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T Lymphocytes Mediate Protection against *Yersinia enterocolitica* in Mice: Characterization of Murine T-Cell Clones Specific for *Y. enterocolitica*

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***Yersinia enterocolitica* is enteropathogenic for humans and rodents, causing intestinal and extraintestinal diseases. The cellular immune response of the infected host has not yet been analyzed in detail. Therefore, we used a parenteral mouse infection model to determine the role of T lymphocytes in immunity against *Y. enterocolitica*. We report the generation and characterization of *Y. enterocolitica*-specific T-cell clones isolated from spleens of intravenously infected C57BL/6 mice. The T-cell clones obtained showed the phenotype of helper T cells (L3T4) or cytotoxic T cells (Lyt2). All T-cell clones were positive for the interleukin-2 (IL-2) receptor (Tac antigen, p55 subunit) and were negative for the γδ T-cell receptor. L3T4$^+$ clones produced small quantities of IL-2 (<1 U/ml) when stimulated with heat-killed *Y. enterocolitica*, whereas Lyt2$^+$ clones produced no or extremely low levels of IL-2. In contrast to IL-2 production, both L3T4$^+$ and Lyt2$^+$ T-cell clones produced considerable quantities of gamma interferon (500 U/ml). When transferred into nonimmune mice, some of the L3T4$^+$, as well as the Lyt2$^+$, T-cell clones could mediate at least partial protection against a challenge of a lethal dose of *Y. enterocolitica*. These data demonstrate for the first time the generation and characterization of *Y. enterocolitica*-specific T-cell clones and provide evidence that T cells may be involved in protection against enteropathogenic *Y. enterocolitica*.**

During the past two decades, *Yersinia enterocolitica* has aroused the interest of clinicians and microbiologists because of its wide range of clinical manifestations and the increasing knowledge about the genetic regulation of its virulence factors. Infection with *Y. enterocolitica* causes intestinal lesions including acute enteritis, enterocolitis, and mesenterial lymphadenitis (7, 43; for a review, see reference 12). In rare cases, particularly in immunocompromised hosts, *Y. enterocolitica* can induce disseminated infection with abscess formation in the spleen, liver, and other organs (8, 63). Moreover, extraintestinal manifestations such as reactive arthritis, erythema nodosum, and uveitis have been associated with intestinal infection of *Y. enterocolitica* (2, 14, 31, 32, 44, 69, 71). The latter sequelae are presumed to result from an immunopathological host reaction, possibly mediated by *Y. enterocolitica*-specific T lymphocytes and/or immune complexes (20, 32, 36). The first evidence for the role of lymphocytes during *Y. enterocolitica* infection was provided by Alonso et al. (3), who demonstrated that the transfer of lymphoid cells from stimulated Peyer's patches of mice infected with *Y. enterocolitica* of serotype O:3 grown at 26°C can induce immunity to *Y. pestis*. However, apart from this study, there are only few data on the cellular immune response against *Y. enterocolitica* (25, 28). Therefore, a systematic analysis is required which may provide more insights into the pathomechanism of *Y. enterocolitica* infection and its sequelae.

The virulence of *Y. enterocolitica*, in humans and rodents, is controlled by chromosomal (*yst*, *inv*, *ail*, and iron uptake system) (15, 29, 30, 38, 50, 51, 59) and extrachromosomal (genes encoding released proteins or *Yersinia* outer proteins, called Yops, and outer membrane proteins, e.g., *Yersinia* adhesin [YadA]) determinants (6, 30; for a review, see references 10 and 11). All *Yersinia* species pathogenic for humans harbor a virulence plasmid, pYV (6, 10, 34, 61). pYV-encoded YadA and Yops mediate pathogenic functions such as resistance to phagocytosis and complement lysis (4, 33, 34, 45, 58, 65), cell adhesion (30, 33, 70), cytotoxicity (19, 49, 61), colonization of the intestine (40), and, as shown recently, tyrosine phosphatase hydrolysis of host proteins (5, 22).

There is evidence from electron and light microscopic studies (21, 27, 28, 33, 45, 65, 66, 70) that enteropathogenic plasmid-bearing *Yersinia* species multiply predominantly extracellularly in lymphatic organs of experimentally infected mice or in cell culture systems. Although this topic is still controversially discussed (52), we suggest that *Y. enterocolitica* is a predominantly extracellularly located pathogen. If this turns out to be true, the host response to *Y. enterocolitica* should differ fundamentally from that to facultative intracellularly located bacteria (e.g., *Listeria monocytogenes*) (23). Therefore, we raised the question of whether specific T lymphocytes are involved in the immune defense against *Y. enterocolitica*. In our study, we used a well-established mouse model (9, 16, 25) for investigating the cellular immune response. C57BL/6 mice were chosen because of their relatively high resistance to *Y. enterocolitica* of serotype O:8 (50% lethal dose [LD_{50}] = 10^5) compared with susceptible mice, e.g., BALB/c mice (LD_{50} = 10^2) (25). Genetic studies dealing with the different susceptibilities of both mouse strains to *Y. enterocolitica* have not yet clarified this phenomenon (26). But the comparison of normal with athymic T-cell-deficient nude mice suggests that thymus-

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dependent immune mechanisms are required for resistance to *Y. enterocolitica* (25). As a first step, we therefore investigated the T-cell response to *Y. enterocolitica* in a *Yersinia*-resistant mouse strain.

In this report, we describe the generation of *Y. enterocolitica*-specific T-cell clones, their phenotypic characteristics, and their functional properties in vitro (specificity of proliferative response, interleukin-2 [IL-2] and gamma interferon [IFN-γ] production) as well as in vivo (protective ability).

MATERIALS AND METHODS

Mice. Female C57BL/6 mice, 6 to 8 weeks old, were purchased from Charles River Wiga (Sulzfeld, Germany). All mice were kept under specific-pathogen-free conditions (positive-pressure cabinet) and provided food and water ad libitum.

Bacteria. Following a passage in C57BL/6 mice, *Y. enterocolitica* of serotype O:8 (strain WA-P) harboring the pYV virulence plasmid (29, 35) were cultivated at 26°C in Luria broth. After 24 h, cultures were diluted 1:20 in Luria broth and cultivated for an additional 4 to 6 h at 26°C as described by Alonso et al. (3). Then cultures were collected by centrifugation and suspended in Luria broth containing 20% glycerol, frozen immediately in 1-ml aliquots in liquid nitrogen, and stored at a concentration of 5×10^8 bacteria per ml.

