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Mycobacterium leprae-Specific Lyt-2$^+$ T Lymphocytes with Cytolytic Activity

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Mice were immunized intradermally with 10^7 irradiated *Mycobacterium leprae* organisms, and draining lymph nodes were collected after 4 weeks. Lymph node cells were restimulated in vitro with soluble *M. leprae* antigen and accessory cells. The resulting T-cell line was propagated in vitro in the presence of *M. leprae* antigen, accessory cells, and interleukin-2-containing supernatants from concanavalin A-stimulated rat spleen cells. Long-term cultured T cells were Thy-1$^+$ L3T4$^-$ Lyt-2$^+$ as revealed by analysis with the fluorescence-activated cell sorter. From this line, T-cell clones with the same phenotype were established. The T-cell clone A4 failed to secret interleukin-2 after stimulation with antigen and accessory cells, and its growth depended on exogeneous interleukin-2. A4 T cells produced gamma interferon in an antigen-specific, *H-2*-restricted, and interleukin-2-dependent way. Importantly, this T-cell clone was capable of lysing bone marrow macrophages presenting *M. leprae* antigen. Other T-cell clones as well as native Lyt-2$^+$ T cells from *M.leprae*-immunized mice were also capable of lysing bone marrow macrophages expressing *M. leprae* antigens. These findings suggest that specific Lyt-2$^+$ T cells participate in the immune response to *M. leprae*. It is postulated that cytolysis of *M. leprae*-infected macrophages or Schwann cells contributes to protection against and pathogenesis of leprosy.

Leprosy is a chronic infectious disease caused by the intracellular microorganism *Mycobacterium leprae* (1, 9). This pathogen belongs to the group of intracellular bacteria which are capable of replicating within nonactivated macrophages (5). Acquired resistance against these microorganisms is mediated by specific T lymphocytes (1, 5). It is generally assumed that activation of bacteriocidal macrophage functions by lymphokines (e.g., gamma interferon [IFN-γ]) represents the major step in the acquisition of resistance against intracellular bacteria, including *M. leprae* (1, 5, 19). These lymphokines are generally produced by helper T cells of L3T4$^+$ (mouse) T4$^+$ (human) phenotype (5, 25). In agreement with this notion, T-cell clones of helper phenotype which produce IFN-γ after antigen stimulation have recently been established from *M. leprae*-immune mice (8, 11) and from tuberculoid leprosy patients with effective T-cell immunity (3). In contrast, Lyt-2$^+$ (mouse) T8$^+$ (human) effector T cells are capable of killing target cells and are thought to be of major importance for resistance against viral infections (5, 25). However, studies performed in the model of experimental listeriosis and tuberculosis of mice have provided evidence to suggest that Lyt-2$^+$ T cells play a relevant role in adoptive protection against intracellular bacteria (15, 16, 20). Furthermore, we have shown that *Listeria monocytogenes*-specific T-cell clones are capable of lysing *L. monocytogenes*-infected macrophages (14). This finding led us to propose that lysis of infected target cells by cytolytic T lymphocytes represents a relevant mechanism of antibacterial protection (14).

M. leprae is an obligate intracellular bacterium capable of escaping from the phagosome (18). Furthermore, *M. leprae* inhabits not only mononuclear phagocytes but also nonmyeloid cells, in particular, Schwann cells. Therefore, it can be envisaged that *M. leprae* is particularly equipped to evade lymphokine-induced bacteriocidal macrophage functions that had been induced by lymphokines and that specific lysis of infected host cells is of special importance for resistance against leprosy. To test this hypothesis, we have generated *M. leprae*-specific Lyt-2$^+$ T-cell lines and clones. Cloned T cells not only produced IFN-γ in an antigen-specific, *H-2*-restricted way, but also lysed macrophages presenting *M. leprae* antigens.

MATERIALS AND METHODS

Mice. Male C57BL/6 and B10.A mice at 8 to 12 weeks of age were used throughout this study.

