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Effective Protection Against *Listeria monocytogenes* and Delayed-Type Hypersensitivity to Listerial Antigens Depend on Cooperation Between Specific L3T4$^+$ and Lyt 2$^+$ T Cells

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Selected L3T4$^-$ and Lyt 2$^-$ T-cell subpopulations from *Listeria monocytogenes*-infected mice were transferred into syngenic recipients, and their capacity to adoptively mediate protection against *L. monocytogenes* and delayed-type hypersensitivity to listerial antigens was determined. Both functions were markedly reduced by pretreatment of cells with either anti-L3T4 or anti-Lyt 2.2 antibodies plus complement, but they could be restored by admixture of the two selected T-cell subsets. Thus, after systemic cell transfer effective protection against *L. monocytogenes* and delayed-type hypersensitivity to listerial antigens depend on cooperation between specific L3T4$^+$ and Lyt 2$^+$ T cells.

Protective immunity to facultative intracellular bacteria, including *Listeria monocytogenes*, depends on specific T cells which activate mononuclear phagocytes for increased bacteriocidal activities (summarized in reference 9). In the murine model, *L. monocytogenes*-specific T cells capable of producing multiple lymphokine activities after antigenic restimulation in vitro are Lyt 1$^+$2$^-$ (11, 13, 14, 18). In contrast, Lyt 1$^+$2$^+$ T cells are required for successful systemic transfer of protection to *L. monocytogenes* and delayed-type hypersensitivity (DTH) to listerial antigens (15). From these findings it could not be decided whether expression of in vivo activities depends on Lyt 2$^+$ T cells alone or whether Lyt 2$^-$ T cells are also involved. Recently it was shown that, in contrast to the Lyt 1 antigen (17), the L3T4 marker and the Lyt 2 marker are reciprocally expressed by class II-restricted T cells and class I-restricted T cells, respectively (5, 6, 19). Therefore, it became possible to test whether in the murine listeriosis model adoptive protection and DTH depend on Lyt 2$^+$ T cells alone or on cooperation between Lyt 2$^+$ and L3T4$^+$ T cells.

Mice (C57BL/6) were infected with approximately 10^4 live *L. monocytogenes* (strain EGD, serotype 1/2a) organisms (15). After 7 days, peritoneal exudates were induced with 1.5 ml of proteose peptone (10%; Difco Laboratories, Detroit, Mich.), and 3 days later cells were harvested. Peritoneal exudate T-lymphocyte-enriched cells (PETLEs) were obtained by passage over nylon wool columns as described previously (16). Afterwards, PETLEs were treated with antibodies and complement as described (16). In short, 3×10^7 PETLEs per ml were incubated with anti-L3T4 antibodies (culture supernatant of the monoclonal hybridoma GK 1.5 [5, 6, 19], final dilution 1:5) or with anti-Lyt 2.2 antibodies (culture supernatant of the monoclonal hybridoma HO 2.2 [8], final dilution 1:5) for 30 min at room temperature. After being washed cells were suspended in preselected rabbit serum as a source of complement and incubated for 30 min at 37°C. These treatments were repeated once. Afterwards, PETLEs were adjusted to equivalent concentrations of the complement-treated control, and equivalent cell numbers were injected intravenously into C57BL/6 mice. One group of mice had been injected intravenously with 10^5 live *L. monocytogenes* organisms 2 h earlier. After 2 days, bacterial numbers in spleens were determined by plating 0.1-ml samples of spleen homogenate at appropriate dilutions on tryptic soy agar (Difco) as described previously (15). The other group of mice was challenged for DTH by subcutaneous injection of 0.05 ml of *L. monocytogenes* soluble antigens into one hind footpad (15). Footpad swelling was measured 24 h later with a dial-gauge caliper (Kröplin, Schlüchtern, Federal Republic of Germany) as described (15).

