobacterium kansasii* or *M. nonchromogenicum* ([14]), and two qualitatively different types of positive responses have been observed in man ([18]).

In view of these observations, we wished to investigate the morphologic description of delayed-type hypersensitivity in patients with leprosy and in controls with the new skin test reagent prepared from armadillo-derived *M. leprae* designated Leprosin A. We present here comprehensive findings of the delayed reactions, employing 1–2 μm Epon-embedded sections studied by light microscopy.

MATERIALS AND METHODS

Test subjects. Forty-seven biopsies from 9 leprosy patients and 3 healthy persons were studied. The leprosy patients were drawn from the Central JALMA Institute for Leprosy, Agra, India, and from Hemerijckx Leprosy Centre, Polambakam, South India. They were classified clinically and histologically according to the Ridley-Jopling scale ([12]). The details of the experiment were explained and their consent to take part was obtained.

Skin test antigen. Leprosy bacilli were isolated from the tissues of infected armadillos ([6]) and the bacilli were broken open by sonic disruption, centrifuged, and the soluble supernatant was standardized on the basis of protein content. This soluble product constituted the skin test reagent. Preparations thus made up were diluted to a concentration of 10 μg/ml protein with borate buffered saline (pH 8.0). The diluted antigenic solution, Leprosin A, was dispensed through a sterile 0.22 μm membrane filter into sterile 1 ml tuberculin vials.

Skin test procedure. Four skin tests of Leprosin A were administered per person, using Gillette Scimitar 1 ml disposable tuberculin syringes and 25 gauge needles, so that each forearm received two skin tests intradermally into the volar aspect. The macroscopic erythema and induration were measured and sequential biopsies were taken at 12, 24, 48, and 72 hr, one from each skin test site. Biopsies were taken from both macroscopically positive and negative reactions to Leprosin A (Table 1).

Biopsy of skin test sites. The forearm was cleaned with 70% alcohol, after which local anesthetic (1% xylocaine) was injected around, but not into, the area to be biopsied. The biopsies were taken with a 4 mm punch, and the specimens were placed in freshly prepared Helly's fixative in a glass bottle for 18–24 hr at room temperature.

Helly's fixative was made immediately before use by combining 100 ml Zenker's stock solution (mercuric chloride 5 g% and

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TABLE 1. *Number of cutaneous biopsies obtained at different intervals after the intradermal inoculation of Leprosin A.*

Disease status	Hours				
	6	12	24	48	72
LL	1	2	3	2	2
BL	—	2	2	2	2
BB	—	1	1	1	1
BT	—	2	2	2	2
TT	1	2	3	2	2
Contact	—	1	1	1	1
Normal	—	—	—	1	2
Total	2	10	12	11	12

potassium dichromate 2.5 g%) with 5 ml 40% formaldehyde. Both Zenker's stock solution and the formaldehyde were stable at room temperature when stored separately ([2]). After 18–24 hr, the fixative was removed and the tissues were washed in tap water overnight. These were then transferred to plastic vials containing 70% alcohol.

Preparation of dilute Epon embedding medium. The Epon embedding medium was prepared freshly during the dehydration period in disposable beakers. The medium was composed of 4.5 ml of Epon 812, 4.5 ml of epoxy araldite 502, 2.0 ml of DER epoxy resin 732, 1.5 ml of Epon curing agent dedecenyl-succinic anhydride (DDSA), and 0.5 ml of Epon curing agent DMP-30 (2,4,6-tri dimethyl amono methyl phenol). Aliquots of this Epon mixture can be stored frozen for at least 2 months in a plugged plastic syringe.

Embedding and polymerization. Polymerization was allowed to proceed at room temperature for 4 hr, at 37°C for 18 hr and, finally, at 60°C for 16 hr.

Sectioning. The cooled block was trimmed with a fine saw, mounted on a microtome, and sectioned on a ¼-inch glass knife on which a boat was created, using dental wax applied to the knife, and filled with distilled water. The 1–2 μm sections were floated on a drop of distilled water on a clean, dry microscope slide and immediately dried and fixed for 10 min on a hotplate set at a low temperature. This effectively expanded the sections and affixed them to the slide in one operation.

