LISTERIA MONOCYTOGENES-REACTIVE T LYMPHOCYTE CLONES WITH CYTOLYTIC ACTIVITY AGAINST INFECTED TARGET CELLS

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The T cell lineage is generally divided into two distinct subpopulations on the basis of their function, phenotype, and genetic restriction (1, 2). In the mouse, helper/inducer T cells are $L3T4^+$, $Lyt\text{-}2^-$, class II–restricted, and secret multiple lymphokines. CTL are $L3T4^-$, $Lyt\text{-}2^+$, class I–restricted, and lyse target cells via direct cell-cell contact. Protective immunity against intracellular bacteria, including *Listeria monocytogenes*, is mediated by specific T cells and expressed by activated macrophages (2). Class II–restricted, Lyt 2^- *L. monocytogenes*–specific T lymphocytes produce many lymphokines in vitro, and adoptive protection against listeriosis has been found to be class II–restricted (3–6). These findings led to the concept that Th are crucial for cellular antibacterial immunity. This concept, however, requires verification because it was also found that successful adoptive protection against listeriosis depends on $Lyt\text{-}2^+$ T cells and on class I compatibility between T cells and recipients (7–9). Whether *L. monocytogenes*–specific $Lyt\text{-}2^+$ T cells reside in the CTL set has not been addressed by these studies. To approach this question we have established $Lyt\text{-}2^+$ T cell clones from *L. monocytogenes*–infected mice and studied their cytotoxic potential against infected phagocytes.

Materials and Methods

Mice. Male C57BL/6, DBA/2, B6.C-$H2^{bm1}$, and B6.C-$H2^{bm12}$ mice, 8–12 wk old, were used. Mice were raised under specific pathogen–free conditions at the Max-Planck-Institute, Freiburg.

Establishment of $Lyt\text{-}2^+$, L. monocytogenes-reactive T cell clones. C57BL/6 mice were intravenously infected with 5×10^4 live *L. monocytogenes* EGD organisms (originally obtained from G. B. Makaness, Saranac Lake, NY) and 6 d later splenic T cells were enriched by passage over nylon wool columns, as described previously (7). T cells were analyzed or sorted at a flow rate of 2,000 cells/s using a Cytofluorograph 50 H (Ortho Diagnostic Systems Inc., Raritan, NJ) as described (8). After washing, positively selected $Lyt\text{-}2^+$ T cells (2×10^4/0.2 ml) were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% FCS, antibiotics, 1 mM glutamine, and 2×10^{-5} M 2-ME in the presence of 2×10^5/0.2 ml *L. monocytogenes*–infected stimulator cells and 10% crude IL-2 in round-bottomed microculture plates (Nunc, Roskylde, Denmark) at 37°C, 7% CO_2. Irradiated (2,200 rad) spleen cells from mice that had been infected

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intravenously with 5×10^5 live *L. monocytogenes* 2 d previously were used as stimulator cells. As a source of crude IL-2, we used unpurified supernatants of Con A– (Pharmacia Fine Chemicals, Uppsala, Sweden) stimulated spleen cells from Lewis rats (6) containing 20 μg/ml α-methyl-mannoside. T cells were cloned in round-bottomed microculture plates (Nunc) in complete IMDM containing 2×10^5/0.2 ml *L. monocytogenes*–infected stimulator cells and 10% crude IL-2. Growing cells were transferred to and expanded in Costar 3524 and 3506 trays at a density of 2×10^4 to 1×10^5 cells per ml as described above.

Determination of Proliferative Responses and of IL-2 and IFN-γ Activities. T cells were purified over a Urovison-Ficoll gradient (ρ-1.077) and 3×10^4/0.2 ml T cells cultured with *L. monocytogenes*–infected or noninfected stimulator cells (2×10^5/0.2 ml) in complete IMDM in round-bottomed plates at 37°C, 7% CO_2. Some cultures were also stimulated with 50 U/0.2 ml human rIL-2. Proliferation responses were measured after 4 d (6). For determination of lymphokine activities, 100 μl supernatants were collected 24 h after initiation of cultures. IL-2 activities were determined using an IL-2-addicted CTL line and IFN activities were determined as described (4). A specific rabbit anti-IFN-γ antiserum was added to some cultures (final dilution, 1:50).

