American Journal of Pathology, Vol. 137, No. 4, October 1990

Rapid Communication

Immunohistologic Assessment of Cytokine Production of Infiltrating Cells in Various Forms of Leprosy

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The aim of this study was to determine cytokines in human leprosy lesions by means of immunohistologic examination. Cryostat sections of skin biopsies from 57 patients with various forms of leprosy were immunostained according to the APAAP method, using monoclonal antibodies against interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-gamma), and, in addition, against CD 1 antigen. Granulomas in biopsies of untreated patients with tuberculoid leprosy showed large amounts of cells positive for IL-1β, TNF-alpha, IFN-gamma, and CD 1, whereas no positive signals could be detected in untreated patients with lepromatous leprosy. However, in those biopsies obtained from lepromatous leprosy patients undergoing chemotherapy, positive staining for cytokines as well as subepidermal Langerhans cells increased to a detectable amount. Remarkably, in tuberculoid leprosy patients, the number of IL-1β–positive cells did not vary under therapy, while the number of TNF-alpha and IFN-gamma reactive cells decreased. These results suggest that immunohistologic determination of cytokines in combination with the assessment of subepidermal Langerhans cells in human leprosy lesions may be used as a parameter for the patient's status of cell-mediated immunity under chemotherapeutic treatment. (Am J Pathol 1990, 137: 749–753)

In human leprosy, granulomatous inflammation affecting the skin and nerves is a prominent clinical manifestation. This chronic infectious disease is characterized by a broad clinical spectrum of host responses,[1] reflecting gradiations in the patient's capacity to develop a specific cell-mediated immune response.

In tuberculoid leprosy, the host mounts a vigorous cell-mediated immune response, exhibiting cutaneous delayed hypersensitivity to *Mycobacterium leprae* antigens, which is suggested to limit the growth of *M. leprae* organisms.[2] At the other end of the spectrum, patients with lepromatous leprosy fail to mount an effective cell-mediated immune response determined by specific anergy to *M. leprae* antigen and many acid-fast bacilli located in the lesions.[3]

Because cytokine production is an outstanding parameter of cell-mediated immunity,[3] we investigated whether the skin lesions of patients with various forms of leprosy differed with regard to cytokine-producing cells to obtain insight into the mechanism of local immune reactivity. As previously described,[4] epidermal Langerhans cells play an important role in the delayed hypersensitivity reaction, and Langerhans hyperplasia[5,6] may be a marker for an intact cell-mediated immune response.

Therefore we studied skin biopsies of patients with various forms of leprosy by immunohistochemistry using monoclonal antibodies to determine Langerhans cells and cytokines produced by infiltrating cells in the lesions.

Material and Methods

Patients

Skin biopsies of 57 patients suffering from leprosy were obtained from the files of the leprosy eradication program

Supported by a grant from the German Leprosy Relief Association, Würzburg, FRG.

Accepted for publication July 27, 1990.

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Table 1. *Patients*

Diagnosis	No. of Untreated Patients	No. of Patients Undergoing Combined Chemotherapy[2]
LL	22	10
BL	1	4
BB	2	1
BT	1	6
TT	7	3

LL, lepromatous leprosy; BL, borderline leprosy; BB, midborderline leprosy; BT, borderline tuberculoid leprosy; TT, tuberculoid leprosy.

that were performed by the Department of Leprosy of the Ministry of Health and Welfare, Asuncion, Paraguay in cooperation with the Forschungsinstitut Borstel.

Patients were classified according to the clinicopathologic criteria of Ridley and Jopling[1] (Table 1). The patients who underwent combined chemotherapy received treatment with rifampicine (10 mg/kg/day), isoniacide (5 mg/kg/day), protionamide (5 mg/kg/day), and dapsone (1.2 mg/kg/day), as previously described by Freerksen.[7] Because of the logistic difficulties, biopsies from treated or untreated cases used for our study were obtained from different patients. Biopsy specimens were snap frozen in liquid nitrogen and stored until use at −80°C.

