Reversal reaction in borderline leprosy is associated with a polarized shift to type 1-like Mycobacterium leprae T cell reactivity in lesional skin. A follow-up study

Verhagen, C.E.; Wierenga, E.A.; Buffing, A.A.M.; Chand, M.A.; Faber, W.R.; Das, P.K.

Publication date
1997

Published in
The journal of immunology

Reversal Reaction in Borderline Leprosy Is Associated with a Polarized Shift to Type 1-Like *Mycobacterium leprae* T Cell Reactivity in Lesional Skin

A Follow-Up Study[1]

Claudia E. Verhagen,*† Eddy A. Wierenga,‡ Anita A. M. Buffing,*† Mohamed A. Chand,† William R. Faber,* and Pranab K. Das[2]*†

Borderline leprosy patients often undergo acute changes in immune reactivity that manifest as reversal reaction (RR) in the course of the disease. RR is associated with an exacerbated local delayed-type cellular immune response to *Mycobacterium leprae* and is responsible for severe tissue damage. We investigated whether RR episodes are associated with a change in T cell subsets in the lesional skin with regard to their cytokine secretion profiles. *M. leprae*-responsive T cell lines and thereafter T cell clones (TCC) were generated from the lesional skin of seven untreated borderline leprosy patients (with or without RR) and again from three of these patients experiencing RR during treatment. The phenotypes of the *M. leprae*-responsive TCC were either $CD4^+$, $CD8^+$, $CD4^-/CD8^+/TCR\gamma\delta^+$, or $CD4^-/CD8^-/TCR\gamma\delta^+$, although most of them were $CD4^+$. Regardless of the clinical status of the untreated patients, a major subset of the *M. leprae*-responsive TCC was type 0-like and produced both IFN-γ and IL-4. Interestingly, in all three patients who experienced a (re)occurrence of RR during treatment after the first analysis, a clear shift to polarized IFN-γ production by the *M. leprae*-responsive TCC (type 1-like) was observed. This shift in T cell subsets was also reflected in the observed decrease in serum IgG and IgM levels of the same patients during RR. These finding indicate that $CD4^+$ *M. leprae*-responsive T cells with a polarized type 1-like phenotype might be responsible for the immune-mediated tissue damage occurring during RR. *The Journal of Immunology,* 1997, 159: 4474–4483.

Leprosy, caused by infection with *Mycobacterium leprae*, manifests as a clinical, histopathologic, and immunologic spectrum with two polar forms of pathology (1–3). At one end of the spectrum, tuberculoid leprosy patients are characterized by resistance to infection, strong cell-mediated immunity with the expression of delayed-type hypersensitivity (CMI-DTH),[3] and weak Ab responses to *M. leprae* Ags. Skin lesions of these patients comprise organized granulomas with predominant infiltration of $CD4^+$ T cells, resulting in the elimination of intracellular *M. leprae* by activated macrophages. At the other end, lepromatous leprosy (LL) patients are characterized by a selective absence of *M. leprae*-specific CMI-DTH responses and by abundant production of polyspecific Abs. Skin granulomas of these patients show disorganized inflammatory infiltrates with a predominant presence of $CD8^+$ T cells and bacilli-loaded foamy macrophages. The largest group of patients, however, is recognized as immunologically unstable borderline leprosy; these patients are classified as borderline tuberculoid (BT), mid-borderline (BB), or borderline lepromatous (BL) leprosy. Due to their unstable immunologic status, 20 to 30% of the borderline leprosy patients undergo immunologic changes, known as reactional states, resulting in clinical and pathologic alterations accompanied by tissue damage (4, 5). One such reactional state is known as reversal reaction (RR), which is recognized by a sudden increase in *M. leprae*-specific CMI-DTH responses accompanied by an influx of $CD4^+$ T cells at the lesional site (5–8). The augmentation of T cell reactivity in RR patients parallels the severe and often irreversible destruction of peripheral nerves (9, 10).

The subdivision of $CD4^+$ T lymphocytes into functionally distinct subsets, e.g., Th1, Th0, and Th2 cells, in both animals and humans has provided a basis for understanding the regulatory role of T cells in intracellular infectious diseases, allergic manifestations, and autoimmune diseases (11–18). Th1 cells, producing IL-2, IFN-γ, and lymphotoxin, sustain CMI-DTH responses, whereas Th2 cells, producing IL-4, IL-5, IL-6, and IL-10, provide B cell help for Ab production. The intermediate subset of pluripotential Th0 cells secretes both type 1- and type 2-related cytokines (19).

Departments of *Dermatology and †Pathology, and ‡Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Received for publication February 19, 1997. Accepted for publication August 4, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Dutch Leprosy Foundation (The Netherlands) and The Q. M. Gastmann Wichers Foundation (The Netherlands) and was conducted under the research programs of ODP/DE1 and ODP/PA2 of The Van Loghem Immunology Institute of the Faculty of Medicine, Academic Medical Center, University of Amsterdam. C.E.V. and A.A.M.B. are recipients of Dutch Leprosy Foundation maintenance grants; E.A.W. received an Independent Research Fellowship from The Royal Netherlands Academy of Arts and Sciences. Preliminary reports of the present work were presented at the following meetings: Joint Meeting of Dutch and British Societies of Immunology, 1995, Brighton, England (*Immunology 86(Suppl. 1):41 (Abstr.)*) and the Third Conference on the Pathogenesis of Mycobacterial Infections, 1996, Stockholm, Sweden.

[2] Address correspondence and reprint requests to Dr. Pranab K. Das, Research Laboratory Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. E-mail address: C.E.Verhagen@AMC.UVA.NL

[3] Abbreviations used in this paper: CMI-DTH, cell-mediated immunity/delayed-type hypersensitivity; LL, lepromatous leprosy; BT, borderline tuberculoid; BB, mid-borderline; BL, borderline lepromatous; RR, reversal reaction; BI, bacterial index; TCL, T cell line; TCC, T cell clone; SI, stimulation index.

0022-1767/97/$02.00

Table I. *Clinical classification of untreated and treated leprosy patients*

Untreated Patients					Treated Patients			
Class	No.	Reactional state (if any)	Lepromin/ Mitsuda (mm)[a]	BI[b]	Treatment	Reactional state (if any)	Months after onset of treatment	BI
BT	P1	None	4 mm	0	MDT	None		
BT	P2	None	8 mm	0	MDT	None		
BT	P3	None	6 mm	0	MDT	None		
BL	P4	None	0 mm	4+	MDT	1st RR	2 mo	0
BL	P5	None	0 mm	4+	MDT	None		
BT	P6	1st RR	NT	4+	MDT/pred	2nd RR[c]	9 mo	0
BB	P7	1st RR	NT	1+	MDT/pred	2nd RR	19 mo	0

[a] Twenty-eight days after injection of whole sonicated *M. leprae* Ags (lepromin H) in uninvolved skin, the erythematous skin reaction was measured in mm.
[b] Fite-Faraco-Wade in situ staining was used to determine the BI (0–6+).
[c] Patient P6 experienced a second RR episode after stopping the prednisone treatment with continuation of multiple drug therapy (MDT).
NT, not tested; pred, prednisone.

It has previously been demonstrated that the vast majority of *M. leprae*-responsive T cell clones (TCC) generated from PBMC or lesional skin of polar paucibacillary tuberculoid leprosy patients with high levels of CMI-DTH responses belong to the subsets of $CD4^+$ IFN-γ producing type 1-like T cells (13, 14), whereas $CD8^+$ IL-4 producing type 2-like TCC predominate in the panel generated from skin lesions of polar multibacillary LL patients (14). This type 2-like $CD8^+$ T cell subset was shown to suppress the *M. leprae*-induced immune reactivity of $CD4^+$ TCC in vitro, although the regulatory role for IL-4 in this suppressive effect could not be confirmed by other investigators (20). On the other hand, recent studies demonstrated that *M. leprae*-responsive T cells from skin or PBMC of leprosy patients may show concomitant expression of IFN-γ and IL-4 (type 0) regardless of their clinical status (21, 22). One reason for the conflicting results between different studies may be discrepancies in the classification of patients due to the difficulties in discriminating between borderline and polar leprosy. An appropriate strategy, therefore, for studying the distinct role of *M. leprae*-responsive T cell subsets in relation to the immunopathologic spectrum of leprosy would be to analyze the cytokine secretion profiles of lesional skin T cells from unstable borderline patients and again when these patients experience immunologic changes in the form of reactional episodes during the course of the disease. The relevance of investigating T cells of lesions is that these cells, due to local Ag-induced activation, probably reflect immune responses closely related to tissue damage (23, 24).

