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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

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SUMMARY

In the present study we examined the functional properties of T-cell clones reactive with *Mycobacterium leprae* and other mycobacterial antigens. Clones isolated from the skin lesions and blood of leprosy patients across the spectrum were exclusively $CD4^+CD8^-$ and expressed the $\alpha\beta$ T-cell receptor. Substantial heterogeneity in the production of cytokines, in particular interleukin-4 (IL-4), was observed, although no striking correlation with clinical status was apparent. A variety of patterns of cytokine secretion distinct from those of T-helper type-1 (Th1) Th2 or Th0, as defined in murine studies, was evident. Most noteworthy was a large number of clones from skin which secreted neither IL-2 nor IL-4, but large amounts of tumour necrosis factor (TNF) and interferon-γ (IFN-γ). Clones isolated from the blood of leprosy patients had a more restricted cytokine secretion profile, and appeared to resemble more closely previously described patterns, including those of high level production of IL-2 and/or IL-4. Virtually all clones, from either skin or blood, produced high levels of IFN-γ, and thus many clones were IL-4 and IFN-γ co-producers. The pattern of cytokine production by skin-derived T-cell clones was significantly affected by the *in vitro* activation status of the cells. Cells enriched in activated blasts tended to produce more IL-4 than small resting cells. In addition, the production of IFN-γ by skin T-cell clones after $\leqslant 10$ weeks of culture was strikingly distinct from that of these clones after 5 months of culture. IL-4 and IFN-γ co-producing clones shifted to a Th2-like pattern with much less IFN-γ secretion, whereas non-IL-4-producing clones secreted much higher levels of IFN-γ after prolonged culture, and became much more Th1-like. However, there was still no correlation between clinical status and pattern of cytokines produced. These results imply that a high fraction of T cells exists in leprosy lesions that is distinct from or that has not yet fully matured into Th1 or Th2 cells.

INTRODUCTION

Leprosy currently affects some 10–12 million people throughout the world, despite considerable progress made in treatment.[1] The disease is quite heterogeneous, and the observed spectrum of clinical features is correlated with different forms of the host immune response. At one end of the spectrum lie tuberculoid patients, who have localized skin and nerve granulomas, measurable T-cell responsiveness *in vitro* or *in vivo*, and some serum antibodies to *Mycobacterium leprae*. At the other end of the spectrum are lepromatous patients who are unable to limit the spread of *M. leprae* which in the absence of chemotherapy may disseminate throughout the body. These patients show little detectable *in vitro* proliferative or *in vivo* skin test responses specific for *M. leprae*, antigens, although serum antibody levels specific to *M. leprae* are very high. The vast majority of individuals who are infected with *M. leprae* clear the organisms with no apparent sign of the disease. These individuals exhibit strong T-cell responsiveness. Collectively these observations have led investigators to focus in particular on *M. leprae* specific T cells.[1–3] It has been proposed that the two polar forms of leprosy reflect the dominance of two major T-cell subpopulations. One population, T-helper type-1 (Th1), secretes interleukin-2 (IL-2) and interferon-γ (IFN-γ) and predominates in tuberculoid leprosy, whereas the other, termed Th2, produces IL-4 and IL-5 and is present primarily in lepromatous leprosy. Recent studies have provided evidence

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Abbreviations: BCG, bacillus Calmette–Guérin (*M. bovis*); BD, blood; BT, borderline tuberculoid; BT/RR, borderline tuberculoid in type I reversal reaction; EBV, Epstein–Barr virus; FCS, fetal calf serum; HGZ, homogenization; IL-2 O/N, IL-2 overnight preculture; LL, lepromatous leprosy; LLs, subpolar lepromatous leprosy; MB, multibacillary; NHS, normal human serum; NMS, normal mouse serum; OPD, orthophenyldiamine; PB, paucibacillary; PPD, purified protein derivative of *Mycobacterium tuberculosis*; SK, skin; Tween, polyoxyethylene (20) sorbitan monolaurate.

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in favour of this hypothesis,[4–6] whereas another has provided contrasting results.[7]

Most investigations utilizing T-cell clones in either murine or human systems have examined panels of clones derived from precultured cell lines.[4–11] However, the degree to which such clones are representative of the entire freshly isolated T-cell population is uncertain. Fewer studies have examined T cells that have been cloned directly from freshly isolated samples without prior *in vitro* antigen selection procedures.[12–15] The primary objective of the present study was to derive antigen-specific $CD4^+$ clones from the skin lesions of chronic leprosy patients utilizing a high-efficiency cloning system. With this alternative strategy, we observed a variety of cytokine-secreting phenotypes distinct from Th1 and Th2 present in both types of lesions. However, the pattern of cytokine production was significantly affected by activation status of the T cells, and after long-term culture many of the clones became much more Th1- or Th2-like. A statistically significant correlation between cytokine production and clinical status could not be demonstrated, even after long-term culture. These results are consistent with the hypothesis that skin granulomas in leprosy may be continuously seeded with relatively uncommitted precursor cells, eventually leading to the presence of many T-cell subsets at various stages of differentiation.

MATERIALS AND METHODS

Patients

Blood and skin biopsies were taken from seven patients and two healthy control individuals. The patients had been admitted to the out-patient examination clinic at the All Africa Leprosy and Rehabilitation Training Center (ALERT; Addis Ababa, Ethiopia). All were newly diagnosed cases with the following classifications based on clinical, laboratory and histological examination: no. 1, borderline tuberculoid in type I reversal reaction (BT/RR); no. 2, borderline tuberculoid (BT); no. 3, lepromatous (LL); no. 5, BT; no. 6, BT; no. 7, subpolar lepromatous (LLs); no. 8, LLs. According to the classification criteria of the World Health Organization (WHO), LL and LLs patients were considered multibacillary (MB) and the others paucibacillary (PB).[16] Healthy controls, nos. 4 and 9, were individuals living in the area without clinical signs of leprosy but who had demonstrated strong *in vitro* T-cell proliferative responses to *M. leprae*.