Staining. From each block, duplicate Epon sections were stained—one with distilled water 1:1 Giemsa for 6 hr at 60°C (pH 5), and the other with 0.4 M sodium phosphate buffer 1:1 Giemsa for 3 hr at 60°C for basophils ([9]). Eosinophils generally had bilobate nuclei and numerous cytoplasmic granules that stained green-blue with the alkaline stain and bright orange-red with the acid stain. Neutrophils had multilobate nuclei and blue-green cytoplasm when stained at pH 8; with the acid stain, small numbers of minute red cytoplasmic granules were visible. Mononuclear cells were nongranulocytic cells, such as lymphocytes and macrophages, and usually had a clear, slightly blue-grey to colorless cytoplasm surrounding a nonsegmented nucleus.

This technique is ideal for quantitative analysis of cellular infiltrates since it affords optimal light microscopic morphology on large blocks of tissue; structural detail normally reserved for electron microscopy is preserved, while the sampling problems inherent in the latter method are avoided. Epon sections also allow identification of fibrin deposits when these are large.

Quantitative analysis of cell infiltrate. In order to quantify the various types of inflammatory cells participating in the lesions and to determine the order of their arrival, detailed cell counts were performed on the sequence of biopsies. The epidermis was relatively spared in contrast to the infiltration of the dermis. The dermal infiltrate was analyzed by counting all of the cells found in a series of 5 swaths taken perpendicular to the skin surface and followed to the deep edge of the biopsy. Swaths, each 100 μm in width, were defined with the aid of an ocular micrometer. The number of cells in each swath counted was recorded, and the differential cell infiltrate was recorded (Table 2). Differential cell counts were divided into: a) mononuclear cells, b) polymorphonuclear neutrophils, c) mast cells, d) plasma cells, and e) eosinophils.

RESULTS

The reactions extended into the deep dermis and frequently involved the subcutis, but spared the epidermis, because of antigen distribution within the skin ([20]). Reactions were characterized by striking perivascular infiltrations of mononuclear cells, neutrophils, mast cells, plasma cells, and eosinophils, and fibrin depositions and microvas-

TABLE 2. *Details of microscopic and gross features of examples of some reactions to Leprosin A skin tests. Numbers of cells counted and their percentage distribution are shown.*