The present study focuses on exploring whether a change in *M. leprae*-responsive T cell subsets in the lesional skin by virtue of shifts in local production of cytokines is associated with RR in borderline leprosy patients. The IFN-γ and IL-4 secretion profile of skin-derived TCC from untreated borderline patients (with or without RR) and that from the same patients experiencing a RR during treatment were studied. We observed that the *M. leprae*-responsive TCC from patients with a RR episode during treatment showed a clear polarized shift to a type 1-like phenotype. These data provide evidence that the host's immune status shifts to increased CMI-DTH during RR, which may play a role in the mechanisms leading to tissue damage.

Materials and Methods

Clinical aspects of the patients studied

Lesional skin biopsies were obtained from a total of seven untreated leprosy patients attending the Dermatology Clinic of our institute. The patients were diagnosed on the basis of the clinical and histopathologic criteria of Ridley and Jopling (1). The bacterial index (BI) and CMI-DTH status of the patients was determined by Fite-Faraco-Wade staining (25) and a Lepromin test (26), respectively, to support the clinical classification. Clinical criteria used for the diagnosis of RR were erythematous swelling of the existing lesions, appearance of new lesions, and onset or worsening of neuritis. The clinical details of the patients studied are summarized in Table I. At the entry of the study were three BT, one BT with RR, one BB with RR, and two BL patients. After obtaining the lesional skin biopsies and heparinized peripheral blood, the patients were given multiple drug therapy. The treatment durations for paucibacillary patients (BI = 0) and multibacillary patients (BI = 1–4+) were a minimum of 6 and 24 mo, respectively. Patients experiencing RR were treated with prednisone. Additional lesional skin biopsies were obtained from three patients (P4, P6, and P7) who experienced RR during the course of treatment. P6 showed a reoccurrence of RR episode due to failure in compliance with prednisone treatment.

Processing of skin biopsies

The fresh lesional skin biopsies (diameter, 4 or 6 mm) were cut in two; one-half was frozen in liquid nitrogen for histopathologic and immunohistochemical analysis, and the other half was used for isolation of infiltrated T cells.

Generation of skin-derived T cell lines (TCL) and TCC

Our study was designed first to generate primary lesional skin-derived TCL with the maintenance of in vivo distribution of phenotypes, followed by isolation of TCC by limiting dilution. The fresh biopsy was incubated in a well of a 24-well culture plate (Costar, Cambridge, MA) that was precoated with fibronectin (10 μg/well; 2 h at 37°C; Sigma Chemical Co., St. Louis, MO) to facilitate the spontaneous migration of infiltrated T cells from the tissue into the culture medium. The culture medium consisted of Iscove's modified Dulbecco's medium (Life Technologies, Paisley, U.K.) supplemented with 10% pooled complement-inactivated NHS (BioWhittaker, Walkersville, MD), 1 mM glutamine (Life Technologies), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). After 3 to 5 days, the migrated T cells were transferred to a new 24-well plate and expanded by mitogenic stimulation with 0.05% PHA (Difco, Detroit, MI) in the presence of 3000-rad-irradiated allogenic feeder cells comprising of PBMC (10^6/well) from two unrelated donors, EBV-transformed B cells (JY; 5×10^4/well), and 10 U/ml rIL-2 (a gift from Eurocetus, Amsterdam, The Netherlands). After 10 days of expansion, the TCL were analyzed for their T cell subset (CD4, CD8, and TCRγδ) constitution and for their responsiveness to *M. leprae* using an Ag-induced stimulation assay as described below. In identical *M. leprae*-induced parallel cultures, rIL-2 (10 U/ml) was added on day 4 to further promote expansion of the *M. leprae*-responsive T cells. After another 8 days, the expanded T cells were cloned by limiting dilution using a protocol described previously (27). After 10 to 17 days, individual TCC were further expanded by mitogenic stimulation in the presence of an irradiated allogenic feeder cell mixture as described above. After 10 days of expansion, TCC were screened for their *M. leprae* responsiveness and further characterized.

M. leprae *responsiveness of TCL and TCC*

The *M. leprae* reactivity of TCL was assayed by culturing 5×10^4 T cells in 100 μl of culture medium in a U-bottom 96-well plate for 4 days in either the absence or the presence of *M. leprae* Ags (5 μg/ml; a preparation of whole sonicated *M. leprae*, lot WHO-CD143, a gift from Dr. M. J.

Table II. *Phenotypic composition and* M. leprae *(MI) responsiveness of lesional skin derived T cell lines generated from untreated borderline leprosy patients (without or with RR) and again from the same patients (re-)experiencing RR during treatment*[a]

	Patient No.	Diagnosis	BI	T Cell Subsets[b]			Poliferation cpm [^{3}H] $\times 10^{3c}$		
				CD4$^+$ (%)	CD8$^+$ (%)	TCRγ/δ^+	−MI	+MI	SI
Untreated	P1	BT	0	85	13	4	0.1	0.8	8
	P2	BT	0	75	25	<1	0.1	1.4	14
	P3	BT	0	75	20	NT	NT	NT	
	P4	BL	4+	17	69	14	0.3	8.4	28
	P5	BL	4+	55	35	10	0.3	2.2	7
	with RR								
	P6	BT	4+	18	45	41	0.2	5.0	25
	P7	BB	1+	80	10	10	0.4	2.6	7
Treated	with RR								
	P6	BT	0	47	23	30	0.1	20.1	201
	P7	BB	0	68	15	13	0.1	25.1	251
	P4	BL	0	56	49	1	0.3	12.1	40

[a] Ten days after the last mitogenic stimulation with an allogenic feeder cell mixture, TCL were used for parallel analyses.
[b] Results were obtained by immunohistochemical single staining of cytospin preparations of TCL that were stained with mAb to CD4, CD8, or TCRγ/δ.
[c] TCL were incubated in the presence or absence of whole sonicated *M. leprae* Ags and autologous PBMC as APC for 72 h, followed by another 16 h incubation with [^{3}H]thymidine. Results are expressed as mean cpm $\times 10^3$ of triplicate cultures. The SI represents the mean cpm of culture +MI divided by the mean cpm of cultures −MI. NT, not tested.

Colston) and 3000-rad-irradiated autologous PBMC (10^5 cells/well) as a source of APC. TCC were tested for *M. leprae* responsiveness under similar conditions but with 5×10^4 autologous PBMC and only for 40 h. In both assays, [^{3}H]thymidine (0.3 μCi/well; Amersham International, Aylesbury, U.K.) was present in the cultures for the last 16 h. Cultures were harvested, and incorporated radioactivity was measured by liquid scintillation counting. The results are expressed as the mean counts per minute of triplicate cultures. The stimulation index (SI) is the mean counts per minute of cultures in the presence of *M. leprae* Ags divided by the mean counts per minute of parallel cultures in the absence of Ags. A culture was considered responsive to *M. leprae* if incorporated radioactivity reached >800 cpm and/or the SI was 10 or higher.

Cytokine production of TCC

To assay Ag-induced cytokine production of TCC, 60 μl of cell-free culture supernatants were collected after 24 h of Ag-specific stimulation, as described above, and stored at −80°C until tested. Cytokine production of TCC was also assayed after mitogenic stimulation by incubating 10^5 cells with immobilized anti-CD3 mAb (OKT3; 1 mg/ml; American Type Culture Collection, Rockville, MD) and soluble PMA (1 ng/ml; Sigma Chemical Co.) in a total volume of 200 μl/well in a flat-bottom 96-well plate (17). After 24 h, 100 μl of cell free supernatants were collected and stored at −80°C until tested. In both assays, the remaining cultures were incubated for an additional 16 h in the presence of [^{3}H]thymidine (0.3 μCi/well) to confirm stimulation.

Measurement of IFN-γ and IL-4

Estimation of IL-4 and IFN-γ concentrations in the supernatants of stimulated TCC and in the supernatant of irradiated autologous PBMC was performed with a specific solid phase sandwich ELISA as described previously (28, 29). The anti-IFN-γ mAb (MD2 and MD1) used in this study were purchased from Innogenetics (Ghent, Belgium). The detection limit for IL-4 was 40 pg/ml, and that for IFN-γ was 50 pg/ml.