Media

Medium used for T-cell cultures consisted of RPMI-1640 supplemented with penicillin, streptomycin, glutamine and 5% heat-inactivated normal human serum (NHS). All transformed cell lines were cultured in the same medium with 10% fetal calf serum (FCS) instead of NHS.

Cell lines

To isolate Epstein–Barr virus (EBV)-transformed cell lines, either fresh or thawed peripheral blood mononuclear cells (PBMC) that had been stored in liquid nitrogen were infected with culture supernatant from the B95–8 cell line.[17] Transformed cells appeared in culture after 1–5 weeks. Cytokine indicator lines L929, WISH and CTLL-1 were obtained from the American Type Tissue Culture Collection (ATTC, Rockville, MD).

Antibodies

Monoclonal antibody (mAb) 64·1, anti-CD3,[18] was kindly provided by Dr P. Martin (University of Washington, Seattle, WA). OKT4 (anti-CD4) and OKT8 (anti-CD8) were obtained from ATTC. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse whole immunoglobulin was purchased from Dakopatts (Glostrup, Denmark). FITC-conjugated anti-$\alpha\beta$ T-cell receptor (TCR) (WT31) was purchased from Becton Dickinson (Belgium), anti-TCRδ1 from T Cell Sciences (Cambridge, MA), and polyclonal antibodies to human IL-2, IL-4 and IFN-γ from Genzyme (Cambridge, MA). Murine mAb to human IL-4 was generously provided by Immunex Corp. (Seattle, WA). DMS-1 (anti-IL-2) hybridoma was a gift of Dr Kendall Smith (Dartmouth University, NH). Monoclonal antibody to human IFN-γ (RU F18) was kindly provided by Roussel UCLAF (France).

Antigens

Whole X-irradiated *M. leprae*, stock CD136, was obtained from Dr Rees (WHO/IMMLEP *M. leprae* Bank, London, UK). Purified protein derivative (PPD) and bacillus Calmette–Guérin (BCG) was purchased from Statens Serum Institute (Copenhagen, Denmark). Recombinant p65 of *M. leprae* was a gift from Dr J. Colston (MRC, London, UK).

Cell isolation

PBMC were isolated by Ficoll–Hypaque density centrifugation. In some cases PBMC were frozen until later use. Cryovials containing $1–5 \times 10^6$ cells in 10% FCS and 10% dimethyl sulphoxide (DMSO) were frozen in styrofoam containers placed at $-70°$ for 1–24 hr. The vials were then transferred to liquid nitrogen storage containers. Cells were thawed and gradually diluted with medium at room temperature before use as described elsewhere.[19] Two methods were employed to isolate skin infiltrating cells from biopsies. The first method, homogenization (HGZ), used a modification of that described by Modlin *et al.*,[20] employing a fine mesh oil screen as a sieve device, while the second method, IL-2 overnight preculture (IL-2 O/N) consisted of mincing the biopsy in small pieces with a sterile scalpel, followed by overnight culture with 20 U/ml of IL-2 (Cetus, Emeryville, CA).[21]

T-cell clone selection

Three procedures were utilized. (1) Cells infiltrating lesion biopsies from patient 1 were isolated by anti-CD3 + antigen preselection. A T-cell line was initiated by culturing 10^4 cells in microwells that had been precoated overnight with a 1 : 5000 dilution of anti-CD3 ascites (mAb 64.1) in phosphate-buffered saline (PBS). Cultures were additionally supplemented with 25% Lymphocult-T (conditioned medium of phytohaemagglutinin (PHA)-stimulated PBMC; Biotest Serum Institut, Germany) and 100 U/ml of IL-2. After 7 days the cultures were fed with fresh medium containing 20 U/ml IL-2. Following 2 additional weeks of growth, the cells were stimulated with PPD (5 μg/ml) and 20 U/ml IL-2 in the presence of autologous 14-day cultured PBMC that had been thoroughly washed to obtain purified macrophage adherent cells. After two such stimulations at biweekly intervals, the line was cloned in the presence of phytohaemagglutanin, irradiated allogeneic PBMC and IL-2, and retested for specific proliferation to the original priming antigen in the presence of either autologous EBV-transformed B cells or cultured adherent cells from thawed PBMC samples.

(2) All clones from blood were isolated by antigen preselection. Cell lines were initiated by stimulating $1{\cdot}5 \times 10^5$ PBMC with the appropriate antigen, either whole *M. leprae*, recombinant *M. leprae* p65, PPD or BCG. Responding cells were then restimulated with the priming antigen and either irradiated (4000 rads) autologous PBMC or autologous irradiated (4000 rads) EBV-transformed B cells. In some cases 20 U/ml IL-2 was added at day 4–6 to boost proliferation. Cell lines maintained in this fashion were then cloned as described above.

(3) Direct cloning was utilized to derive clones from the skin biopsies of patients 5–8. One hundred, 30, 10 and three responder cells/well were added to 8–16 replicate wells of a flat-bottomed 96-well microtitre plate, to which 75 000 irradiated autologous PBMC had been added. Cultures contained antigen, 50 U/ml IL-2, and 25% Lymphocult-T. Trace levels of PHA had been removed from the Lymphocult-T preparation by prior passage over a protein A column to which rabbit anti-PHA (Dakopatts) had been adsorbed. This substantially reduced background T-cell proliferation in the absence of antigen. After 1 week, cultures were fed with fresh medium containing IL-2. Following the second week wells were scored microscopically for the presence of large phase bright cells. Wells that were scored positive were estimated to have at least 1000 cells, whereas wells scored negative had no readily detectable blasts. Wells originally given irradiated PBMC alone contained only scattered cell debris and very large plastic-adherent cells, probably macrophages surviving the radiation. All cells isolated in this manner were subject to one additional stimulation with the priming antigen, IL-2 and autologous irradiated cultured PBMC as a source of antigen-presenting cells (APC). Clones from patients 7 and 8 were further subcloned in the presence of 0·1% PHA, 10^5 irradiated allogeneic PBMC, and 20 U/ml IL-2. Precursor frequencies were calculated by maximum likelihood estimation as described by Taswell.[22]

