INFECTION AND IMMUNITY, July 1984, p. 234–241
0019-9567/84/070234-08$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 45, No. 1

Acquired Resistance to Facultative Intracellular Bacteria: Relationship Between Persistence, Cross-Reactivity at the T-Cell Level, and Capacity to Stimulate Cellular Immunity of Different *Listeria* Strains

STEFAN H. E. KAUFMANN

Max-Planck-Institut für Immunbiologie, D-7800 Freiburg, and Institut für Medizinische Mikrobiologie, Freie Universität Berlin, D-1000 Berlin, Federal Republic of Germany

Received 20 January 1984/Accepted 16 April 1984

C57BL/6 mice were infected with different strains of *Listeria* sp., and bacterial survival in spleens was assessed. Six strains (EGD, NCTC 5348, ATCC 19113, ATCC 19114, NCTC 10527, and ATCC 19116) were able to persist in spleens (persistent strains), whereas with five other strains (ATCC 19111, ATCC 19119, ATCC 33090, ATCC 33091, and ATCC 14870), only few if any bacteria were demonstrable after infection with up to 10^8 organisms (nonpersistent strains). Immunization of mice with persistent listeriae induced strong immune responses as determined in vitro (antigen-induced proliferation and interleukin production) and in vivo (protection and delayed-type hypersensitivity), whereas immunization with nonpersistent bacteria resulted in weaker responses. On the other hand, T lymphocytes from mice immunized with live organisms of the persistent strain EGD were stimulated equally well by heat-killed listeriae of all strains. Furthermore, three T-cell clones which were able to adoptively mediate antibacterial protection in vivo could be stimulated by heat-killed organisms of persistent as well as nonpersistent *Listeria* strains. It is concluded that both persistent and nonpersistent listeriae express antigenic epitopes which are recognized by protective T cells, although nonpersistent strains are not effective in inducing cellular immune responses due to rapid elimination in the host.

Acquired resistance to facultative intracellular bacteria normally requires immunization with live microorganisms (3, 7). Although it is now well established that protection to these pathogens is mediated by specific T lymphocytes and that replicating antigens are generally more potent in stimulating cellular immunity than nonreplicating ones (3, 7), the optimal conditions for effective vaccination against intracellular infections remain poorly understood. Infection of mice with *Listeria monocytogenes* has been used extensively for studying acquired resistance to facultative intracellular bacteria. It has been shown that protective immunity is T-cell dependent (2, 18, 23) and that Lyt 1^+23^+ as well as Lyt 1^+23^- T cells are involved (11–15). By using cloned T cells, it has also been demonstrated that a single T-cell population is able to effect different biological functions in vitro and in vivo (9–11). In these, as well as numerous other studies, *L. monocytogenes* EGD was employed. This strain has been made highly virulent for the mouse by Mackaness (19). However, the genus *Listeria* consists of several species and strains of different virulence for experimental animals and humans, and these strains can be distinguished by the use of appropriate antisera (6, 25, 26). The need of live microorganisms for vaccination against facultative intracellular pathogens makes it important to know whether avirulent bacteria are suitable for stimulation of specific T cells mediating protection against virulent strains of the same species. Consequently, in the present study it was attempted to ascertain whether a relationship exists between the persistence of different *Listeria* strains, their cross-reactivity on the T-cell level, and their capacity to stimulate cellular immune responses.

MATERIALS AND METHODS

Mice. Male C57BL/6 and (C57BL/6 × DBA/2)F1 mice were used at 8 to 14 weeks of age. BALB/c mice of either sex were used when they were 3 to 4 weeks old. Animals were bred under specific pathogen-free conditions and fed ad libitum at the Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany.

Bacteria and bacterial antigens. The *Listeria* species and strains as well as their serotypes are summarized in Table 1. *L. monocytogenes* EGD was originally obtained from G. B. Mackaness, Saranac Lake, N.Y., and its virulence was maintained by continuous mouse passage (14). All other strains were kindly provided by H. Hof, Würzburg, Federal Republic of Germany. Serotyping of strain EGD was kindly carried out by H. Hof. *Listeria* strains were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 18 h and afterwards were stored frozen in 0.5-ml portions at −70°C. Bacterial numbers were determined by plating 0.1-ml portions of serial 1:10 dilutions on Trypticase soy plates (BBL) and counting CFU after incubation at 37°C for 24 to 36 h (14). *Listeria* organisms were heat-killed by incubating ca. 10^9 bacteria per ml (suspended in phosphate-buffered saline) at 61°C for 60 min (15). Heat-killed *Listeria* (HKL) organisms were stored in 1-ml samples at −70°C. Soluble antigen from *L. monocytogenes* EGD was obtained from a cell-free culture supernatant as described previously (14). The protein content of the soluble antigen was 100 μg/ml.

Determination of bacterial persistence in infected spleens. Mice were infected intravenously (i.v.) with various num-

TABLE 1. *Listeria* strains used

Species	Strain	Serotype
L. monocytogenes	EGD[a]	1/2a
L. monocytogenes	ATCC 19111, NCTC 7973[b]	1/2a
L. monocytogenes	NCTC 5348[b]	1/2c
L. monocytogenes	ATCC 19113, NCTC 5105[b]	3a
L. monocytogenes	ATCC 19114, NCTC 5214[b]	4a
L. monocytogenes	NCTC 10527[b]	4b
L. monocytogenes	ATCC 19116[b]	4c
L. monocytogenes	ATCC 19119[b]	5
L. innocua	ATCC 33090, NCTC 11288[b]	6a
L. innocua	ATCC 33091, NCTC 11289[b]	6b
L. denitrificans	ATCC 14870[b]	NA[c]

[a] Originally obtained from G. B. Mackaness.
[b] Kindly provided by H. Hof.
[c] NA, No serotype available.

bers of listeriae, and 1 to 4 days later bacterial numbers in spleens were determined. Organs were homogenized, 0.1-ml samples of appropriate dilutions were plated out on Trypticase soy plates (BBL), and CFU were counted 24 h later (14).