Phenotyping of lesional T cells in situ and in cultures

An immunohistochemical single staining on frozen biopsy sections (6 μm) and cytospin preparations of TCL was performed with a streptavidin/biotin immunoperoxidase method (30). Briefly, sections were preincubated with sodium azide to inhibit endogenous peroxidase activity, followed by incubation with normal goat serum (Dakopatts, Glostrup, Denmark). Primary mouse mAb to CD3 (Becton Dickinson, Mountain View, CA), CD4 (Becton Dickinson), CD8 (Dakopatts), and TCR$\gamma\delta$ (T Cell Diagnostics, Woburn, MA) were applied to the specimens, followed by consecutive incubation with biotinylated rabbit anti-mouse Ig (Dakopatts) and streptavidin-biotinylated horseradish peroxidase complex (Dakopatts). Peroxidase activity was visualized using 3-amino-9-ethyl carbazole (Sigma Chemical Co.) as a substrate, and the samples were counterstained with hematoxylin. The percentages of CD4$^+$, CD8$^+$, and TCR$\gamma\delta^+$ T cells in the TCL were determined by light microscopy by counting at least 500 to 1000 cells in the cytospin preparation. The percentages of CD8$^+$ and TCR$\gamma\delta^+$ T cells within the CD3$^+$ T cell population in the lesions were determined by light microscopy in three randomly selected fields of sequential sections. The CD4$^+$ T cell subset within the CD3$^+$ T cell population was not determined by single staining methods due to cross-reactivity with other cells types, i.e., monocytes and Langerhans cells. The percentage of CD4$^+$ T cells was calculated by subtracting the percentages of CD8$^+$ and TCR$\gamma\delta^+$ from that of CD3$^+$ cells. The cell typing of TCC was performed by FACS analysis (FACScan, Becton Dickinson) or fluorescence microscopy. Suspended cells (5×10^4) were either directly labeled with FITC-conjugated mouse mAb to CD4 (Becton Dickinson) or CD8 (Dakopatts) or with an unlabeled mouse mAb to TCR$\gamma\delta$ followed by FITC-conjugated rabbit anti-mouse Ig (Dakopatts).

Measurement of serum Ig levels (IgM, IgE, total IgG, and the IgG subclasses)

The serum concentration of polyclonal IgM was determined by a Behring Nephelometer-Analyzer, and polyclonal IgE was determined by ELISA (31). Levels of total IgG and the IgG subclasses were determined as described previously (32).

Statistics

Two group comparisons of IFN-γ/IL-4 ratios were analyzed by the Mann-Whitney test. Values were considered significantly different at $p < 0.05$.

Results

Characterization of lesional skin-derived TCL from borderline leprosy patients

TCL were generated from one part of the lesional skin biopsies of seven untreated borderline leprosy patients (P1–P7) and again from the lesions of three of these patients (P4, P6, and P7) at the time they experienced a (re)occurrence of RR during treatment. The compositions of the various T cell types (CD4$^+$, CD8$^+$, and TCR$\gamma\delta^+$) in the in vitro cultured TCL are summarized in Table II. All TCL consisted of variable percentages of CD4$^+$, CD8$^+$, and TCR$\gamma\delta^+$ T cells. To compare the compositions of T cell types in the in vitro TCL with the in situ distribution in the biopsy, immunohistochemical analyses were performed on sequential sections of the frozen counterparts of the biopsies. The distributions of T

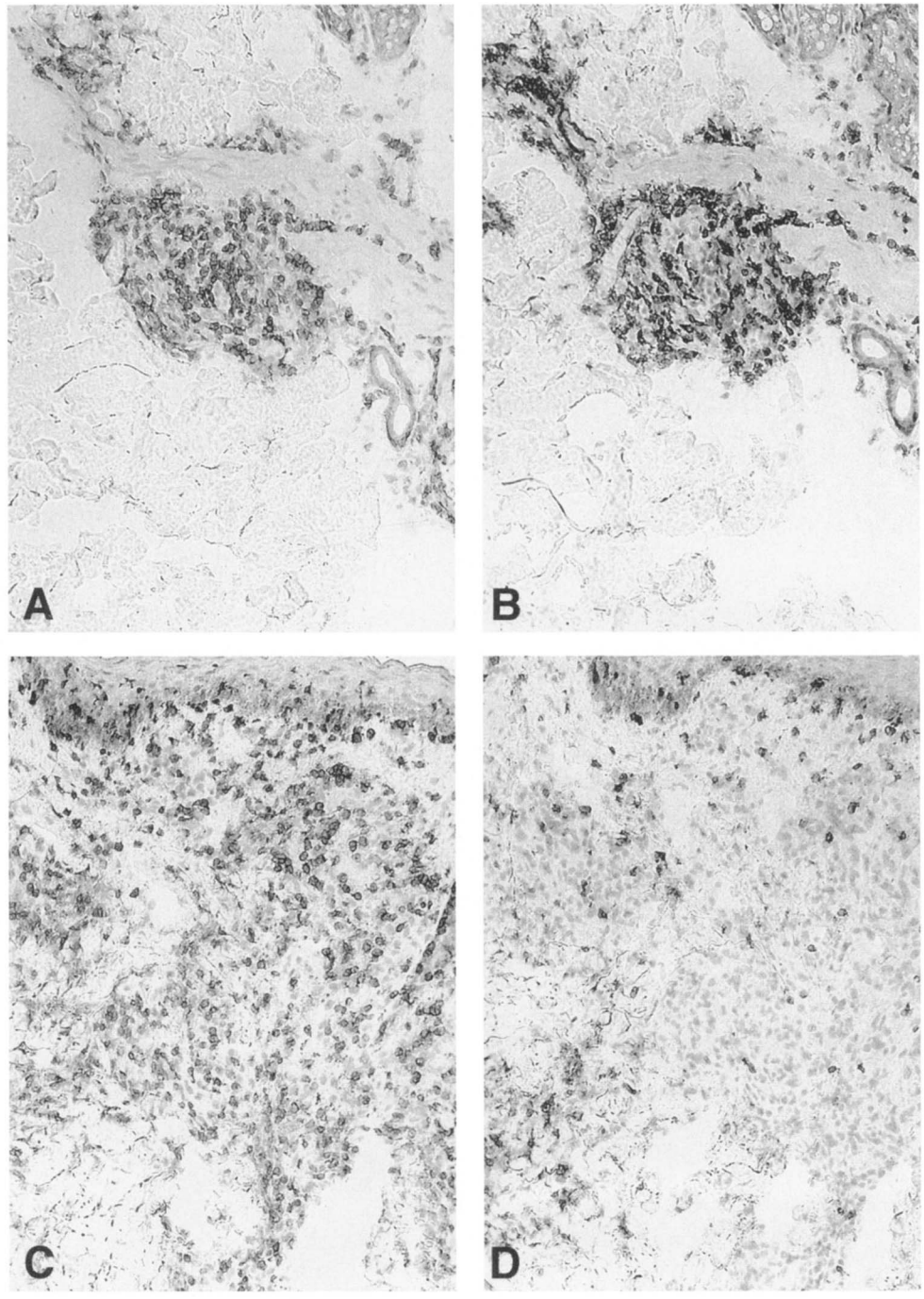

FIGURE 1. Representative examples of immunohistochemical analysis of lesional skin biopsies from a borderline leprosy patient before treatment and with RR during treatment. Sequential skin sections were stained with mAb to CD3 (*A* and *C*) or CD8 (*B* and *D*). The sections were counterstained with hematoxylin (magnification, ×115). Sequential sections of the lesional skin of P4 (BL) before treatment (*A* and *B*) show that a significant percentage of the $CD3^+$ cells in the lesions are $CD8^+$. Sequential sections of the lesional skin of P4 experiencing RR during treatment (*C* and *D*) show that fewer $CD3^+$ cells were $CD8^+$ compared with the untreated situation.

cell types in both the TCL and the counterpart biopsy were similar for each patient (data not shown). Interestingly, experiencing RR in the course of the disease appeared to be accompanied by an increase in the percentage of $CD4^+$ T cells (see Table II and Fig. 1). In general, $CD4^+$ T cells predominated in untreated paucibacillary BT (P1, P2, and P3) and RR lesions (P7) as well as in treated RR lesions (P4, P6, and P7), whereas $CD8^+$ T cells were usually found in significant numbers in untreated multibacillary (BI = 4+; P4, P5, and P6) lesions. As an exception, TCL generated from the untreated and treated lesions of P6 consisted of a significant number of $TCR\gamma\delta^+$ T cells (41 and 30%, respectively) that were not seen in such high numbers in the lesions from which they originated.

