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The Cellular Content of Dermal Leprous Granulomas: An Immuno-histological Approach[1]

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Leprosy is a chronic infectious disease in which the long lasting presence of bacilli within the cells of the host's immune system gives rise to many immunopathologic disturbances ([6, 17]). Patients with tuberculoid leprosy have a relatively high resistance, as demonstrated by a positive lepromin skin test and the formation of immunologic granulomas ([4]), with an epithelioid differentiation of macrophages and a high content of lymphocytes. Patients with lepromatous leprosy exhibit, both *in vivo* and *in vitro*, a selective T cell deficiency towards *Mycobacterium leprae* antigens. The dermal granulomas of these patients contain few lymphocytes, and their mononuclear phagocytes differentiate into Virchowian cells, harboring numerous bacilli.

Erythema nodosum leprosum (ENL) is an immunopathologic complication seen in about half of the patients with lepromatous leprosy. Its pathological picture includes vasculitis and the presence of polymorphonuclear leukocytes. Because of the demonstration of immunoglobulins and complement deposits around vessels in some ENL lesions, ENL has generally been considered as an immune complex disease ([22]).

In recent years, it has become possible to identify different subsets among the thymus-derived lymphocytes. The availability of monoclonal antibodies specific for all T cells, T cells with a helper/inducer phenotype, and T cells with a suppressor/cytotoxic phenotype has allowed in-depth studies of the role of these T cells in human diseases ([1]).

Regarding leprosy, the study of T cell subsets with monoclonal antibodies has permitted significant progress in the understanding of some immunopathologic abnormalities ([21]). T suppressor cells are increased in the peripheral blood of bacillary, untreated, lepromatous patients. Circulating T suppressor cells are at normal levels in treated lepromatous patients, and we have shown a significant correlation between the bacterial load and the helper/suppressor ratio. This ratio can be considered as an expression of the equilibrium between the two major cellular populations involved in cell-mediated immune responses.

We have also shown that patients with ENL are unique among lepromatous patients in that their suppressor cells are significantly decreased, whatever their bacterial load. This decrease is transient, and it is associated with an increase of T cell responses *in vitro*. There is now accumulating evidence that imbalances in T cell subpopulations are important in the understanding of ENL ([2, 7, 9, 21]).

As stated above, the morphology and cellular composition of the cellular infiltrate of the cutaneous lesions in leprosy depends on the intensity of cell-mediated immunity. This work has been undertaken to find out whether the T cell imbalances evidenced in the blood of leprosy patients can also be demonstrated at the site of the pathologic lesions. We have studied the cellular content of leprous granulomas at various stages of the disease, using monoclonal antibodies.

PATIENTS AND METHODS

Patients. Twenty-two patients, followed as outpatients at the Malta building in Paris, were selected for this study. The following characteristics of the patients are shown in Table 1: sex; age; geographical origin; type of the disease, according to Ridley and Jopling's criteria ([16]); duration and type of ther-

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TABLE 1. *General characteristics of the leprosy patients.*

Patient no.	Sex	Age (yr)	Geographical origin	Form of leprosy	Duration of therapy	BI (MI in %)
1	M	16	Guyana	BT	Irregular	0
2	M	10	Senegal	BT	NT[a]	0
3	M	16	Cambodia	TT	NT	0
4	M	20	Vietnam	TT	NT	0
5	M	41	Senegal	LL[b]	NT	3.50 (1%)
6	F	44	La Réunion	LL[b]	NT	4.75 (60%)
7	M	40	India	LL[b]	NT	3.25 (80%)
8	M	43	Comorean Islands	LL[b]	NT	1.75 (1%)
9	M	44	Senegal	LL[b]	R.[c]P.[d]D.[e] 2 weeks	4.50 (1%)
10	M	32	West Indies	LL[b]	R.P.C.[f] 2 mos.	3.25 (0%)
11	M	19	Guyana	LL[b]	R.P.D. 2 mos.	2.25 (0%)
12	M	23	Comorean Islands	LL[g]	R.P.C. 15 mos.	2.25 (0%)
13	M	65	Vietnam	BL[g]	R.P.C. 12 mos.	2.50 (0%)
14	M	40	West Indies	LL[g]	R.P.C. 12 mos.	1.50 (0%)
15	F	44	La Réunion	LL[g]	R.P.D. 12 mos.	3.00 (0%)
16	F	39	Portugal	LL[g]	R.P.C. 16 mos.	2.5 (0.1%)
17	M	64	Vietnam	LL, ENL	R.P.C. 36 mos.	0
18	M	42	Vietnam	LL, ENL	R.P.D. 60 mos.	2.25 (0%)
19	M	17	Cambodia	BL, ENL	R.P.D. 6 mos.	3.75 (0%)
20	M	23	West Indies	BL, ENL	NT	2.25 (0%)
21	F	18	Cambodia	LL, ENL	R.P.C. 20 mos.	2.50 (0%)
22	F	37	Spain	LL, ENL	R.D. 60 mos.	2.00 (0%)

