Effective Antibacterial Protection Induced by a *Listeria Monocytogenes*-Specific T Cell Clone and Its Lymphokines

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The capacity of the murine *Listeria monocytogenes*-specific T cell clone 9-36-1 and of lymphokines derived therefrom to induce antibacterial protection in vivo was studied. Clone 9-36-1 was stimulated to proliferate and to produce lymphokines by in vitro culture with syngeneic accessory cells and heat-killed *L. monocytogenes*. Although 9-36-1 cells were highly active in vitro, intravenous transfer of the cells resulted in marginal protection against a systemic infection with *L. monocytogenes*. In contrast, 9-36-1 cells injected subcutaneously together with *L. monocytogenes* into the footpad induced marked protection in syngeneic, but not in allogeneic, mice. Multiplication of *Salmonella typhimurium* was not reduced by the T cell clone. Studies with ^{51}Cr-labeled T cells indicated that the low activity of intravenously transferred cells was due to an altered migration pattern. Lymphokines produced by 9-36-1 cells in vitro induced protection against *L. monocytogenes* in syngeneic recipient mice. Lymphokine-induced protection was also demonstrable in allogeneic recipients and against *S. typhimurium*. These findings suggest that the *L. monocytogenes*-specific T cell clone 9-36-1, although unable to immigrate into sites of bacterial deposition, had retained its ability to mobilize antibacterial defense mechanisms once present at the site of reaction.

Listeriosis of mice has been widely used as experimental model for protective immunity to infections with facultative intracellular bacteria (summarized in reference 2). It has become clear from these studies that effective defense mechanisms are induced by a subpopulation of specific T lymphocytes that are able to activate mononuclear phagocytes through lymphokine secretion at the site of microbial deposition. The ability to clone and propagate *Listeria monocytogenes*-specific T lymphocytes has allowed studies on the role of the T cell in antibacterial immunity on a single-cell level (4). It can be shown that a cloned T cell population is capable (i) of producing and inducing different interleukins and of helping B cells after antigen specific, H-2IA-restricted stimulation in vitro, as well as (ii) of mediating delayed-type hypersensitivity (DTH) and antibacterial protection in H-2IA-compatible recipient mice (4). These studies favor the idea of a common T cell mediating multiple biological functions in antibacterial immunity. However, although these T cell lines were highly active in vitro, high cell numbers were required for intravenous (i.v.) transfer of antibacterial immunity (4). The question, therefore, remained open as to whether or not these T cell populations are responsible for protection. In the present study, the protective activity of a recloned *L. monocytogenes*-specific T cell subline was

investigated in more detail. Evidence is presented (i) that this T cell clone had retained its capacity to effectively mobilize antimicrobial defense mechanisms by lymphokine secretion once present at the site of listerial implantation, and (ii) that the weak antibacterial activity of the cloned T cells after i.v. cell transfer was due, at least in part, to their aberrant migration pattern as compared with the cells they originated from, i.e., unselected *L. monocytogenes*-immune peritoneal exudate T lymphocyte-enriched cells (PETLEs).

MATERIALS AND METHODS

Mice. Female C57BL/6 and Balb/c mice were used at 10 to 12 weeks of age.

Bacteria and bacterial antigens. The virulence of *L. monocytogenes* and *Salmonella typhimurium* was maintained by continuous mouse passage as described previously (7). *L. monocytogenes* cells were heat killed by incubation in a water bath at 61°C for 60 min and stored at −70°C until use (4a, 5).

Cloning and propagation of *L. monocytogenes*-specific T cells. The *L. monocytogenes*-specific T cell clone 9-36-1 used in this study was derived from the cloned *L. monocytogenes*-specific T cell line 9-3 (4). The cloning and propagation procedures with this line as well as its characteristics have been described elsewhere (4). Clone 9-36-1 was obtained by recloning 200 T cells in double-layer soft agar (15) in the presence of 10^5

TABLE 1. Proliferation and interleukin induction by clone 9-36-1[a]

9-36-1 T cells	Accessory cells	Antigen	Proliferative response ([^{3}H]TdR uptake; cpm/3 × 10^4 T cells)[b]	Interleukin activity ([^{3}H]TdR uptake; cpm/2 × 10^4 ConA-activated cells)[b]
+	+	HKL	74,600	26,400
−	+	HKL	900	260
+	−	HKL	1,800	1,500
+	+	None	2,500	1,700
+	+	PPD	2,100	2,000

[a] Clone 9-36-1 T cells (3 × 10^4/0.2 ml) were cocultured with 10^5 accessory cells (2,800 R-irradiated spleen cells) and 10^8 HKL cells or 5 μg of purified protein derivative of tuberculin (PPD).