Propogation of T-cell clones and proliferation assays

T-cell clones were selected from cloning wells at input cell concentrations resulting in $<25\%$ of the wells containing proliferating cells. The probability of clonality based on the Poisson equation[22] was calculated to be $\geqslant 0{\cdot}86$ for clones from patients 5 and 6, and $\geqslant 0{\cdot}98$ for all other clones. 10^3–10^4 T cells were placed with 0·03–0·1% PHA, 10^5 irradiated allogeneic PBMC, and 20 U/ml IL-2 in round-bottomed microtitre wells. After 5–7 days the cells were harvested and subcultured for an additional 7–16 days in 16 mm 24-well plates with IL-2-containing medium. The second subculture both promoted further expansion and reversion to small resting cells that had little capacity to respond to feeder cells and IL-2 without antigen or mitogen. Alternatively, cells were propagated with a similar protocol using antigen and autologous feeder cells in place of PHA and allogeneic feeder cells. A haemocytometer counting chamber was used to assess proliferative responses of the cells. Greater than 100 cells were counted from each of two replicate samples.

Immunofluorescence

T cells were stained with the appropriate dilution of test or control mAb in PBS–sodium azide for 30 min at 4°. After washing, the cells were incubated for 30 min at 4° with a 1 : 20 dilution of FITC-conjugated rabbit anti-mouse immunoglobulin. Washed cells were placed onto poly-L-lysine-coated glass slides, fixed with 0·5% paraformaldehyde, mounted with glycerol and examined under a Leitz fluorescent microscope. Alternatively, cells were stained, fixed and analysed by a Becton-Dickinson FACScan flow cytometer.

Induction of lymphokine secretion

Cloned T cells were harvested 14–21 days after the previous stimulation with PHA or antigen, and thoroughly washed prior to use. 2×10^5 cells were cultured in anti-CD3-coated flat-bottomed wells of a 96-well microtitre plate in 200 μl of RPMI medium supplemented with 10% FCS instead of NHS. The wells had been coated with anti-CD3 by overnight incubation with 100 μl of a 1 : 5000 dilution of mAb 64·1 ascites in 0·1 M carbonate–bicarbonate buffer, pH 9·5, and then washed with PBS. After the cells were incubated at 37° for 24 hr, the cell-free supernatant was harvested and stored at −70° until analysis.

Measurement of cytokines

Capture ELISA was used to detect IL-2, IL-4 and IFN-γ. The basic procedure utilized in each assay was as follows: microtitre plates were first coated overnight with 10 μg/ml murine mAb specific for the cytokine of interest in carbonate–bicarbonate buffer. Following washing with PBS–Tween, and blocking with PBS–0·5% gelatin, cytokine-containing samples were added for 2 hr. The following reagents were then added sequentially, alternating with washing with PBS–Tween: polyclonal rabbit anti-cytokine antibodies at a 1 : 80 dilution in PBS–Tween for 2 hr; 1 : 1000 dilution of biotinylated goat anti-rabbit immunoglobulin (Sigma, St Louis, MO) in PBS–Tween (pre-adsorbed on a normal mouse serum–protein A column to remove cross-reactive anti-mouse immunoglobulin) for 1 hr; 1 : 1000 dilution of streptavidin peroxidase (Sigma) in PBS–Tween for 30 min. Finally OPD (1 mg/ml, Sigma) was added with 0.06% hydrogen peroxide for 2 min, and the reaction stopped with 2 M sulphuric acid. Absorbance was read at 405 nm. Cytokine concentrations in cell supernatants were determined by extrapolation from standard curves performed with recombinant cytokines. IFN-γ was detected utilizing mAb RU F18 and rabbit anti-human IFN-γ as the second capture antibodies. One per cent normal mouse serum (NMS) was added together with rabbit anti-IFN-γ. The sensitivity of the assay was determined to be approximately 10 U/ml (0·4 ng/ml). For measurement of IL-2, protein A-purified DMS-1 murine anti-human IL-2 mAb and rabbit anti-IL-2 were utilized. IL-2-containing supernatants were added for 24 hr instead of 2 hr. The sensitivity of this test was determined to be 5 U/ml (1·66 ng/ml). IL-4 levels were determined as indicated in the general outline above, utilizing murine monoclonal anti-IL-4 (Immunex) and rabbit anti-IL-4 as the capture reagents. Sensitivity was 3 U/ml (0·1 ng/ml).

A bioassay for IFN activity was performed using protection of the cytopathic effect of vesicular stomatitis virus (VSV) on the WISH cell line.[23] WISH cells were plated out at 10^4 cells/well, to which serial dilutions of test supernatant were added. After 24 hr the plates were washed, and a 1% solution of VSV was added to the wells for an additional 24 hr. Cell viability was determined by microscopic examination. TNF was measured by its cytopathic effect on murine L929 cells in the presence of 1 μg/ml actinomycin D.[24] Cytotoxicity was determined after 24 hr by microscopic examination. IL-2 was assessed by the

viability of the IL-2-responsive T-cell line CTLL-1 indicator cells, as described elsewhere.[25] The limit of sensitivity was 0·5–1 U/ml for all bioassays. When directly comparable, ELISA and bioassays gave similar results.

RESULTS

Isolation of T-cell clones

We derived 28 mycobacteria-specific T-cell clones from leprosy patients and control individuals. The patients' clinical status, and the T-cell clones' tissue of origin and method of isolation, are summarized in Table 1. Clones from blood were isolated by standard procedures. The cells from skin were extracted either by homogenization, as described elsewhere,[20] or by overnight culture of intact tissue in the presence of IL-2.[21] In general, the latter method resulted in greater recoveries and viabilities. FACScan analysis of IL-2-cultured cells indicated that virtually all were small resting cells, and such cells did not incorporate tritiated thymidine. Thus, we believe short-term culture in the presence of IL-2 and FCS promotes emigration from the tissue mass, as well as maintaining viability, without inducing proliferation. Once cells had been extracted from tissue, they were cultured in the presence of various combinations of activation stimuli. In general, cells from skin responded poorly to antigenic or mitogenic stimuli, which readily induced proliferation of blood-derived T cells. If skin cell cultures were supplemented with high concentrations of IL-2 and other cytokines present in T-cell-conditioned medium in addition to antigen or mitogen, growth was more consistently induced (R.C. Howe, unpublished observations). In line with the results of Modlin *et al.*,[26] we observed that an average of 1 cell in 10 from PB skin lesions was *M. leprae* reactive, and such precursors could also be identified in MB lesions, although at frequencies at least fivefold lower (R.C. Howe, manuscript in preparation). The proliferative reactivity towards mycobacterial antigens of the 28 clones from both skin and blood is illustrated in Figs 1 and 2. All clones were found to express high levels of CD4 molecules and TCR $\alpha\beta$.