The TCL were analyzed for *M. leprae* responsiveness by testing their abilities to proliferate upon stimulation with whole sonicated *M. leprae* in the presence of autologous PBMC as APC. As presented in Table II, all lines were *M. leprae* responsive, with varying SI. The SI appeared to be relatively higher in lines isolated from lesional skin with RR during treatment, suggesting a higher frequency of responding T cells in these lesions.

Table III. *Phenotypic distribution of* M. leprae*-responsive and -nonresponsive T cell clones generated from lesional skin-derived* M. leprae*-responsive TCL of untreated borderline leprosy patients (without or with RR) and of the same patients when experiencing RR during treatment*[a]

	Patient No.	Diagnosis	Number of *M. leprae*-responsive TCC/Total Number of TCC[b]				% $CD4^+$ of *M. leprae* responsive TCC
			Total	$CD4^+$	$CD8^+$	TCR-$\gamma\delta^+$	
Untreated	P1	BT	40/72	40/71	0/1	0/0	100
	P2	BT	19/41	17/27	2/14	0/0	89
	P4	BL	14/80	11/19	3/58	0/3	79
	P5	BL	0/77	0/41	0/20	0/16	
	with RR						
	P6	BT	34[c]/59	30/38	0/0	0/4	[c]
	P7	BB	45/77	45/77	0/0	0/0	100
Treated	with RR						
	P6	BT	12/87	11/15	1[d]/41[e]	1[d]/31[e]	92
	P7	BB	33/69	33/62	0/4[d]	0/4[d]	100
	P4	BL	36/47	32/35	1/9	3/3	89

[a] Ten days after the last mitogenic stimulation (0.05% PHA) with an allogenic feeder cell mixture, TCC were used for parallel analyses.
[b] TCC were phenotyped by flow cytometry after being stained with mAb to CD4, CD8, and TCR-γδ, and *M. leprae* responsiveness was assayed by incubation of TCC in the presence or absence of whole sonicated *M. leprae* Ags and autologous PBMC as APC for 40 h, followed by another 16-h incubation with [^{3}H]thymidine.
[c] Within this panel, 17 TCC were not phenotyped, of which 4 were *M. leprae* responsive.
[d] One TCC is double positive for CD8 and TCR-γδ.
[e] Three TCC are double positive for CD8 and TCR-γδ.

Generation and analysis of M. leprae*-responsive TCC*

To investigate the *M. leprae*-responsive T cell subset within the lesional TCL, TCC were generated by limiting dilution. The cloning procedure allowed the generation of TCC of different phenotypes ($CD4^+$, $CD8^+$, and TCRγ/δ^+), as summarized in Table III. In most cases, except P6, the phenotypic distribution of TCC reflected that in the original lines (Table II). Within the panel of TCC from P6 (untreated), 17 TCC (labeled b) did not grow in sufficient quantities for phenotypic analysis. This may tentatively explain the discrepancy between the phenotypic distribution among TCC and the TCL in this particular case. All TCC were screened for *M. leprae* responsiveness by testing their abilities to proliferate upon stimulation with whole sonicated *M. leprae* presented by autologous PBMC as APC. With the exception of P5, *M. leprae*-responsive TCC were generated from the TCL of all patients, as shown in Table III. Regardless of the clinical status of the patient, the majority of the *M. leprae*-responsive TCC was $CD4^+$ (between 79–100%). All *M. leprae*-responsive TCC with $CD4^-/CD8^+$/TCR$\gamma\delta^+$ or $CD4^-/CD8^-$/TCR$\gamma\delta^+$ phenotypes were generated from patients with RR during treatment (P4 and P6).

IFN-γ and IL-4 levels of M. leprae*-responsive TCC from untreated borderline leprosy patients*

To determine the type 1-, type 0-, or type 2-like phenotypes of the *M. leprae*-responsive TCC, their IFN-γ and IL-4 production upon Ag-induced stimulation was measured by ELISA. In every experiment the production of IFN-γ and IL-4 by irradiated autologous PBMC alone (in the presence of Ag) was below the detection threshold. Figure 2 shows the absolute IFN-γ and IL-4 production levels of the *M. leprae*-responsive TCC from the untreated borderline patients. A small number of *M. leprae*-responsive TCC produced both cytokines in quantities <200 pg/ml and were excluded from this figure. Regardless of the clinical status of the untreated patient, the majority of TCC produced both IFN-γ and IL-4 (type 0-like). However, a considerable number of TCC from BT patients (P1, P2, and P6, with or without RR) predominantly produced IFN-γ over IL-4 (type 1-like), whereas a number of TCC from a BL patient (P4) produced dominantly IL-4 over IFN-γ (type 2-like). From the BL patient, two $CD8^+$ TCC were generated that produced predominantly IFN-γ. These results indicate that in the lesions of untreated borderline leprosy patients $CD4^+$ *M. leprae*-responsive type 0 T cells are the main subset, whereas a predominant presence of type 1-like or type 2-like $CD4^+$ *M. leprae*-responsive T cells within the lesions may parallel the CMI-DTH status of the patient.

M. leprae*-responsive TCC from patients with RR shift to the type 1-like cytokine secretion profile*

The CMI-DTH status of borderline patients strongly increases during RR (5–7). In this respect, a change in local T cell subsets, characterized by a shift in the cytokine secretion profile, may be associated with developing RR in the lesions. To test this assumption, the IFN-γ/IL-4 secretion profiles of the *M. leprae*-responsive TCC from the lesions of three patients (P4, P6, and P7) who experienced a (re)occurrence of RR during treatment were compared with those from lesions of the same patients before treatment. Since nonspecific stimulation (with anti-CD3 mAb plus PMA) of the *M. leprae*-responsive TCC gave similar IFN-γ/IL-4 ratios but induced higher absolute cytokine levels compared with *M. leprae*-induced stimulation (see Fig. 2) and to minimize the requirement of autologous PBMC, we analyzed the cytokine profiles of the *M. leprae*-responsive TCC upon mitogenic stimulation, as presented in Figure 3. In all three patients the cytokine secretion profiles of the *M. leprae*-responsive T cell population shifted to dominant IFN-γ production when experiencing RR during the course of treatment. We considered TCC that produced IFN-γ but no IL-4 (<40 pg/ml) and TCC with an IFN-γ/IL-4 ratio of > 20 to be type 1-like. According to these criteria, the percentage of type 1-like TCC in the *M. leprae*-responsive T cell population had significantly increased in all three patients during the course of treatment (increase in percentage of type 1-like TCC: P6 from 24 to 42%; P7 from 4 to 89%; P4 from 8 to 71%). Upon further comparative