[a] NT = no treatment.
[b] Untreated, nonreactional lepromatous leprosy.
[c] R = rifampin.
[d] P = prothionamide.
[e] D = dapsone.
[f] C = clofazimine.
[g] Treated, nonreactional lepromatous leprosy.

apy; and bacterial status. The five-group classification of leprosy patients was chosen because, in our experience, the subdivision of the LL group in two subgroups ([14]) is not always easy to perform. The bacterial index (BI) was expressed according to the logarithmic scale of Ridley ([15]); the morphological index is the mean percentage of homogeneous bacilli in the skin and mucosal smears.

Patients were divided into four groups: 1) 4 tuberculoid patients (2 TT and 2 BT); 2) 7 nonreactional LL patients who had received little (less than two months) or no treatment; 3) 5 nonreactional lepromatous patients (4 LL, 1 BL) who had been treated for 12–16 months with a combined chemotherapy, comprising daily intake of rifampin (600 mg), prothionamide (375 mg), and dapsone (50–100 mg) or clofazimine (100 mg); and 4) 6 lepromatous patients with active erythema nodosum leprosum (ENL). These patients were classified along the leprosy spectrum before the onset of ENL, according to the clinical and histological features of their cutaneous lesions: 2 were BL

TABLE 2. *Monoclonal antibodies used to identify cells of the dermal granulomas.*

Monoclonal antibody (class)	Cell recognized	Source	Working solution (μg/ml)
Leu 4 (IgG 1)	80–95% of peripheral T cells	Becton-Dickinson	20
Leu 3a (IgG 1)	Helper/inducer subset of T cells	Becton-Dickinson	40
OKT8 (IgG 2a)	Suppressor/cytotoxic subset of T cells	Ortho	2.5
Leu M1 (IgM)	>95% of granulocytes and 80–100% of circulating monocytes (does not react with macrophages)	Becton-Dickinson	40

[a] The identification of circulating T cells was performed with OKT3, OKT4, and OKT8 antibodies, as previously described ([2]).

and 4 LL—3 of them (Nos. 18, 19, 20) had moderate ENL with cutaneous nodules and no systemic symptoms (Table 1). The three other patients had severe ENL. The duration of therapy prior to the investigation is shown in Table 1. Biopsies were performed prior to any administration of anti-ENL drugs. For each patient, the cutaneous biopsy and the blood sampling were performed on the same day.

Skin biopsies. A 4 mm or 6 mm punch biopsy specimen was taken from a typical, papular lesion of each patient. For ENL patients, a nodule of ENL was chosen as the site of the biopsy. Half of the specimen was fixed in formalin for hematoxylin and eosin (H&E) staining. The other half was snap frozen in liquid nitrogen and stored at −70°C for a few days until processing.