Anti-CD3-induced lymphokine production by T-cell clones isolated from skin biopsies

Table 2 summarizes experiments analysing anti-CD3-induced

Table 1. Derivation and specificity of T-cell clones

Clone	Patient no.	Diagnosis	Tissue	Priming antigen	Method of Cell isolation	Method of Clone isolation	Additional reactivity (if known)
6-23	1	BT/RR	SK	PPD	HGZ	Anti-CD3	
6-26	1	BT/RR	SK	PPD	HGZ	Anti-CD3	
6-31	1	BT/RR	SK	PPD	HGZ	Anti-CD3	
6-34	1	BT/RR	SK	PPD	HGZ	Anti-CD3	
28-1	5	BT	SK	Mlep	IL-2 O/N	Direct cloning	
28-2	5	BT	SK	Mlep	IL-2 O/N	Direct cloning	
28-3	5	BT	SK	Mlep	IL-2 O/N	Direct cloning	
28-4	5	BT	SK	Mlep	IL-2 O/N	Direct cloning	
29-1	6	BT	SK	Mlep	IL-2 O/N	Direct cloning	
29-2	6	BT	SK	Mlep	IL-2 O/N	Direct cloning	
29-4	6	BT	SK	Mlep	IL-2 O/N	Direct cloning	Mlep p65
30-1	7	BL	SK	PPD	IL-2 O/N	Direct cloning	Mlep
30-2	7	BL	SK	PPD	IL-2 O/N	Direct cloning	Mlep p65
30-3	7	BL	SK	Mlep	IL-2 O/N	Direct cloning	
30-4	7	BL	SK	Mlep	IL-2 O/N	Direct cloning	
31-2	8	BL	SK	Mlep	IL-2 O/N	Direct cloning	PPD
9-1	1	BT/RR	BD	PPD	Ficoll	Ag preselect	Mlep
9–3	1	BT/RR	BD	PPD	Ficoll	Ag preselect	Mlep p65
7-3	2	BT	BD	PPD	Ficoll	Ag preselect	Mlep
7-4	2	BT	BD	PPD	Ficoll	Ag preselect	Mlep
7-6	2	BT	BD	PPD	Ficoll	Ag preselect	Mlep
8-6	3	LL	BD	PPD	Ficoll	Ag preselect	
10-3·1	4	CON	BD	Mlep	Ficoll	Ag preselect	
25-10	9	CON	BD	Mlep	Ficoll	Ag preselect	
26-1	9	CON	BD	Mlep p65	Ficoll	Ag preselect	PPD
2-5	9	CON	BD	PPD	Ficoll	Ag preselect	
2-11	9	CON	BD	BCG	Ficoll	Ag preselect	PPD
2-12	9	CON	BD	BCG	Ficoll	Ag preselect	PPD

Samples from skin (SK) or blood (BD) were prepared by HGZ, IL-2 O/N or Ficoll density centrifugation (Ficoll). The original selecting mycobacterial antigen was *M. leprae* (Mlep), BCG or PPD. Clones were isolated directly from tissue (direct cloning), following short-term culture with antigen (Ag preselect), or after short-term stimulation with immobilized anti-CD3 followed by PPD (anti-CD3). See the Materials and methods for details.

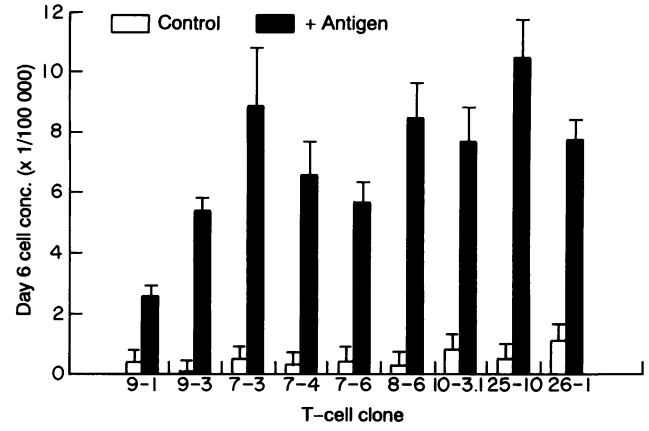

Figure 1. Reactivity of blood-derived T-cell clones to mycobacterial antigens. T-cell clones from PBMC were isolated as described in the Materials and Methods. 10^4 cells were cultured with or without the priming antigen (Table 1), 20 U/ml IL-2 and autologous APC. After 6 days, the cell concentration of replicate wells was measured with a haemocytometer. Autologous irradiated PBMC were used as APC for clones 10–3·1, 25–10 and 26–1, whereas the remaining clones were cultured with autologous EBV-transformed B cells as APC. Antigens used were *M. leprae* (5×10^6), PPD (5 μg/ml) or *M. leprae* p65 (5 μg/ml).

IL-4 production by clones isolated from the skin biopsies of three PB and two MB patients. Clones clearly differed in the amounts of IL-4 secreted. IL-4-producing clones secreted from 10 to 100 U/ml IL-4, whereas non-producing clones produced < 3 U/ml. However, there was no correlation between IL-4 production and clinical status, as assessed by Fisher's exact test ($P = 0{\cdot}37$; Table 3).

IL-2 production by these clones was consistently low (Table 2). Only three of 16 clones produced detectable IL-2, and two of these clones also produced IL-4. The results also showed that a large number of clones isolated from skin produced neither IL-4 nor IL-2.