Animal infection and enumeration of CFU in spleen tissue. For infection, a *Y. enterocolitica* stock suspension was thawed and washed twice in sterile phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% normal mouse serum. This commonly used procedure was mainly chosen because of the excellently reproducible infection doses revealed. The survival rate after freezing and thawing was more than 97% as determined by plating for CFU before and after freezing. After appropriate dilution, bacteria were injected into the lateral tail vein in a volume of 0.1 ml of PBS. For induction of *Y. enterocolitica*-specific T lymphocytes, mice were infected with 1×10^4 bacteria (0.1 LD_{50}). In T-lymphocyte transfer experiments, 5×10^5 bacteria (five times the LD_{50}) were used as the challenge to achieve a lethal infection for determination of the protective capacity of T cells transferred (see below). The actual number of bacteria administered was determined by plating 0.1 ml of serial 1:10 dilutions on Mueller-Hinton agar and counting for CFU after incubation for 36 h at 26°C. The number of bacteria present in mice infected with *Y. enterocolitica* was determined after aseptic removal and homogenization of each spleen in 5 ml of sterile PBS containing 0.1% Tergitol TMN 10 (Fluka, Buchs, Switzerland) and 0.1% bovine serum albumin (E. Merck AG, Darmstadt, Germany) with tissue homogenizers (glass potter) (Bellco, Feltham, England). This procedure inhibited the aggregation of microorganisms as determined by microscopy. A 0.1-ml sample of appropriate 1:10 dilutions was plated out, and the next day CFU were counted as described above. The lower limit of detectable splenic CFUs by this method was 50 ($\log_{10} 50 = 1.7$).

Antigen preparation. Bacteria were cultivated as described above and heat killed by incubation for 1 h at 60°C. Sterility was controlled by cultivation in Luria broth. Heat-killed *Y. enterocolitica* was collected by centrifugation, washed twice, and resuspended in PBS. For T-cell stimulation experiments, a suspension of heat-killed *Y. enterocolitica* corresponding to 10 μg of protein per ml was used (48). To determine the specificity of T-cell clones, heat-killed *Salmonella typhimurium* (clinical isolate 2943) and heat-killed *Listeria monocytogenes* (strain EGD, serotype 1/2a) were used as controls in the same concentrations.

Cell culture medium and supplements. Cells were cultured in Click/RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5×10^{-5} M 2-mercaptoethanol, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% heat-inactivated fetal calf serum (Biochrom).

As a source of IL-2, we used the supernatant of spleen mononuclear cells (SMNC) from Lewis rats (2×10^6/ml) cultured with 5 μg of concanavalin A (ConA) (Pharmacia, Uppsala, Sweden) per ml for 30 h. The collected supernatant was supplemented with 100 mM methyl-α-D-mannopyranoside (Roth, Karlsruhe, Germany), filtered until sterile, and stored in aliquots at −20°C.

Purification of splenic T cells and generation of T-cell lines and clones. After various time intervals postinfection (p.i.), mice were killed by carbon dioxide asphyxiation. Spleens were aseptically removed, and single-cell suspensions were prepared. Erythrocytes were lysed by a short incubation in 0.2 M NH_4Cl. Cells were purified by Ficoll (Biochrom) density gradient centrifugation, washed, and resuspended in warm (37°C) medium, and T cells were enriched by passage through a nylon wool column (39) equilibrated with warm medium. The eluted nonadherent cell population contained 85 to 95% Thy1.2-positive cells as determined by fluorescence-activated cell sorter (FACS) analysis (see below). Purified splenic T cells (10^7) were cultured in the presence of 2×10^7 irradiated (3,000 rads) syngeneic nonimmune SMNC as antigen-presenting cells (APC), 10 μg of heat-killed *Y. enterocolitica* per ml, and 10% IL-2-containing ConA supernatant in 25-cm^2 flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere of 5% CO_2. Once a week irradiated feeder cells and IL-2-containing supernatant and every second week antigen were added to the cultures. After 7 weeks of culture, *Y. enterocolitica*-specific T cells were purified by Ficoll density gradient centrifugation and cloned by limiting dilution in round-bottom microculture plates containing 10^6 irradiated (3,000 rads) SMNC, 100 μg of heat-killed *Y. enterocolitica* per ml, and 10% IL-2-containing ConA supernatant in a total volume of 0.2 ml per well. Seven days later, 25 U of recombinant mouse IL-2 (Boehringer GmbH, Mannheim, Germany) was added to each well, and after another 7 days, wells containing proliferating cells could be detected. Wells exhibiting clonal growth were expanded into 24-well macroculture plates (Costar, Cambridge, Mass.) in the presence of 10^6 irradiated SMNC, 10 μg of heat-killed *Y. enterocolitica* per ml, and 10 U of recombinant IL-2 per ml. T cells cocultured with feeder cells and restimulated with antigen every 12 to 15 days were maintained in culture for 5 months. For control experiments, we used the T-cell clone 12.B.9, specific for ovalbumin. This clone was generated and cultured under the same conditions as described by Reske-Kunz and Rüde (64) and was restimulated every 14 to 21 days with 50 μg of ovalbumin (Sigma, Munich, Germany) per ml.

Proliferation assay. Purified T cells (5×10^4 per well) were cultured with 2×10^5 irradiated (3,000 rads) SMNC as APC and 10 μg of heat-killed *Y. enterocolitica*, *S. typhimurium*, or *L. monocytogenes* per ml in 0.2 ml of medium in round-bottom microculture plates (Nunc). For one week before and during this procedure, no exogenous IL-2 was added to the cultures. After 3 days, cultures were pulsed with 0.5 μCi (= 18.5 kBq per well) of [^{3}H]thymidine (Dupont, NEN Research Products, Homburg, Germany) per well for 16 h.

Samples were collected with a 96-well cell harvester (Pharmacia LKB, Turku, Finland), and [^{3}H]thymidine uptake was measured in a liquid scintillation counter (Betaplate; Pharmacia LKB).

IL-2 assay. Cells were cultured under conditions described above, and after 24 h, supernatants of single wells were collected and assayed for IL-2 activity. Supernatant (0.1 ml) was added to 50 μl of medium containing 1.5×10^4 cells of a cytotoxic T lymphocyte line (CTLL; American Type Culture Collection; ATCC TIB 209) (17) in round-bottom microculture plates (5% CO_2, 37°C). Twenty-four hours later, CTLL were pulsed with 0.5 μCi of [^{3}H]thymidine for 8 h, harvested, and measured as described above. Serial dilutions of recombinant mouse IL-2 served as a positive control and reference values to estimate the actual IL-2 production of the T-cell clones in units per milliliter (standard curve, see Fig. 3).