Determination of Cytotoxic Activity. Mice were injected intraperitoneally with 10^6 live *L. monocytogenes*, with 2 ml 10% proteose peptone, or remained untreated. Bone marrow cells from C57BL/6 mice were cultured in Teflon bags according to Munder et al. (10). After 9 d, bone marrow macrophages (BMMØ) were infected with 10^7/ml live *L. monocytogenes* or 5×10^6/ml *Mycobacterium bovis*, strain Bacillus Calmette-Guerin (BCG), overnight. Peritoneal cells or BMMØ were washed vigorously, labeled with ^{51}Cr for 80 min, and used as target cells. In some experiments, ^{51}Cr-labeled P815 and YAC tumor cells were also used as targets. T cells were added to 5×10^3 ^{51}Cr-labeled target cells at the E/T ratios indicated in the figures (7). After 4–6 h, the percentage of ^{51}Cr released from target cells was determined in triplicate samples and calculated according to the following equation: percent ^{51}Cr release = 100 X (experimental release-baseline release)/(maximal release-baseline release).

Results

IFN-γ Secretion by L. monocytogenes–reactive Lyt-2⁺ T cell clones. Positively selected Lyt 2^+ T cells from *L. monocytogenes*–infected mice were grown in the presence of in vivo–infected stimulator cells and exogeneous IL-2. Using this protocol, we established six clones that had the phenotype Thy-1^+, $L3T4^-$, Lyt-2^+, as revealed by analysis with the fluorescence-activated cell sorter. Lyt-2^+ T cell clones were stimulated with normal or infected stimulator cells ± human rIL-2. Normal and infected stimulator cells alone failed to induce T cell proliferation, as well as secretion of IL-2 (data not shown). Although human rIL-2 alone induced T cell proliferation (data not shown), long-term growth of the clones depended on the presence of *L. monocytogenes*–infected stimulator cells and subcultures grown in the presence of IL-2 alone ceased to divide and died after 3 to 4 weekly restimulations. As shown in Table I, cultures of cloned T cells, infected stimulator cells, and human rIL-2 produced significant IFN activities, while similar cultures with normal stimulator cells did not. The IFN activities could be abrogated by addition of a specific anti-IFN-γ antiserum and hence were of the IFN-γ type. In the absence of cloned T cells, infected stimulator cells already produced low amounts of IFN, which could not be neutralized by the anti-IFN-γ antiserum, indicating that it was of the IFN-α/β type. Thus, the Lyt-2^+ T cell clones derived from *L. monocytogenes*–infected mice produced IFN-γ in an antigen plus IL-2-dependent manner.

Cytolytic Activity of L. monocytogenes–reactive Lyt2⁺ T cell Clones against Infected Target Cells. As shown in Fig. 1, the T cell clones significantly lysed peritoneal

TABLE I

IFN-γ Secretion of L. monocytogenes–reactive Lyt-2⁺ T Cell Clones

Culture condition	IFN secretion by T cell clone*						
	None	1D7	1D11	1D12	1E6	1E11	1F1
Normal stimulator cells	<3	<3	<3	<3	<3	<3	<3
Normal stimulator cells + human rIL-2	<3	<3	<3	<3	<3	<3	<3
Infected stimulator cells	3	3	3	3	3	3	3
Infected stimulator cells + human rIL-2	3	27	81	27	9	27	27
Human rIL-2	ND	<3	<3	<3	<3	<3	<3
Infected stimulator cells + human rIL-2 + anti–IFN-γ antiserum	3	3	3	3	3	3	3

* T cells were cultured with L. monocytogenes–infected or normal stimulator cells in the presence or absence of human rIL-2 and supernatants were tested for IFN-activities, as described in Materials and Methods.