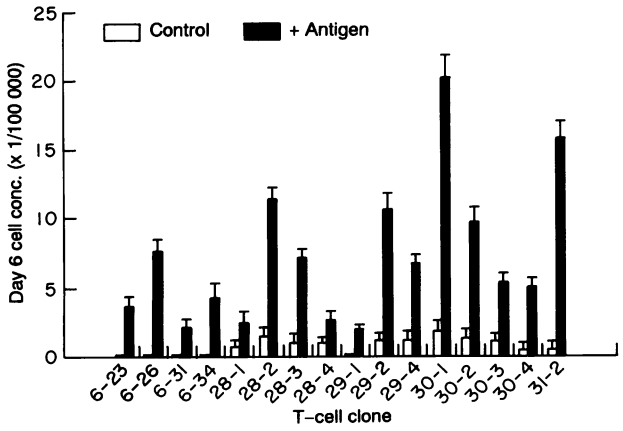

Figure 2. Reactivity of skin-derived T-cell clones to mycobacterial antigens. T-cell clones from skin were cultured and assayed as described in Fig. 1. Autologous EBV-transformed B cells were utilized as APC for clones 6–23, 6–26 and 6–34. APC for the other clones were autologous 7-day cultured plastic-adherent cells from PBMC.

All clones produced TNF and all but one produced high (84–1500 U/ml) levels of IFN-γ. There was considerable quantitative variation from clone to clone. However, there was no striking correlation between production and clinical status. The one clone, 30–3 (isolated from a lepromatous individual), that did not produce substantial IFN-γ (levels ≤ 10 U/ml) did secrete high levels of IL-4, low levels of TNF and no IL-2.

Anti-CD3-induced lymphokine production by blood-derived clones

The levels of IL-2 and IL-4 produced by clones isolated from blood are shown in Fig. 3a. Most clones produced IL-2 and those clones that did not produce IL-2 produced IL-4. Some clones produced both cytokines. These results contrast sharply with the results of clones isolated from the skin of leprosy patients (Fig 3b), a lower fraction of which produced detectable IL-2. The difference in the fraction of IL-2-secreting clones among blood and skin was determined to be statistically significant ($P = 0{\cdot}01$; Fisher's exact test; Table 3).

We determined that all blood-derived clones produced TNF at levels > 120 U/ml, and all but one clone produced IFN-γ at levels between 100 and 600 U/ml. This clone, 26–1, isolated from a control individual, produced very high levels of IL-4 (710 U/ml) and moderate levels of IL-2 (22 U/ml), but low IFN-γ levels (< 10 U/ml). We also observed that most of the IL-4-secreting clones isolated from blood had been primed to soluble antigens and selected in the presence of EBV-transformed B cells, whereas the non-IL-4-producing clones had been selected in the presence of PBMC and intact mycobacterial organisms. This difference was highly significant ($P = 0{\cdot}02$; Fisher's exact test, Table 3).

Lymphokine production by antigen-activated cloned T cells

In order to assess whether the pattern of cytokine production in response to anti-CD3 was similar to that induced by mycobacterial antigens, we tested four of the clones that were capable of cross-reactive recognition of PPD (in the presence of autologous EBV-transformed B cells). The results are summarized in Table 4. In general, the patterns of cytokine production in response to antigen (PPD) were similar to that stimulated by anti-CD3, although the levels produced were somewhat lower in response to antigen.

Lymphokine production by resting versus partially activated cells

All of the above experiments were performed utilizing T cells isolated 2–3 weeks following previous mitogen stimulation. Analysis of the forward light scatter properties, as well as molecules associated with activation indicated that such cells were small sized and expressed reduced levels of HLA-DR (33 ± 10% brightly staining cells; $n = 9$). In contrast, cells taken 6 days after previous mitogen stimulation were much larger in size and had higher levels of HLA-DR (78 ± 16%, $n = 9$). In order to assess whether the pattern of cytokine production of these T-cell clones was related to cell activation, we compared the levels of cytokine produced by cells isolated either 6 or 20 days after previous mitogen stimulation. Figure 4 depicts the levels of IL-4 (*x*-axis) and IFN-γ (*y*-axis) produced

Table 2. Lymphokine production by leprosy skin lesion-derived *M. leprae*-reactive T-cell clones

Clone*	Lymphokine production			
	IL-4†	IFN-γ	TNF	IL-2
BT/RR				
6-23	< 3	> 256	NT	3(< 2–5)
6-26	< 3	> 256 (130– > 256)	NT	< 2
6-31	< 3	> 256	NT	< 2
6-34	< 3	> 256	NT	< 2
BT				
28-1	< 3	314 (128–500)	70 (32–108)	< 1
28-2	< 3	94 (60–128)	412 (324–500)	< 1
28-3	< 3	84 (18–150)	81 (54–108)	< 1
28-4	10	250	237 (180–300)	16
BT				
29-1	57 (37–78)	169 (100–280)	150 (128–162)	< 1
29-2	8 (4–11)	377 (128–500)	204 (128–324)	< 1
29-4	67 (34–125)	478 (256–650)	82 (32–162)	3(< 1–8)
LL				
30-1	23 (23–23)	1500 (1000–2000)	118 (108–128)	< 1
30-2	< 3	545 (90–1000)	21 (12–32)	< 1
30-3	10 (5–15)	3 (< 4–10)	91 (54–128)	< 1
30-4	47 (42–52)	1325 (650–2000)	145 (128–162)	< 1
LL				
31-2	< 3	412 (350–475)	27 (18–36)	< 1

*T cells (2×10^5) were stimulated for 24 hr in anti-CD3-coated microwells. The cell-free supernatants were obtained and assayed for lymphokine content. No lymphokines were detected in supernatants of cells cultured without anti-CD3.