IFN-γ ELISA. Supernatants of T cells cultured as described above were harvested after 24 h. For determination of the IFN-γ content, we used a capture enzyme-linked immunosorbent assay (ELISA) according to the method described by Slade and Langhorne (67). ELISA microtiter plates (96 well; Greiner, Frickenhausen, Germany) were coated with anti-mouse IFN-γ antibody AN-18.17.24 (hybridoma cell line kindly provided by J. Langhorne, Max-Planck-Institut für Immunbiologie, Freiburg, Germany). Nonspecific binding sites were blocked with PBS containing 1% bovine serum albumin (Sigma) and 0.1% NaN_3. After being washed three times with PBS–0.05% Tween 20 (Merck), serially diluted duplicates from pooled triplicate supernatants were incubated overnight. After the contents were flicked out and three wash steps were done, a biotin-labeled anti-mouse IFN-γ antibody, R4-6A2 (hybridoma from American Type Culture Collection; ATCC HB 170), was added to the wells. After incubation and three wash steps, an avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP Kit; Camon, Wiesbaden, Germany) was added and incubated for 30 min. The signal was developed after final wash steps with *p*-nitrophenyl phosphate disodium as the substrate (Sigma) in diethanolamine buffer. Optical density (OD) was measured at 405 and 490 nm with an ELISA reader (Dynatech, Stuttgart, Germany). Negative controls were performed without primary or secondary antibody. Blank values (controls with cell culture medium instead of T-cell supernatants) were subtracted from all other ODs obtained. Both anti-IFN-γ antibodies were purified from hybridoma supernatants by using protein G-Sepharose 4 Fast Flow (Pharmacia LKB) and fast protein liquid chromatography (FPLC; Pharmacia LKB). R4-6A2 was coupled to NHS-biotin (Sigma) as described by Goding (18). The standard curve (see Fig. 4) resulted from serial dilutions of recombinant murine IFN-γ (kindly provided by G. Adolf, Bender, Vienna, Austria). Units of IFN-γ from T-cell supernatants were finally calculated from the straight-line portion of the standard curve.

Immunofluorescence analysis. Purified T cells were washed twice in PBS containing 2% fetal calf serum and stained for phenotypic characterization with the following antibodies: anti-Thy1.2 (30-H12), anti-L3T4 (GK1.5), and anti-Lyt2 (53-6.7) (all purchased from Becton Dickinson, Heidelberg, Germany). All these antibodies were used as conjugates with fluorescein isothiocyanate or phycoerythrin. Fluorescein isothiocyanate-conjugated anti-IL-2 receptor (Tac antigen, p55 subunit) antibody (7D4) was a kind gift from A. Schimpl (Institut für Immunbiologie, Würzburg, Germany). Anti-γδ T-cell-receptor antibody (UC7 13D5) (hybridoma was provided by J. A. Bluestone, University of Chicago), purified from cell culture supernatants by using protein G-Sepharose 4 Fast Flow and FPLC, was coupled with a biotin-conjugated goat anti-hamster antibody (Medac, Hamburg, Germany) and streptavidin-phycoerythrin (Becton Dickinson). Labeling procedures were performed at 4°C for 30 min, and after three final wash steps, cells were analyzed by cytofluorometry with a FACScan (Becton Dickinson).

Determination of protection by in vivo transfer of T cells. Fourteen days after antigen stimulation, T-cell lines and clones were purified by Ficoll density gradient centrifugation, washed, and resuspended in PBS containing 0.5% mouse serum. Cell viability was determined by trypan blue exclusion. A total of 5×10^6 to 10×10^6 cells per mouse was transferred into nonimmune mice by injection into the lateral tail vein. One day later, mice were challenged with a lethal dose (5×10^5 [five times the LD_{50}]) of viable *Y. enterocolitica* by intravenous injection. To determine the extent of protection, spleens of infected animals were removed, homogenized, and plated out as described above. Protection (log protection) was assessed by subtracting the mean $\log_{10}$ CFU per spleen of the test groups from the mean $\log_{10}$ CFU per spleen of the control group. To control specificity of transferred T cells and to exclude bystander effects, we transferred T-cell clone 12.B.9, specific for ovalbumin, or challenged mice with *S. typhimurium* (S2943) in control experiments.

RESULTS

Establishment and phenotypic characterization of T-cell clones. T cells from spleens of C57BL/6 mice infected with 10^4 (0.1 LD_{50}) *Y. enterocolitica* (strain WA-P) were isolated between days 7 and 24 p.i. Bulk cultures were set up, containing 1×10^7 purified T cells, 2×10^6 irradiated nonimmune syngenic SMNC as APC, 10 μg of heat-killed *Y. enterocolitica* as the antigen, and 10% IL-2-containing ConA supernatant per 10 ml of culture medium. Three T-cell lines from spleens removed on days 14, 20, and 24 p.i. could be obtained by regular feeding, by antigen stimulation, and by adding IL-2-containing ConA supernatant or recombinant murine IL-2 to the culture medium (see Materials and Methods). Attempts to establish *Y. enterocolitica*-specific T-cell lines from spleens at day 7 or 10 p.i. failed. The most stable cell line (line 14.6, established on day 20 p.i.) contained 95% L3T4$^+$ and 5% Lyt2$^+$ T cells as determined by FACS analysis. T cells positive for the γδ T-cell receptor could not be detected. Limiting dilution cloning of this T-cell line revealed 17 T-cell clones, of which 13 showed the Thy1.2$^+$ L3T4$^+$ phenotype (helper T cells) and 4 showed the Thy1.2$^+$ Lyt2$^+$ phenotype (cytotoxic T cells). All T-cell clones obtained were negative for the γδ T-cell receptor and positive for the IL-2 receptor (Tac antigen, p55 subunit). Figure 1 shows two representative stainings of both L3T4$^+$ (Fig. 1A) and Lyt2$^+$ (Fig. 1B) T-cell clones analyzed by FACScan. The T-cell clone 12.B.9 was generated from lymph nodes of an ovalbumin-immunized mouse by using conditions similar to those described previously (64). The phenotype of this clone was Thy1.2$^+$ L3T4$^+$ IL-2 receptor positive. For subsequent experiments ([^{3}H]thymidine uptake, IL-2 assay, IFN-γ ELISA) presented below, 10 randomly chosen clones were characterized. For T-lymphocyte transfer experiments, six representative clones were chosen.