Time of biopsy (hr)	Mononuclears		Plasma cells		Neutrophils		Mast cells		Eosinophils		Total counted	Induration (mm)
					Negative contact 98							
12	310	52.5%	10	1.7%	55	9.3%	35	6%	180	30.5%	590	0
24	105	70%	0		10	7%	15	10%	20	13%	150	0
48	415	72%	25	4.5%	40	7%	50	8.5%	45	8%	575	0
72	157	71%	6	3%	24	11%	18	8%	15	7%	220	0
					Positive healthy control 48P							
48	105	97%	0		0		3	3%	0		108	20
					Positive healthy control 103							
72	930	98%	0		20	2%	0		0		950	10.5
					Positive healthy control 105							
72	856	83%	92	9%	59	6%	26	2%	0		1033	16
					Negative lepromatous (LL) patient 96							
12	96	27.7%	2	0.6%	230	66.5%	2	0.6%	16	4.6%	346	0
24	180	59%	12	4%	104	34%	8	3%	0		304	0
48	232	80%	5	1.7%	51	17.6%	2	0.7%	0		290	0
72	326	85.3%	8	2.1%	40	10.5%	8	2.1%	0		382	0
					Negative lepromatous (LL) patient 84							
12	105	39.2%	20	7.5%	120	44.8%	8	3%	15	5.6%	268	0
24	158	48.2%	8	2.4%	122	37.2%	20	6.1%	20	6.1%	328	0
48	280	79.5%	20	5.7%	48	13.6%	4	1.1%	0		352	0
72	198	78.6%	6	2.4%	40	15.9%	8	3.2%	0		252	0
					Negative borderline lepromatous (BL) patient 108							
12	60	39.5%	10	6.6%	62	40.8%	5	3.3%	15	9.9%	152	0
24	92	51.7%	12	6.7%	64	36%	10	5.6%	0		178	0
48	102	53.1%	10	5.2%	55	28.6%	25	13.0%	0		192	0
72	168	80.8%	10	4.8%	20	9.6%	10	4.8%	0		208	0
					Positive borderline lepromatous (BL) patient 101							
12	76	25.6%	16	5.4%	178	59.9%	2	0.7%	25	8.4%	297	10
24	160	31.4%	10	2%	215	42.2%	50	9.8%	75	14.6%	510	10.5
48	145	33.3%	15	3.5%	165	37.9%	25	5.7%	85	19.6%	435	12.5
72	220	36.7%	0		145	24.2%	10	1.7%	225	37.4%	600	13
					Positive tuberculoid (TT) patient 97							
12	435	65%	0		65	9.7%	20	2.9%	150	22.4%	670	16
24	650	82.9%	0		20	2.5%	15	1.9%	100	12.7%	785	17.5
48	745	85.6%	0		25	2.9%	20	2.3%	80	9.2%	870	22.5
72	482	84.4%	0		24	4.2%	25	4.4%	40	7%	571	20
					Positive tuberculoid (TT) patient 95							
12	35	9.9%	0		45	12.7%	15	4.2%	260	73.2%	355	10
24	85	54.8%	0		10	6.5%	0		60	38.7%	155	12.5
48	235	69.1%	0		55	16.2%	5	1.5%	45	13.2%	340	13.5
72	425	85%	0		0		25	5%	50	10%	500	15
					Negative borderline (BB) patient 88							
12	42	52%	4	5%	11	14%	3	4%	20	25%	80	4.5
24	144	85%	3	2%	0		0		22	13%	169	11
48	152	94%	0		0		0		10	6%	162	7
72	163	93%	0		0		0		12	7%	175	0
					Positive borderline tuberculoid (BT) patient 83							
12	150	60%	0		75	30%	5	2%	20	8%	250	11
24	272	68%	0		75	18.7%	15	3.8%	38	9.5%	400	15.5
48	304	79.6%	0		40	10.5%	20	5.2%	18	4.7%	382	15
72	426	81.2%	0		52	9.9%	30	5.7%	17	3.2%	525	15
					Positive borderline tuberculoid (BT) patient 93							
12	40	26.7%	0		85	56.7%	0		25	16.7%	150	10
24	100	35%	0		150	52.4%	10	3.5%	26	9.1%	286	11.5
48	285	77%	0		40	10.8%	15	4.1%	30	8.8%	370	10
72	240	84.2%	0		0		35	12.3%	10	3.5%	285	8

cular alterations were included in this infiltrate (Figs. 6–10).

Polymorphonuclear neutrophil infiltrations at 12 hr are shown in Figure 1. Patients with lepromatous (LL), borderline lepromatous (BL), and borderline tuberculoid (BT) leprosy had a high mean percentage of cells compared to those with borderline (BB) or tuberculoid (TT) leprosy or healthy contacts. The percentage of neutrophils decreased considerably by 24, 48, and 72 hr except in the BL and LL patients and in contacts in whom a high percentage of neutrophils was observed at 72 hr (Table 2). These cells were observed in normal subjects at 24 and 48 hr. This finding is in contrast to the study by Turk, *et al.* ([19]) which showed an absence of neutrophils in normal skin treated with tuberculin. Figure 2 shows the progressive striking increase of mononuclear cell infiltrations in all subjects in contrast to the inverse relationship of neutrophils.

Figure 3 shows the plasma cells in the cellular infiltration deposited at the site of the Leprosin A injection. Absence of plasma cells was observed in TT and BT leprosy. The mean percentage of plasma cells in BL patients was highest at 72 hr.

Figure 4 shows the mast cell response in the delayed reaction to Leprosin A. The granules were variable in fully developed reactions and were reduced in numbers.