Bacteria and bacterial antigens. Irradiated *M. leprae* organisms (batch CD20) and soluble *M. leprae* proteins (batches CD34 and CD48) were kindly provided by R. J. W. Rees, Harrow, U.K., through the World Health Organization-Immunology of Leprosy (WHO-IMMLEP) program and had been prepared acording to protocol 1/79 WHO document TDR, IMMLEP-SWG (S) 80.3. Purified protein derivative was purchased from the State Serum Institute, Copenhagen, Denmark. Live *M. bovis* organisms, strain BCG Phipps, were originally provided by R. J. North, Trudeau Institute, New York, N.Y., and grown in Dubos Middlebrook medium (Difco Laboratories) supplemented with albumin and Tween 80. Bacterial numbers were determined by plating 1:10 dilutions on Middlebrook agar plates (Difco).

Establishment of *M. leprae*-specific T-cell lines and clones. C57BL/6 mice were immunized with 10^7 irradiated *M. leprae* organisms intradermally into the left and right hind flanks. After 4 weeks, draining lymph nodes were harvested and single cell suspensions were prepared. Lymph node cells (2×10^5/ml) were cultured in the presence of soluble *M. leprae* antigen (1 μg/ml), and 2×10^5 irradiated (3,300 R) spleen cells per ml were used as accessory cells (AC). Cultures were performed in Iscove modified Dulbecco medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics (GIBCO Laboratories) in Costar 3506 trays at 37°C and 7% CO_2. After 1 week of culture, cells were restimulated with AC, antigen, and 10% interleukin-2 (IL-2)-containing supernatant (SN)

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from concanavalin A-activated rat spleen cells (13). T-cell cultures were restimulated every 3 to 4 days by an alternate cycle of SN alone or of antigen, AC, and SN. After 8 weeks of culture, cells were cloned under limiting-dilution conditions in the presence of AC, antigen, and SN in round-bottom microtiter plates (Nunc). Cloned T cells were expanded as described above.

Phenotype analysis. Cells were purified over a Ficoll-Hypaque gradient (Pharmacia, Inc.) and then with anti-Thy-1.2 (Olac), anti-L3T4 (GK1.5 [2]), or anti-Lyt-2.2 (HO2.2 [4]) monoclonal antibodies and afterwards with fluorescein isothiocyanate-coupled goat anti-mouse or anti-rat immunoglobulin antiserum (Dianova) as described previously (15). After being washed, cells were analyzed on an Ortho Cytofluorograph 50 H fluorescence-activated cell sorter (Ortho Diagnostic Systems).

Determination of proliferation and IL-2 and IFN-γ activities. T cells were purified over a Ficoll-Hypaque gradient, and afterwards, 10^4 T cells per 0.2 ml were cultured with 2×10^5 AC and 2 μg of soluble *M. leprae* antigen per 0.2 ml with or without 10 U of recombinant IL-2 (rIL-2) per 0.2 ml in round-bottom microculture plates. After 3 days, 1 μCi of [^{3}H]thymidine (The Radiochemical Centre) was added, and 18 h later [^{3}H]thymidine incorporation was determined. Alternatively, 10^4 T cells per 0.2 ml were stimulated with AC, antigen, and rIL-2 as described above, and 24 h later supernatants were collected. IL-2 activities were determined on an IL-2-addicted T-cell line as described previously (12), and IFN activities were assessed on L929 cells infected with vesicular stomatitis virus as described before (10). As a control, the titer of rIFN-γ was determined in parallel. In some experiments, a specific anti-IFN-γ rabbit antiserum was added at a final dilution of 1:50.

Generation of BMMϕ. Bone marrow cells were obtained from both femura of normal mice, and 10^5 cells per ml were cultured in hydrophobic Teflon bags (Biofolia 25; Haereus) in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 1 mM sodium pyruvate, 10% fetal calf serum, 5% horse serum, 30% L929 conditioned medium, and antibiotics (GIBCO) at 37% and 10% CO_2 (19). Bone marrow macrophages (BMMϕ) were used after 9 days of culture (17).