Specificity of T-cell clones. At 12 to 14 days after antigen restimulation, T-cell clones were purified from dead feeder cells by Ficoll density gradient centrifugation and incubated

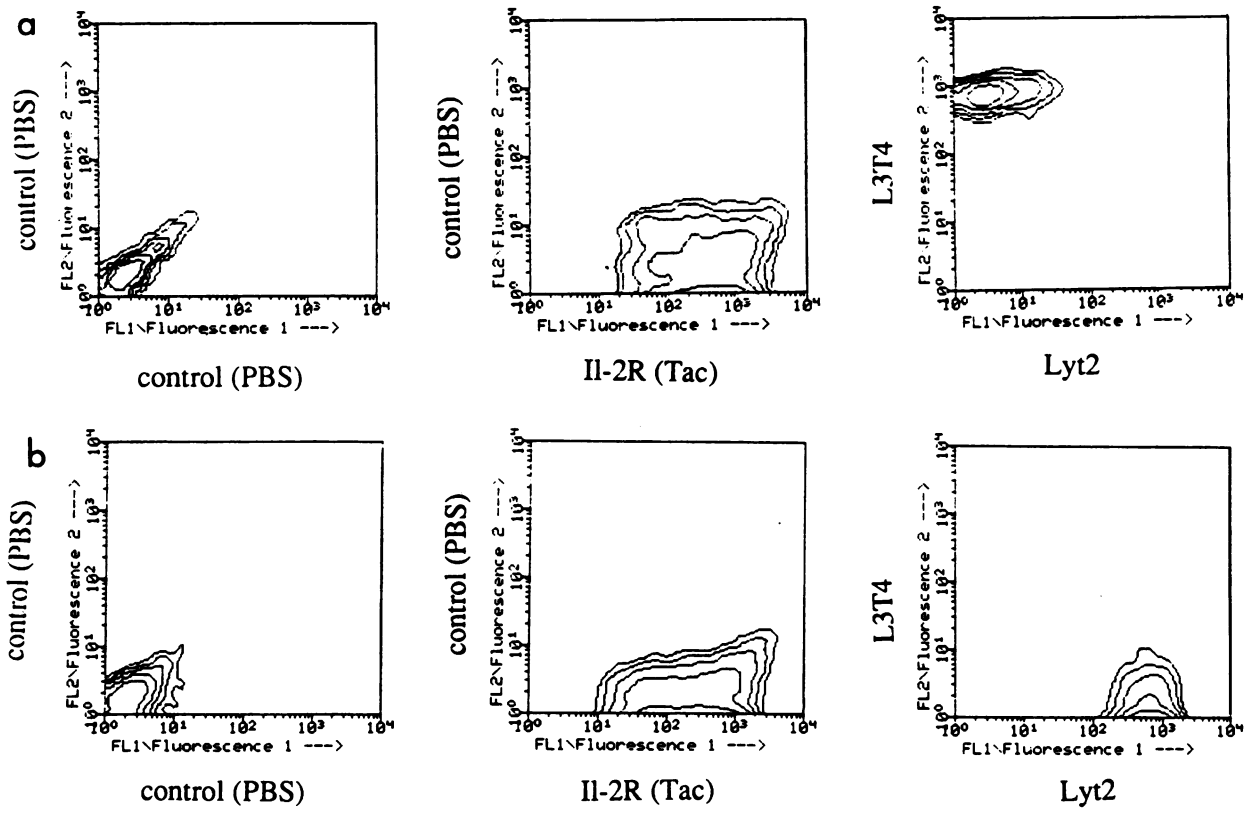

FIG. 1. Fluorocytometric analysis of two representative T-cell clones specific for *Y. enterocolitica*. (a) Clone 14.6.4F7. (b) Clone 14.6.1C1. Fluorescence 1 (*x* axis), green. Fluorescence 2 (*y* axis), red. Panel 1, control staining (PBS without antibody). Panel 2, anti-IL-2 receptor (IL-2R) (green staining in fluorescence 1, control staining [PBS] in fluorescence 2). Panel 3, simultaneous staining of L3T4 (red, fluorescence 2) and Lyt-2 (green, fluorescence 1).

with irradiated SMNC as APC and various amounts of antigen without the addition of exogenous IL-2 for determination of the proliferative response to heat-killed *Y. enterocolitica*. Proliferation of T-cell clones depended on the presence of APC and antigen. Antigen concentrations of 10 μg of heat-killed *Y. enterocolitica* per ml of culture medium induced a significant proliferative response (Fig. 2). Therefore, this concentration was used in all subsequent experiments. The comparison of the proliferative responses of various T-cell clones to heat-killed *Y. enterocolitica* revealed distinct differences (Table 1). Lyt2$^+$ and some L3T4$^+$ T-cell

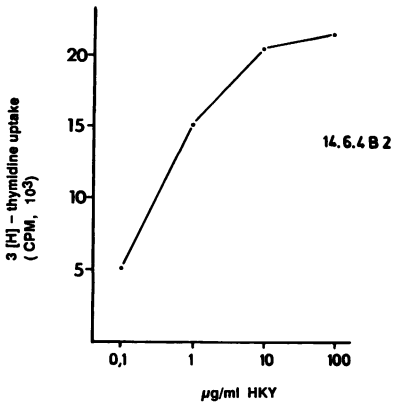

FIG. 2. Proliferative response of T-cell clone 14.6.4B2 to various concentrations of heat-killed *Y. enterocolitica* (HKY) in the presence of irradiated APC was determined on day 3 of incubation by incorporation of [^{3}H]thymidine. Values represent the means of triplicate cultures.