Eosinophils were the only granulocytes to participate in this delayed reaction (Fig. 5). They were occasionally seen in vessel lumina and in the perivascular cuffs of mononuclear cells. They had distinctive orange-red granules of constant size that filled the cytoplasm and often obscured a bilobate nucleus. In several biopsies they accounted for 25–50% of the total dermal cellular infiltrate at 12 hr. In such reactions it was not unusual to observe large numbers of eosinophil granules lying free in the dermis. The mean percentage of eosinophils decreased with time, except in one BL patient whose eosinophils steadily increased to 14.6% at 24 hr, 19.6% at 48 hr, and 37.4% at 72 hr (Table 2). In the LL patients no eosinophils were detected at 24, 48, or 72 hr. One patient with a clinical and histological diagnosis of TT and another with LL from Polambakam (South India) were biopsied at 6 and 24 hr. In both, eosinophils were present in the cellular infiltrate at 6 and 24 hr, indicating that eosinophils peaked between 6–12 hr ([1]). In contrast to the findings described above, biopsies from 3 normal subjects (2 at 72 hr and 1 at 48 hr) failed to show eosinophils in their cellular infiltrates.

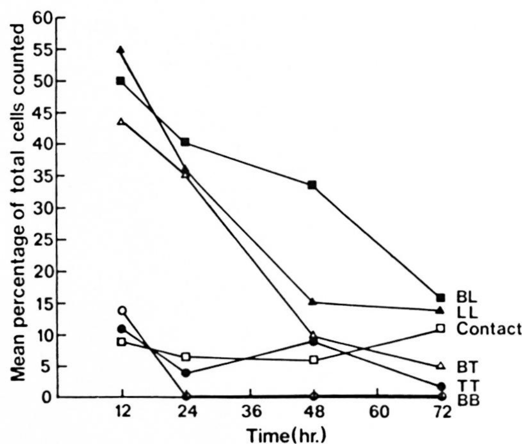

FIG. 1. Polymorphonuclear neutrophil response in skin biopsies elicited by Leprosin A in leprosy patients and controls.

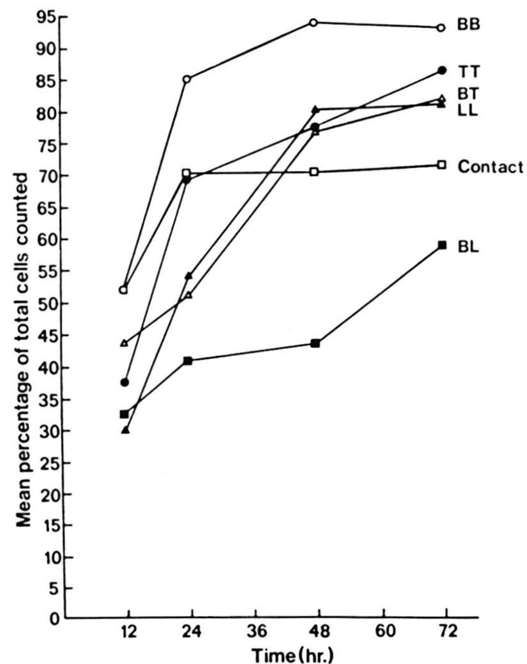

FIG. 2. Mononuclear cell response in skin biopsies elicited by Leprosin A in leprosy patients and controls.

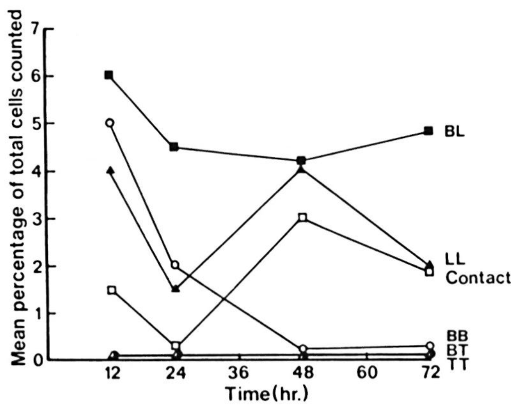

FIG. 3. Plasma cell response in skin biopsies elicited by Leprosin A in leprosy patients and controls.