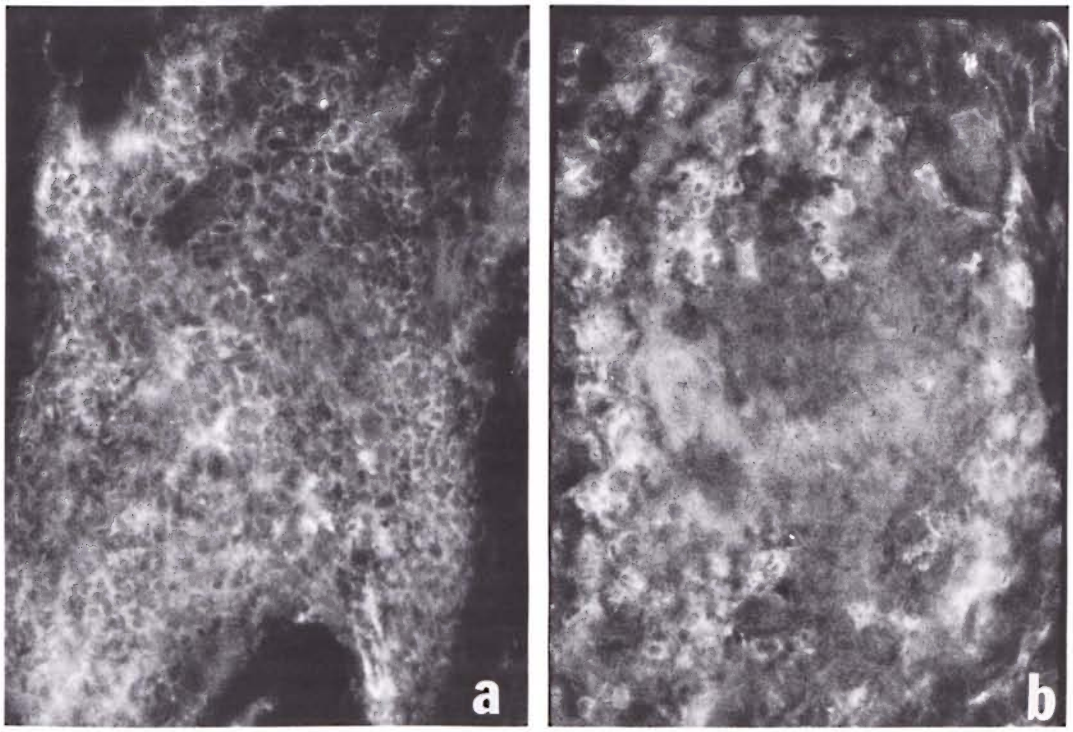
Immunofluorescence technique. Four μm cryostat sections were placed on microscope slides, air dried, fixed in acetone at −20°C for 10 min, and incubated with the monoclonal antibodies at optimal dilution for 1 hr at room temperature. This dilution for each antibody was determined by preliminary chessboard titration ([3]). After washing, a FITC-conjugated, goat anti-mouse antibody was added for 1 hr. Control sections included the omission of the primary antibody, and the use of irrelevant antibodies, followed by incubation with the goat anti-mouse antiserum. The characteristics of the monoclonal antibodies used for the identification of the cells of the cutaneous infiltrates are shown in Table 2. OKT3 and OKT4 antibodies could not be used for the study of the cutaneous infiltrates because of weak, irregular staining.

Analysis of cellular content of granulomas. The slides were examined with a Leitz Orthoplan microscope equipped for epi-il-

TABLE 3. *T cell content of dermal granulomas expressed as mean ± S.D. by arbitrary surface unit*[a].

T cell	Group 1 Tuberculoid (4)[b]	Group 2 Untreated lepromatous (7)	Group 3 Lepromatous (5)	Group 4 ENL (6)
Leu 4+ cells/SU	21.8 ± 1.7	13.6 ± 1.1[c]	12.4 ± 1.07[c]	17.8 ± 1.8[d]
Leu 3a+ cells/SU	12.4 ± 0.9	6.3 ± 0.8[e]	11.9 ± 1.2	13.9 ± 1.7
OKT8+ cells/SU	8.8 ± 1.0	11.1 ± 0.9	8.22 ± 1.0	6.4 ± 1.1[f]

[a] See text.
[b] Number of patients in group.
[c] Significantly different from Group 1, $p < 0.01$, Student's t test.
[d] Significantly different from Group 3, $p < 0.05$, Student's t test.
[e] Significantly different from Groups 1, 3, and 4, $p < 0.01$, Student's t test.
[f] Significantly different from Group 2, $p < 0.01$, Student's t test.