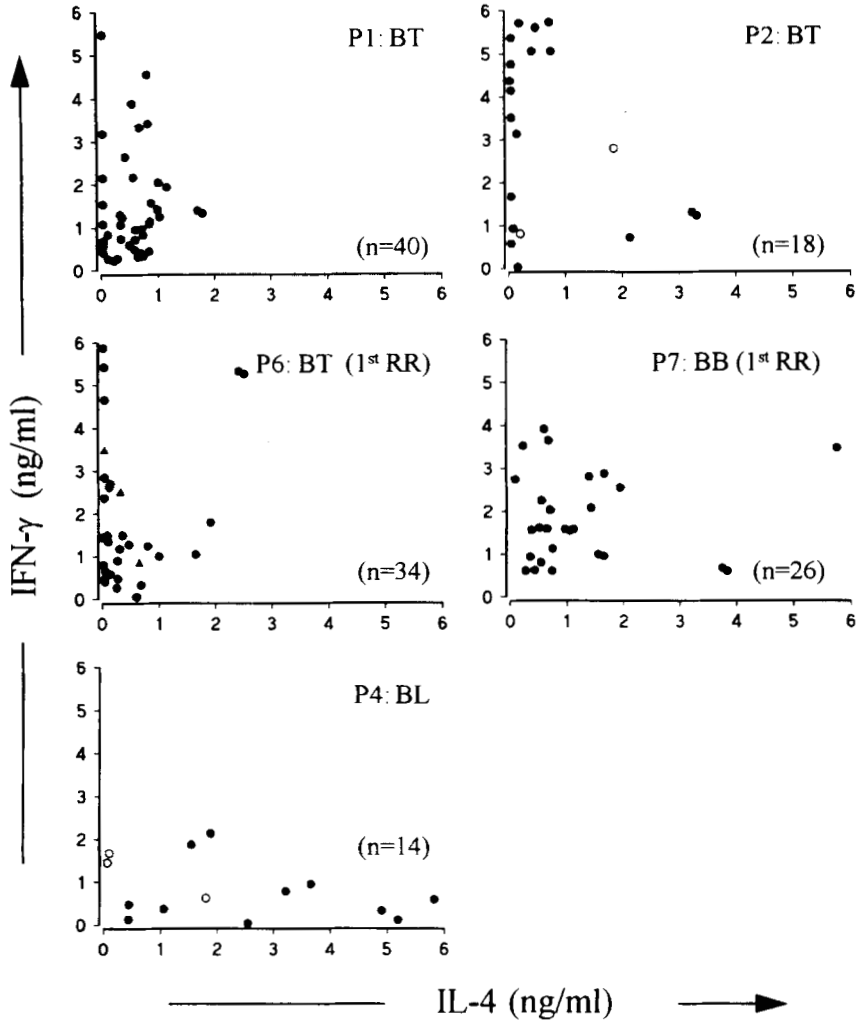

FIGURE 2. The cytokine secretion profiles of *M. leprae*-responsive TCC generated from lesional skin. *M. leprae*-responsive TCC were generated from *M. leprae*-responsive TCL derived from lesional skin biopsies of untreated borderline leprosy patients. Ten days after the last mitogenic stimulation with an allogenic feeder cell mixture, TCC (5×10^4/well) were stimulated with whole sonicated *M. leprae* Ags and irradiated autologous PBMC as APC (5×10^4/well). Twenty-four-hour supernatants were collected and analyzed for IFN-γ and IL-4 production by ELISA. The absolute IFN-γ and IL-4 levels (nanograms per milliliter) secreted by $CD4^+$ (●), $CD8^+$ (○), and those TCC that were not phenotyped (see the text and Table III; ▲) are presented. The production of both IFN-γ and IL-4 by irradiated autologous PBMC alone (in the presence of *M. leprae* Ags) was below the detection level of the assay.

analysis we found that the IFN-γ/IL-4 ratios of the *M. leprae*-responsive TCC from the same patient before treatment and (re)-experiencing RR were significantly different (P6, $p < 0.04$; P7 and P4, $p < 0.001$). The mean values of IFN-γ/IL-4 ratios of the *M. leprae*-responsive T cell populations are shown in Figure 3. This shift to type 1-like immune reactivity occurred despite individual clinical variations with respect to the onset of RR and treatment compliance.

Cytokine secretion profiles of M. leprae*-nonresponsive TCC from the same lesions*

During the cloning procedure, a considerable number of *M. leprae*-nonresponsive TCC ($CD4^+$, $CD8^+$, or $TCR\gamma\delta^+$) were generated from all lesions, as determined by a *M. leprae*-induced proliferation assay (Table III). An alternative readout for *M. leprae* responsiveness, particularly in the case of $CD8^+$ TCC, is the measurement of cytokine production upon incubation with *M. leprae* and autologous APC where a proliferative response was absent (14). We applied this method for all the nonproliferative TCC ($CD4^+$, $CD8^+$, or $TCR\gamma\delta^+$). None of the TCC showed IFN-γ or IL-4 production above the detection level upon incubation with whole sonicated *M. leprae* and autologous APC, and these TCC were considered true nonresponders.

To ascertain whether the shift to a type 1-like phenotype occurs specifically in *M. leprae*-responsive TCC, we also investigated the IFN-γ/IL-4 secretion profile of bystander *M. leprae*-nonresponsive TCC upon nonspecific mitogenic stimulation as an internal control. In contrast to the *M. leprae*-responsive TCC, nonresponsive TCC from all lesions, before or during treatment, were coproducers of IFN-γ/IL-4 in varying proportions and showed heterogenic phenotypes (type 1-, type 0, or type 2-like; data not shown). Furthermore, their IFN-γ/IL-4 ratios were analyzed comparatively with those of the *M. leprae*-responsive TCC generated from the same TCL. We found that the IFN-γ/IL-4 ratios from the nonresponsive TCC differed significantly from those of the *M. leprae*-responsive TCC that were characterized by a polarized phenotype (P6: BT + second RR, $p < 0.001$; P7: BB + second RR, $p < 0.001$; P4: BL untreated, $p < 0.05$).

Serum levels of IgM, IgE, total IgG, and IgG subclasses

It is well recognized that the increased activity of type 1-like T cells may be inhibitory for B cell functions in lymphoid organs and may lead to decreased Ig levels in serum (33, 34). To investigate the consequence of the shift of *M. leprae*-specific T cells toward the type 1-like phenotype during RR, the Ig levels (IgG, IgG isotypes, IgM, and IgE) in the serum of the patients experiencing a RR during treatment were compared with those in the serum of the same patients obtained before treatment. The serum levels of IgG, IgG1, and IgM are presented in Figure 4. We found that untreated multibacillary patients (P4, P6, and P7) had higher levels of IgG and IgM compared with the untreated BT patients (P1, P2, and P3), of which all serum values were within the normal range. When P4,

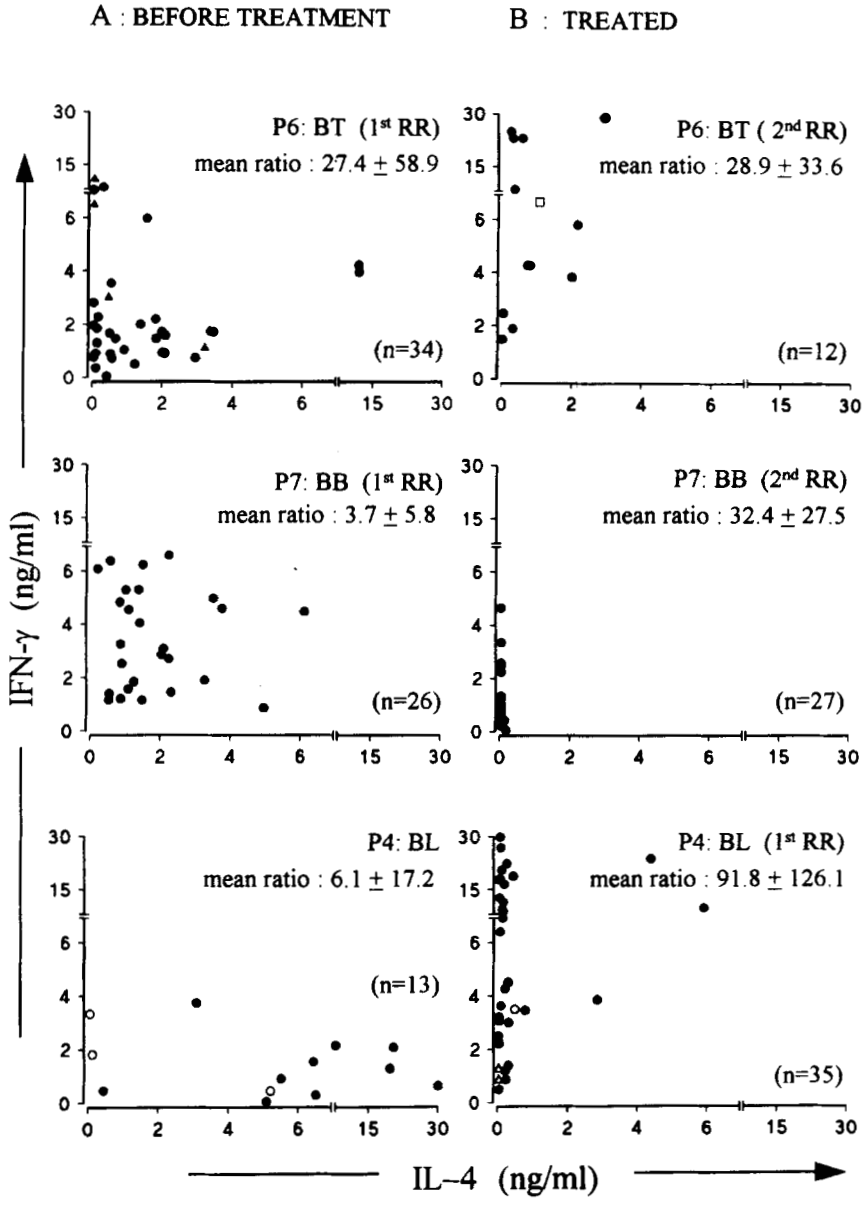

FIGURE 3. The cytokine secretion profiles of *M. leprae*-responsive TCC shift toward type 1-like phenotype during RR. TCC were generated from lesional skin-derived *M. leprae*-responsive TCL from borderline leprosy patients before treatment (*A*) and from the same patients experiencing RR during treatment (*B*). Ten days after the last mitogenic stimulation with an allogenic feeder cell mixture, TCC (10^5/well) were stimulated with PMA and anti-CD3 mAb. Twenty-four-hour supernatants were collected and analyzed for IFN-γ and IL-4 production by ELISA. The absolute IFN-γ and IL-4 levels (nanograms per milliliter) produced by $CD4^+$ (●), $CD8^+$ (○), $CD4^-/CD8^-/TCR\gamma\delta^+$ (△), $CD8^+/TCR\gamma\delta^+$ (□), and TCC with unidentified phenotype (see the text and Table III; ▲) are presented. Mean (±SD) IFN-γ/IL-4 ratios are given. The IFN-γ/IL-4 ratios differed significantly between the *M. leprae*-responsive TCC from the same patient before treatment and with (re-)experiencing RR (P6, $p < 0.04$; P7 and P4, $p < 0.001$).