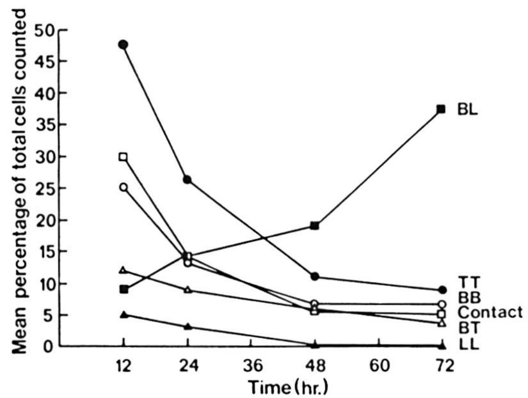

FIG. 5. Eosinophil response in skin biopsies elicited by Leprosin A in leprosy patients and controls.

"Negative" Leprosin A reactions. Reactions to the same intradermal skin test were regarded as negative if they measured less than 5 mm in induration, according to standard convention. Five such patients (1 contact, 3 LL, 1 BL) were biopsied at different time intervals being grossly negative. Microscopically, all of these "negative" lesions had cellular infiltrates that did not differ quantitatively (except for the absence of eosinophils after 24 hr in BL and LL patients) from those in positive reactions. Several of these lesions could not have been distinguished microscopically from much larger grossly positive reactions.

DISCUSSION

In tuberculosis, the development of the purified protein derivative of *M. tuberculosis* has helped in the understanding of the epidemiology of the disease. The use of soluble skin test antigens in leprosy are based on this model. Lepromin is a useful test for

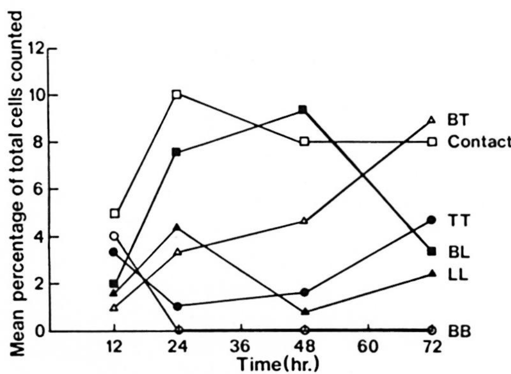

FIG. 4. Mast cell response in skin biopsies elicited by Leprosin A in leprosy patients and controls.

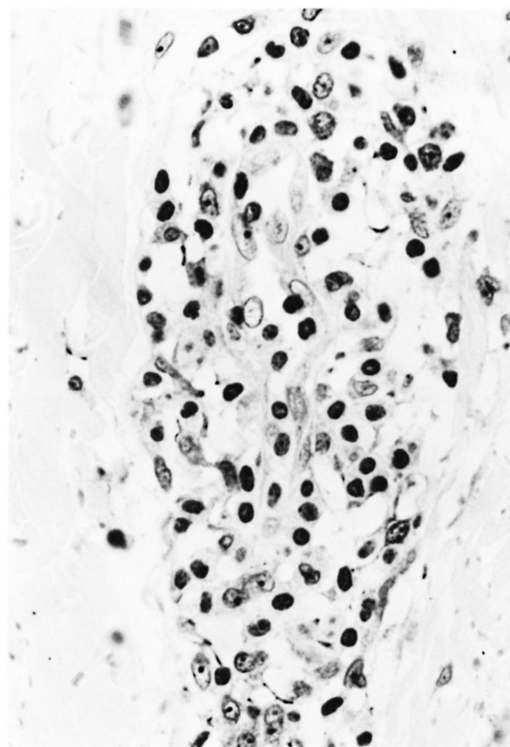

FIG. 6. Perivascular infiltrate, mainly composed of lymphocytes and monocytes, at a reaction to Leprosin A in a normal person at 48 hours (1 μm section, Giemsa stain ×560).