FIGS. 1a and 1b. A tuberculoid granuloma from Patient 4. Fig. 1a. Cells with the helper phenotype, labeled by the Leu 3a antibody, are numerous and diffusely distributed within the granuloma. Fig. 1b. Cells with the suppressor/cytotoxic phenotype, labeled by the OKT8 antibody, are confined at the periphery of the granuloma (×250).

lumination (Ploëm) and microphotography (Orthomat). Significant cellular areas of the granulomas were photographed at ×250 magnification on similar adjacent sections stained with different monoclonal antibodies. The 24 × 36 mm photographic slides were then projected on a screen. The total surface of a slide is about 74,800 μm^2; it was divided into 12 squares. Each of these squares has a 6230 μm^2 surface; this area is called the "surface unit" (SU).

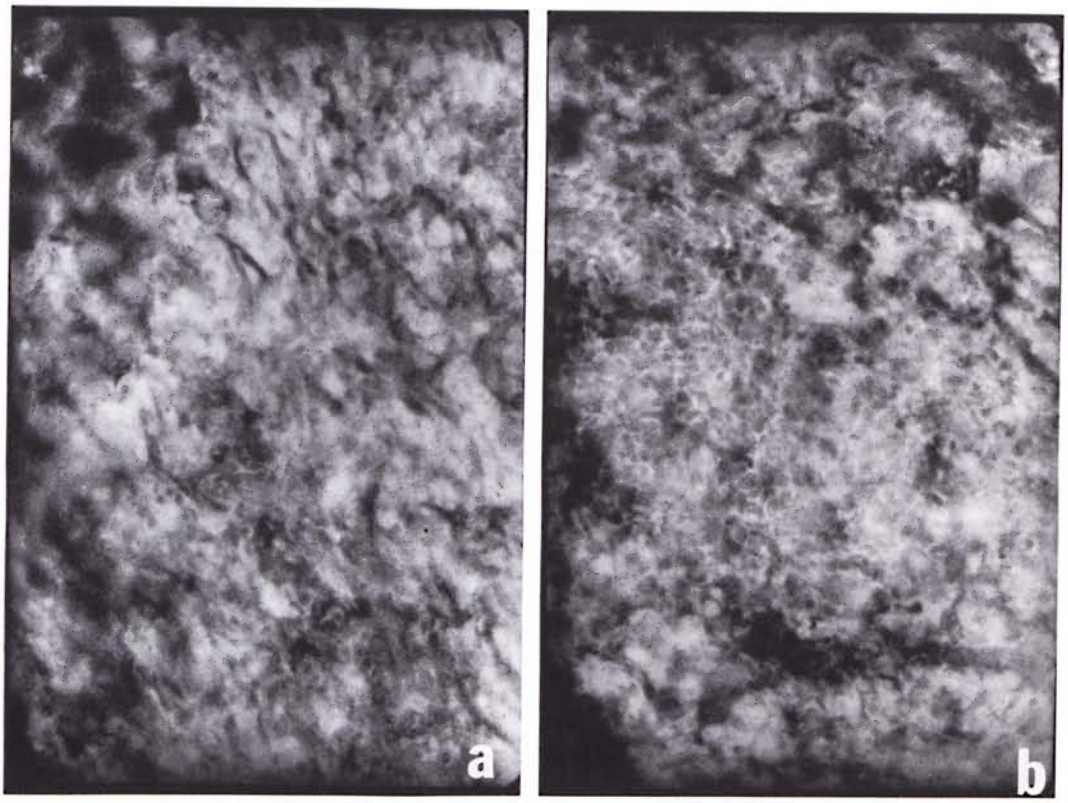
The labeled cells were enumerated on approximately 10 SU of the granulomas, on 3–5 photographs for each of the monoclonal antibodies used. The mean of the counts of all the SU examined is shown in Table 3. All enumerations were performed by the same observer (B.F.). Preliminary studies showed that careful observations of the slides gave reproducible results on repeated countings. At the time of the counting, the observer was unaware of the name and status of the patients.