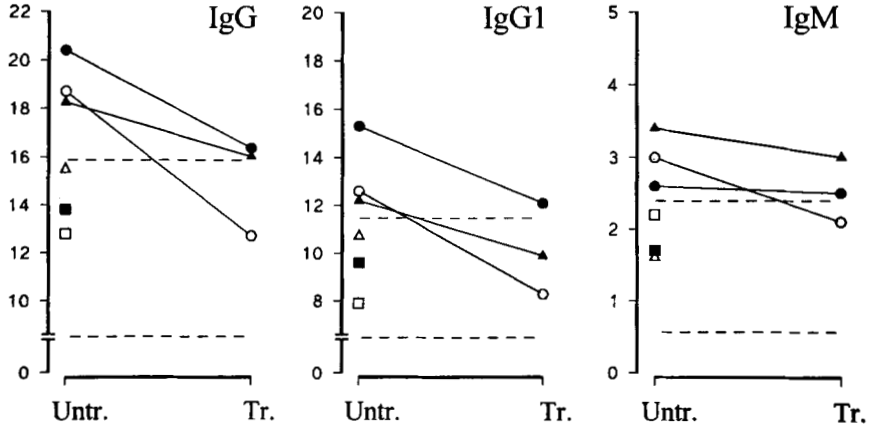

FIGURE 4. Decrease in Ig levels (IgG, IgG1, and IgM) in the serum of untreated multibacillary borderline leprosy patients when experiencing RR during treatment. IgG, IgG1, and IgM serum levels of three paucibacillary patients (P1:BT, □; P2:BT, ■; P3:BT, △) and three untreated multibacillary patients and again when these patients experienced RR during treatment (P6:BT, ●; P7:BB, ○; P4:BL, ▲) are presented. The area in between the dashed lines is the normal range.

P6, and P7 experienced a RR during treatment, the initially high serum levels of total IgG and IgM had decreased to levels almost within the normal ranges. The decrease in IgG levels was mainly due to the decrease in the IgG1 subclass. The responses of IgG2 and IgG3 decreased very little, but were within the normal ranges (data not shown). The levels of IgE and IgG4 remained unchanged

during a RR episode and were within the normal ranges (data not shown).

Discussion

An increasing number of infectious diseases are known to be regulated through functionally distinct T cell subsets (type 1-, type 0-, and type 2-like) that are recognized by their cytokine secretion profile (15). The importance of such T cell subsets with respect to infection with an intracellular parasite may best be illustrated by the model in which inbred mice strains are infected with *Leishmania major* (reviewed in Ref. 35). Resistance strains control the disease by activation of type 1-like T cells, whereas susceptible mice were found to develop activated type 2-like T cells. The dominant regulatory role of these type 1- and type 2-like T cells was distinctly illustrated by the fact that the immune status of both mouse strains was convertible by adding neutralizing Abs against either IFN-γ or IL-4, respectively.

Likewise, borderline leprosy with its changeable immune status provides a unique model to study the regulatory role of T cell subsets in humans. These patients often show exacerbated CMI-DTH responses (RR) in the course of the disease accompanied by tissue damage. It is tempting to speculate that pluripotential *M. leprae*-responsive type 0-like T cells form the central T cell subset in the pathogenesis of borderline leprosy, and changes in the immune status of these patients may involve a selective shift toward polarized type 1- or type 2-like phenotypes. The focal point of the present study is to address this issue. To our knowledge no studies concerning the importance of different T cell subsets in the regulation of the changeable immune status of borderline leprosy in the course of the disease have been reported.

The novelty of our study was, therefore, based on a follow-up study, so that the dynamic role of T cell subsets from the lesional skin, representing the in vivo disease-related immune reactivity, could be established in relation to immunologic changes in the patients. Consequently, primary TCL from the lesional site of the skin were generated without preselection using a protocol that allows culture of spontaneously migrating T cells from the skin biopsies. These short term cultured TCL from biopsy materials of seven patients showed a T cell subset distribution ($CD4^+$, $CD8^+$, and $TCR\gamma\delta^+$) similar to that in the in vivo situation, with the exception of only one patient (P6). In general, a predominance of $CD4^+$ T cells was found in untreated paucibacillary and RR lesions, and a predominance of $CD8^+$ T cells was found in untreated multibacillary lesions. These results are in concordance with earlier reports (8, 23, 36). Further evidence for the generation of in vivo correlated TCL is the observation that the percentage of $CD4^+$ T cells in the lesions of two multibacillary patients at the time of a RR increased during treatment, and such an increase was similar in the generated TCL from the same lesions. In the case of P6, the TCL obtained both before and during treatment consisted of high percentages of $TCR\gamma\delta^+$ T cells that were not observed in such numbers in the lesions. Since $TCR\gamma\delta^+$ T cells are known to be promiscuously cytotoxic (37), it is possible that in these exceptional TCL, these $TCR\gamma\delta^+$ T cells overgrew other T cell subsets due to autologous killing. In support of such an assumption is a report on $TCR\gamma\delta^+$ T cell-mediated killing of autologous $CD4^+$ T cells in bovine paratuberculosis infection (38) and the observation that in this particular patient (P6) the number of $CD4^+$ *M. leprae*-responsive TCC from the TCL of the lesion with RR during treatment was the lowest of that in all patients included in this study. However, additional research is needed to validate such an assumption.

Lymphoproliferative analyses showed that all TCL were *M. leprae* responsive regardless of the immune status of the patient, including those from the lesions of BL patients who did not show a Mitsuda skin reaction. This finding is compatible with those of other studies suggesting the presence of *M. leprae*-responsive T cells in multibacillary lesions based on the possibility of inducing CMI-DTH responses by intralesional injection of IL-2 (39). The association between local CMI-DTH responses and the presence of *M. leprae*-responsive T cells in the lesional site is also evident from the observation that TCL from lesions with RR during treatment exhibited the strongest proliferation. This finding might be regarded as similar to those reported by previous investigators (6, 7). However, those studies were conducted with peripheral blood and may not necessarily reflect the local immune reactivity.

The highly efficient cloning procedure applied in the present study yielded a distribution of $CD4^+$, $CD8^+$, and $TCR\gamma\delta^+$ TCC closely resembling the respective primary TCL, with the exception of P6 (untreated). The phenotypic diversity of TCC from most of the cloning experiments indicate that in vitro selection was minimal. As stated in the results, in the case of P6 (untreated), 17/59 TCC could not be phenotyped and may thus explain the discrepancy between the phenotypic distribution among TCC and the TCL. The vast majority of the *M. leprae*-responsive TCC from each line were $CD4^+$ (from 79–100%), and only a few were $CD8^+$, $CD4^-/CD8^+/TCR\gamma\delta^+$, or $CD4^-/CD8^-/TCR\gamma\delta^+$. The majority of the $CD4^+$ *M. leprae*-responsive TCC regardless of the CMI-DTH status of the untreated borderline patient, had a type 0-like cytokine secretion profile. In addition, type 1-like *M. leprae*-responsive TCC were observed at the paucibacillary BT side (with or without RR), and type 2-like *M. leprae*-responsive TCC were observed at the multibacillary BL side of the clinical spectrum. In this respect our results are in agreement with the report describing that both indiscriminate and polarized *M. leprae*-responsive T cell subsets can be found in the blood of leprosy patients (22). These results suggest that in the lesions of untreated borderline patients, *M. leprae*-responsive T cells belong to the central $CD4^+$ type 0-like subset, whereas more polarized phenotypes may determine the CMI-DTH status of the patient.