Determination of cytolytic T-cell activity. Cytolysis was determined by a neutral red incorporation assay by the method of Parish and Müllbacher (21), with BMMϕ as target cells. T cells were purified over a Ficoll-Hypaque gradient. BMMϕ (10^5 per well) were cultured with T cells at different effector/target ratios with or without antigen in flat-bottom microculture plates (Nunc) at 37°C and 7% CO_2. After overnight incubation, medium was discarded; after washing, 0.2 ml of warm 0.036% neutral red (Sigma Chemical Co.) was added. After 30 min at 37°C, the dye was discarded, and target monolayers were washed twice with phosphate-buffered saline and then lysed with 0.1 ml of 100 mM acetic acid in 50% ethanol. Optical density was measured at 570 nm with reference at 630 nm, using a micro-enzyme-linked immunoassay autoreader MR580 (Dynatech Industries, Inc.). Percent lysis was calculated by the formula: $100 \times$ (control − experimental/control).

RESULTS

Establishment of an Lyt-2$^+$ T-cell line from *M. leprae*-immunized mice. Lymph node cells from *M. leprae*-immunized mice were cultured in the presence of AC, *M. leprae* antigen, and IL-2-containing SN. After 4 weeks, the phenotype of this line was determined by fluorescence-activated cell sorter analysis as being Thy-1$^+$ L3T4$^-$ Lyt-2$^+$ (Fig. 1). Activation of this T-cell line was antigen and IL-2 dependent as indicated by the fact that the T cells produced IFN only after stimulation with *M. leprae* antigen, AC, and rIL-2 and that, in the absence of any of these stimuli, IFN was not detectable (data not shown). The T-cell line was cloned under limiting dilution conditions and four clones were estabished. The four clones were Thy-1$^+$ L3T4$^-$ Lyt-2$^+$ as revealed by fluorescence-activated cell sorter analysis (data not shown). One of these clones, designated A4, was studied more extensively.