† Mean (and range) for two to three experiments are shown for each clone. IL-2 and IFN-γ were assayed by both ELISA and bioassays, TNF was determined by bioassay only; IL-4 content was measured by ELISA only.

by 10 of the clones. Five of the six T-cell clones that produced detectable levels of IL-4 at day 20, produced three- to fivefold higher concentrations when assayed at day 6. IFN-γ and TNF production was generally unchanged or somewhat reduced. The remaining clones did not produce detectable IL-4 at any time. However, three of four such clones produced 3–10-fold higher levels of IFN-γ at day 6 than at day 20 following previous stimulation. Thus, among blast-enriched populations

Table 3. Fisher's exact probability analysis of the frequency of clones with cytokine-secreting phenotypes

	IL-4 production and clinical status*				IL-2 production and tissue origin†				IL-4 production by blood-derived clones and antigen selection‡		
	$IL\text{-}4^+$	$IL\text{-}4^-$	Total		$IL\text{-}2^+$	$IL\text{-}2^-$	Total		$IL\text{-}4^+$	$IL\text{-}4^-$	Total
PB	4	7	11	Skin	3	13	16	SOL	8	0	8
MB	3	2	5	Blood	8	4	12	ORG	1	3	4
Total	7	9	16	Total	11	17	28	Total	9	3	12
		$P = 0{\cdot}366$				$P = 0{\cdot}014$				$P = 0{\cdot}018$	

* The total number of clones falling into four categories: IL-4-producing clones from PB patients; IL-4-producing clones from MB patients; IL-4-non-producing clones from PB; and IL-4-non-producing clones from MB. When the data were analysed by Fisher's exact probability test, there was no association between clinical status and IL-4 production ($P = 0{\cdot}366$).

† The number of clones in each of four categories defined by tissue origin (blood versus skin) and IL-2 production (detectable versus non-detectable). The numbers of IL-2-producing clones were significantly higher in blood ($P = 0{\cdot}011$).

‡ The number of blood-derived clones in groups defined by IL-4 production and the *in vitro* method utilized in the selection of the line prior to cloning, either selection by soluble antigens in the presence of EBV-transformed B cells (Sol), or selection by intact organisims in the presence of blood plastic-adherent cells (Org). IL-4-producing clones were most frequently selected by soluble antigens in the presence of EBV-transformed B cells.

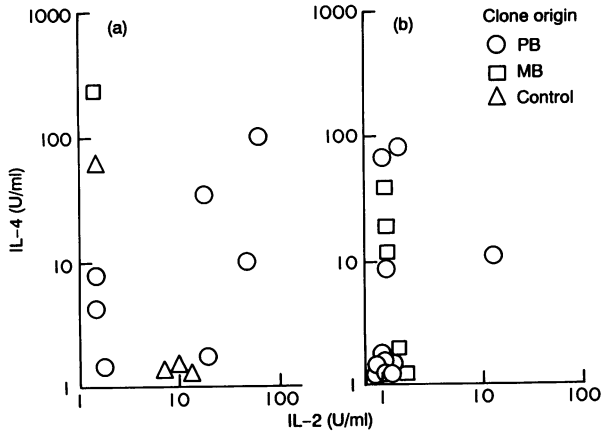

Figure 3. Comparison of the levels of IL-2 and IL-4 produced by T-cell clones from blood and skin. T-cell clones were isolated as described in Table 1 and the Materials and Methods. Cells were washed thoroughly and stimulated for 24 hr with plastic immobilized anti-CD3. The supernatant was harvested and assayed for IL-2 and IL-4 by ELISA or bioassay. (a) IL-4 production as a function of IL-2 production present within the supernatant of each of 12 clones isolated from blood; (b) the same from the 16 skin-derived clones. The cluster of symbols at the lower left of (b) consists of seven circles and two squares.

there was a modest tendency for IL-4-producing T clones to shift more towards a Th2-like pattern of cytokine secretion, and for non-IL-4-producing clones to become more Th1-like.

Lymphokine production by long-term cultured cells

Figure 5 compares the levels of IL-4 (x-axis) and IFN-γ (y-axis) produced by clones after $\geqslant 5$ months of after culture, with average levels produced at $\leqslant 10$ weeks. Although levels of IL-4 did not change in any consistent pattern, the IFN-γ-producing capacity of the clones shifted dramatically, in a characteriztic pattern for each patient. Thus, skin clones from BT patient 5 had greatly increased IFN-γ production, whereas all the clones from BT patient 6, and LL patients 7 and 8, produced substantially lower levels of IFN-γ. Thus, five of eight of these clones (from patients 7–9) had shifted to a pattern of cytokine secretion very similar to Th2 cells, one maintained a Th2-like pattern, and the other two clones produced little IL-4 or IFN-γ.

Table 4. Cytokine production by antigen-activated T-cell clones

Clone	Stimulus*	IL-4†	IL-2	IFN-γ
7-6	PPD	25	NT	128
	Media	<3	NT	<8
30-1	PPD	5 (4–5)	<2	256 (256–256)
	Media	<3	<2	<8
30-2	PPD	<3	<2	768 (512–1024)
	Media	<3	<2	<8
31-2	PPD	<3	<2	392 (256–512)
	Media	<3	<2	<8

* Cloned cells were harvested and cultured at 5×10^4 cells/well in the presence of 100 μg/ml PPD and 5×10^4 irradiated autologous EBV-tranformed B cells in 200 μl of media with 10% FCS. Higher doses of T cells, PPD and EBV B cells were used to increase cytokine levels. After 36 hr the supernatants were harvested and assayed for cytokine content.

† IL-4 and IL-2 were assayed by ELISA, and IFN-γ by bioassay. Results are expressed as the mean and range of two experiments for clones 30–1, 30–2 and 31–2. The results shown for clone 7–6 are from one experiment. This clone produced 96 U/ml IL-4 and 94 U/ml IFN-γ in response to immobilized anti-CD3.

NT, not tested.