Antibodies

The following monoclonal antibodies were used for phenotyping of infiltrating cells (recognized specificity in brackets): monoclonal Fib 3 (IL-1β)[8]; TNF-E (TNF-alpha)[9] was provided by Dr. Adolf, Boehringer Institut, Vienna, Austria; GZ 4 (IFN-gamma)[10] was provided by Dr. Zahn, Thomae Research Laboratories, Biberach, FRG; and Okt 6 (anti-CD 1) was a product of Ortho Diagnostic Systems GmbH, Neckargemünd, FRG. Rabbit anti-mouse immunoglobulin antiserum was a product of Dakopatts, Copenhagen, Denmark. Alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes were prepared according to Cordell.[11]

All primary antibodies were of IgG_1 subclass and used at concentrations between 0.6 and 33 μg/ml. The secondary antibody used was exhaustively preabsorbed with insolubilized human serum proteins. It showed no cross-reactivity with any human tissue constituent.

Immunostaining

Cryostat frozen sections, cut to 4 to 6 μm, were fixed in acetone for 30 minutes, followed by fixation in chloroform for 30 minutes. After fixation the sections were incubated with the monoclonal antibody for 30 minutes and immunostained according to the APAAP method[11] with New Fuchsin development. Sections were preincubated with rabbit normal serum for 30 minutes to block nonspecific binding of the monoclonal antibodies to Fc receptors. Finally, slides were counterstained with hematoxylin and mounted.

All immunostaining samples were controlled by 1) the use of secondary reagents only to confirm their species specificity; 2) the development of alkaline phosphatase alone to preclude staining due to endogenous enzyme activity; and 3) the use of murine primary control monoclonal antibodies, eg, mouse antibody directed toward the cell proliferation antigen detected by Ki 67.[12] All control assays consistently yielded the expected negative results and thus are not mentioned further. A semiquantitative analysis of the stained tissues was performed by counting a minimum of three microscopic high-power fields.

Results

The results of immunostainings of skin biopsies from 57 patients with various forms of leprosy with antibodies against IL-1β, TNF-alpha, and IFN-gamma are summarized in Table 2. Generally there was an increase of cytokine-positive cells located in the granulomas from lepromatous forms to tuberculoid forms of leprosy. In biopsies of untreated lepromatous leprosy patients, only a few IL-1β–positive cells were detectable, while no TNF-alpha– or IFN-gamma–positive cells could be demonstrated by means of immunohistologic study (Figure 1). Similar results were seen in the borderline lepromatous forms. Undergoing chemotherapy, cytokine-positive cells, predominantly IL-1β reactive cells, increased to a detectable

Table 2. *Cytokine-positive Cells in Skin Biopsies of Untreated and Treated Leprosy Patients*

	Cells Positive for		
	IL-1β	TNF-alpha	IFN-gamma
Untreated LL	±	−	−
Treated LL	+	±	±
Untreated BL	+	−	−
Treated BL	++	±	±
Untreated BB	−	−	−
Treated BB	−	−	−
Untreated BT	±	±	±
Treated BT	+	±	±
Untreated TT	+++	++	++
Treated TT	+++	±	±

LL, lepromatous leprosy; BL, borderline lepromatous leprosy; BB, borderline leprosy; BT, borderline tuberculoid leprosy; TT, tuberculoid leprosy.
(−), negative; (±), single positive cells; (+), 0%–5% positive cells; (++), 5%–20% positive cells; (+++), >20% positive cells.