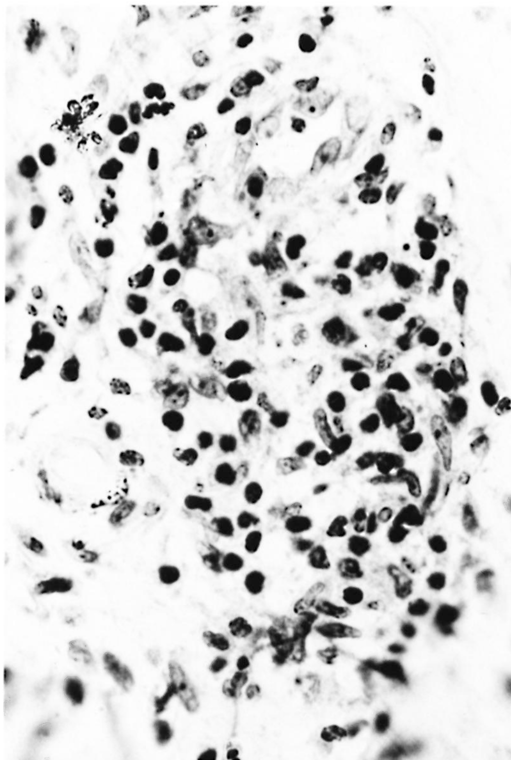

FIG. 7. Photomicrograph of a 1 μm Epon-embedded section of a 72-hour reaction to Leprosin A in a TT patient. Perivascular infiltrate mainly consists of mononuclear cells. Hypertrophy of endothelial cells is seen (Giemsa stain ×560).

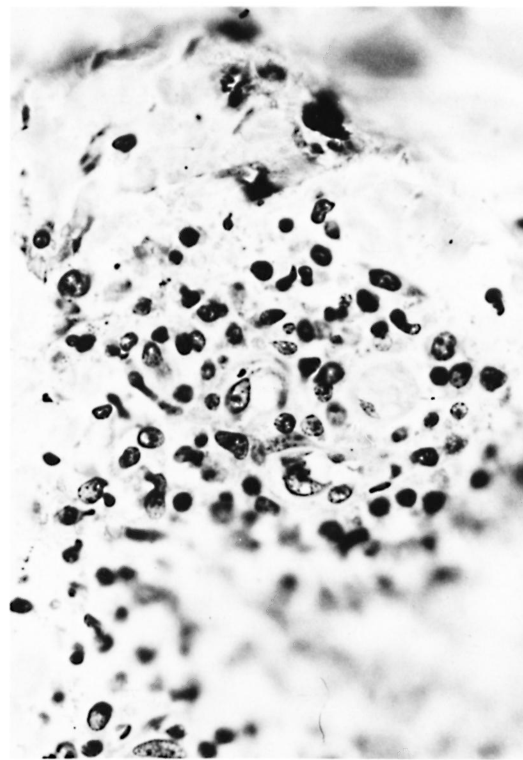

FIG. 8. Mononuclear cellular infiltrate in a 48-hour reaction to Leprosin A in a contact whose macroscopic reaction was negative at all times (1 μm Epon section, Giemsa stain ×560).

the study of patients and contacts but has little value in epidemiological studies. The recent concern to develop a vaccine against leprosy has renewed interest in developing soluble skin test reagents derived from *M. leprae* for the study of leprosy. The basic assumptions of skin tests based on cell-mediated hypersensitivity are that the antigen content of the skin tests are minimal to avoid sensitization of the test subjects, and the histological findings are of perivascular infiltration of mononuclear cells. In our study, sequential biopsies of Leprosin A test sites indicated that Leprosin A induced a characteristic delayed-type hypersensitivity response in patients, contacts, and normal persons.

Of particular interest in the present study, apart from the microscopic changes observed at positive Leprosin A sites, were the cellular infiltrates seen in "negative" Leprosin A reactions. Cellular infiltration at the "negative" sites did not differ qualitatively from those with positive reactions, suggesting that even the smallest indurations are positive reactions.

Although, inasmuch as possible, armadillo tissue is removed from the leprosy bacilli prior to their sonication in the preparation of the reagent, the certainty exists that some antigenic molecules of armadillo origin must persist. The possibility remains that this might be the cause of cellular infiltrates at the "negative" sites.

In this study, the striking findings include the absence of basophils, the presence of eosinophils, degranulation of eosinophils and mast cells, and deposition of fibrin in the reticular dermis. Eosinophils are not commonly seen in normal skin, but it is not unusual for patients with allergic, neoplastic, and parasitic diseases to develop blood eosinophilia or to have infiltrates in which these cells predominate ([3, 10]).