The most interesting aspect of the present study is that three patients (P4, P6, and P7) suffered from (re)occurrence of RR during treatment and could be re-examined. This rare opportunity was exploited by comparing the cytokine profiles of the lesional *M. leprae*-responsive TCC from the same patients before and during RR. For all three patients the results revealed a clear shift of cytokine profiles from the $CD4^+$ *M. leprae*-responsive TCC toward a type 1-like phenotype during RR. The results are in agreement with those of other studies that showed that on the basis of increased IFN-γ mRNA detection by reverse transcription-PCR (40) or in situ hybridization (41), a type 1-like cytokine response is involved in RR. However, from those studies conclusions could not be drawn about whether this type 1-like reactivity was due to the T cell subset in total or selectively to those specific to *M. leprae*. In this context it is apparent from the present study that a shift to type 1-like immune reactivity did not occur among the *M. leprae*-nonresponsive T cells generated in parallel from the same lesions (data not shown). The bystander TCC from both untreated and treated lesions had heterogeneous phenotypes (type 1-, type 0-, or type 2-like), and their IFN-γ/IL-4 ratios significantly differed from those of polarized *M. leprae*-responsive T cells from the same lesions. In addition, the selective shift of the *M. leprae*-responsive T cells argues against a major influence of type 1-skewing microenvironmental cytokines produced by irradiated allogenic feeder cells or autologous PBMC.

Since IFN-γ contributes to the host's defense against mycobacterial infection by facilitating the intracellular microbial killing in macrophages (42), this shift of the $CD4^+$ *M. leprae*-responsive TCC toward a polarized type 1-like phenotype may be directly correlated with the decreased bacterial load in the lesions seen during RR. Besides the bactericidal activity during RR, cytotoxic immune reactivity against macrophages and Schwann cells is recognized as well. Indeed, $CD4^+$ *M. leprae*-responsive TCC with cytotoxic capacities have been described previously (43, 44), and this increased cytotoxic capacity of T cells is usually associated with the type 1-like phenotype (45). The polarized type 1-like $CD4^+$ *M. leprae*-responsive T cells in the lesions may, therefore, be responsible for local cytotoxic activities leading to tissue damage in RR. Additionally, type 1-like effector T cells may exert their influence systemically through regional lymph node and lymphatics (46). We observed that with RR during treatment the initially high serum levels of polyclonal total IgG, IgG1, and IgM decreased almost to the normal ranges. Another consequence of the general shift toward a cytotoxic type 1-like phenotype during RR may, therefore, be a decreased humoral response (33, 34). This may also occur in the skin, as based on earlier observations by others that *M. leprae*-specific Abs are produced endogenously in BL/LL granuloma (47).

Another aspect of the present study is that some $CD8^+$ and/or $TCR\gamma\delta^+$ TCC proliferated upon stimulation with *M. leprae*. $CD8^+$ *M. leprae*-specific TCC reported previously were not found to proliferate in response to *M. leprae* Ags, but exerted a suppressive effect on $CD4^+$ *M. leprae*-responsive T cells that was thought to be due to the release of IL-4 (14). In contrast, in our study three of four $CD8^+$ *M. leprae*-responsive TCC had a type 1-like phenotype. In the mouse, mycobacterial Ag-specific Schwann cell killing by $CD8^+$ T cells has been reported (48), and it could, therefore, be implied that the presently reported *M. leprae*-responsive $CD8^+$ type 1-like TCC are cytotoxic rather than suppressor cells. Similarly, $TCR\gamma\delta^+$ TCC may be involved in mycobacterial immunity by cytotoxic action (49). In this context we investigated the cytotoxic potential of a number of TCC in a MHC-nonrestricted cytotoxic assay by the method described previously (33). Indeed, the type 1-like $CD8^+$ TCC as well as the $CD4^-/CD8^-/TCR\gamma\delta^+$ TCC showed cytolytic potential, whereas the type 2-like $CD8^+$ TCC did not (data not shown). Further functional studies are needed to determine what possible role different T cell subsets can have in such cytotoxic events and to what extent these activities are responsible for tissue damage such as degeneration of macrophages and Schwann cells (a major complication of leprosy pathology) as well as killing of B cells.

In conclusion, the present study suggests that the dynamic immunopathologic states of borderline leprosy patients are associated with the polarization of cytokine secretion by *M. leprae*-responsive T cells during RR episode. It is well known that since the introduction of multiple drug therapy for leprosy, RR occurs frequently after treatment (10). One of the main goals in leprosy research is to elucidate the pathomechanism of RR in relation to immune reactivity and to develop laboratory markers for early diagnosis of risk for RR. In this regard, the present report showed a shift of $CD4^+$ *M. leprae*-responsive T cells to a polarized type 1-like phenotype and decreased humoral immunity paralleled by increased CMI-DTH activity. We are presently investigating whether monitoring in vitro IFN-γ/IL-4 secretion of lesional TCL in the course of the disease combined with serum Ig levels could be used as a predictive assay for the early diagnosis of RR. In addition, future research will focus on the parameters that induce the cytokine shift during RR. Understanding such mechanisms may provide more effective tools to develop therapeutic strategies to control pathologic complications in leprosy.

Acknowledgments

We thank Dr. M. J. Colston of the National Institute for Medical Research (Mill Hill, London, U.K.) for providing *M. leprae* sonicated extracts. Dr. S. O. Stapel (Allergy Department, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and M. J. de Nooijer (Immunology Laboratory Unit, Academic Medical Center, Amsterdam) are acknowledged for their help with determining the serum Ig titers. We thank Dr. G. Koopman, Drs. P. Kalinski, and Prof. Dr. J. J. Weening for critically reading the manuscript and for their helpful suggestions.

References

1. Ridley, D. S., and W. H. Jopling. 1966. Classification of leprosy according to immunity: a five-group system. *Int. J. Lepr. 34:255.*
2. Bloom, B. R., and T. Godal. 1983. Selective primary health care: strategies for control of disease in the developing world. V. Leprosy. *Rev. Infect. Dis. 5:765.*
3. Das, P. K., and J. M. Grange. 1993. Mycobacteria in relation to tissue immune response and pathogenesis. *Rev. Med. Microbiol. 4:15.*
4. Ridley, D. S. 1969. Reactions in leprosy. *Lepr. Rev. 40:77.*
5. Godal, T., B. Myrvang, D. R. Samuel, W. F. Ross, and M. Lofgren. 1973. Mechanism of 'reactions' in borderline tuberculoid (BT) leprosy. *Acta. Pathol. Microbiol. Scand. 236(Suppl.):45.*
6. Barnetson, R. StC., G. Bjune, J. M. H. Pearson, and G. Kronvall. 1976. Cell mediated and humoral immunity in 'reversal reaction'. *Int. J. Lepr. 44:267.*
7. Bjune, G., R. St. C. Barnetson, D. S. Ridley, and G. Kronvall. 1976. Lymphocyte transformation test in leprosy: correlation of the response with inflammation of lesions. *Clin. Exp. Immunol. 25:85.*
8. Modlin, R. L., J. F. Gebhard, C. R. Taylor, and T. H. Rea. 1983. In situ characterization of T lymphocyte subsets in the reactional states of leprosy. *Clin. Exp. Immunol. 53:17.*
9. Lockwood, D. N. J., S. Vinayakumar, J. N. A. Stanley, K. P. W. J. McAdam, and M. J. Colston. 1992. Clinical features and outcome of reversal (type 1) reactions in Hyderabad, India. *Int. J. Lepr. 61:8.*
10. Lienhardt, C., and P. E. M. Fine. 1994. Type 1 reaction, neuritis and disability in leprosy: what is the current epidemiological situation? *Lepr. Rev. 65:9.*
11. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
12. Romagnani, S. 1991. Human T_H1 and T_H2 subsets: doubt no more. *Immunol. Today 12:256.*
13. Haanen, J. B. A. G., R. de Waal Malefijt, P. C. M. Res, E. M. Kraakman, T. H. M. Ottenhoff, R. R. P. de Vries, and H. Spits. 1991. Selection of a human T helper type 1-like T cell subset by mycobacteria. *J. Exp. Med. 174:583.*
14. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science 254:279.*
15. Kaufmann, S. H. E. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol. 11:129.*
16. Kapsenberg, M. L., E. A. Wierenga, J. D. Bos, and H. M. Jansen. 1991. Functional subsets of allergen-reactive human $CD4^+$ T cells. *Immunol. Today 12:392.*
17. Parronchi, P., D. Macchia, M.-P. Piccinni, P. Bisswas, C. Simonelli, E. Maggi, M. Ricci, A. A. Ansari, and S. Romagnani. 1991. Allergen- and bacterial antigen-specific T cell clones established from atopic donors show a different profile of cytokine production. *Proc. Natl. Acad. Sci. USA 88:4538.*
18. Mosmann, T. R., and S. Sad. 1996. The expanding universe of T cell subsets: Th1, Th2 and more. *Immunol. Today 17:139.*
19. Firestein, G. S., W. D. Roeder, J. A. Laxer, K. S. Townsend, C. T. Weaver, J. T. Hom, J. Linton, B. E. Torbett, and A. L. Glasebrook. 1989. A new murine $CD4^+$ T cell subset with an unrestricted cytokine profile. *J. Immunol. 143:518.*
20. Mutis, T., E. M. Kraakman, Y. E. Cornelisse, J. B. G. Haanen, H. Spits, R. R. P. De Vries, and T. H. M. Ottenhoff. 1993. Analysis of cytokine production by *Mycobacterium*-reactive T cells: failure to explain *Mycobacterium leprae*-specific nonresponsiveness of peripheral blood T cells from lepromatous leprosy patients. *J. Immunol. 150:4641.*
21. Howe, R. C., A. Wondimu, A. Demissee, and D. Frommel. 1995. Functional heterogeneity among $CD4^+$ T-cell clones from blood and skin lesions of leprosy patients: identification of T-cell clones distinct from Th0, Th1 and Th2. *Immunology 84:565.*
22. Misra, N., A. Murtaza, B. Walker, N. P. S. Narayan, R. S. Misra, V. Ramesh, S. Singh, M. J. Colston, and I. Nath. 1995. Cytokine profile of circulating T cells of leprosy patients reflects both indiscriminate and polarized T-helper subset: T-helper phenotype is stable and uninfluenced by related antigens of *Mycobacterium leprae*. *Immunology 86:97.*
23. Van Voorhis, W. C., G. Kaplan, E. N. Sarno, M. A. Horwitz, R. M. Steinman, W. R. Levis, N. Nogueira, L. S. Hair, C. R. Gattass, B. A. Arrick, and Z. A. Cohn. 1982. The cutaneous infiltrates of leprosy: cellular characteristics and predominant T-cell phenotypes. *N. Engl. J. Med. 307:1593.*