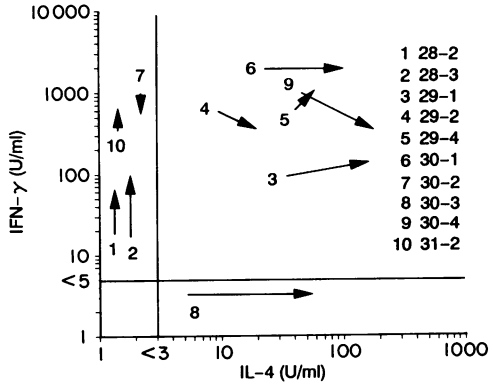

Figure 4. IFN-γ and IL-4 production by blast-enriched versus small cell-enriched populations. Cloned T cells were harvested either 6 days (blast enriched) or 20 days (resting cell enriched) after the previous stimulation with PHA, irradiated allogeneic feeder cells and IL-2. Cells were washed thoroughly and added to anti-CD3-coated microwells. Supernatants were harvested after 24 hr and IL-4 and IFN-γ measured by ELISA. The data from two experiments were averaged. The limit of detection was 5 U/ml and 3 U/ml for IFN-γ and IL-4, respectively. Each arrow represents the activity of one clone. The end-point of the arrow's tail represents cytokine activity of resting cell-enriched populations, whereas the tip of the head of the arrow depicts cytokine activity of blast-enriched cells. For example, at day 20 post-stimulation clone 3 (29-1) produced an average of 100 U/ml IFN-γ and 33 U/ml IL-4 in response to anti-CD3, whereas day 6 cells produced 130 U/ml IFN-γ and 170 U/ml IL-4.

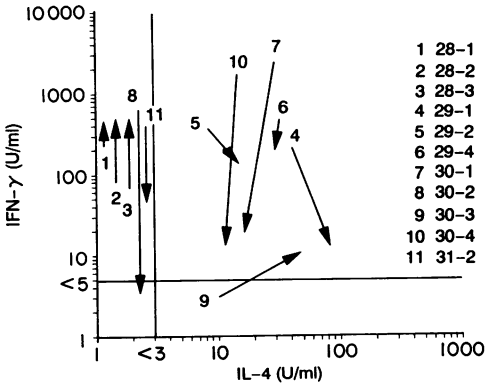

Figure 5. IFN-γ and IL-4 production by cloned T cells after short-term and long-term culture. T-cell clones were harvested 14–21 days after previous stimulation with PHA, irradiated allogeneic feeder cells and IL-2. Cells were washed and stimulated with anti-CD3, and supernatants collected, assayed for cytokine activity, and the data expressed as described in Fig. 4. The end-point of the arrow's tail indicates activity assayed at $\leqslant 10$ weeks of culture, whereas the tip of the arrow's head represents levels produced after $\geqslant 5$ months culture.

Separate experiments indicated that proliferation of the T-cell clones to the priming antigen was unaffected by long-term culture (data not shown). Significantly, the eventual pattern of cytokine synthesis was still not strictly correlated with clinical status of the patients, because clones from both PB and MB lesions had undergone this shift towards a Th2 pattern. Histological reassessment of a skin biopsy of BT patient 6 taken adjacent to that used to isolate the T-cell clones confirmed the presence of conventional epithelioid granuloma typical of BT patients (T.L. Miko, unpublished observations). Thus, there can be no question that all clones from a BT lesion had a clear IL-4-secreting pattern.

DISCUSSION

The goal of the current study was to examine the cytokine-synthesizing properties of a panel of CD4 T-cell clones derived from leprosy skin lesions and blood, and compare these properties with the clinical status of the patients from which they were derived. The important findings are as follows. (1) There was clear heterogeneity in the pattern of cytokine production, and although conventional Th0-, Th1- and Th2-like clones could be identified, a number of other patterns were apparent. (2) Several variables were found to influence the pattern of cytokine production, including the method of T-cell clone selection, the state of T-cell activation, and duration in long-term culture. In particular, prolonged culture over many months resulted in shifts in cytokine synthesis towards more Th1- or Th2-like patterns. (3) T cells isolated from the skin appeared to have reduced capacity to secrete IL-2, as compared with T-cell clones from blood. (4) There was no absolute correlation between cytokine production and clinical status, even after long-term culture. Thus, IL-4-producing clones were identified in lesions and blood of both PB and MB patients. Some of these results are in agreement and others contrast with other studies in human and animal models.

Of the lymphokines we examined, the production of IL-4 was most clearly heterogeneous from clone to clone, and in this respect our results agree with many other studies of murine and human clones.[6–10] However, one of the distinct features associated with murine IL-4-secreting Th2 clones is the failure to produce IFN-γ.[7] In our study, only two of 15 IL-4-producing clones conformed to this classification. The remainder produced high levels of IFN-γ, and they could be further subdivided into IL-2-producing or -non-producing groups.

Clones or cell populations with these and a number of other phenotypes distinct from Th1 and Th2 have in fact been identified in several more recent studies.[13–15,27–29] These findings have led to the hypothesis that Th1 and Th2 arise as a result of repeated antigen stimulation, either *in vitro* during the propagation of T-cell lines,[7,15] or *in vivo* during chronic diseases. Other patterns are produced by cells at earlier stages of differentiation.[30] A number of cytokines has been identified that affect this process both *in vivo* and *in vitro*.[31,32]

Two series of experiments suggested that the pattern of lymphokine production by our clones could be modulated *in vitro*. First, we observed that the ratio of IL-4 to IFN-γ was affected significantly by the time in the growth cycle at which the cells were assayed. IL-4-secreting clones produced higher levels of IL-4, and IL-4 non-secreting clones produced higher levels of IFN-γ, if populations enriched in activated blasts rather than small resting cells were induced by anti-CD3. Previous investigations have observed or proposed that distinct patterns of cytokines may be produced by activated and resting T cells.[28,33] More dramatic shifts towards Th1-like and Th2-like phenotypes were apparent when cytokine patterns were compared after many months in culture, with IFN-γ secretion being principally affected. It is not known what factors mediate this process in culture, but a striking result was that the shift in IFN-γ production was clearly lesion specific, even though all the clones were expanded in long-term culture under identical conditions. This argues strongly against the possibility that such changes were induced randomly in culture. One interpretation of this finding is that the cells *in situ* had irreversibly entered Th1 and Th2 differentiation pathways, but were isolated prior to full maturation.