[b] Means of three determinations; standard deviation, <20%. [^{3}H]TdR, [^{3}H]thymidine.

syngeneic accessory cells and 10^7 heat-killed *L. monocytogenes* (HKL) cells as described previously (4). Clone 9-36-1 cells were expanded and propagated in vitro in Click medium supplemented with 5% selected fetal calf serum (GIBCO Europe, Glasgow, United Kingdom) and 5% semipurified T cell growth factor. The preparation of T cell growth factor has been described previously (4). Cultures contained approximately 10^6 9-36-1 cells, 10^5 irradiated (2,800 R) spleen cells as accessory cells, and 10^8 HKL cells in a total volume of 1 ml. They were kept at 37°C in 5% CO$_2$ in air and fed every 3 to 4 days.

L. monocytogenes-immune PETLEs. Mice were infected i.v. with approximately 5 × 10^3 live *L. monocytogenes* cells, and 6 days later, peritoneal exudates were induced with 5% casein (7). After another 2 days, cells were harvested and PETLEs were obtained by passage over nylon wool columns (3) as described previously (7).

In vitro characterization of clone 9-36-1. The assays for *L. monocytogenes*-specific T cell proliferation and interleukin induction have been described in detail elsewhere (4). In short, 3 × 10^4 cloned T cells were cultured with 10^5 syngeneic irradiated (2,800 R) spleen cells and 10^8 HKL cells in a total volume of 0.2 ml of Click medium in the absence of T cell growth factor. After 24 h, supernatants were collected and substituted with fresh medium. After another 2 days, cell cultures were pulsed with 1 μCi of [^{3}H]thymidine (Radiochemical Centre, Amersham, England), and radioactivity incorporation was determined after another 18 h. Supernatants (20%) were tested for interleukin activity on concanavalin A (ConA)-activated spleen cells as described previously (4).

Adoptive antibacterial protection with T cells. Two assays were performed. Systemic antibacterial protection was assessed in mice infected i.v. with approximately 5 × 10^4 live *L. monocytogenes* cells 2 h before i.v. T cell transfer. After 2 days, spleens were removed and homogenized as described previously (7). For local protection assays, T cells were mixed with 5 × 10^5 live *L. monocytogenes* or 1 × 10^5 live *S. typhimurium* cells in a total volume of 0.05 ml and injected subcutaneously (s.c.) into one hind footpad of recipient mice. After 2 days, feet were removed, rinsed with alcohol (70%), and homogenized with a tissue grinder (Ultra Turrax; Ika, Staufen, Federal Republic of Germany). In both assay systems, bacterial numbers were determined by plating 0.1-ml samples of homogenate at appropriate dilutions on Trypticase

soy agar (BBL Microbiology Systems, Cockeysville, Md.) as described previously (7). Antibacterial protection is expressed as the difference between the log$_{10}$ numbers of bacteria in spleens or footpads of T cell recipients and the log$_{10}$ numbers in controls.

Passive antibacterial protection with supernatants. Clone 9-36-1 cells (3 × 10^4) were cultured with 1 × 10^5 syngeneic accessory cells and 1 × 10^8 HKL cells in a total volume of 1 ml of Click medium in the absence of antibiotics and T cell growth factor. Alternatively, 10^6 normal spleen cells were cultured in the presence of 1 μg of ConA in a total volume of 1 ml. After 24 h, supernatants were collected and filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass.). These conditions are known to result in production of macrophage activating factor (for a review, see reference 14). Live *L. monocytogenes* cells (5 × 10^5) or *S. typhimurium* cells (1 × 10^5) were suspended in 0.05 ml of supernatant and injected s.c. into one hind footpad of recipient mice. Alternatively, 5 × 10^4 bacteria were suspended in 0.2 ml of supernatant and injected i.v. After 2 days, bacterial numbers in footpads or spleens were determined as described above.

Labeling of T cells with ^{51}Cr. T cells (10^7/ml; clone 9-36-1 or *L. monocytogenes*-immune PETLEs) were incubated with 50 μCi of Na$_2$^{51}CrO$_4$ (Radiochemical Centre) per ml under gentle shaking at 37°C for 60 min as described previously (6). ^{51}Cr-labeled T cells were washed three times and injected i.v. into recipient mice. At different time intervals, lungs, livers, and spleens were removed and radioactivities were counted. Data are expressed as percentages of radioactivity injected.