TABLE 1. Proliferative response of T-cell clones to different bacterial antigens[a]

Clone	Phenotype	[^{3}H]thymidine uptake (10^3 cpm) (S.I.) with the following bacterial antigen[b]:			
		HKY	HKL	HKS	None
14.6.1F12	Lyt2$^+$	29.374 (30.9)	1.062 (1.1)	2.755 (2.9)	0.950
14.6.1C1	Lyt2$^+$	4.991 (18.3)	1.442 (5.3)	1.268 (4.7)	0.272
14.6.4G6	L3T4$^+$	55.518 (5.8)	11.116 (1.2)	14.080 (1.9)	9.563
14.6.4B2	L3T4$^+$	31.202 (70.4)	0.730 (1.6)	5.469 (12.3)	0.443
14.6.1D1	L3T4$^+$	68.670 (536)	0.347 (2.7)	8.654 (67.6)	0.128
14.6.1B8	L3T4$^+$	42.114 (81.3)	0.445 (<1)	3.542 (6.8)	0.518
14.6.4F7	L3T4$^+$	32.155 (109)	0.468 (1.6)	2.136 (7.2)	0.295
14.6.4A8	L3T4$^+$	36.042 (55.1)	0.515 (<1)	1.614 (2.5)	0.654
14.6.3E9	L3T4$^+$	68.871 (138)	1.194 (2.4)	4.608 (9.3)	0.497
14.6.4G10	L3T4$^+$	21.520 (133)	0.384 (2.4)	1.247 (7.7)	0.162

[a] T-cell clones (5×10^4 per well) were cultured with 10 μg of heat-killed *Y. enterocolitica* (HKY), *L. monocytogenes* (HKL), or *S. typhimurium* (HKS) or without bacterial antigen in the presence of 2×10^5 irradiated syngenic SMNC as APC. Cultures were pulsed with [^{3}H]thymidine after 3 days of incubation (see Materials and Methods).

[b] Values are the means of triplicate cultures. Standard deviations were less than 30%. (S.I. = mean of antigenic proliferation/mean of nonantigenic spontaneous proliferation.)

TABLE 2. Proliferative response and IL-2 and IFN-γ production of ovalbumin-specific T-cell clone 12.B.9[a]

Antigen[b]	[3H]thymidine uptake[c] (10^3 cpm) (S.I.)		IFN-γ production (U/ml) (S.I.)[e]
	Clone 12.B.9	CTLL[d]	
Ovalbumin	20.212 (173)	93.717 (281)	285 (285)
HKY	0.349 (2.9)	0.179 (<1)	6.3 (6.3)
HKL	0.166 (1.4)	0.141 (<1)	0 (0)
HKS	0.265 (2.3)	0.131 (<1)	10.5 (10.5)
None	0.117	0.334	1

[a] T-cell clone 12.B.9 was cultured and the assays were performed as described in Tables 1, 3, and 4 and in Materials and Methods.
[b] HKY, heat-killed *Y. enterocolitica*; HKL, heat-killed *L. monocytogenes*; HKS, heat-killed *S. typhimurium*.
[c] Values are the means of triplicate cultures.
[d] IL-2 production of T cells was assayed by [3H]thymidine uptake of CTLL as described in Table 3. Values are the means of triplicate cultures.
[e] OD values of the IFN-γ ELISA are expressed as units of IFN-γ per milliliter according to the standard curve (Fig. 4). Values are the means of duplicates.

TABLE 3. IL-2 production of different T-cell clones after stimulation with various bacterial antigens[a]

Clone	Phenotype	[3H]thymidine uptake (10^3 cpm) of CTLL with the following bacterial antigen[b]:			
		HKY	HKL	HKS	None
14.6.1F12	Lyt2+	0.040 (<1)	0.059 (1.3)	0.072 (1.6)	0.045
14.6.1C1	Lyt2+	0.844 (2.8)	0.358 (1.2)	0.384 (1.3)	0.301
14.6.4G6	L3T4+	9.518 (5.2)	1.491 (<1)	1.611 (<1)	1.838
14.6.4B2	L3T4+	1.227 (5.7)	0.290 (1.3)	0.846 (3.9)	0.215
14.6.1D1	L3T4+	4.358 (24)	0.282 (1.6)	0.556 (3.1)	0.178
14.6.1B8	L3T4+	1.831 (2.2)	0.489 (<1)	0.187 (<1)	0.826
14.6.4F7	L3T4+	3.503 (6.1)	1.139 (2.0)	0.871 (1.5)	0.577
14.6.4A8	L3T4+	5.708 (5.2)	1.187 (1.1)	1.973 (1.8)	1.091
14.6.3E9	L3T4+	1.809 (9.3)	0.154 (<1)	0.166 (<1)	0.194
14.6.4G10	L3T4+	7.721 (7.5)	0.812 (<1)	1.359 (1.3)	1.023

[a] IL-2 production of antigen-stimulated T-cell clones was assayed by culturing 1.5×10^4 IL-2-dependent CTLL with 0.1 ml of T-cell clone culture supernatants for 24 h. Proliferation of CTLL was determined by [3H]thymidine incorporation (see Materials and Methods). Supernatant containing serial dilutions of recombinant mouse IL-2 served as reference values and a positive control.
[b] Values are the means of triplicate cultures. Standard deviations were less than 30%. HKY, heat-killed *Y. enterocolitica*; HKL, heat-killed *L. monocytogenes*; HKS, heat-killed *S. typhimurium*.

clones showed only a weak or moderate proliferative response to heat-killed *Y. enterocolitica*, unless IL-2 was added to the cultures. Addition of exogenous IL-2 (5 U/ml) improved proliferation of T-cell clones but also increased the nonspecific proliferative response of T-cell clones to other antigens tested (data not shown). This effect was most distinct in Lyt2+ clones. To determine the specificity of the proliferative response to antigen, we incubated clones in parallel with heat-killed *Y. enterocolitica*, *L. monocytogenes*, and *S. typhimurium* or without antigen (Table 1). As is evident from Table 1, most T-cell clones showed a specific proliferation upon exposure to heat-killed *Y. enterocolitica*. However, in comparison with heat-killed *Y. enterocolitica*, some cross-reactivity to heat-killed *S. typhimurium* and *L. monocytogenes* could be observed with several clones. Stimulation with heat-killed *S. typhimurium* (gram-negative bacteria) elicited a higher response than did stimulation with heat-killed *L. monocytogenes* (gram-positive bacteria). This pattern of reactivity is best assessed by comparing the stimulation indices (S.I.) for the different bacterial antigens. S.I. were much higher for heat-killed *Y. enterocolitica* (most clones, 30 to 140) than for heat-killed *S. typhimurium* (most clones, <10) or heat-killed *L. monocytogenes* (most clones, <3). T-cell clone 12.B.9 was specific for ovalbumin (S.I. = 173), and stimulation with heat-killed *Y. enterocolitica*, *L. monocytogenes*, or *S. typhimurium* did not induce a significant proliferation (S.I. = 1.4 to 2.9) (Table 2).