24. Modlin, R. L., J. Melancon-Kaplan, S. M. M. Young, C. Pirmez, H. Kino, J. Convit, T. H. Rea, and B. R. Bloom. 1988. Learning from lesions: patterns of tissue inflammation in leprosy. *Proc. Natl. Acad. Sci. USA 85:1213.*
25. Ridley, D. S. 1985. *Skin Biopsy in Leprosy*, 2nd Ed. Ciba-Giegy Ltd., Basel.
26. Dugan, E., R. L. Modlin, and T. H. Rea. 1985. An in situ immunohistological study of Mitsuda reactions. *Int. J. Lepr. 53:404.*
27. Wierenga, E. A., M. Snoek, J. D. Bos, H. M. Jansen, and M. L. Kapsenberg. 1990. Comparison of diversity and function of housedust mite-specific T lymphocyte clones from atopic and nonatopic donors. *Eur. J. Immunol. 20:1519.*
28. Van der Meide, P. H., M. Dubbeld, and H. Schellekens. 1985. Monoclonal antibodies to human immune interferon and their use in a sensitive solid-phase ELISA. *J. Immunol. Methods 79:293.*
29. Van der Pouw-Kraan, T. C. T. M., I. Rensink, and L. A. Aarden. 1993. Characterization of monoclonal antibodies to human IL-4: application in an ELISA and differential inhibition of IL-4 bioactivity on B cells and T cells. *Eur. Cytokine Netw. 4:343.*
30. Hsu, S. M., L. Raine, and H. Fanger. 1981. Use of avidin biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem. 29:577.*
31. Stallman, P. J., and R. C. Aalberse. 1977. Estimation of basophil-bound IgE by quantitative immunofluorescence microscopy. *Int. Arc. Allergy Appl. Immunol. 54:9.*
32. Out, T. A., E. A. van de Graaf, N. J. van den Berg, and H. M. Jansen. 1991. IgG subclasses in bronchoalveolar lavage fluid from patients with asthma. *Scand. J. Immunol. 33:719.*
33. Wierenga, E. A., M. Snoek, H. M. Jansen, J. D. Bos, R. A. W. van Lier, and M. L. Kapsenberg. 1991. Human atopen-specific types 1 and 2 T helper cell clones. *J. Immunol. 147:2942.*
34. Del Prete, G. F., M. de Carli, M. Ricci, and S. Romagnani. 1991. Helper activity for immunoglobulin synthesis of T helper Type 1 (Th1) and Th2 human T cell clones: help of Th1 clones is limited by their cytolytic capacity. *J. Exp. Med. 174:809.*
35. Reiner, S. L., and R. M. Locksley. 1995. The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol. 13:151.*
36. Narayan, R. B., S. Laal, A. K. Sharma, L. K. Bhutani, and I. Nath. 1984. Differences in predominant T cell phenotypes and distribution pattern in reactional lesions of tuberculoid and lepromatous leprosy. *Clin. Exp. Immunol. 55:623.*
37. Janeway, C. A., Jr., B. Jones, and A. Hayday. 1988. Specificity and function of T cells bearing γδ receptors. *Immunol. Today 9:73.*
38. Chiodini, R. J., and W. C. Davis. 1992. The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent $CD4^+$ activity. *Microb. Pathog. 13:447.*
39. Kaplan, G., N. K. Mathur, C. K. Job, I. Nath, and Z. A. Cohn. 1989. Effects of multiple interferon gamma injections on the disposal of *Mycobacterium leprae*. *Proc. Natl. Acad. Sci. USA 86:8073.*
40. Yamamura, M., X.-H. Wang, J. D. Ohmen, K. Uyemura, T. H. Rea, B. R. Bloom, and R. L. Modlin. 1992. Cytokine patterns of immunologically mediated tissue damage. *J. Immunol. 149:1470.*
41. Cooper, C. L., C. Mueller, T.-A. Sinchaisri, C. Pirmez, J. Chan, G. Kaplan, S. M. M. Young, I. L. Weissman, B. L. Bloom, T. H. Rea, and R. L. Modlin. 1989. Analysis of naturally occurring delayed-type hypersensitivity reactions in leprosy by in situ hybridization. *J. Exp. Med. 169:1565.*
42. Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon-γ as the lymphokine that activates human macrophages oxidative metabolism and antimicrobial activity. *J. Exp. Med. 158:670.*
43. Kaleab, B., T. H. M. Ottenhoff, P. Converse, E. Halapi, G. Tadesse, M. Rottenberg, and R. Kiessling. 1990. Mycobacterial-induced cytotoxic T cells as well as nonspecific killer cells derived from healthy individuals and leprosy patients. *Eur. J. Immunol. 20:2651.*
44. Del Carmen Sasiain, M., S. de la Barrera, F. Minnucci, R. Valdez, M. M. de Elizalde de Bracco, and L. M. Balina. 1992. T-cell-mediated cytotoxicity against *Mycobacterium* antigen-pulsed autologous macrophages in leprosy patients. *Infect. Immun. 60:3389.*
45. Mutis, T., Y. E. Cornelisse, and T. H. M. Ottenhoff. 1993. Mycobateria induce $CD4^+$ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. *Eur. J. Immunol. 23:2189.*
46. Streilein, J. W. 1978. Lymphocyte traffic, T cell malignancies and the skin. *J. Invest. Dermatol. 71:167.*
47. Lai A Fat, R. F. M., M. Chan Pin Jin, R. Van Fewth, and M. Harboe. 1980. In vitro synthesis of antimycobacterial antibodies in biopsies from skin lesions of leprosy patients. *Infect. Immunol. 27:227.*
48. Steinhoff, U., and S. H. E. Kaufmann. 1988. Specific lysis by $CD8^+$ alpha/beta T cells expressing *Mycobacterium leprae* antigens. *Eur. J. Immunol. 18:969.*
49. Janis, E. M., S. H. E. Kaufmann, R. H. Schwartz, and D. M. Pardoll. 1989. Activation of γδ T cells in the primary immune response to *Mycobacterium tuberculosis*. *Science 244:713.*