For immunofluorescence analysis of *L. monocytogenes*-immune PETLEs, 2×10^6 cells were treated with anti-Thy 1.2 antibodies (diluted 1:100; Olac, Bicester, United Kingdom), anti-L3T4 antibodies (diluted 1:5), or anti-Lyt 2.2 antibodies (diluted 1:5) in 200 μl of medium at room temperature for 30 min. Anti-Thy-1.2- and anti-Lyt-2.2-treated cells were incubated with fluorescein isothiocyanate (FITC)-coupled goat anti-mouse immunoglobulin (diluted 1:50) and anti-L3T4-treated cells with FITC-coupled goat anti-rat immunoglobulin (diluted 1:50) for 30 min at room temperature. These antisera were obtained from Dianova, Hamburg, Federal Republic of Germany. Incubations were performed in RPMI 1640 medium with 5% fetal calf serum and 0.02% NaN_3. Afterwards, cells were washed three times, suspended in phosphate-buffered saline with 5% fetal calf serum and 0.02% NaN_3, and analyzed on an Ortho cytofluorograph 50H (Ortho Diagnostic Systems, Westwood, Calif.).

For mixed leukocyte cultures of selected T cells, spleen cells from C57BL/6 mice were purified over nylon wool columns and then treated with anti-L3T4 or anti-Lyt 2.2 antibodies and complement as described above. Equivalents of 6×10^5 cells were cocultured with 3×10^6 X-irradiated (3,300 rads) DBA/2 stimulator cells in 2 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, kanamycin, HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 2-mercaptoethanol (GIBCO Laboratories, Grand Island, N.Y.) in macrotiter plates (Nunc, Roskylde, Denmark). On day 4, cytotoxic T-lymphocyte activity was assessed. T cells were added to 2×10^3 ^{51}Cr-labeled P 815 tumor cells at the effector to target cell ratios indicated in Table 2. The percentage of ^{51}Cr released from target cells was determined with triplicate samples and calculated according to the following equation:

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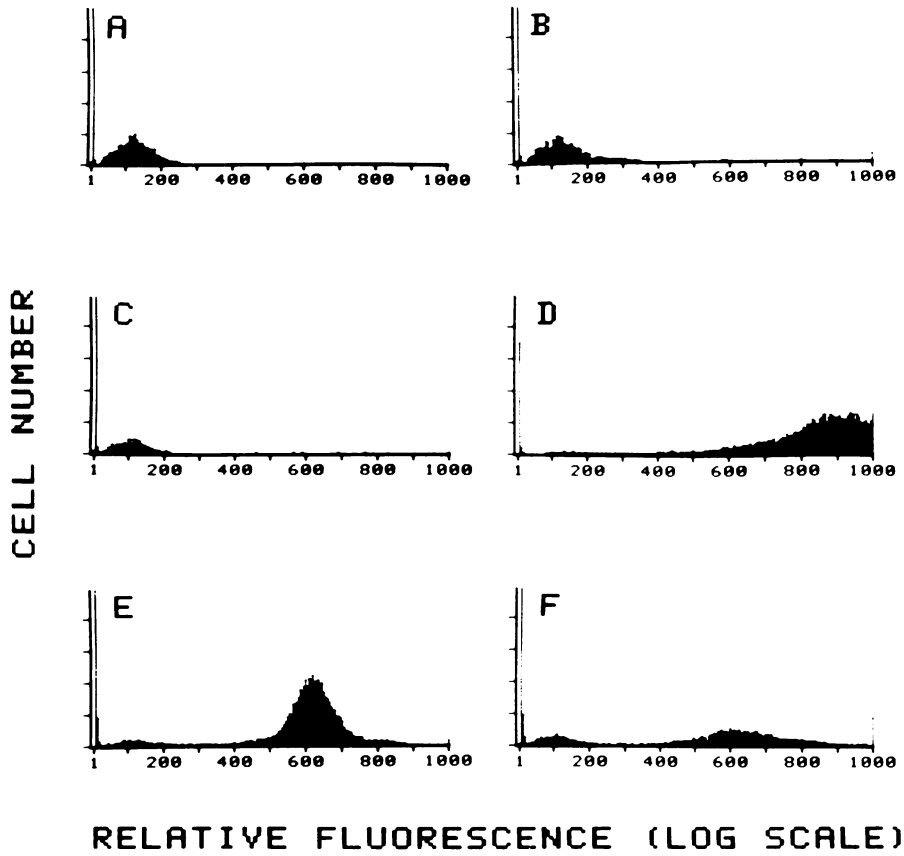

FIG. 1. Fluorescence analysis of *L. monocytogenes*-immune PETLEs. PETLEs were stained as described in the text. (A) No treatment (0.0%); (B) FITC-goat anti-mouse immunoglobulin (3.0%); (C) FITC-goat anti-rat immunoglobulin (2.4%); (D) anti-Thy 1.2 plus FITC-goat anti-mouse immunoglobulin (91.6%); (E) anti-L3T4 plus FITC-goat anti-rat immunoglobulin (61.9%); (F) anti-Lyt 2.2 plus FITC-goat anti-mouse immunoglobulin (27.7%). Values in parentheses are the percentage of positive PETLEs.