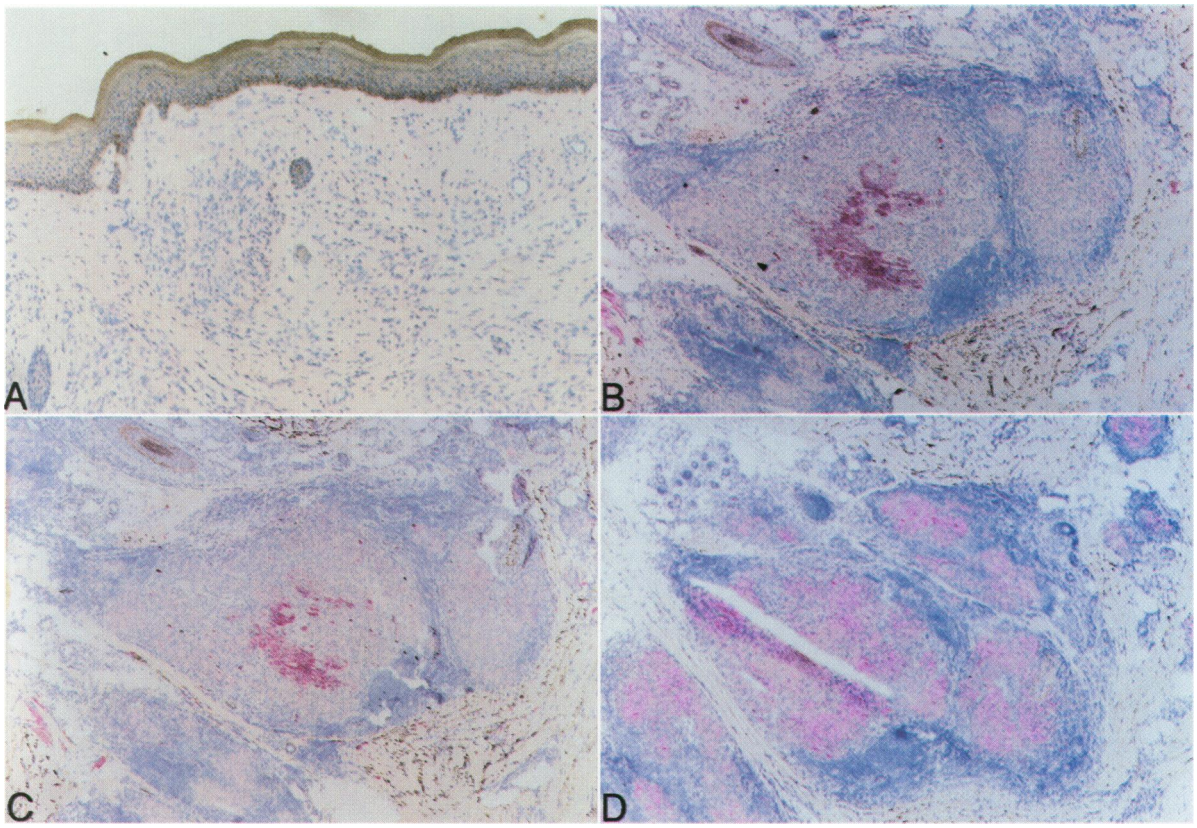

Figure 1. *Representative immunohistologic assessment of cytokines in a case of untreated lepromatous leprosy* (A) *and in a case of tuberculoid leprosy* (B–D). *As demonstrated in* A *for TNF-alpha, no cytokines could be demonstrated in cases of untreated lepromatous leprosy. Epitheloid cells in the center of the granuloma exhibit IL-1β* (B) *and TNF-alpha* (C) *reactivity. IFN-gamma–positive cells* (D) *were distributed throughout the granuloma (APAAP staining, hematoxylin counter staining, ×20).*

amount in lepromatous and borderline lepromatous forms.

In cases of mid-borderline leprosy, no cytokine production could be immunoenzymatically detected. Granulomas in biopsies classified as borderline tuberculoid leprosy contained only a few cytokine-positive cells, whereas granulomas of untreated tuberculoid patients contained large amounts of material that could be immunostained using the monoclonal antibodies against IL-1β, TNF-alpha, and IFN-gamma.