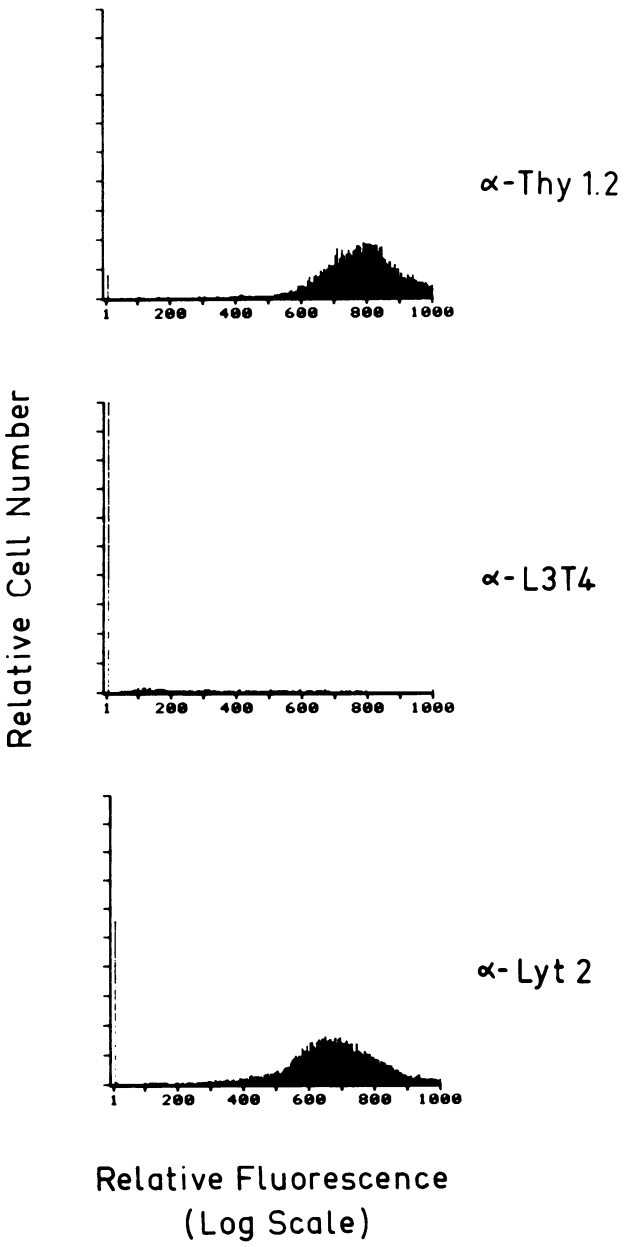

FIG. 1. Fluorescence analysis of long-term cultured T cells from *M. leprae*-immune mice. T cells were stained as described previously (15). Control treatments with fluorescein isothiocyanate-coupled goat anti-mouse or anti-rat immunoglobulin stained <3% of the T cells.

***M. leprae*-specific IFN-γ secretion by A4 T cells.** A4 T cells

TABLE 1. Lack of *M. leprae*-induced proliferation and IL-2 secretion by T-cell clone A4[a]

Culture condition	Proliferative response (cpm)	IL-2 activity (cpm)
T cells alone	734	155
T cells + AC	1,269	281
T cells + *M. leprae* antigen	848	164
T cells + AC + *M. leprae* antigen	1,420	73
T cells + AC + *M. leprae* antigen + rIL-2	22,458	ND[b]
T cells + rIL-2	28,373	ND

[a] T cells (10^4) were stimulated with 2×10^5 syngenic AC, 2 μg of soluble *M. leprae* antigen, and 10 U of rIL-2, and proliferative responses and IL-2 activities were determined as described in Materials and Methods.
[b] ND, Not determined.

were stimulated by AC and *M. leprae* antigen with or without rIL-2, and proliferative responses were determined. In the absence of rIL-2, proliferation by A4 T cells was negligible (Table 1), indicating that growth of A4 T cells was strictly IL-2 dependent. Although no differences in T-cell growth were seen in short-term cultures with or without antigen, long-term cultures of A4 T cells ceased to grow after several restimulations with SN or rIL-2 alone. Furthermore, A4 T cells failed to secrete IL-2 after stimulation by AC plus *M. leprae* antigen (Table 1). Cultures of A4 T cells, AC, *M. leprae* antigen, and rIL-2 produced significant IFN activities (Table 2). IFN secretion was antigen specific and IL-2 dependent, since in the absence of either IL-2 or antigen lower IFN activities were found. The IFN activity could be abrogated by the addition of a specific anti-IFN-γ antiserum, indicating that it was of the IFN-γ type.

***H-2*-restricted IFN-γ secretion by A4 T cells.** To evaluate whether IFN-γ secretion by *M. leprae*-specific A4 T cells was *H-2* restricted, AC from C57BL/6 (*H-2*^{b}) and congenic B10.A (*H-2*^{a}) mice were used. A4 T cells were stimulated by AC, antigen, and rIL-2, and IFN activities were determined. The data depicted in Table 3 show that IFN secretion was only stimulated by syngenic, not congenic, *H-2*-incompatible AC. This finding suggests that recognition of *M. leprae* antigen by A4 T cells was *H-2* restricted.

***M. leprae*-specific cytolysis by cloned T cells.** We wanted to assess whether cloned *M. leprae*-specific T cells were capable of lysing antigen-presenting host cells. Macrophages are

TABLE 2. *M. leprae*-specific IFN-γ secretion by T-cell clone A4[a]

Culture condition	IFN activity (U)
T cells alone	<1
T cells + AC	<1
T cells + *M. leprae* antigen	<1
T cells + AC + *M. leprae* antigen	9
T cells + AC + *M. leprae* antigen + rIL-2	81
T cells + AC + PPD	<1
T cells + AC + PPD + rIL-2	1
T cells + rIL-2	9
T cells + AC + *M. leprae* antigen + rIL-2 + anti-IFN-γ antiserum	<1

[a] T cells (10^4) were cultured with 2×10^5 irradiated syngenic spleen cells, antigen (2 μg), and rIL-2 (10 U), and supernatants were tested for IFN activities. As a control, rIFN-γ was titrated in parallel. PPD, Purified protein derivative.