Determination of cellular immunity to *Listeria* organisms in vitro. Mice were immunized i.v. with different *Listeria* strains, and 7 days later peritoneal exudates were induced by intraperitoneal injection of 10% proteose peptone (Difco Laboratories, Detroit, Mich.). Cells were harvested after another 3 days and peritoneal exudate T-lymphocyte-enriched cells (PETLE) were obtained by passage over nylon wool columns (8) as described previously (12, 13, 15). Plastic adherent peritoneal exudate cells from nonimmune mice, which were injected with 1.5 ml of 10% proteose peptone 3 days before harvest, were used as accessory cells (12, 13, 15). Cells were cultured in complete Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (Reheis Chemical Co., Phoenix, Ariz.), 2×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 1% penicillin, and 1% streptomycin (GIBCO Europe, Glasgow, United Kingdom).

Proliferative responses of *Listeria*-immune T cells (15) were determined as follows: *Listeria*-immune PETLE (2×10^5) were cultured with 2×10^3 accessory cells and 2×10^8 HKL in flat-bottomed microculture plates (Becton Dickinson Labware, Oxnard, Calif.) in a total volume of 0.2 ml of complete DMEM for 4 days at 37°C in 7% CO_2 in air. Eighteen hours before cell harvest, cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (Radiochemical Centre, Amersham, U.K.). The incorporation of radioactivity served as a measurement of T-cell proliferative responses.

Interleukin production by *Listeria*-immune T-cell cultures (12, 13, 15) was determined as follows: *Listeria*-immune PETLE (2×10^5) were cultured with 2×10^5 accessory cells and 2×10^8 HKL in flat-bottomed microculture plates (Becton Dickinson Labware) in 0.2 ml of complete DMEM at 37°C in 7% CO_2 in air. After 24 h, supernatants were collected and assayed for interleukin activity on thymocytes from young BALB/c mice. In short, thymocytes (2×10^5) were cultured in round-bottomed microculture plates (Nunc, Roskylde, Denmark) in 0.2 ml of complete DMEM containing 25% supernatant for 3 days at 37°C in 7% CO_2 in air; the last 18 h of culture was in the presence of 1 μCi of [^{3}H]TdR. Thymocyte proliferation served as the measure of interleukin activity.

Determination of cellular immunity to *Listeria* organisms in vivo. Delayed-type hypersensitivity (DTH) (14) was determined as follows: mice were immunized i.v. with different *Listeria* strains. After 7 days, mice were injected subcutaneously with 50 μl of soluble *Listeria* antigen into one hind footpad, and footpad swelling was determined 24 h later with a dial gauge caliper (Kröplin, Schlüchtern, Federal Republic of Germany). Data are expressed as differences (in units of 0.1 mm) between injected and uninjected contralateral footpad.

Protection (14) of *Listeria* organisms was determined as follows: mice which were immunized i.v. with different *Listeria* strains on day 8 were infected i.v. with either the homologous strain or the highly virulent strain EGD on day 0. On day 2, spleens were removed, and bacterial numbers were determined as described above. Data were calculated by the following formula: $\log_{10}$ (protection) = $\log_{10}$ (bacterial number in spleens of nonimmunized animals) − $\log_{10}$ (bacterial numbers in spleens of immunized animals).

***Listeria*-specific T-cell clones.** Mice (C57BL/6) were immunized with 2×10^5 live *L. monocytogenes* EGD subcutaneously in the tailroot. After 8 days, inguinal and para-aortic lymph nodes were removed, and single-cell suspensions were prepared. A total of 5×10^6 lymph node cells per ml were cultured with 10^6 HKL per ml in 5 ml of complete DMEM in 25-cm^2 flasks (Nunc) in an upright position at 37°C in 7% CO_2 in air. After 6 days, cells were washed, and 10^6 cells per ml were recultured with 10^6 irradiated (2,200 rad) spleen cells and 10^6 HKL per ml. After another 6 days, cells were transferred to Costar 3506 trays (Costar, Data Packaging, Cambridge, Mass.). *Listeria*-specific T cells were propagated in these trays in 5 ml of complete DMEM in the presence of 10^6 irradiated (2,200 rad) spleen cells and 10^6 HKL per ml at 37°C in 7% CO_2 in air and were fed every 6 to 8 days.

After 9 weeks of culture, *Listeria*-specific T cells were cloned at limiting dilution in round-bottomed microculture plates (Nunc) containing 2×10^5 irradiated (2,200 rad) spleen cells, 2×10^6 HKL, and 5% T-cell growth factor (TCGF) in 0.2 ml of complete DMEM. The preparation of TCGF from supernatants of concanavalin A-activated rat spleen cells has been described elsewhere (10). T cells were plated in replicates of 96 wells at threefold dilutions from 1,000 to 0.3 cells per well. After 8 days, wells containing proliferating cells were identified with an inverted microscope (frequency of positive wells, 1 in 25). Growing cells at dilutions with frequencies of less than 10% were recloned at a concentration of 0.3 cells per well under the conditions described above. Cloned T cells were expanded in the presence of 10^6 irradiated (2,200 rad) spleen cells, 10^6 HKL, and 5% TCGF per ml. Cloned T cells were fed once a week and propagated as described above. Although cell growth was more marked in the presence of exogeneous TCGF, significant growth was also observed in its absence.