Enumeration of circulating T cell subsets. Circulating T cells, T helper cells, and T suppressor cells were evaluated in the peripheral blood using a technique which has previously been described in detail ([2]). Monoclonal antibodies used were the OKT3, OKT4, and OKT8 antibodies.

Statistical analysis. Comparisons of the results between the four groups of patients were made using Student's *t* test.

RESULTS

Tuberculoid leprosy. Histological examination of the tuberculoid lesions showed well circumscribed dermal granulomas with epithelioid cells and numerous lymphocytes. The immunofluorescent staining of the T cells with the monoclonal antibodies directed against the various subsets showed that T helper cells are diffusely distributed in the granuloma (Fig. 1a); on the other hand, cells with a T suppressor cytotoxic phenotype were strikingly confined at the periphery of the granulomas, with a typical ring-like pattern (Fig. 1b). In the enumeration of



FIGS. 2a and 2b. Two adjacent sections of a dermal granuloma from a nonreactional, untreated lepromatous patient. In this patient (No. 5), Leu 3a+ cells (Fig. 2a) are five times less numerous than OKT8+ cells (Fig. 2b) (×250).

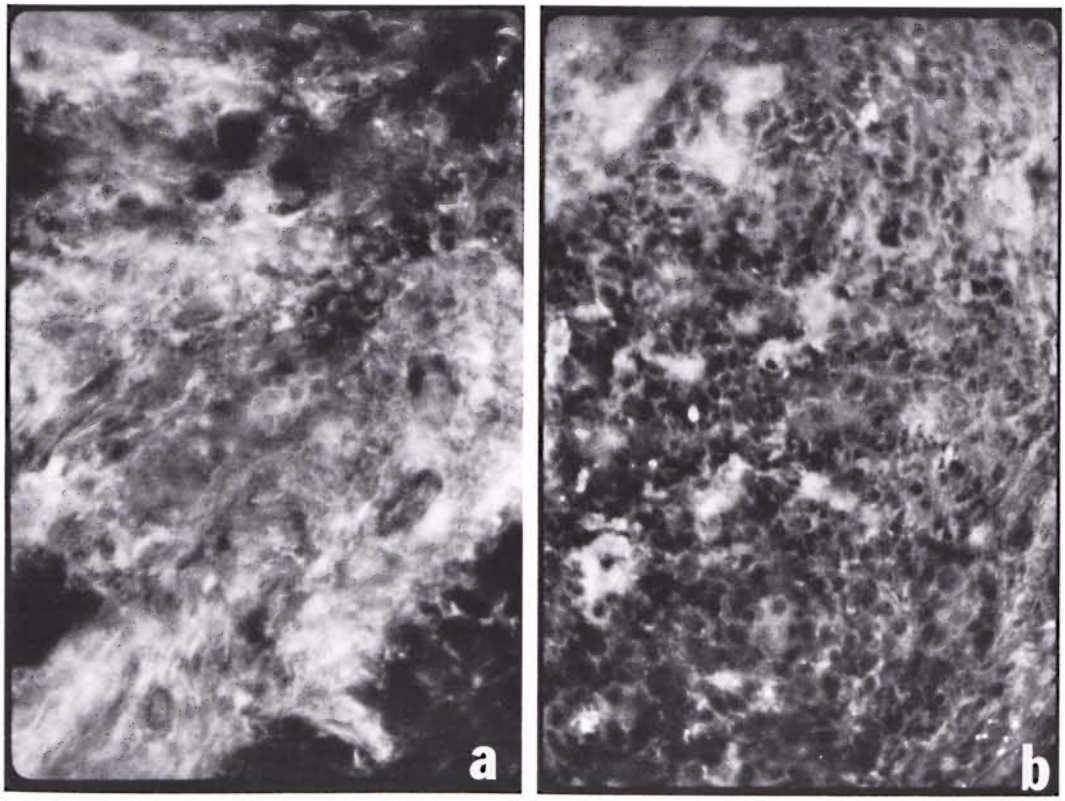
the cells in the dermis (Table 3), it can be seen that T helper cells outnumber T suppressor cells.