TABLE 3. *H-2*-restricted IFN secretion by *M. leprae*-specific T-cell clone A4[a]

Stimulation	IFN activity (U)
AC (*H-2*^{b})	<1
AC (*H-2*^{b}) + *M. leprae* antigen	<1
AC (*H-2*^{b}) + *M. leprae* antigen + rIL-2	81
AC (*H-2*^{a})	<1[a]
AC (*H-2*^{a}) + *M. leprae* antigen	<1[a]
AC (*H-2*^{a}) + *M. leprae* antigen + rIL-2	3[a]

[a] T cells (10^4) were stimulated with 2×10^5 AC from C57BL/6 (*H-2*^{b}) or B10.A (*H-2*^{a}) mice in the presence of 2 μg of soluble *M. leprae* antigen and 10 U of rIL-2. IFN activities were determined in supernatants of 24-h cultures. As a control, rIFN-γ was titrated in parallel.

a natural habitat of *M. leprae* organisms and are capable of processing and presenting *M. leprae* antigens (1). Recently, we have shown that *L. monocytogenes*-infected BMMϕ can serve as targets for antigen-specific T-cell lysis (14). Therefore, T cells were cultured together with BMMϕ in the presence or absence of *M. leprae* antigen, and their cytolytic activity was determined in a modified neutral red incorporation assay (21). A4 T cells lysed BMMϕ presenting *M. leprae* antigens (Fig. 2), whereas BMMϕ presenting *M. bovis* BCG organisms were not affected. In contrast, an *M. bovis* BCG-specific T-cell line could lyse *M. bovis* BCG-pulsed BMMϕ, demonstrating that this antigen could be presented adequately (Fig. 2). These findings suggest that the T-cell clone A4 possessed *M. leprae*-specific cytolytic activity. Furthermore, A4 T cells failed to lyse YAC cells as determined in a conventional ^{51}Cr-release assay, indicating that they were devoid of natural killer activity (data not shown). The *M. bovis* BCG-specific line lysed BMMϕ pulsed with *M. leprae* (data not shown), indicating cross-reactivity between *M. bovis* and *M. leprae* on the population level. So far, we have tested six other *M. leprae*-specific Lyt-2$^+$ T-cell clones

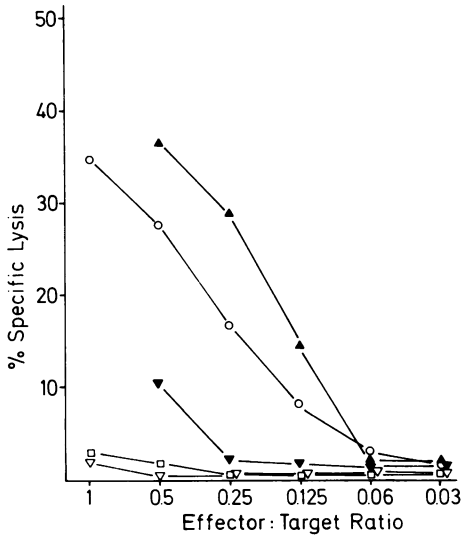

FIG. 2. Cytolytic activity of *M. leprae*-specific T-cell clone A4. BMMϕ (10^5) were cultured with 2 μg of *M. leprae* antigen (○), 2×10^6 killed *M. bovis* BCG (□), or without antigen (▽) at the effector/target ratios indicated. As a control, the cytolytic activity of an *M. bovis*-specific T-cell line against BMMϕ with (▲) or without (▼) *M. bovis* was also determined. Specific lysis was determined and calculated as described in Materials and Methods.