Determination of biological activities of *Listeria*-specific T-cell clones. *Listeria*-specific T-cell clones were propagated for more than 16 weeks and were cultured in the absence of TCGF for at least 3 weeks before the experiments reported here were carried out. After centrifugation through Urovison-Ficoll (density, 1.077) for 15 min at 700 × *g*, 3×10^4 cells were cultured with 2×10^5 irradiated (2,200 rad) spleen cells and 10^8 HKL of the different strains in round-bottomed microculture plates (Nunc) in complete DMEM at 37°C in 7% CO_2 in air. After 3 days of culture, cells were pulsed with 1 μCi of [^{3}H]TdR and harvested 18 h later. For evaluation of their protective activity, 3×10^5 cloned *Listeria*-specific T cells, together with 2×10^5 live organisms of *L. monocytogenes*, were injected subcutaneously into one hind footpad

of recipient mice in a total volume of 0.05 ml (9). After 2 days, feet were removed, disinfected with alcohol (70%), and homogenized with a tissue grinder (Ultra Turrax, IKA, Staufen, Federal Republic of Germany). Samples of 0.1 ml of homogenate of appropriate 10-fold dilutions were plated on Trypticase soy agar (BBL). Protection is expressed as the difference between the $\log_{10}$ numbers of bacteria in footpads of T-cell recipients and $\log_{10}$ numbers of bacteria in controls.

RESULTS

Persistence of different *Listeria* strains in spleens of infected animals. To determine the persistence of the different *Listeria* strains used, C57BL/6 mice were infected with 10^4 to 10^5 organisms per 0.2 ml i.v., and 1, 2, and 4 days later bacterial numbers in spleens were determined. After this time period, significant numbers of bacteria still persisted in spleens of mice infected with *L. monocytogenes* EGD (serotype 1/2a), NCTC 5348 (serotype 1/2c), ATCC 19113 (serotype 3a), ATCC 19114 (serotype 4a), NCTC 10527 (serotype 4b), and ATCC 19116 (serotype 4c) (Fig. 1). On the other hand, in the case of *L. monocytogenes* strains ATCC 19111 (serotype 1/2a) and ATCC 19119 (serovar 5), *Listeria innocua* strains ATCC 33090 (serotype 6a) and ATCC 33091 (serovar 6b), as well as *Listeria denitrificans* strain ATCC 14870, no microorganisms were demonstrable on day 4. Even after infection with ca. 10^8 organisms of the latter strains, only few, if any, bacteria persisted in the spleens (Fig. 1). At these high inocula, the *L. monocytogenes* strains EGD, NCTC 5348, ATCC 19113, ATCC 19114, NCTC 10527, and ATCC 19116 caused death of the mice within a few days. On day 4 of infection, comparable numbers of bacteria were found in spleens of mice infected with ca. 10^8 organisms of *Listeria* sp. strains ATCC 33091 and ATCC 14870 and with 10^4 to 10^5 organisms of strains ATCC 19113 and ATCC 19114. Therefore, in another experiment, mice were infected with strains ATCC 19113 (1×10^5 organisms), ATCC 19114 (8×10^4 organisms), ATCC 33091 (1×10^8 organisms), and ATCC 14870 (7×10^7 organisms), and bacterial numbers were determined 6 days later. Although no bacteria were demonstrable with strains ATCC 33091 and ATCC 14870, 300 to 600 organisms were still present in spleens of mice infected with strains ATCC 19113 and ATCC 19114 (data not shown). It becomes apparent from these results that the different strains of *Listeria* sp. can be separated into two distinct groups: one group of bacteria that are capable of persisting in spleens of infected animals and another group of bacteria that are readily eliminated by the host. Hereafter, the former group will be designated persistent strains, and the latter group will be designated nonpersistent strains.

Immunogenicity of different *Listeria* strains. In the next set of experiments, the capacity of the *Listeria* strains to induce cellular immunity was analyzed in vitro and in vivo. PETLE from (C57BL/6 × DBA/2)F1 mice immunized with the different strains were cultured with HKL of the homologous strain or strain EGD, and proliferation and interleukin production were assessed. The persistent strains EGD, NCTC 5348, ATCC 19113, ATCC 19114, NCTC 10527, and ATCC 19116 induced marked T-cell responses, whereas the nonpersistent strains ATCC 19111, ATCC 19119, ATCC