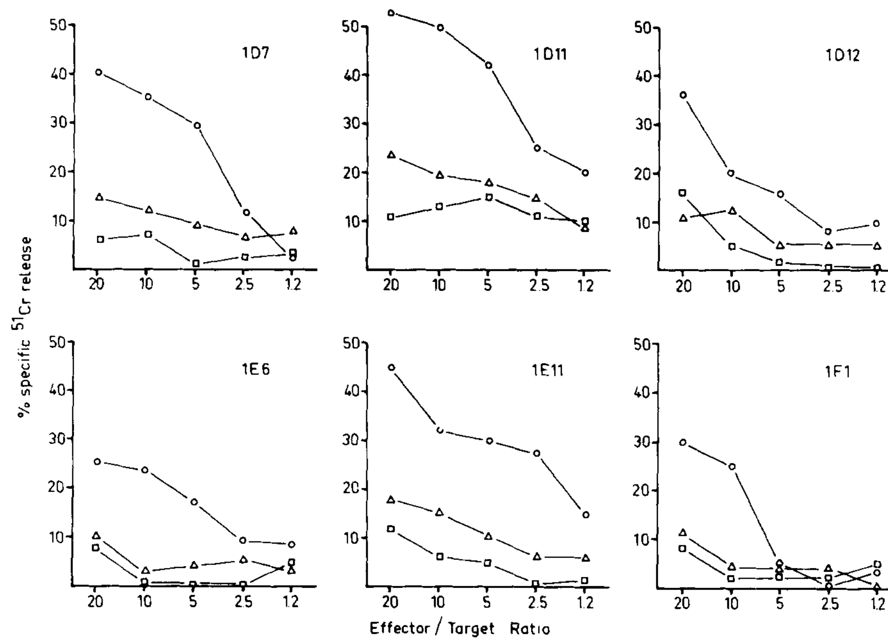

FIGURE 1. Cytolytic activity of *L. monocytogenes*–reactive Lyt-2⁺ T lymphocyte clones. T cell clones were tested for their cytolytic activity against ^{51}Cr-labeled peritoneal cells (PC). (○) *L. monocytogenes*–infected PC; (△) proteose peptone–elicited PC; (□) untreated PC. Values are means of triplicate wells. SD <10%.

cells from *L. monocytogenes*–infected mice as compared with noninfected peritoneal cells. Two clones, 1D7 and 1F1, were further studied. These clones failed to lyse P 815 and YAC target cells, and did not kill peritoneal cells from *L. monocytogenes*–infected DBA/2 mice (Fig. 2*A*). This finding indicated that the killing was genetically restricted. We next determined whether lysis was H-2-restricted and antigen-specific. The two clones killed *L. monocytogenes*–infected peritoneal cells from the H-2I-A mutant B6.C-H2^{bm12}, but not those from the H-2K mutant B6.C-H2^{bm1} (Fig. 2*B*). Furthermore, the clones killed BMMØ that had been infected with *L. monocytogenes* in vitro, but not uninfected or BCG-