IL-2 production by T-cell clones. To determine IL-2 production, we stimulated T-cell clones with various antigens in the presence of irradiated APC without the addition of exogenous IL-2 as described above. After 24 h of incubation, 0.1 ml of supernatant per well was collected and analyzed for its IL-2 content with an IL-2-dependent cell line (CTLL). Proliferation of CTLL was measured by [3H]thymidine uptake (Table 3; for a standard curve, see Fig. 3). As shown in Table 3, most T-cell clones indeed produced IL-2 when stimulated with the appropriate antigen (heat-killed *Y. enterocolitica*), although only very small quantities were produced. No significant IL-2 production could be detected when T cells were stimulated with heat-killed *L. monocytogenes* or *S. typhimurium* or when APC and antigen only without T cells were incubated. The maximum amount of IL-2 production was measured on day 1 after stimulation. From day 2 on, the detectable amount of IL-2 decreased rapidly (data not shown). Lyt2+ clones showed no or extremely low IL-2 production. But even supernatants of L3T4+ clones also contained only small IL-2 quantities, at best, 1 U/ml of culture medium. Independently of the amount of IL-2 production, all T-cell clones expressed the IL-2 receptor as revealed by FACS analysis (Fig. 1). S.I. of IL-2 production for the different bacterial antigens did not show as specific a difference as did S.I. of the proliferative response. The ovalbumin-specific T-cell clone 12.B.9 produced significant amounts of IL-2 when stimulated with ovalbumin (approximately 12 U/ml). But when stimulated with heat-killed *Y. enterocolitica*, *L. monocytogenes*, or *S. typhimurium*, there was no detectable IL-2 production in supernatants of the ovalbumin-specific T-cell clone 12.B.9 (Table 2).

IFN-γ production by T-cell clones. All T-cell clones were cultured and stimulated as described above. Supernatants of triplicate wells were harvested and pooled. IFN-γ content was measured by a capture ELISA with the monoclonal antibodies R4-6A2 and AN-18.17.24 and an avidin-biotin-alkaline phosphatase complex. According to the values (OD)

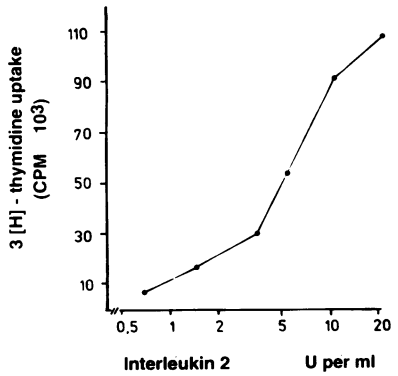

FIG. 3. Titration curve for recombinant IL-2 measured by [3H]thymidine uptake of CTLL (1.5×10^4 per well). Values represent the means of triplicate cultures.

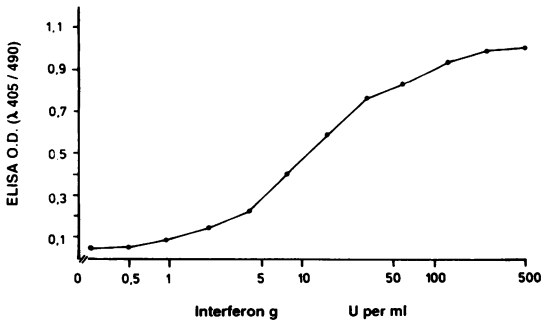

FIG. 4. Titration curve for recombinant IFN-γ measured by ELISA as described in Materials and Methods. Values represent the means of triplicate determinations. Units of IFN-γ in T-cell supernatants were calculated only from the straight-line portion of the standard curve.

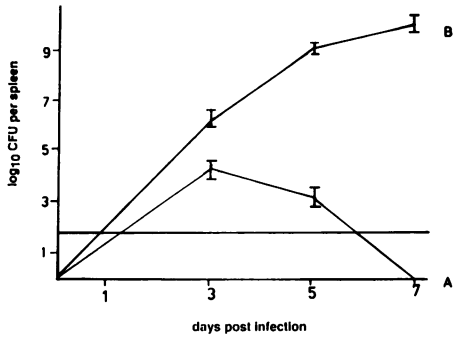

FIG. 5. Transfer of 10^7 T cells (T-cell line 14.6) per mouse (A) 1 day prior to challenge (day 0) with 5×10^5 *Y. enterocolitica* (strain WA-P). (B) Control group (mice received PBS 1 day prior to infection instead of T cells). Values represent the means of three animals. The horizontal line indicates the limit of detectable CFU (limit, 50 CFU; $\log_{10} 50 = 1.7$).

of the standard curve obtained with recombinant IFN-γ (Fig. 4), we expressed the OD values of the T-cell supernatants in units of IFN-γ per milliliter. As shown in Table 4, both L3T4$^+$ and Lyt2$^+$ T-cell clones produced considerable amounts of IFN-γ, some of them even more than 500 U/ml. Production of IFN-γ was maximal at days 1 and 2 after stimulation (data not shown). The S.I. of the production of IFN-γ following incubation of T-cell clones with different bacterial antigens were, like IL-2 production, not as specific for heat-killed *Y. enterocolitica* as were the S.I. of the proliferative response ([^{3}H]thymidine uptake) to heat-killed *Y. enterocolitica*. In contrast, supernatants from cultures of APC plus bacterial antigens or APC plus bacterial antigens and ovalbumin-specific T-cell clone 12.B.9 (Table 2) did not contain detectable amounts of IFN-γ in the former case and did contain insignificant amounts (6 to 10 U/ml) in the latter case.