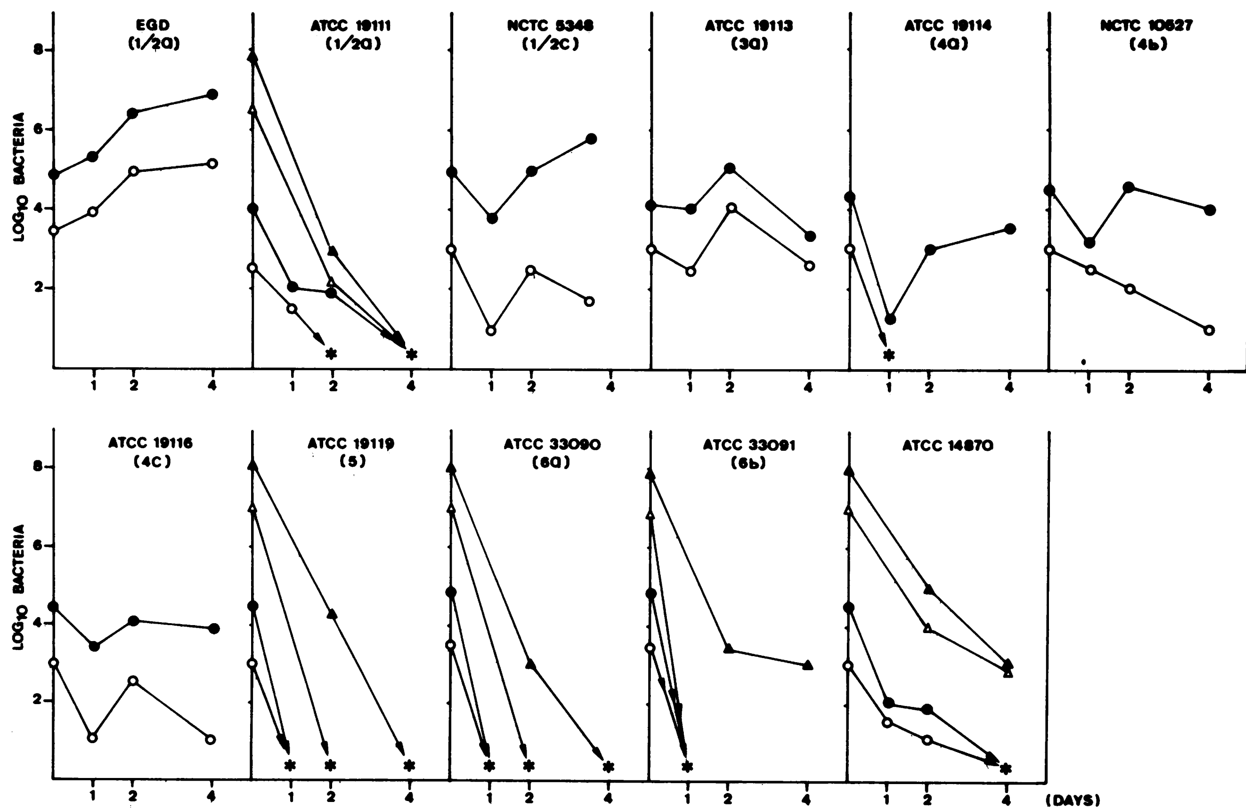

FIG. 1. Growth curves of different *Listeria* strains in spleens of C57BL/6 mice. Mice were infected with different numbers of viable *Listeria* organisms, and bacterial numbers in spleens were determined on various days thereafter, as described in the text. Each point represents the mean values of four mice; standard deviation, <15%. *, Not determinable (<100 organisms per spleen). Serotypes are indicated in parentheses.

TABLE 2. Proliferative responses and interleukin production by PETLE from mice immunized with different *Listeria* strains

Listeria strain used for immunization		Persistence	Proliferative response ([^{3}H]TdR uptake [cpm/2 × 10^5 PETLE])[a]		Interleukin production ([^{3}H]TdR uptake [cpm/10^6 thymocytes])[a]	
Strain	Serotype		Homologous antigen	EGD antigen	Homologous antigen	EGD antigen
EGD	1/2a	Persistent	154,700	154,700	53,400	53,400
ATCC 19111	1/2a	Nonpersistent	22,200	22,900	3,600	2,100
NCTC 5348	1/2c	Persistent	99,200	138,200	19,200	20,200
ATCC 19113	3a	Persistent	130,600	147,700	28,700	26,100
ATCC 19114	4a	Persistent	121,700	128,200	29,800	35,700
NCTC 10527	4b	Persistent	148,600	145,900	31,000	33,900
ATCC 19116	4c	Persistent	139,200	143,000	28,200	30,500
ATCC 19119	5	Nonpersistent	40,800	37,400	2,000	3,400
ATCC 33090	6a	Nonpersistent	23,100	24,300	2,500	1,800
ATCC 33091	6b	Nonpersistent	20,500	22,300	5,900	4,600
ATCC 14870	NA[b]	Nonpersistent	21,700	22,500	2,700	2,300

[a] PETLE from mice immunized with different *Listeria* strains were cultured with accessory cells, and HKL of the homologous strain or EGD and proliferative responses or interleukin production were assessed. In the absence of antigen, proliferative responses were <2,000 cpm and interleukin activity was <1,000 cpm. Values are means of three determinations; standard deviation, <20%.

[b] NA, No serotype available.

33090, ATCC 33091, and ATCC 14870 stimulated significantly lower responses (Table 2). Furthermore, within all groups, T cells responded equally well to the homologous and heterologous (strain EGD) antigens (Table 2).

For evaluation of in vivo activities, (C57BL/6 × DBA/2)F1 mice were vaccinated with *Listeria* strains (ca. 5 × 10^4 organisms per 0.2 ml i.v.), and afterwards, protection against the highly virulent *Listeria* sp. strain EGD as well as DTH responses to soluble antigens of strain EGD were assessed. Only those strains which stimulated T cells for strong in vitro activities were able to induce significant protection and DTH responses in vivo (Table 3). Even when mice were inoculated with higher numbers (10^8 to 10^9 live organisms) of the nonpersistent *Listeria* strains, only marginal immune responses were obtained. Thus, protection against the virulent strain EGD was at least 100-fold lower in mice vaccinated with a high number of organisms of the nonpersistent strains ATCC 19111, ATCC 19119, ATCC 33090, ATCC 33091, and ATCC 14870 as compared with mice vaccinated with 10^4 live organisms of strain EGD. Comparable results were obtained when PETLE from mice injected with high numbers of nonpersistent listeriae were tested for antigen-induced proliferation and interleukin production in vitro (data not shown). Hence, a close correlation existed between the persistence of *Listeria* strains and their capacity to induce cell-mediated immune responses. These data indicate that the presence of high concentrations of bacteria over a limited time period is not sufficient for stimulation of T-cell-dependent immunity and that bacterial survival in the host is required for a considerable period of time.