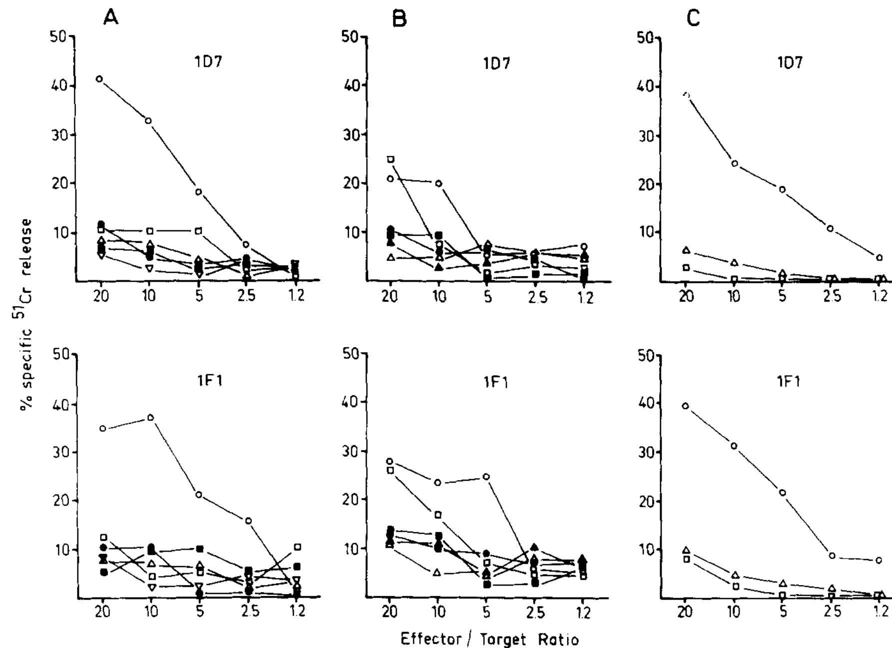

FIGURE 2. Antigen specificity and H-2 restriction of *L. monocytogenes*-reactive Lyt-2$^+$ T lymphocyte clones. Clone 1D7 and 1F1 were tested for their cytolytic activity against different ^{51}Cr-labeled target cells. *A*: (○) PC from *L. monocytogenes*-infected C57BL/6 mice, (●) PC from untreated C57BL/6 mice, (□) PC from *L. monocytogenes*-infected DBA/2 mice, (■) PC from untreated DBA/2 mice, (△) YAC-1, (▽) P815. *B*: (○) PC from *L. monocytogenes*-infected C57BL/6 mice, (●) PC from untreated C57BL/6 mice, (△) PC from *L. monocytogenes*-infected B6.C-H2^{bm1} mice, (▲) PC from untreated B6.C-H2^{bm1} mice, (□) PC from *L. monocytogenes*-infected B6.C-H2^{bm12} mice, (■) PC from untreated B6.C-H2^{bm12} mice. *C*: (○) *L. monocytogenes*-infected C57BL/6 BMMØ, (□) *M. bovis* BCG-infected C57Bl/6 BMMØ, (△) uninfected C57Bl/6 BMMØ. Values are means of triplicate wells SD <10%.

infected BMMØ (Fig. 2*C*). BCG-infected BMMØ were killed by short-term cultured T cell lines from BCG-infected mice (data not shown).

Discussion

In the present study, Lyt-2$^+$ T cell clones that lysed *L. monocytogenes*-infected macrophages and produced IFN-γ after stimulation with infected stimulator cells plus IL-2 are described. The T cell clones failed to lyse peritoneal cells from infected B.6C-H2^{bm1} mice and syngeneic BMMØ infected with the intracellular pathogen, *M. bovis* BCG. We conclude that the T cell clones recognized *L. monocytogenes* determinants in association with H-2K-encoded molecules on the surface of infected macrophages, and hence are similar to virus-specific and alloreactive CTL and distinct from natural or lymphokine-activated killer cells. Peritoneal cells have also been used successfully as targets for virus-specific and alloreactive, class I-restricted CTL (11).

Human CTL from BCG-vaccinated volunteers, which kill autologous antigen-labeled target cells better than allogeneic ones, have been described (12). However, in this study the restriction class and phenotype of the CTL were not determined. Chen-Woan and McGregor (13) have observed the generation of

$OX8^+$ CTL in *L. monocytogenes*–infected rats that killed a variety of target cells in the absence of an apparent antigen specificity and genetic restriction. Using short-term cultured Lyt-2^+ T lymphocytes from *L. monocytogenes*–infected mice, we observed less stringent target specificity as well (De Libero, G., and S. H. E. Kaufmann, manuscript submitted for publication). We therefore assume that *L. monocytogenes* infection stimulates multiple cytotoxic T cell precursors with varying recognition stringency and that class I–restricted *L. monocytogenes*–specific CTL belong to a rare population that can only be identified on the clonal level.

What could be the in vivo relevance of specific Lyt-2^+ T cells in antibacterial resistance? Several possibilities may be considered. In vivo, the cytolytic activity of the cells may be irrelevant, the major in vivo function being IFN-γ secretion. Because *L. monocytogenes*–reactive $L3T4^+$ T cells also produce IFN-γ (4), the function of both subsets in acquired antibacterial resistance would be similar. Alternatively, the cytolytic activity is an important effector mechanism in vivo: (*a*) Intracellular bacteria preferentially multiply within the mononuclear phagocyte system (2), and hence destruction of host cells harbouring bacteria could already lead to reduced microbial multiplication; (*b*) mononuclear cells markedly differ in their bacteriocidal potential. For example, Kupffer cells phagocytize intracellular bacteria but are refractory to further activation of bacteriocidal mechanisms (14). In this situation, intracellular bacteria are protected from more effective killing mechanisms and lysis of phagocytes would facilitate bacterial uptake by immigrant monocytes with high bacteriocidal potential; (*c*) CTL cause nucleus destruction and DNA-fragmentation of target cells (15), and a similar internal mechanism could facilitate killing of bacteria within phagocytes. The release of endogeneous molecules (proteolytic enzymes and/or monokines) from lysed macrophages could further contribute to the inflammatory reaction at the site of bacterial implantation. The availability of T cell clones with specific cytolytic activity against infected macrophages will allow us to test these alternatives experimentally.

Summary

Lyt-2^+ T cell clones were established from *Listeria monocytogenes*–infected mice. The clones secreted IFN-γ after stimulation with spleen cells from *L. monocytogenes*–infected mice plus IL-2. Spleen cells from normal mice were not stimulatory. Furthermore, cloned T cells lysed *L. monocytogenes*–infected macrophages. Cytolysis was antigen-specific and H-2K-restricted. These findings suggest a role for specific cytotoxic T cells in the immune response to intracellular bacteria.

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