TABLE 4. Lysis of BMMϕ expressing *M. leprae* antigen by native *M. leprae*-immune T cells[a]

Target	% Specific lysis at given effector/target ratio		
	1:1	0.5:1	0.25:1
BMMϕ alone	12	3	1
BMMϕ + *M. leprae*	44	31	8

[a] BMMϕ (10^5) were cultured with T cells at the effector/target cell ratios indicated in the presence or absence of 2 μg of soluble *M. leprae* antigen, and specific lysis was determined.

derived from either the line described here or another independent line and observed *M. leprae*-specific cytolysis by four of six clones (data not shown).

Lysis of BMMϕ expressing *M. leprae* antigen by native T cells from *M. leprae*-immunized mice. The possibility was considered that cloned Lyt-2^+ T cells had acquired their cytolytic activity during long-term in vitro propagation. To contest this assumption, lymph node cells from mice immunized intradermally with killed *M. leprae* organisms 4 weeks previously were restimulated in vitro with AC, *M. leprae* antigen, and SN. After 8 days of culture, these cells were >95% Lyt-2^+ L3T4$^-$. These T cells were purified over Ficoll-Hypaque, and their cytolytic activity was determined. The T cells lysed BMMϕ only in the presence of *M. leprae* antigen (Table 4). In contrast, T cells from normal mice or from *L. monocytogenes*-immune mice failed to lyse *M. leprae*-primed BMMϕ. Lyt-2^+ T-cell lines from *L. monocytogenes*-immune mice were capable of lysing *L. monocytogenes*-primed BMMϕ (G. De Libero and S. H. E. Kaufmann, J. Immunol., in press). These findings suggest that in vivo immunization was essential for the generation of *M. leprae*-specific Lyt-2^+ T cells with cytolytic activity. Native T cells from *M. leprae*- or *L. monocytogenes*-immune mice without being restimulated in vitro failed to lyse target cells expressing the homologous antigen. These findings indicate that the T cells acquired their antigen-specific cytolytic potential after in vivo immunization and that expression of cytolytic activity required triggering by antigen or helper factors or both.

DISCUSSION

In the present study, long-term cultured T-cell lines and clones were derived from *M. leprae*-immunized mice. These T cells were *M. leprae* specific based on the finding that they reacted with *M. leprae* antigen but not with *M. bovis* BCG or with purified protein derivative. Furthermore, *M. leprae* antigen was recognized in association with *H-2*-encoded molecules since cells of *H-2*^{b} but not those of the congenic *H-2*^{a} haplotype could serve as AC.

In the absence of exogeneous IL-2, these T cells failed to replicate, and antigen-induced IFN secretion was markedly increased by addition of exogeneous IL-2, indicating that the A4 T-cell clone was strictly IL-2 dependent. This finding is in accordance with those of others (i) that most Lyt-2^+(T8$^+$) T cells fail to secrete IL-2 after antigen stimulation and depend on exogeneous IL-2, which in situ is supplied by L3T4$^+$(T4$^+$) T cells (24), and (ii) that IFN-γ secretion by Lyt-2^+(T8$^+$) T cells requires IL-2 as a second signal in addition to antigen (22). However, we cannot exclude that the A4 T cells had acquired their IL-2 dependence during in vitro propagation.

The *M. leprae*-specific T-cell lines and clones described here were from mice that had been immunized intradermally with *M. leprae*. It has been shown that this immunization scheme protects mice from subsequent *M. leprae* infection (23). Previously, we generated T-cell lines and clones from mice immunized subcutaneously with killed *M. leprae* in incomplete Freund adjuvant which had the characteristics of helper T cells (8, 11). Thus, immunization of mice with irradiated *M. leprae* leads to the generation of both L3T4$^+$ helper and Lyt-2^+ cytolytic T cells. Whether the different modes of immunization had an influence on the preferential induction of either T-cell type remains to be clarified.