Cross-reactivity between different *Listeria* strains on the level of unselected T-cell populations. For evaluation of cross-reactivity between different *Listeria* strains on the level of unselected T cells, PETLE from (C57BL/6 × DBA/2)F1 mice immunized with *L. monocytogenes* EGD were cultured with HKL of the different strains, and proliferation in these cultures was determined. T cells immune to strain EGD responded equally well to heat-killed organisms of all *Listeria* strains, as well as to soluble antigen from strain EGD, but not to purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Table 4). Thus, in immune, heterogeneous T-cell populations, a striking cross-reactivity between persistent and nonpersistent strains exists. However, these findings do not determine whether different determinants are recognized by different lymphocyte clones or, alternatively, whether a single common epitope is recognized by those T

TABLE 3. Protection and DTH in mice immunized with different *Listeria* strains

Listeria strain used for immunization		Persistence	Log_{10} protection[a]		DTH (0.1 mm)[b]
Strain	Serotype		Homologous strain	Strain EGD	
EGD	1/2a	Persistent	3.5 ± 0.93	3.5 ± 0.61	15.3 ± 0.85
ATCC 19111	1/2a	Nonpersistent	ND[c]	1.0 ± 0.51	2.1 ± 1.20
NCTC 5348	1/2c	Persistent	1.5 ± 0.66	2.0 ± 0.25	12.0 ± 0.72
ATCC 19113	3a	Persistent	3.3 ± 0.74	3.4 ± 0.33	8.2 ± 1.03
ATCC 19114	4a	Persistent	3.0 ± 1.21	3.2 ± 0.92	14.3 ± 0.79
NCTC 10527	4b	Persistent	3.8 ± 0.82	2.7 ± 0.84	12.7 ± 1.51
ATCC 19116	4c	Persistent	3.4 ± 0.43	3.1 ± 0.72	14.5 ± 0.98
ATCC 19119	5	Nonpersistent	ND	1.3 ± 0.86	4.0 ± 0.84
ATCC 33090	6a	Nonpersistent	0.6 ± 0.39	1.2 ± 0.51	2.3 ± 0.55
ATCC 33091	6b	Nonpersistent	0.6 ± 0.57	1.2 ± 0.67	6.6 ± 0.32
ATCC 14870	NA[d]	Nonpersistent	ND	0.7 ± 0.35	5.8 ± 0.77

[a] Mice were immunized with different *Listeria* strains on day 8 and infected with 7 × 10^4 organisms of strain EGD on day 0. Bacterial numbers in spleens were determined on day 2. Values are means of five determinations ± standard deviation.

[b] Mice were immunized with different *Listeria* strains on day 8 and challenged with soluble listerial antigen on day 0. DTH responses were evaluated 24 h later. Values are means of five determinations ± standard deviation.

[c] ND, Not determinable (<100 organisms per spleen).

[d] NA, Not available.

cells in the unselected population which mediate antibacterial protection.

Cross-reactivity between different *Listeria* strains on the clonal level. The availability of antigen-specific T-cell clones has allowed analysis of antigenic cross-reactivity on the single-cell level. Three T-cell clones from C57BL/6 mice and specific for *L. monocytogenes* EGD were cultured with different strains, and proliferative responses were assessed. Clones 26.1.1 and 26.1.2 were stimulated to proliferate by all but one strain (ATCC 19114; see Table 6). In the case of clone 26.1.2, the response to strain ATCC 19113 was also somewhat reduced. On the other hand, clone 26.1.3 reacted equally well to all *Listeria* strains (see Table 6). Furthermore, the three clones were stimulated by soluble antigen from strain EGD but not by PPD (see Table 6). Thus, epitopes recognized by T-cell clones were expressed by different *Listeria* strains, irrespective of their capacity to persist in the host. It is of importance that these three T-cell clones were able to adoptively mediate antilisterial resistance as assessed in a local transfer system. This system was used because it has been shown recently (9) that after systemic transfer, *L. monocytogenes*-specific T-cell clones are trapped in the lungs of recipient mice and express only weak antibacterial activity. A total of 3×10^5 cells of all three clones were able to protect mice against live *L. monocytogenes* EGD to a significant degree (Table 5). Because it was found that two of the T-cell clones were able to distinguish between two persistent *Listeria* strains in vitro (Table 6), it became possible to ascertain the specificity of antibacterial resistance on the single-cell level. In fact, clones 26.1.1 and 26.1.2 which did not cross-react with *L. monocytogenes* ATCC 19114 in vitro were also unable to transfer protection against this strain, whereas clone 26.1.3 was able to do so (Table 5). These findings demonstrate that protective T-cell clones can react with antigenic epitopes shared by persistent and nonpersistent *Listeria* strains. Thus, nonpersistent strains expressed epitopes which are recognized by T cells responsible for acquired resistance, although they did not induce effective protective immunity by themselves.