The relationship between mononuclear

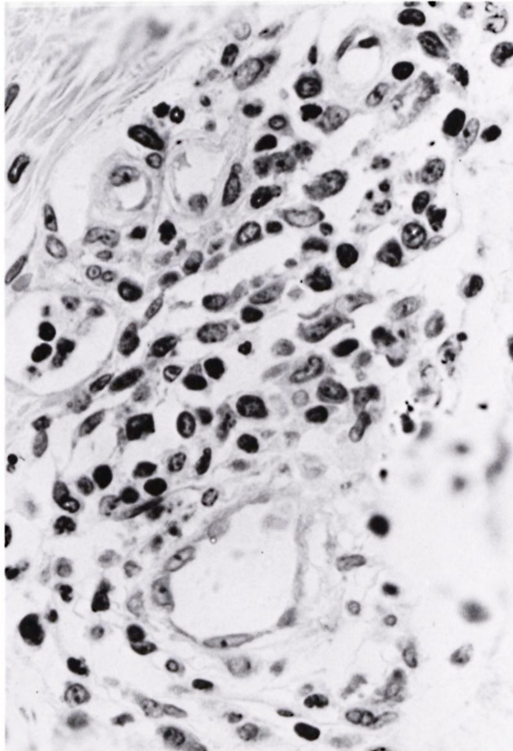

FIG. 9. Reaction to Leprosin A in a BT patient, at 24 hours, whose macroscopic response was positive at all times (1 μm Epon section, Giemsa stain ×560).

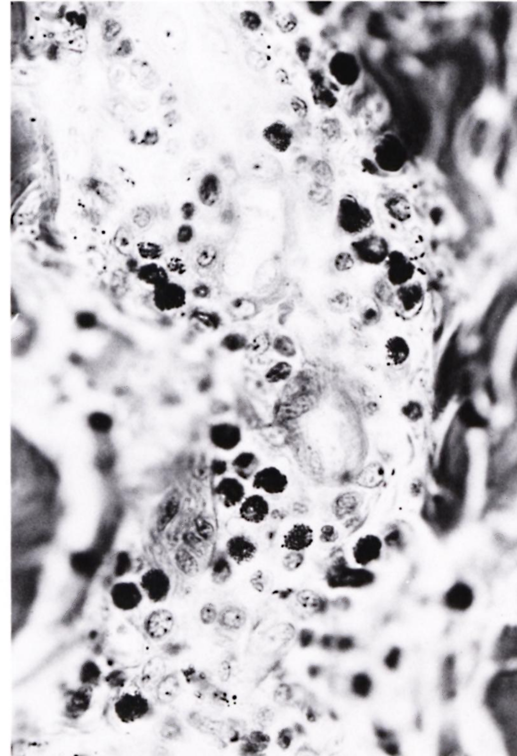

FIG. 10. To demonstrate eosinophils in a delayed cutaneous reaction to Leprosin A at 72 hours in a BL patient (1 μm Epon section ×560).

cells, mast cells, and eosinophils is poorly understood. Nevertheless, eosinophils are clearly implicated as effector cells in some disease states ([12]) and this might apply to leprosy.

A possible explanation for the presence of eosinophils in our subjects could be raised blood eosinophil counts due to parasitic infection, and that the eosinophils may be supplementing the functions of basophils and mast cells. (Unfortunately, blood pictures are not available for our patients). Alternatively, it could be that responses to Leprosin A are usually associated with eosinophils. Most of the work in this field has concentrated on responses to tuberculin, to which positive responses can be of two types: a) the response classically associated with Koch incorporating a necrotic element and b) the response identified by Debré and Bonnet ([5]), described by Mackanness ([11]), and highlighted by Rook ([13, 14]) as the possible true basis of protective immunity, in which necrosis does not play a part. Responses to Leprosin A always appear to be of this latter type ([17]).