RESULTS

Characteristics of the *L. monocytogenes*-specific T cell clone 9-36-1. Clone 9-36-1 was derived from the *L. monocytogenes*-specific T cell line 9-3, which is restricted by the H-2IA sublocus of the major histocompatibility complex (4) and bears the phenotype Thy 1$^+$ Lyt 1$^+$2$^-$ (unpublished data). These properties are characteristic of helper T cells. In accordance with previous data (4), the coculture of low numbers of 9-36-1 cells with syngeneic accessory cells and homologous antigen resulted in antigen-specific proliferative responses and interleukin production in vitro (Table 1). On the other hand, high numbers
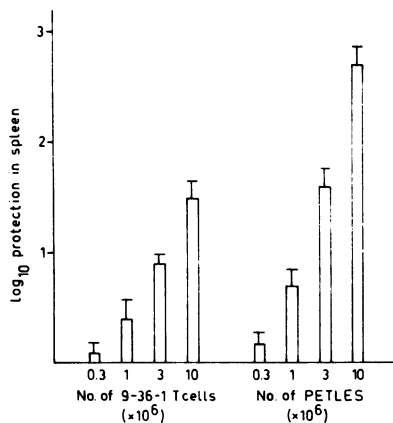
FIG. 1. Systemic transfer of protection against *L. monocytogenes* by clone 9-36-1 or by *L. monocytogenes*-immune PETLEs. T cells were injected i.v. 2 h after i.v. infection with 5×10^4 live *L. monocytogenes* cells. After 2 days, protection in spleens was determined. Means of five determinations ± standard deviations are shown.

of cells had to be injected i.v. for successful adoptive protection against a systemic infection with *L. monocytogenes* (Fig. 1). On the basis of cell numbers, 9-36-1 cells were less protective than PETLEs from *L. monocytogenes*-immune mice (Fig. 1).

Migratory pattern of clone 9-36-1. Evidence has been presented previously that T cells demonstrate an aberrant migration pattern in vivo after in vitro propagation (1, 12). To assess the influence of the migration pattern of clone 9-36-1 on its antibacterial activity, the distribution of ^{51}Cr-labeled 9-36-1 cells was compared with that of *L. monocytogenes*-immune PETLEs after i.v. injection into normal recipient mice. A 10-fold-higher amount of ^{51}Cr accumulated in the lungs of recipients of 9-36-1 cells as compared with recipients of PETLEs, whereas in spleens, a reciprocal relationship was observed (Table 2). Only small differences were found in livers. These data indicate that clone 9-36-1 exhibited an altered migration pattern.

TABLE 2. Migration patterns of 9-36-1 T cells and *L. monocytogenes*-immune PETLEs[a]

| Time (h) | % of ^{51}Cr injected [b] | | | | | |
| | 9-36-1 T cells | | | PETLEs | | |
	Lung	Spleen	Liver	Lung	Spleen	Liver
2	44.8	1.6	20.5	4.3	14.7	22.9
10	32.0	3.0	39.0	2.8	22.0	20.4
24	10.2	4.5	41.6	1.6	29.3	22.5

[a] ^{51}Cr-labeled 9-36-1 T cells or *L. monocytogenes*-immune PETLEs (3×10^5/0.2 ml) were injected i.v.
[b] Means of four determinations.

Effective local antibacterial protection by clone 9-36-1. To avoid the trapping of 9-36-1 cells at sites irrelevant to antimicrobial deposition, the cells were injected locally together with live *L. monocytogenes* into the footpad. In this local protection system, clone 9-36-1 was severalfold more effective than *L. monocytogenes*-immune PETLEs on the basis of cell numbers (Fig. 2). Protection was not observed in Balb/c mice or against the unrelated bacterium *S. typhimurium* (Table 3). When both pathogens were administered together with 9-36-1 cells, protection was demonstrable not only against *L. monocytogenes* but also against *S. typhimurium* (Table 3). These data show that low cell numbers of clone 9-36-1 are capable of activating antibacterial defense mechanisms in vivo after appropriate interaction with antigenic determinants derived from live *L. monocytogenes*.