It is noteworthy that most leprosy patients have been chronically infected for years prior to diagnosis.[2,3] If the differentiation of Th1 and Th2 cells was driven by chronic exposure to infectious agents, as has been suggested,[30] the Th1- and Th2-like patterns that we eventually observed should have been immediately apparent upon short-term culture. However, it is conceivable that, in chronic infections, relatively uncommitted T cells may be continuously recruited into the granuloma. This view is similar to the proposal of Bendelec & Schwartz,[33] who suggested that recent thymic emigrants may express multiple lymphokines upon exposure to antigen within inflammatory foci, and, depending on strength of antigen stimulus as well as activation state of the T cell, further differentiate into Th1 or Th2 types. Accordingly, at any given time, one might expect to find T cells present within diseased tissues at various stages of maturation. Our data are entirely consistent with this possibility.

In addition to the IL-4/IFN-γ co-secreters, the most striking departure from Th1 and Th2 patterns among clones assayed after short-term culture was the generally low IL-2 production by clones from skin. Only three of 16 clones produced IL-2, and at levels less than that made by clones from blood. Thus many clones could be identified which produced neither IL-2 nor IL-4. However, these clones were capable of strong antigen-specific proliferation provided IL-2 was added exogenously. Their functional potential was underscored by their ability to produce high levels of IFN-γ and TNF, two cytokines implicated in a number of important protective or pathological manifestations of the immune response.[34,35] The finding of few IL-2-producing clones was also consistent with our difficulties in inducing proliferation of freshly isolated cells from skin. In contrast, clones from blood could be derived easily, and a high frequency had the capacity to secrete IL-2. Those that did not secrete IL-2 produced IL-4, and some produced both. It is difficult to make a definitive comparison between blood and skin because the clones from these sources were selected by different procedures. None the less, it is tempting to speculate that, in addition to antigen, there may be important tissue-specific components that influence the activation status or cytokine-synthesizing potential of T cells. Such components could include different types of APC, for example Langerhans' cells,[36] extracellular matrix components, such as collagen, fibronectin, and laminin,[37] or other factors or hormones acting within tissue microenvironments.[38]

We did not observe a strong correlation between IL-4 production and the clinical status of the individual from which the clone was derived. Clones isolated from the blood of both

MB and PB patients produced IL-4. Among skin-derived clones, 36% from PB lesions and 60% from MB patients' lesions produced detectable IL-4. Many more clones would have to be analysed in order for these differences to reach statistic significance. These results are consistent with some studies,[4] but contrast sharply with others[5,6,39] who found IL-4 mRNA or IL-4-producing T cells primarily from lepromatous blood and lesions.

There are several possible reasons for these discrepancies. First, the method of clone selection may significantly affect the results obtained. In particular, our selection method for skin T-cell clones was designed to isolate cells present in the numerical majority, and we used primarily antigen-specific proliferation as the selection criterion. Panels of clones used by other investigators have included clones selected for their ability to suppress T-cell proliferation.[6,10,40] These clones themselves did not proliferate to *M. leprae* antigen,[10,40] so such clones would not have been identified by our approach.

Second, our approach makes a significant assumption, namely that the *in vitro* functional properties of purified T cells reflect in some way *in vivo* activity of the entire population. Studies examining cytokine levels by immunohistological methods[41] or mRNA levels by *in situ* hybridization[42] or polymerase chain reaction (PCR) techniques[5,39] clearly circumvent this problem. Such methods assume, however, that measured levels are produced by antigen-specific T cells, an assumption that is not necessarily valid considering most T cells in lesions cannot be demonstrated to be *M. leprae* specific[26] (R.C. Howe, manuscript in preparation) and may have undergone activation prior to their homing to the inflammatory site.[43] None the less, if we take the results of both approaches at face value, the implication is that only the summed effect of all cell–cell and cell–tissue interactions results in either a Th1 or Th2 pattern, whereas analysis of the individual T cells uncovers more extensive and less predictable heterogeneity. One of the many mechanisms that might contribute to such a phenomenon is 'cross-regulation',[44] the ability of Th1 and Th2 cells to inhibit the expression of the other. Thus it is difficult to exclude the possibility that the IL-4-producing T cell clones that we identified in BT lesions and blood may have been functionally suppressed *in vivo*, and their latent activity only revealed upon separation from the influence of other cells.

Third, the nature of the patients themselves may vary substantially from study to study. Among our PB patients we selected BT or borderline tuberculoid leprosy patients, and it is known that these individuals are prone to shifting their clinical status.[2,3] One cannot ignore the possibility that such patients may have once had a more lepromatous form of the disease. However, if this were correct it would also imply that the generation of *M. leprae*-reactive T cells with IL-4-producing potential may be more strongly correlated with the priming environment than the existing clinical state of the granuloma(s). It could thus be argued that Th1 and Th2 patterns occur as secondary events rather than directly influencing granuloma formation and maintenance.

In addition, the clinical forms of leprosy could be very significantly influenced by many epidemiological variables which are often difficult to evaluate, such as the dose of *M. leprae*, duration of the disease, or socio-economic status.[2,3] In particular, it is difficult to assess the effects of other infections superimposed on leprosy. Several investigators have shown that T cells from mice immunized with infectious organisms known to evoke either Th1-like or Th2-like responses secreted different patterns of cytokines in response to polyclonal, antigenic or alloantigenic stimuli.[15,45] Consistent evidence in human studies has been provided by Parronchi *et al.*,[11] who reported that differences in cytokine production by *M. tuberculosis*-specific clones correlated with the presence of another concurrent disease such as atopic allergy. These studies imply that the immune response to one immunogen or infectious agent can influence the response to an antigenically unrelated one. By analogy, a high percentage of the population in Ethiopia is heavily exposed to parasite infections,[46] many of which may go undetected and be relatively harmless clinically but which could influence the functional phenotype and frequency of *M. leprae*-specific T cells. Such a hypothesis, while readily accommodating discrepancies between laboratories, none the less underscores the heterogeneity of cytokine secretion patterns which could exist in humans, particularly in poor, underdeveloped countries.

In summary, our analysis of lymphokine secretion by *M. leprae*-specific T cells suggests that such cells are substantially more heterogeneous than previously suspected. Our data do not correspond exactly with any of the previous models of helper T-cell subpopulations, but they are more consistent with proposals that suggest that inflammatory foci may be continuously seeded with relatively uncommitted precursor cells, which then give rise to multiple subpopulations under the influence of locally produced factors.

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