As shown in Figure 1, IL-1β– and TNF-alpha–positive cells were located in the center of different granulomas, while IFN-gamma–positive cells were distributed throughout the granulomas. As revealed by light microscopy, most of the IL-1β–positive cells appeared to be positive also for TNF-alpha. Remarkably, in patients with tuberculoid leprosy undergoing chemotherapy, the number of IL-1β–positive cells did not vary, while the number of TNF-alpha and IFN-gamma reactive cells decreased.

In accordance with reports from Collings[13] and Narayanan,[14] the distribution of Langerhans cells was characteristically different in tuberculoid to lepromatous forms of leprosy. As shown in Figure 2, in tuberculoid forms of leprosy we could demonstrate large amounts of subepidermal CD-1–positive cells. In cases of lepromatous leprosy, no CD-1–positive subepidermal cells were observed, whereas under chemotherapeutic treatment a considerable amount of subepidermal Langerhans cells could be detected.

Discussion

The aim of our study was to immunohistologically assess cytokine production in skin biopsies from various forms of leprosy. In untreated cases of lepromatous leprosy, only a very small number of IL-1β–positive cells could be detected, while TNF-alpha and IFN-gamma reactivity was virtually absent. Interestingly these findings correlate with our recent *in vitro* observations that demonstrate the low capacity of *M. leprae* to induce TNF-alpha in macrophages compared to other species of mycobacteria.[15]

In patients undergoing chemotherapy, cytokine production increased to a detectable amount.

In contrast to observations of Volc-Platzer et al,[16] who demonstrated a significant number of IL-1β–positive macrophages in lepromatous lesions and a lack of IL-1β reactivity in cellular infiltrates of tuberculoid leprosy our finding