Effective antibacterial protection by clone 9-36-1-derived lymphokines. Because cultures of cloned *L. monocytogenes*-specific T cells have been found to be a rich source of biologically active factors (4; S. H. E. Kaufmann, H. Hahn, R. Berger, and H. Kirchner, Eur. J. Immunol., in press), in the following experiment the question was asked of whether lymphokines derived from 9-36-1 cells in vitro are also protective in vivo. Supernatants of cultures of 9-36-1 cells were highly potent in protecting syngeneic as well as allogeneic recipient mice against local and systemic infection with *L. monocytogenes* (Table 4). Local multiplication of *S. typhimurium* was also reduced after s.c. administration of supernatants. In contrast, injection of supernatants of ConA-activated spleen cells (which contained macrophage activating factor as assessed by activation of tumoricidal macrophages in vitro
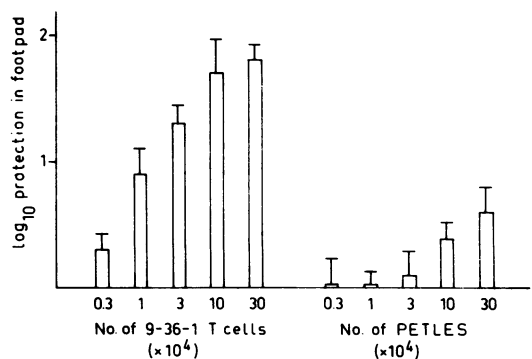

FIG. 2. Local transfer of protection against *L. monocytogenes* by clone 9-36-1 or by *L. monocytogenes*-immune PETLEs. T cells were injected s.c. together with 5×10^5 live *L. monocytogenes* cells. After 2 days, protection in footpads was determined. Means of five determinations ± standard deviations are shown.

TABLE 3. Specificity of protection induced by clone 9-36-1[a]

Strain of recipient mice	Challenge infection	Log$_{10}$ protection in footpad[b] ± SD
C57B1/6	L. monocytogenes	1.1 ± 0.21
C57B1/6	S. typhimurium	−0.1 ± 0.13
BALB/c	L. monocytogenes	0.3 ± 0.18
C57B1/6	L. monocytogenes + S. typhimurium	1.2 ± 0.26 (L. monocytogenes) 0.8 ± 0.17 (S. typhimurium)

[a] Clone 9-36-1 cells (3 × 10^4), together with 5 × 10^5 live L. monocytogenes or 1 × 10^5 live S. typhimurium cells or both, were injected s.c. in a total volume of 0.05 ml into one hind footpad.
[b] Means of five determinations.

[data not shown]) or of the nonspecific irritant proteose peptone (10%) did not affect multiplication of L. monocytogenes. When supernatants of 9-36-1 T cell cultures were administered locally together with soluble listerial antigen, no demonstrable DTH reactions developed (data not shown), although cloned L. monocytogenes-specific T cells are able to mediate DTH (4).

DISCUSSION

Protective immunity to facultative intracellular bacteria depends on cooperative events between T cells and mononuclear phagocytes at the site of bacterial deposition (summarized in reference 2). Recently, cloned H-2IA-restricted, L. monocytogenes-specific T cell lines have been established (4) which are highly active in vitro but exert only marginal in vivo activity after systemic transfer upon syngeneic recipient mice. The present study shows that the L. monocytogenes-specific T cell clone 9-36-1 is capable of effectively protecting mice against a local L. monocytogenes infection when administered s.c. together with the infective agent, although the same T cell clone exerted only weak antibacterial activity after i.v. administration. These findings indicate (i) that clone 9-36-1 cells could interact with appropriately presented antigenic determinants derived from viable L. monocytogenes, and (ii) that 9-36-1 cells can activate mononuclear cells for increased bacteriocidal activity once they are present at the site of bacterial multiplication.

The low activity after i.v. transfer probably resulted from the inability of clone 9-36-1 to enter inflammatory foci. At least in part, this seems to be due to a trapping of the T cell clone in the lungs of recipient mice. Alterations in the migration pattern have been observed with several cytolytic as well as noncytolytic T cell clones and lines (1, 12), indicating that the aberrant migration pattern is a more generalized effect resulting from cloning and in vitro propagation procedures. On the other hand, evidence has been presented earlier that infection with facultative intracellular bacteria can induce both short-lived T lymphocytes able to enter inflammatory foci and long-lived T lymphocytes unable to migrate into inflammatory foci directly (10, 11, 13). Furthermore, it has been shown recently that after i.v. transfer, L. monocytogenes-immune PETLEs of the Lyt 1$^+$23$^+$ phenotype are required for antibacterial protection, whereas Lyt 1$^+$23$^-$ PETLEs alone can interact with antigen-presenting macrophages in vitro (4a, 5, 7–9) and also can induce local protection when administered s.c. together with live L.