$\%^{51}Cr$ release = $100 \times (a - b)/(c - b)$, where a is ^{51}Cr release from target cells in the presence of cytotoxic T cells, b is ^{51}Cr release from target cells alone, and c is maximal ^{51}Cr release from target cells.

Immunofluorescence analysis revealed that *L. monocytogenes*-immune PETLEs were 89% Thy 1.2^+, 60% L3T4$^+$, and 25% Lyt 2.2^+ (Fig. 1), indicating that the L3T4 and Lyt 2 molecules were reciprocally expressed by *L. monocytogenes*-immune PETLEs. The data depicted in Table 1 show that pretreatment of *L. monocytogenes*-immune PETLEs with either anti-L3T4 or anti-Lyt 2.2 antibodies plus complement significantly reduced adoptive protection against *L. monocytogenes* and DTH to listerial antigens, although some DTH was induced by selected L3T4$^+$ T cells alone. On the other hand, the mixed population consisting of L3T4$^+$ and Lyt 2^+ T cells was capable of transferring effective protection and DTH. These findings suggest that after systemic transfer neither L3T4$^+$ nor Lyt 2^+ T cells alone were able to induce effective protection and DTH and that both activities depended on cooperation between these two T-cell subpopulations. To validate the effectivity of the antibody-plus-complement treatments, the capacity of selected T-cell

TABLE 1. Effect of anti-L3T4 and anti-Lyt 2.2 antibodies and complement on adoptive transfer of protection against live *L. monocytogenes* organisms and DTH to listerial antigens by *L. monocytogenes*-immune T cells[a]

Group and treatment	T-cell subset	Log_{10} viable bacteria in spleens	Log_{10} protection in spleen	DTH (0.1 mm)
1. Complement	Unselected	5.63 ± 0.33	1.22	6.0 ± 0.71
2. Anti-L3T4 and complement	Lyt 2^+	6.56 ± 0.42	0.29	2.2 ± 0.45
3. Anti-Lyt 2.2 and complement	L3T4$^+$	6.59 ± 0.16	0.26	3.6 ± 0.34
4. Mixture of groups 2 and 3 (1:1)	L3T4$^+$ and Lyt 2^+	5.39 ± 0.34	1.46	6.8 ± 0.84
5. No PETLEs transferred		6.85 ± 0.36		2.4 ± 0.55

[a] Equivalents of 3×10^6 *L. monocytogenes*-immune PETLEs were transferred intravenously. Means of five animals ± SD. Significant differences obtained ($P < 0.005$, Student's t test) for group 1 versus groups 2, 3, and 5.

populations to generate H-2^d-specific cytotoxic T cells in a mixed leukocyte culture was determined. Neither L3T4$^+$ T cells nor Lyt 2^+ T cells alone was able to generate cytotoxic responses, but only the mixed cell population consisting of L3T4$^+$ and Lyt 2^+ T cells was able to do so (Table 2). Abrogation of cytotoxic T-cell responses in L3T4- but not in Lyt 2.2-treated cultures could be reversed by addition of exogeneous interleukin 2 containing supernatant from concanavalin A-stimulated rat spleens (data not shown). Thus, the antibodies used were of the predicted specificities.

T-cell subpopulations with different biological functions are usually discriminated on the basis of expression of exclusive surface molecules. In mice it was originally thought that the Lyt 1 and Lyt 2 antigens would serve this purpose (2). However, more intensive studies showed that all T cells carry the Lyt 1 marker, although at various densities (17). Recently, another murine T-cell surface molecule, L3T4, has been identified which, in contrast to the Lyt 1 determinant, shows reciprocal expression with the Lyt 2 antigen (5, 6, 19). Furthermore, it was found that L3T4$^+$ T cells recognize antigen in association with class II molecules, whereas Lyt 2^+ T cells recognize antigen plus class I molecules (5, 19). Hence, class II-restricted helper T cells are L3T4$^+$ and class I-restricted cytotoxic T cells are Lyt 2^+. It has therefore become possible to analyze the relative role of distinct T-cell subsets in immune responses in which Lyt 1^+2^+ T cells are involved.