Protection of nonimmune mice by transfer of T cells. The ability of T-cell lines and clones to transfer immunity to *Y. enterocolitica* in nonimmune mice was tested by injecting 5×10^6 to 10×10^6 T cells specific for *Y. enterocolitica* 1 day prior to intravenous challenge with a lethal dose (5×10^5 [five times the LD_{50}]) of *Y. enterocolitica*. As shown in Fig. 5, the transfer of T-cell line 14.6 led to effective protection against *Y. enterocolitica* (log protection, >7). In contrast to control mice, which showed severe disease with massive abscess formation in the spleen and liver, mice receiving immune T cells prior to infection rapidly eliminated *Y. enterocolitica* from tissues and histologically exhibited only insignificant lesions after 3, 6, and 9 days p.i. (data not shown). Some clones derived from this T-cell line were also tested for their protective capacity (Table 5). Interestingly, transfer of a single clone was not as protective as the T-cell line 14.6. Finally, some of the T-cell clones showed no protective ability at all. To exclude a nonspecific bystander effect in our in vivo experiments, we transferred the ovalbumin-specific T-cell clone 12.B.9 in another experiment 1 day prior to infection. Data presented in Table 5 show that these ovalbumin-specific T cells had only insignificant protective ability against a challenge with *Y. enterocolitica*. In a second control experiment, we transferred T cells specific for *Y. enterocolitica* (a mixture of three T-cell clones) 1 day prior to a challenge with either *Y. enterocolitica* or *S. typhimurium*. As shown in Table 6, the transferred *Y. enterocolitica*-specific T cells mediated specific protection against a challenge with *Y. enterocolitica* (log protection =

TABLE 4. IFN-γ production by different T-cell clones after stimulation with various bacterial antigens[a]

Clone	Phenotype	IFN-γ production (U/ml) (S.I.) with the following bacterial antigen[b]:			
		HKY	HKL	HKS	None
14.6.1F12	Lyt2$^+$	150 (<1)	242 (1.5)	177 (1.1)	163
14.6.1C1	Lyt2$^+$	565 (15.3)	53 (1.4)	168 (4.5)	37
14.6.4G6	L3T4$^+$	502 (4.2)	150 (1.3)	225 (1.9)	120
14.6.1D1	L3T4$^+$	418 (2.5)	460 (2.7)	240 (1.4)	170
14.6.1B8	L3T4$^+$	386 (4.0)	83 (<1)	160 (1.7)	96
14.6.4F7	L3T4$^+$	134 (3.5)	22 (<1)	51 (1.3)	38
14.6.3E9	L3T4$^+$	78 (2.0)	26 (<1)	40 (1.0)	39
14.6.4H6	L3T4$^+$	290 (16.1)	30 (1.7)	80 (4.4)	18
14.6.4C2	L3T4$^+$	170 (9.4)	19 (1.1)	65 (3.6)	18

[a] T-cell clones were cultured as described in Table 2 (see also Materials and Methods). Supernatants of triplicate wells were harvested and pooled after 24 h of incubation.

[b] Values are means of duplicates of appropriate serial dilutions of supernatants. The values (OD) revealed in the IFN-γ ELISA are expressed as units of IFN-γ per milliliter according to the linear portion of the standard curve (Fig. 4) with recombinant murine IFN-γ. HKY, heat-killed *Y. enterocolitica*; HKL, heat-killed *L. monocytogenes*; HKS, heat-killed *S. typhimurium*.

TABLE 5. Protection against *Y. enterocolitica* by transfer of T-cell clones[a]

Clone	Phenotype	Log protection
14.6.1F2	Lyt2$^+$	2.47
14.6.1B8	L3T4$^+$	3.06
14.6.4F7	L3T4$^+$	4.26
14.6.4A8	L3T4$^+$	0.00
14.6.3E9	L3T4$^+$	2.95
14.6.4G10	L3T4$^+$	4.52
12.B.9	L3T4$^+$	0.40[b]

[a] T-cell clones (5×10^6 to 10×10^6 cells per mouse) were transferred 1 day prior to a challenge of a lethal dose (5×10^5) of living *Y. enterocolitica* (strain WA-P). Number of CFU per spleen was determined at day 5 p.i. (see Materials and Methods). Control mice for determination of log protection received PBS instead of T cells prior to infection. Values of log protection are the means of two to three animals.

[b] As a control for specificity of protection, the ovalbumin-specific T-cell clone 12.B.9 was transferred 1 day prior to the challenge with *Y. enterocolitica* as described above. Log protection was determined on day 6 p.i. The indicated value represents the mean of three animals.

TABLE 6. Specificity of protection by transfer of *Y. enterocolitica*-specific T-cell clones[a]

Pretreatment	Challenge	Log_{10} CFU/spleen[b]	Log protection
PBS	*S. typhimurium*	7.9 ± 0.34	
T-cell transfer	*S. typhimurium*	8.2 ± 0.69	0
PBS	*Y. enterocolitica*	8.97 ± 0.42	
T-cell transfer	*Y. enterocolitica*	5.47 ± 0.70[c]	3.5

[a] Three T-cell clones (clones 14.6.4G10, 14.6.1D1, 14.6.1B8) were pooled, and 10×10^6 T cells per mouse were transferred 1 day prior to challenge with a lethal dose of *Y. enterocolitica* (5×10^5) or *S. typhimurium* S2943 (5×10^4). Control groups received PBS instead of T cells.

[b] Number of CFU per spleen was determined at day 5 p.i. Values represent the means of three animals per group (± standard deviation). Log protection was determined as described in Materials and Methods.

[c] Experimental value differs from the respective control group (PBS instead of T cells) at $P < 0.01$ by the Student t test.

3.5) but not against a challenge with *S. typhimurium* (log protection = 0).

DISCUSSION

The primary aim of our study was to investigate whether T cells are involved in the immune response against enteropathogenic *Y. enterocolitica*. This question is particularly attractive because this pathogen is believed to multiply predominantly extracellularly in host tissues (27, 28, 66, 70), in contrast to *L. monocytogenes* or *Mycobacterium tuberculosis*, which have been proven to be facultative intracellular bacteria. Moreover, there is evidence that T lymphocytes may be involved in immunopathological host responses to *Y. enterocolitica*, causing sequelae such as reactive arthritis (36).

In our approach to characterize the T-cell response to *Y. enterocolitica*, we used the C57BL/6 mouse model and *Y. enterocolitica* of serotype O:8, strain WA-P, as the infectious agent for the following reasons. (i) Serotype O:8 is the most virulent strain of *Y. enterocolitica*. Since we have constructed several chromosomal mutants (concerning the iron uptake system) (29) and plasmid mutants (concerning Yops and YadA [32]) abrogating mouse lethality, we can use these mutants in subsequent studies. (ii) Experiments with normal C57BL/6 and athymic T-cell-deficient C57BL/6 nude mice (25) provided evidence for the assumption that a thymus-dependent immune response is critical in resistance to *Y. enterocolitica*. Since C57BL/6 mice are relatively resistant to *Y. enterocolitica* compared with other mouse strains (e.g., BALB/c) (25, 26), we started the analysis of the T-cell response to *Y. enterocolitica* in C57BL/6 mice. (iii) Pathogenesis and histomorphology of infection with *Y. enterocolitica* in mice closely resemble those in humans (9). Thus, this model might provide evidence for a better understanding of the pathogenesis of *Y. enterocolitica* infection and its associated lesions in humans.