TABLE 4. Protection by clone 9-36-1-derived lymphokines[a]

Material injected	Strain of recipient mice	Challenge infection	Log$_{10}$ protection[b] ± SD
Clone 9-36-1 supernatant (s.c.)	C57B1/6	L. monocytogenes (s.c.)	1.4 ± 0.23 (footpad)
Clone 9-36-1 supernatant (s.c.)	BALB/c	L. monocytogenes (s.c.)	1.5 ± 0.17 (footpad)
Clone 9-36-1 supernatant (s.c.)	C57B1/6	S. typhimurium (s.c.)	0.9 ± 0.10 (footpad)
ConA-supernatant (s.c.)	C57B1/6	L. monocytogenes (s.c.)	0.1 ± 0.17 (footpad)
Proteose peptone (s.c.)	C57B1/6	L. monocytogenes (s.c.)	−0.4 ± 0.05 (footpad)
Clone 9-36-1 supernatant (i.v.)	C57B1/6	L. monocytogenes (i.v.)	2.1 ± 0.24 (spleen)
ConA-supernatant (i.v.)	C57B1/6	L. monocytogenes (i.v.)	0.0 ± 0.09 (spleen)
Proteose peptone (i.v.)	C57B1/6	L. monocytogenes (i.v.)	−0.3 ± 0.11 (spleen)

[a] Mice were injected with 5 × 10^4 live L. monocytogenes cells i.v., 5 × 10^5 live L. monocytogenes cells s.c., or 1 × 10^5 live S. typhimurium cells s.c., suspended in 0.2 ml (i.v. injection) or 0.05 ml (s.c. injection) of the material indicated.
[b] Means of five determinations.

monocytogenes (unpublished data). These findings suggested requirements for a regulatory interrelationship between distinct T cell populations in the systemic mediation or regulation of antibacterial immunity under physiological conditions which would have been surmounted by transfer of increasing numbers of 9-36-1 cells.

Administration of supernatants of 9-36-1 cell cultures resulted in protection against *L. monocytogenes* as well as the unrelated intracellular bacterium *S. typhimurium* in syngeneic and allogeneic mice. These lymphokines were effective after s.c., as well as i.v., injection. Although it appears likely from these results that 9-36-1 cells are capable of secreting high levels of lymphokines relevant for antibacterial immunity upon appropriate stimulation, it is unclear whether the in vivo activity resided in a single or multiple biochemical entities. Supernatants of ConA-stimulated spleen cells had no antibacterial activity in vivo, although in accordance with published data (for a review, see reference 14), they contained macrophage activating factor as assessed by activation of tumoricidal macrophages in vitro. Therefore, quantitative or qualitative differences (or both) between lymphokines produced by ConA-stimulated cultures and those produced by cultures of *L. monocytogenes*-specific T cell clones have to be considered. It has been shown recently that cultures of cloned *L. monocytogenes*-specific T cells contain a variety of factors with potent in vitro activities, including interleukin 1 and 2 (4), interferon-γ (Kaufmann et al., in press), and macrophage activating factor (unpublished data).

Local injection of lymphokines together with soluble listerial antigen did not induce DTH reactions, although cloned *L. monocytogenes*-specific T cells can do so (4). Elicitation of DTH reactions was attempted with soluble antigen, whereas live *L. monocytogenes* organisms were used for protection studies. Attraction of mononuclear phagocytes by bacteria or factors of bacterial origin has been described previously (16), and recently it has been suggested that *L. monocytogenes* might serve as a second signal required for macrophage activation by lymphokines (R. D. Schreiber, H. K. Ziegler, E. Calamai, and E. R. Unanue, Fed. Proc. 40:1002, 1981). It was found that live *L. monocytogenes* but not soluble listerial antigen elicited marked signs of inflammation in the footpads (data not shown), which presumably facilitated the accumulation of mononuclear cells. Lymphokine-dependent mechanisms, however, have to be considered essential for macrophage activation at a later stage because nonspecific inflammation alone had no protective effect. Alternatively, lymphokines might have been less effective in attracting and activating macrophages than

the cloned T cells themselves, and therefore did not suffice for elicitation of a DTH reaction.

The data presented here show that *L. monocytogenes*-specific T cells can be cloned and propagated in vitro for long periods of time without loss of their capacity to mobilize protective effector mechanisms once they are present at sites of microbial multiplication. However, their propensity to immigrate into inflammatory foci seems to be affected, at least in part, due to an aberrant migration pattern. At present, the possibility of overcoming this failure by appropriate propagation procedures is being investigated. Such T cell clones as well as their lymphokines might then become helpful in the evaluation of vaccination procedures against facultative intracellular pathogens.

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