Although the number of subjects we have studied is very small, one interpretation of the proportion of eosinophils (Fig. 5) could be that the early phase of a non-necrotic ("*Listeria*") response ([14]) is associated with the attraction of eosinophils which soon degranulate, whether or not a clinically positive reaction occurs, or the reaction is regulated out by homeostatic suppressor mechanisms.

However, some of those studied might have been expected to have a necrotic ("Koch") response ([14]), were such a thing possible to this reagent, and indeed the early infiltrate with neutrophils seen in LL, BL, and BT patients (Fig. 1) may herald the phenomenon. Homeostasis, of necessity, needs to control such a possibility since a necrotic response to antigens of *M. leprae* occurring in disseminated or multibacillary disease

would be catastrophic. Perhaps some leprosy reactions indicate minor breakdowns in this all-important homeostatic control, and the rather bizarre cellular changes seen in our single Leprosin-A-positive BL case may be an example. A similar lack of responsiveness to tuberculin is sometimes seen in patients with renal tuberculosis in which florid necrosis would be extremely damaging. This active control of reaction must not be confused with tuberculin "anergy" seen in very advanced tuberculosis.

Certainly the expression of cell-mediated immunity as a positive skin test is the end result of an extremely complex series of integrated reactions. However much one may speculate, our results indicate the lack of knowledge of histological changes associated with antigen recognition and their regulation occurring in different parts of the leprosy spectrum and/or, for that matter, in healthy persons.

SUMMARY

Punch biopsies taken 12, 24, 48, and 72 hours after skin testing with Leprosin A have been used to prepare ultrathin sections for the identification and enumeration of infiltration cells. The study was performed on small numbers of both healthy persons and leprosy patients with various forms of the disease living in India. Similar cells were found to infiltrate both positive and negative responses to the skin test reagent, although there were quantitative differences.

The most striking findings were the absence of the expected basophils and an infiltration of eosinophils which proceeded to degranulate. This was especially noticeable in a healthy leprosy contact and in patients at the tuberculoid end of the leprosy spectrum, whether or not they produced positive skin reactions to Leprosin A. In patients at the lepromatous end of the spectrum, infiltrates were largely neutrophils.

RESUMEN

Se tomaron biopsias con sacabocado a las 12, 24, 48 y 72 horas después de la inyección dérmica de leprosina A en pacientes con lepra y en contactos sanos. Las biopsias se usaron para preparar cortes ultradelgados y para enumerar células infiltrantes. Aunque se encontraron células similares en los infiltrados tanto de las respuestas dérmicas positivas como de las negativas, hubieron diferencias cuantitativas.

Los hallazgos más sorprendentes fueron la ausencia de basófilos y la presencia de eosinófilos en proceso de desgranulación. Esto fue particularmente notable en un contacto sano y en pacientes tuberculoides al extremo del espectro independientemente de que éstos dieran o no respuestas dérmicas positivas a la leprosina A. En los pacientes lepromatosos polares los infiltrados fueron predominantemente neutofílicos.

RÉSUMÉ

Des biopsies ponctuelles prélevées 12, 24, 48, et 72 heures après une épreuve cutanée à la Léprosine A, ont été utilisées pour préparer des coupes ultrafines, en vue de l'identification et du comptage des cellules d'infiltration. Cette étude a été menée sur un petit nombre d'individus sains et de malades de la lèpre, ces derniers atteints de différentes formes de la maladie, et tous vivant en Inde. Que la réponse à l'antigène cutané soit positive ou négative, on a observé que les infiltrats étaient constitués de cellules semblables; des différences quantitatives étaient cependant notées.

Ce qui était le plus frappant était l'absence inattendue de cellules basophiles, et la présence d'une infiltration à cellules éosinophiles, en voie de perdre leurs granules. Ceci était particulièrement notable chez un contact de malade, lui-même en bonne santé, et chez des malades présentant une lèpre à l'extrémité tuberculoïde du spectre clinique de la maladie; et ceci n'était pas en rapport avec le résultat de la réaction à la Leprosine A. Chez les malades présentant une lèpre se situant à l'extrémité lépromateuse du spectre clinique, les infiltrats étaient principalement constitués de neutrophiles.

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