Infection of mice with *L. monocytogenes* results in the generation of specific Lyt 2^+ and Lyt 2^- T cells. *L. monocytogenes*-immune Lyt 2^- T cells are class II restricted (7) and after antigenic restimulation secrete multiple lymphokines (11, 13, 14, 18). On the other hand, after systemic transfer, protection against *L. monocytogenes* and DTH to listerial antigens depend on Lyt 2^+ T cells (15). Because these T cells also express high densities of the Lyt 1 molecule, it was not possible to answer the question whether Lyt 1^+2^+ T cells alone are sufficient for induction of protection and DTH reactions or whether additional *L. monocytogenes*-specific Lyt 2^- T cells are required. The availability of anti-L3T4 antibodies has now allowed us to address this question. The data presented here demonstrate that cooperation between *L. monocytogenes*-specific L3T4$^+$ and Lyt 2^+ T cells is required for effective protection and DTH after systemic cell transfer.

With respect to the genetic restriction of adoptive protection against *L. monocytogenes*, controversial data have been published (1, 3, 4, 20, 21). In mice, Zinkernagel et al. (21) have shown that protection is class II restricted, whereas the studies of Cheers and Sandrin (4) point to class I restriction. In rats, a major role for class II-restricted cytotoxic cells in the immune response to *L. monocytogenes* was also observed (20). Our findings would indicate that both genetic-restriction patterns are involved.

As to the functional role of the two T-cell subsets, it is most likely that *L. monocytogenes*-immune L3T4$^+$ T cells are of the helper type and participate in protection and DTH by secreting multiple lymphokine activities which affect other T-cell subsets as well as mononuclear phagocytes (11, 13, 14, 18). Indeed, selected L3T4$^+$ T cells could induce a small degree of DTH (Table 1), and cloned class II-restricted *L. monocytogenes*-specific T cells which secrete multiple lymphokines in vitro can induce some protection and DTH after systemic transfer but only at very high cell concentrations (10, 12; S. H. E. Kaufmann, *in* A. J. L. Macario and E. Conway de Macario (ed.), *Monoclonal Antibodies Against Bacteria*, in press; S. H. E. Kaufmann, *in* H. V. Boehmer and W. Haas (ed.), *T Cell Clones*, in press). (As shown previously [10], *L. monocytogenes*-specific T-cell clones exert an aberrant migration pattern which may also contribute to their low activity.) Furthermore, lymphokines produced by these T-cell clones as well as recombinant interferon-γ can protect mice against *L. monocytogenes* infection (10, 16a; Kaufmann, *Monoclonal Antibodies Against Bacteria*, in press). Under physiological conditions, however, L3T4$^+$ T cells seem to cooperate with Lyt 2^+ T cells in the induction of effective protection and DTH. Although the role of Lyt 2^+ T cells remains unclear, evidence for their involvement in granuloma formation has been presented recently (17a). Whether Lyt 2^+ T cells express cytotoxic activity against *L. monocytogenes*-infected target cells is currently under investigation.

TABLE 2. Effect of anti-L3T4 and anti-Lyt 2.2 antibodies and complement on the generation of cytoxic T cells in mixed leukocyte culture[a]

Group and treatment	T-cell subset	Specific lysis (%^{51}Cr release) at[b]:			
		50:1	25:1	12:1	6:1
1. Complement	Unselected	96	68	46	34
2. Anti-L3T4 + complement	Lyt 2^+	5	4	3	0
3. Anti-Lyt 2.2 + complement	L3T4$^+$	3	5	5	2
4. Mixture of groups 2 and 3 (1:1)	L3T4$^+$ and Lyt 2^+	105	87	70	54

[a] Equivalents of 6×10^5 T cells were cultured with 3×10^6 irradiated DBA/2 spleen cells, and cytotoxic responses were determined on day 4.
[b] Ratio of effector to target cells.

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