Nonreactional, untreated lepromatous leprosy. The histological appearance of the lepromatous granulomas showed many foamy histiocytes and few lymphocytes, diffusely distributed in the dermis. On fluorescein-labeled sections, histiocytes appeared with diffuse, weak staining, easily distinguishable from the network of specifically stained lymphocytes. In these patients, T cells were present in the dermis in a diffuse pattern, and it was not possible to delineate a typical distribution such as that observed in tuberculoid lesions. The enumerations of labeled cells are shown in Table 3. The most important finding is that in untreated lepromatous patients, T suppressor cells are, as a mean, twice as numerous as T helper cells (Fig. 2).

Nonreactional, treated lepromatous leprosy. The total T cell content of the granulomas in these patients was not different from the untreated patients (Table 3), but the T helper cells are more numerous here, and the H/S ratio in the dermis is significantly higher than the H/S ratio of untreated patients, and is close to the H/S ratio of tuberculoid patients. In two instances, cells with the T suppressor phenotype were often seen around well-defined areas, but it was not as clear-cut a pattern as that observed in tuberculoid patients.

ENL patients. The total number of T cells in ENL nodules was higher than in any other lepromatous granuloma (Fig. 3). Cells with the T suppressor phenotype were rare and T helper cells were numerous, resulting in an increase of the H/S ratio. No particular distribution of the two T cell subsets was found. It must be stressed that the preponderance of T helper cells was also marked in the untreated ENL patient (No. 20).

Two other observations could be made in ENL nodules: a) In three instances,



FIGS. 3a and 3b. The T cell content of the dermal granulomas of two untreated lepromatous patients. Fig. 3a. Patient No. 7 is a nonreactional patient with few Leu 4+ cells. Fig. 3b. Patient No. 20 is an ENL patient with many Leu 4+ cells (×250).

OKT8+ cells were found in the epidermis. b) Leu M1+ cells were found in all of the ENL granulomas with an apparently uneven distribution. Their number varied from 5–7 SU. In the lesions of patients from the other groups, there was less than 1 Leu M1+ cell/SU. With our technique, it was not possible to find a relationship between Leu M1+ cells and the dermal structures (vessels).

T cell subsets in peripheral blood. Results of the enumerations of T cell subsets in the peripheral blood are shown in Table 4. The changes in T cell subpopulations are similar to the ones observed in the dermal granulomas.

DISCUSSION

T cell mediated immunity is of paramount importance in the pathogenesis of leprosy. When anti-T cell monoclonal antibodies became available, we ([2, 21]) and others ([9]) studied the distribution of T cells in the blood of patients with various forms of leprosy. These studies showed that imbalances of T cell subsets may be important in many aspects of the disease. The most striking findings are: a) T suppressor cells are increased in nonreactional, bacillary leprosy and the T cell populations return to normal when the patients are free of bacilli. b) ENL is associated with a transient decrease in T suppressor cells ([21]).

The study of the cells infiltrating the leprous lesions is likely to provide additional data of interest, inasmuch as the histological pattern of leprosy reflects the cellular immunity of the patient. Modlin, *et al.* ([8]), using an immunoperoxidase technique, recently described two immuno-histological patterns in granulomatous lesions. a) In tuberculoid leprosy, suppressor cells are confined to areas surrounding the center of the granuloma and helper cells are in close contact to epithelioid cells within the granuloma. b) In lepromatous leprosy, cells with the helper and the suppressor phenotypes

TABLE 4. *T helper/T suppressor ratios in patients' blood*[a] *and skin.*[b]

H/S ratio	Group 1 Tuberculoid	Group 2 Untreated lepromatous	Group 3 Lepromatous	Group 4 ENL
Blood	1.42 ± 0.07	0.91 ± 0.15[c]	1.88 ± 0.24	1.97 ± 0.16[d]
Skin	1.65 ± 0.3	0.58 ± 0.08[e]	1.56 ± 0.21	2.72 ± 0.8