Perhaps the most remarkable finding of this study was that cloned T cells as well as native T cells from *M. leprae*-immune mice, after restimulation in vitro, lysed macrophages presenting *M. leprae* antigen. To demonstrate antigen-specific cytolysis, we used BMMϕ as target cells. These cells have already been used successfully for the demonstration of antigen-specific cytolytic activity of Lyt-2^+ T-cell lines and clones from *L. monocytogenes*-infected mice (14; De Libero and Kaufmann, in press). Thus, BMMϕ seem to be particularly suitable for processing and presenting bacterial antigens to cytolytic T cells.

In a widely accepted concept to explain the mechanisms underlying cellular resistance against infectious agents, it has been postulated that Lyt-2^+(T8$^+$) T cells with cytolytic activity play a major role in the defense against viral infections, whereas L3T4$^+$(T4$^+$) T cells with helper activity are thought to be the prominent mediators of protection in intracellular bacterial diseases (5, 25). It has been argued in a teleological way that lysis of virus-infected cells is protective because viral replication strictly depends on intact host cells. On the other hand, most intracellular bacteria can multiply in the intra- and extracellular compartments. Hence, activation of microbicidal functions would be more adequate for the defense of bacterial infections. Our finding that host cells presenting *M. leprae* antigens can be lysed by specific T lymphocytes of the Lyt-2^+ phenotype suggests that cytolytic mechanisms are involved in leprosy.

Lysis of host cells could contribute to acquired resistance against leprosy for the following reasons. *M. leprae* cannot be grown in vitro, indicating that also in an in vivo situation this bacterium is an obligate parasite which can only replicate inside viable host cells (1, 9, 18). Thus, destruction of infected cells could already restrict growth of *M. leprae*. Furthermore, *M. leprae* resides not only in professional phagocytes, but also in nonmyeloid host cells, particularly in Schwann cells (1, 6). The latter cells as well as certain tissue macrophages have a weak antibacterial potential and hence are refractory to lymphokine activation. In this case, cytolysis and concomitant release of *M. leprae* could facilitate their uptake by monoclear phagocytes with high antibacterial potential, e.g., immigrant blood monocytes. Through lymphokine activation these cells could then become capable of destroying intracellular *M. leprae* organisms in a more efficient way. The signals required for the latter step could be provided by helper T cells, since *M. leprae*-specific L3T4$^+$(T4$^+$) T cells have been found to secrete macrophage-activating lymphokines including IFN-γ (3, 8, 11). Cytolytic Lyt-2^+(T8$^+$) T cells could further facilitate lymphokine-mediated activation because, as shown here, they can produce IFN-γ as well. However, IFN-γ secretion by Lyt-2^+ T cells seems to depend on IL-2-producing L3T4$^+$ T cells, because unlike *M. leprae*-specific L3T4$^+$ T-cell clones (8, 11), Lyt-2^+ T cells failed to secrete IL-2 and IFN-γ after antigenic stimulation. At the same

time, lysis of infected target cells could result in tissue destruction. Hence, a single mechanism could contribute to both protection and pathogenesis.

It has been shown that Lyt-2$^+$ T cells are required for successful transfer of protection against murine listeriosis and tuberculosis (15, 16, 20) and that *L. monocytogenes*-specific Lyt-2$^+$ T-cell clones can lyse infected target cells (14). Therefore, participation of cytolytic Lyt-2$^+$(T8$^+$) T cells may be a general mechanism of acquired resistance against intracellular bacteria. In the listeriosis model, it has also been found that L3T4$^+$ T cells are required for effective protection (7, 14). In summary, these findings suggest that both T-cell subsets are involved in acquired resistance against intracellular bacterial pathogens. Due to their preferential replication in Schwann cells and their capacity to escape from the phagosome (18), *M. leprae* may be particularly resistant against lymphokine-induced bacteriocidal functions, and cytolytic mechanisms may gain special importance in protection against and pathogenesis of leprosy.

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