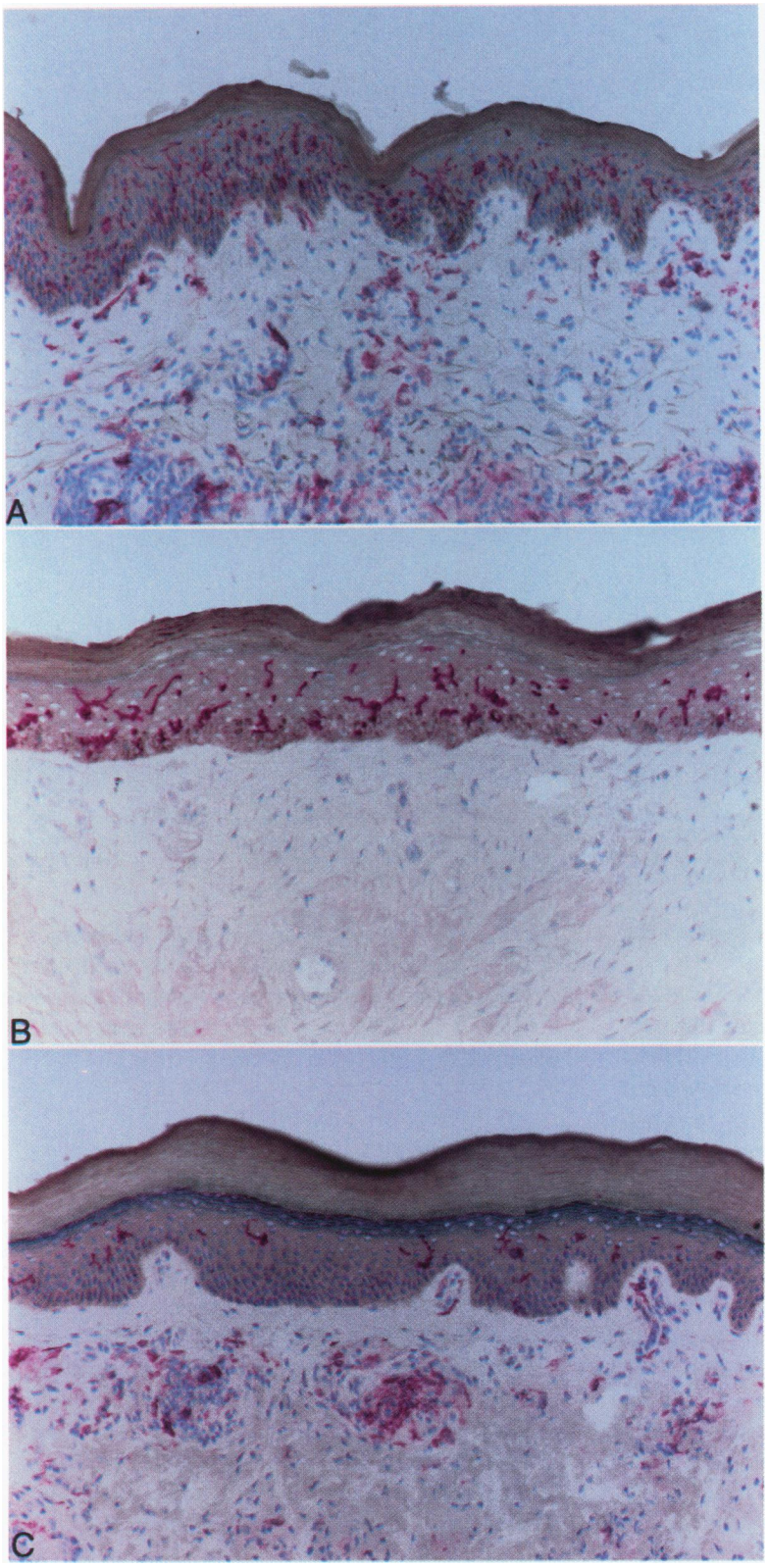

Figure 2. *Immunostaining of Langerhans cells in representative skin lesions from patients with leprosy (APAAP staining, hematoxylin counterstaining, ×60).* **A:** *Presence of subepidermal Langerhans cells in a case of untreated tuberculoid leprosy.* **B:** *Lack of subepidermal Langerhans cells in an untreated case of lepromatous leprosy.* **C:** *Presence of subepidermal Langerhans cells in a case of lepromatous leprosy being treated with chemotherapy.*

of a failure of IL-1β reactivity in lepromatous lesions suggests that this part of the macrophage function may be defective in lepromatous leprosy. Using the monoclonal antibody Fib 3, we can demonstrate large amounts of IL-1β reactivity in cellular infiltrates from tuberculoid lesions, which suggests an intact macrophage activation and stimulation of T lymphocytes. While Volc-Platzer demonstrated IFN-gamma reactivity in epitheloid cells located in the center of granulomas, our observations suggest a more distributed arrangement of these positive cells, as demonstrated in Figure 1.

Using CD 1 as a marker for Langerhans cells, we confirmed and extended the recent findings of Collings[13] and Narayanan[14,17] that the subepidermal distribution of CD-1–positive cells is characteristic of the different forms of leprosy. In addition, our findings demonstrate that lepromatous leprosy patients treated with chemotherapy had a remarkable increase of CD-1–positive subepidermal cells when compared with untreated patients. In the light of our findings, it is tempting to speculate that chemotherapy has restored the capacity of Langerhans cells to bind antigen, to migrate to the subepidermal tissue, and subsequently to present the antigen to other infiltrating cells, resulting in the initiation of the cytokine cascade.

In conclusion, our results demonstrate that skin lesions of patients with various forms of leprosy differ with regard to cytokine-producing cells, indicating that the unresponsiveness of lepromatous leprosy patients may be based on lymphokine dysregulation.[3,18] Thus in the light of our findings it is tempting to assume that determination of cytokine production and phenotyping of subepidermal Langerhans cells in skin lesions from human leprosy patients may be used as parameters for the patient's status of cell-mediated immunity and thus may be helpful in monitoring the therapeutic response of leprosy patients. Of course we are aware of the fact that further investigations, using follow-up biopsies from patients before and during chemotherapy, are needed to verify this assumption.

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Acknowledgments

The authors thank Angelika Arnoldi and Susanne Tiedemann for technical assistance.