[a] OKT4+/OKT8+ cells.
[b] Leu 3a+/OKT8+ cells.
[c] Significantly different from Groups 1, 3, and 4, $p < 0.05$, Student's t test.
[d] Significantly different from Group 1, $p < 0.01$, Student's t test.
[e] Significantly different from Groups 1, 3, and 4, $p < 0.01$, Student's t test.

are both diffusely distributed among the histiocytes. The same pattern of distribution was found by Narayanan, *et al.* ([10]). In contrast, Van Voorhis, *et al.* ([19]) found that the suppressor cells were generally dispersed in either tuberculoid or lepromatous lesions.

Our results confirm that tuberculoid lesions are unique in that cells with the suppressor phenotype are not found in the center of the granulomas but only in a peripheral "ring-like" localization. Modlin, *et al.* ([8]) found the same immuno-histological pattern in the cutaneous lesions of sarcoidosis, another disease with immunological granulomas.

In lepromatous leprosy, no clear-cut difference can be found between the localizations of helper and suppressor cells in the granulomas. These cells, however, vary quantitatively according to the form of the disease. Semi-quantitative evaluations of the T cells in the granulomas have already been described ([7,10,19]). Van Voorhis, *et al.* ([19]) used an immunofluorescence technique and estimated, on serial sections, the percentage of positive cells after labeling with each monoclonal antibody, as compared with the total leukocyte content determined by labeling with a monoclonal antibody directed against a leukocyte common antigen. They found very important variations in the T cell content of the granulomas. Lepromatous lesions contained more than 90% suppressor cells, tuberculoid lesions contained 80–90% helper cells, and these patterns did not change after long-term treatment.

Narayanan, *et al.* ([10]) also used an immunofluorescence technique. These authors quantitated the cells by assessing the percentage of positive, fluorescent cells as compared to total cells in the same field visualized under phase contrast. They found that the ratio of OKT4+/OKT8+ cells ranged from 1.2–5.0 in the tuberculoid lesions and from 0.2–1.0 in the lepromatous lesions.

Modlin, *et al.* ([7]) used an immunoperoxidase technique to visualize the monoclonal antibodies on T cells. Estimating the percentages of each of the T cell subsets by comparing them to the total number of cells in the granulomas, they found that T helper cells outnumbered T suppressor cells in the granulomas of tuberculoid leprosy and ENL patients, in contrast with lepromatous patients.

With our technique, we have found that the quantitation of cells under the microscope is a difficult task, needing long-term exposure with subsequent fading. Furthermore, the slides cannot be adequately preserved. The use of 24 × 36 mm photographic slides of the significant areas of the granulomas projected on a screen allows easier and more reproducible countings. This method has, in our hands, yielded satisfying results ([5]). The enumerations of fluorescent cells showed that in tuberculoid leprosy there is an overall preponderance of helper cells. In untreated, nonreactional leprosy, we found that suppressor cells outnumbered helper cells, which were significantly few.

Lepromatous patients who received an effective therapy for 12–16 months but still had cutaneous lesions exhibited a different immuno-histological pattern. In these patients, the total number of T cells was not different from untreated patients but the T helper cells were more numerous and the H/S ratio was >1. Using the α-naphthyl-acetate-esterase staining of lymphocytes, we have previously shown ([5]) that T helper cells

do indeed increase in the lepromatous granulomas of treated patients. This seems to occur in all patients, even if they do not present the clinical symptoms of a reversal reaction.

It is apparent from Table 3 that in lepromatous patients the sum of the Leu 3a+ (helper/inducer) cells and the OKT8+ (suppressor/cytotoxic) cells is more than the number of Leu 4+ cells. This may be due to inadequate staining by the Leu 4 antibody, which is specific for 80–95% of T lymphocytes, or to the presence of immature T lymphocytes. It has been shown, using the monoclonal antibodies of the OKT series, that only the more functionally mature subset of thymocytes bears the pan-T marker ([13]). In addition, immature T lymphocytes may bear both the helper/inducer and the suppressor/cytotoxic phenotypes, in contrast with normal peripheral T cells ([13]).