DISCUSSION

In the present study, experimental infection of mice with a panel of *Listeria* strains was used for analysis of a possible relationship between persistence, cross-reactivity, and immunogenicity of facultative intracellular bacteria. The results of the experiments suggest that both persistent and nonpersistent bacteria express antigenic determinants recognized by protective T-cell populations and that bacterial persistence in the host is a crucial prerequisite for the development of acquired cellular resistance. After systemic infection with facultative intracellular bacteria, the majority of microorganisms are entrapped in the reticuloendothelial system, and one prominent feature of these pathogens is their capacity to survive after engulfment. Hence, virulence of facultative intracellular bacteria is intimately related with their persistence in the mononuclear phagocyte system of the host. As has become clear from the pioneering work of Dubos (4) and Mackaness (19–21), in intracellular bacterial infections, such as tuberculosis and listeriosis, enumeration of bacterial numbers in infected organs (e.g., spleens) rather than determination of lethal doses represents the most suitable quantitative analysis of virulence. Accordingly, in the present study persistence of different *Listeria* strains in spleens of C57BL/6 mice was used as a measurement of their virulence. It was found that the genus *Listeria* can be separated into two distinct groups: strains EGD, NCTC 5348, ATCC 19113, ATCC 19114, NCTC 10527, and ATCC 19116 belong to the persistent group, and strains ATCC 19111, ATCC 19119, ATCC 33090, ATCC 33091, and ATCC 14870 belong to the nonpersistent group. Although some data about the virulence of different *Listeria* strains for mice can be found in the literature (1, 6, 16, 17, 26, 30), a systematic survey is not available, and comparison of the published findings is extremely difficult because different strain designations have often been used. In a recent study (30), virulence of several *Listeria* strains for NMRI mice was investigated. The data presented here are in agreement with these findings in that they define serotype 4b as persistent and strain ATCC 33091 as nonpersistent. However, the authors (30) found that strain ATCC 19113 was eliminated in NMRI mice, whereas in the present study this strain could survive in C57BL/6 mice. Because mouse strains express marked differences in their susceptibility to *Listeria* infection (27), the use of different mouse strains may account for this discrepancy.

The possibility of developing monoclonal antibodies directed against infectious agents has facilitated passive immu-

TABLE 4. Proliferative responses by *Listeria*-immune PETLE after in vitro stimulation with different *Listeria* strains

Listeria antigen		Persistence	Proliferative response ([^{3}H]TdR uptake [cpm/2 × 10^5 cells])[a]
Strain	Serotype		
Control			1,200
EGD	1/2a	Persistent	22,700
ATCC 19111	1/2a	Nonpersistent	16,200
NCTC 5348	1/2c	Persistent	25,000
ATCC 19113	3a	Persistent	21,900
ATCC 19114	4a	Persistent	22,600
NCTC 10527	4b	Persistent	20,100
ATCC 19116	4c	Persistent	24,300
ATCC 19119	5	Nonpersistent	18,800
ATCC 33090	6a	Nonpersistent	24,600
ATCC 33091	6b	Nonpersistent	25,700
ATCC 14870	NA[b]	Nonpersistent	24,500
Soluble antigen from strain EGD[c]			14,400
PPD[c]			1,600

[a] *L. monocytogenes* EGD-immune PETLE were cultured with accessory cells, and HKL of different strains and proliferative responses were determined by [^{3}H]TdR incorporation. Values are means of three determinations; standard deviation, <20%.

[b] NA, No serotype available.

[c] Soluble antigen of strain EGD was used at 2 μg, and PPD was used at 5 μg.

TABLE 5. Adoptive protection by *Listeria*-specific T-cell clones[a]

T-cell clone	Log_{10} protection against *Listeria* sp. strain EGD[b]	Log_{10} protection against *Listeria* sp. ATCC 19114[b]
26.1.1	1.6 ± 0.38	0.2 ± 0.50
26.1.2	1.3 ± 0.50	0.4 ± 0.44
26.1.3	1.8 ± 0.21	1.9 ± 0.52

[a] Cloned *Listeria*-specific T cells (3×10^5) together with 2×10^5 *L. monocytogenes* EGD or strain ATCC 19114, respectively, were injected subcutaneously into one hind footpad, and bacterial numbers were determined 2 days later.

[b] Values are means of five determinations ± standard deviation.

nization with pure antibody preparations, as well as characterization and purification of protective antigens to be used for active vaccination. Although this will be of great impact on immunotherapy of infections with extracellular bacteria, the situation is more complex with facultative intracellular infections due to the T-cell dependence of protection against the latter. Because T and B cells often recognize different epitopes, protective antigens of facultative intracellular pathogens would best be characterized with T-cell populations of defined biological activities. Therefore, unselected T-cell populations, as well as T-cell clones, were employed as tools. By using this approach, a striking cross-reactivity between the different *Listeria* strains was observed with in vivo-primed T-cell populations. When tested on the clonal level, two clones recognized 10 of 11 *Listeria* strains, and one clone reacted with all 11 strains after appropriate antigen presentation. It is not clear from these studies whether this cross-reactivity is due to a preferential presentation by macrophages of certain listerial determinants. Also, it remains to be clarified whether the majority of *Listeria*-reactive T cells recognize these cross-reactive epitopes or whether, in addition, T-cell populations exist which are specific for epitopes unique for the *Listeria* strain used for immunization. A limiting dilution assay system (5) is currently being developed to answer this question.

The *Listeria*-specific T-cell clones transferred antilisterial protection to recipient mice (see Table 5). Thus, both persistent and nonpersistent *Listeria* strains expressed epitopes which were recognized by protective T cells and therefore fall under the definition of protective antigens. The results also indicate that protective antigens were retained on the soluble listerial antigen used. Recently, a monoclonal antibody which binds to this soluble antigen as well as to the different *Listeria* strains was identified (S. H. E. Kaufmann and L. J. Wrazel, manuscript in preparation) and which might help in the purification of cross-reactive and protective *Listeria* antigens.