ENL lesions are different from other lepromatous lesions. First, the total T cell content of these lesions is higher, intermediate between nonreactional lepromatous and tuberculoid lesions. Secondly, the T helper cells predominate and the suppressor cells are few. The same pattern of ENL lesions has been recently described by Modlin, *et al.* ([5]). From the quantitative point of view, ENL and tuberculoid lesions have in common a high content of T cells and an elevated H/S ratio. It has been shown previously that patients with ENL do not exhibit the same deficiency in cellular immunity as nonreactional lepromatous patients: significant differences between patients with and without ENL have been documented in DNCB sensitization ([11, 20]), the ability to generate cytotoxic T cells *in vitro* ([18]), the inhibition of macrophage migration ([12]), and circulating T helper and suppressor cells ([2, 9, 21]). The results of the present study emphasize the importance of T cell immunity in ENL ([2, 7, 9, 21]).

Enumerations of the T cell subsets in the peripheral blood showed variations parallel to the findings of cutaneous enumerations but less important. Thus, imbalances of circulating T cell subsets (as described earlier), particularly in ENL, do not seem to result from a selective trapping of T cells within the cutaneous lesions.

Cellular immunity is important in all stages of leprosy. The immuno-histological analysis of the dermal leprous granulomas shows that a pattern of efficient T cell mediated immunity can be identified in tuberculoid leprosy. It also shows that important variations in T cells exist in three different events of lepromatous leprosy; nonreactional, untreated patients have decreased helper cells, which are found at a "normal" level in treated patients. In ENL patients, the most striking finding is a decrease in the number of suppressor cells. Similar imbalances in T cell subpopulations have also been demonstrated in the blood ([9, 21]), but the histological analysis provides a more precise insight into the cellular immune disturbances of leprosy.

SUMMARY

An indirect immunofluorescence technique with monoclonal antibodies has been used to identify T cells, T helper cells, T suppressor cells, and granulocytes in the dermal granulomas of 22 patients with leprosy. In tuberculoid leprosy, T helper cells predominated and T suppressor cells were located at the periphery of well-circumscribed granulomas. In lepromatous leprosy, the two subsets of T cells were numerous in treated patients. In ENL lesions, T cells were more numerous and T helper cells predominated. Enumerations of the T cell subsets in the dermis and in the blood showed similar changes, but these changes were quantitatively more marked in the dermal granulomas.

RESUMEN

Se usó una técnica de inmunofluorescencia indirecta con anticuerpos monoclonales para identificar a las células T, a las células T cooperadoras, a las células T supresoras, y a los granulocitos, en los granulomas dérmicos de 22 pacientes con lepra. En la lepra tuberculoide predominaron las células T cooperadoras y las células T supresoras estuvieron localizadas en la periferia de granulomas bien circunscritos. En la lepra lepromatosa las dos subpoblaciones de células T fueron numerosas en los pacientes tratados. En las lesiones ENL las células T fueron más numerosas y predominaron las células T cooperadoras. Las enumeraciones de las subpoblaciones de células T en la dérmis y en la sangre, mostraron cambios similares, pero estos cambios fueron cuantitativamente más marcados en los granulomas dérmicos.

RÉSUMÉ

On a utilisé une technique indirecte d'immunofluorescence avec des anticorps monoclonaux pour iden-

tifier les cellules "T helper", et les cellules "T suppressor", de même que les granulocytes dans les granulomes du derme, chez 22 malades atteints de lèpre. Dans la lèpre tuberculoïde, les cellules "T helper" prédominairent, et les cellules "T suppressor" étaient situées à la périphérie de granulomes bien circonscrits. Dans la lèpre lépromateuse, les deux sous-ensembles de cellules T étaient nombreux chez les malades traités. Dans les lésions d'érythème noueux lépreux (ENL) les cellules T étaient plus nombreuses, et les cellules "T helper" étaient en plus grand nombre. Le comptage des sous-ensembles de cellules T dans le derme et dans le sang a révélé des altérations semblables, mais ces modifications étaient quantitativement plus prononcées dans les granulomes du derme.

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