Protective antigens were expressed by persistent as well as nonpersistent *Listeria* strains. Nonetheless, both types differed markedly with respect to their capacity to stimulate T-cell-dependent immunity. Immunization with up to 10^9 nonpersistent bacteria resulted in lower T-cell responses, as compared with immunization with less than 10^5 persistent organisms. This held true for the four different activities tested, namely, T-cell proliferation and interleukin production in vitro as well as DTH and antibacterial resistance in vivo. This coherent pattern provides further support for the idea of a common cellular mechanism being responsible for different activities of antibacterial immunity (10, 19, 20).

After infection with high numbers of nonpersistent listeriae, only few if any live organisms were demonstrable in spleens 2 to 4 days later, and no bacteria were found 6 days after infection. It is possible that the rapid abridgement of bacteria was responsible for the failure to effectively induce T-cell responses and that the presence of bacteria only during the first days of infection was not sufficient for the development of optimal protective cellular immunity. Although cellular immune responses induced by nonpersistent *Listeria* organisms were constantly lower than those in mice infected with persistent bacteria, comparable numbers of bacteria were found in spleens 4 days after infection with ca. 10^8 organisms of the nonpersistent strains ATCC 33091 and ATCC 14870 and with 10^4 to 10^5 organisms of several persistent strains (e.g., ATCC 19113 and ATCC 19114). It may therefore be speculated that the events leading to T-cell-dependent immunity are more complex and that the percentage of bacteria surviving in spleens rather than the absolute numbers of survivors was important for the strength of the resulting response. Indeed, the number of persistent bacteria

TABLE 6. Proliferative responses by *Listeria*-specific T-cell clones after in vitro stimulation with different *Listeria* strains

Listeria antigen		Persistence	Proliferative response ([^{3}H]TdR uptake [cpm/3×10^4 T cells])[a]		
Strain	Serotype		Clone 26.1.1	Clone 26.1.2	Clone 26.1.3
Control			900	1,250	710
EGD	1/2a	Persistent	16,700	17,400	55,700
ATCC 19111	1/2a	Nonpersistent	29,500	45,200	56,000
NCTC 5348	1/2c	Persistent	26,600	27,500	53,600
ATCC 19113	3a	Persistent	20,300	11,000	50,300
ATCC 19114	4a	Persistent	3,150	2,400	38,300
NCTC 10527	4b	Persistent	19,550	28,800	43,900
ATCC 19116	4c	Persistent	31,900	29,200	52,800
ATCC 19119	5	Nonpersistent	20,300	31,500	53,100
ATCC 33090	6a	Nonpersistent	16,900	26,400	44,800
ATCC 33091	6b	Nonpersistent	22,400	36,900	62,100
ATCC 14870	NA[b]	Nonpersistent	26,100	35,000	69,200
Soluble antigen from strain EGD[c]			11,200	9,800	14,500
PPD[c]			1,120	980	1,260

[a] Cloned *Listeria*-specific T cells were cocultured with accessory cells, and HKL of different *Listeria* strains and proliferative responses were determined by [^{3}H]TdR incorporation. Values are means of three determinations; standard deviation, <20%.

[b] NA, Not available.

[c] Soluble antigen of strain EGD was used at 2 μg, and PPD was used at 5 μg.

present on day 4 was similar to the number in the inoculum, whereas in the case of nonpersistent strains, more than 99.9% of the inoculum had already been eliminated. These findings, of course, do not rule out the possibility that higher degrees of cell-mediated immunity can be induced by further raising the inoculum of nonpersistent *Listeriae* to increase the number of survivors (30).

It has become clear from different experimental approaches that protective T cells are not generated before day 2 of infection and that maximum T-cell formation takes place between days 3 and 6 (24, 31). T-cell activation seems to follow the replacement of neutrophils by mononuclear phagocytes and the development of granulomas at the site of bacterial implantation (22). In view of these findings, it appears likely that the host was unable to present nonpersistent listeriae to the T-cell compartment in an appropriate way because the great majority of microorganisms were already eliminated by phagocytes that were unable to process and present bacterial antigens. On the other hand, persistent bacteria could have survived long enough to be taken up by macrophages and presented to the T-cell system.

It has been known for a long time that killed listeriae are unable to stimulate protective immunity (21). Although evidence has been presented (28) that under certain experimental precautions killed listeriae may be used as vaccines, this issue has been questioned recently (29). On the other hand, attenuated bacteria have generally been accepted as a suitable source for vaccination against intracellular infections. The experiments described here cast some doubt on the universality of this issue and suggest a marked inferiority of nonpersistent bacteria as compared with persistent organisms in inducing antibacterial immunity. Construction of improved vaccines against facultative intracellular infections should, therefore, concentrate on both the characterization and purification of antigens recognized by the T-cell set mediating antibacterial immunity and the development of adjuvants suitable for optimal stimulation of protective T cells.

ACKNOWLEDGMENTS

I am grateful to H. Hof, Würzburg for supplying the different *Listeria* strains and for serotyping strain EGD. I thank K. Eichmann, J. Cooper, and M. M. Simon for critically reading the paper, B. Brokmeier and L. J. Wrazel for helpful technical assistance, and H. Kuttler and R. Schneider for typing the manuscript.

LITERATURE CITED

1. **Audurier, A. P., P. Pardo, J. Marly, and F. Lantier.** 1980. Experimental infection of mice with *Listeria monocytogenes* and *L. innocua*. Ann. Microbiol. (Paris) **131B**:47–57.
2. **Blanden, R. V., and R. E. Langman.** 1972. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. Scand. J. Immunol. **1**:379–391.
3. **Collins, F. M., and S. G. Campbell.** 1982. Immunity to intracellular bacteria. Vet. Immunol. Immunopathol. **3**:5–66.
4. **Dubos, R. J.** 1954. Biochemical determinants of microbial diseases. Harvard University Press, Cambridge, Mass.
5. **Eichmann, K., J. Goronzy, U. Hamann, P. H. Krammer, R. C. Kuppers, I. Melchers, M. M. Simon, and G. Zahn.** 1982. Clonal analysis of helper and cytolytic T cells. Multiple, independently regulated precursor sets at frequencies suggesting a limited repertoire, p. 234–244. In C. G. Fathman and F. W. Fitch (ed.), Isolation, characterization and utilization of T lymphocyte clones. Academic Press, Inc., New York.
6. **Gray, M. L., and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30**:309–382.
7. **Hahn, H., and S. H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1250.
8. **Julius, H. H., E. Simpson, and L. A. Herzenberg.** 1973. A rapid method for the isolation of functional thymus derived murine lymphocytes. Eur. J. Immunol. **3**:645–649.
9. **Kaufmann, S. H. E.** 1983. Effective antibacterial protection induced by a *Listeria monocytogenes*-specific T cell clone and its lymphokines. Infect. Immun. **39**:1265–1270.
10. **Kaufmann, S. H. E., and H. Hahn.** 1982. Biological functions of T cell lines with specificity for the intracellular bacterium *Listeria monocytogenes* in vitro and in vivo. J. Exp. Med. **155**:1754–1765.
11. **Kaufmann, S. H. E., H. Hahn, R. Berger, and H. Kirchner.** 1983. Interferon production by *Listeria monocytogenes*-specific T cells active in cellular antibacterial immunity. Eur. J. Immunol. **13**:265–268.
12. **Kaufmann, S. H. E., H. Hahn, and M. M. Simon.** 1982. T-cell subsets induced in *Listeria monocytogenes*-immune mice: Ly phenotypes of T cells interacting with macrophages in vitro. Scand. J. Immunol. **16**:539–542.
13. **Kaufmann, S. H. E., H. Hahn, M. M. Simon, M. Röllinghoff, and H. Wagner.** 1982. Interleukin 2 induction in Lyt $1^{+}23^{-}$ T cells from *Listeria monocytogenes*-immune mice. Infect. Immun. **37**:1292–1294.
14. **Kaufmann, S. H. E., M. M. Simon, and H. Hahn.** 1979. Specific Lyt 123 T cells are involved in protection against *Listeria monocytogenes* and in delayed-type hypersensitivity to listerial antigens. J. Exp. Med. **150**:1033–1038.
15. **Kaufmann, S. H. E., M. M. Simon, and H. Hahn.** 1982. Regulatory interactions between macrophages and T-cell subsets in *Listeria monocytogenes*-specific T-cell activation. Infect. Immun. **38**:907–913.
16. **Kautter, D. A., S. J. Silverman, W. G. Roessler, and J. D. Drawdy.** 1963. Virulence of *Listeria monocytogenes* for experimental animals. J. Infect. Dis. **112**:167–180.
17. **Khan, M. A., A. Seaman, and M. Woodbine.** 1973. The pathogenicity of *Listeria monocytogenes*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **224**:355–361.
18. **Lane, F. C., and E. R. Unanue.** 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. J. Exp. Med. **153**:1104–1112.
19. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–406.
20. **Mackaness, G. B.** 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. **129**:973–992.
21. **Miki, K., and G. B. Mackaness.** 1964. The passive transfer of acquired resistance to *Listeria monocytogenes*. J. Exp. Med. **120**:93–103.
22. **North, R. J.** 1970. The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection. J. Exp. Med. **132**:521–534.
23. **North, R. J.**, 1973. The mediators of anti-*Listeria* immunity as an enlarged population of short-lived, replicating T cells. Kinetics of their production. J. Exp. Med. **138**:342–355.
24. **North, R. J., P. A. Berche, and M. F. Newborg.** 1981. Immunologic consequences of antibiotic-induced abridgement of bacterial infection: effect on generation and loss of protective T cells and level of immunologic memory. J. Immunol. **127**:342–346.
25. **Seeliger, H. P. R.** 1958. Listeriose, 2nd ed. Johann Ambrosius Barth Verlag, Leipzig, German Democratic Republic.
26. **Seeliger, H. P. R.** 1975. Serovariants of *Listeria monocytogenes* and other *Listeria* species. Acta Microbiol. Acad. Sci. Hung. **22**:179–181.
27. **Skamene, E., L. Kongshavn, and D. H. Sachs.** 1979. Resistance to *Listeria monocytogenes* in mice: genetic control by genes that are not linked to the H-2 complex. J. Infect. Dis. **139**:228–231.
28. **Van Der Meer, C., F. M. A. Hofhuis, and J. M. N. Willers.** 1977.

Killed *Listeria monocytogenes* vaccine becomes protective on addition of polyanions. Nature (London) **269**:594–595.

29. **Wirsing von König, C. H., H. Finger, and H. Hof.** 1982. Failure of killed *Listeria monocytogenes* vaccine to produce protective immunity. Nature (London) **297**:233–234.
30. **Wirsing von König, C. H., B. Heymer, H. Hof, and H. Finger.** 1983. Course of infection and development of immunity in experimental infection of mice with *Listeria* serotypes. Infect. Immun. **40**:1170–1177.
31. **Zinkernagel, R. M., R. V. Blanden, and R. E. Langman.** 1974. Early appearance of sensitized lymphocytes in mice infected with *Listeria monocytogenes*. J. Immunol. **112**:496–501.