In the present report, we described the generation of *Y. enterocolitica*-specific T-cell lines and clones and their phenotypic and functional characterization. For this purpose, splenic T cells of mice infected with 10^4 *Y. enterocolitica* organisms were isolated, expanded in vitro by stimulation with heat-killed *Y. enterocolitica* in the presence of irradiated SMNC as APC and IL-2-containing ConA supernatant or recombinant murine IL-2, and cloned by limiting dilution. The T-cell clones obtained showed the phenotype of helper T cells ($L3T4^+$) or cytotoxic T cells ($Lyt2^+$). All clones were positive for the IL-2 receptor (Tac antigen, p55 subunit) and negative for the γδ T-cell receptor. The induction of proliferative responses to antigen depended on the presence of APC and was specific for heat-killed *Y. enterocolitica*, although some cross-reaction to heat-killed *S. typhimurium* and, to a much lesser extent, to heat-killed *L. monocytogenes* could be detected, particularly when exogenous IL-2 was added to the cultures. The IL-2-driven nonspecific proliferation of $Lyt2^+$ T-cell clones in particular is a well-known phenomenon in in vitro culture of T-cell clones. $Lyt2^+$ clones did not produce significant amounts of IL-2 when stimulated with antigen and therefore needed exogenous IL-2 as a growth factor in vitro. $L3T4^+$ clones produced various amounts of IL-2 after antigenic stimulation, but even in supernatants of the clone producing the most IL-2, we could not detect more than 1 U of IL-2 per ml of culture medium. In contrast to IL-2 production, most $L3T4^+$, as well as $Lyt2^+$, T-cell clones produced large quantities of IFN-γ, some of them even more than 500 U/ml. This pattern of cytokine production, that is, low IL-2 but high IFN-γ production, has also been found in a study characterizing rat T lymphocytes specific for *Bacteroides gingivalis* (41). According to the subtypes of murine helper T lymphocytes described by Mosmann et al. (54), the $L3T4^+$ T-cell clones obtained in this study are probably all T_H1 cells. Whether this reflects the predominant induction of this T-cell subtype during infection with *Y. enterocolitica* or whether this is due to an in vitro artifact resulting from the culture conditions used (e.g., stimulating antigen, cytokines in the ConA supernatant) remains to be investigated. However, from other infection models with C57BL/6 mice, e.g., experimental leishmaniasis (46, 53, 55, 68), the first assumption may be the more probable one.

To determine the specificity of the T-cell clones described here, we used different bacterial antigens (heat-killed *Y. enterocolitica*, *L. monocytogenes*, and *S. typhimurium*) for in vitro stimulation in our experiments. As is evident from Table 1, most T-cell clones showed a marked specificity for stimulation with heat-killed *Y. enterocolitica*. The S.I. for most T-cell clones was 30 to 140 for heat-killed *Y. enterocolitica*, <10 for heat-killed *S. typhimurium*, and <3 for heat-killed *L. monocytogenes*. In contrast, the S.I. for IL-2 and IFN-γ production (Tables 3 and 4) was not as specific for heat-killed *Y. enterocolitica* when compared with the S.I. for the proliferative response. Since the ovalbumin-specific control T-cell clone 12.B.9 did not produce comparable quantities of IL-2 or IFN-γ upon stimulation with bacterial antigens (Table 2) nor did irradiated APC incubated with bacterial antigens alone produce detectable amounts of IL-2 and IFN-γ, a mitogenic or a polyclonal T-cell-stimulating effect in vitro can be excluded. Thus, this discrepancy remains unclear.

When transferred into nonimmune mice 1 day prior to the challenge, T-cell line 14.6, as well as some T-cell clones, could mediate protection against a challenge with a lethal dose of *Y. enterocolitica*. In our opinion, this is the most striking evidence that *Y. enterocolitica*-specific T cells may also play an important role in the protective immune response to *Y. enterocolitica*. However, T cells transferred to nonimmune mice 1 or 2 days after challenge with a lethal dose of *Y. enterocolitica* were no longer able to mediate protection, probably because in this phase of infection tissue lesions have already reached critical dimensions with overwhelming bacterial growth. Thus, further experiments are required, using sublethal doses for monitoring the bacterial proliferation kinetics in infected organs. Data shown in

Tables 5 and 6 indicate that the protection mediated by *Y. enterocolitica*-specific T cells was specific for infection with *Y. enterocolitica*, since ovalbumin-specific T cells did not mediate protection against a challenge with *Y. enterocolitica* nor did *Y. enterocolitica*-specific T-cells mediate protection against a challenge with *S. typhimurium*.

The large quantities of IFN-γ production by some T-cell clones suggest that IFN-γ is an important mediator of the host response against *Y. enterocolitica*. IFN-γ is known to be a potent activator of resident macrophages, leading to expression of major histocompatibility complex class II molecules, downregulation or loss of transferrin receptors, and increase of bactericidal properties (1). Thus, the IFN-γ-producing T-cell clones described here might be able to activate macrophages, which in turn might be able to control growth of *Y. enterocolitica*. In our experiments, however, there was no correlation between protective capacity of T-cell clones in vivo and IFN-γ production in vitro. Thus, from these results, it is not yet clear which effector function T cells do have in vivo during infection with *Y. enterocolitica* in mice. In contrast to T-cell line 14.6, the protective capacity of most T-cell clones derived from this T-cell line was relatively low. This might be due to an in vitro selection of T-cell clones recognizing antigens which are irrelevant in vivo because of an inappropriate antigen used in vitro (bacteria grown at 26°C). On the other hand, this could be due to a synergistic effect of different T-cell subtypes of the T-cell line 14.6, indicating that different T-cell subsets and thus different T-cell-mediated functions are required during infection with *Y. enterocolitica*.

Current models of immunity to bacterial infections (23) propose the separation of bacteria into two categories: (i) facultative intracellular bacteria, represented by, e.g., *M. tuberculosis*, *L. monocytogenes*, and *Salmonella typhi*, which are eliminated by a specific acquired cellular immune response (T lymphocytes in cooperation with macrophages); (ii) extracellular bacteria, e.g., all cocci and most members of the family *Enterobacteriaceae*, which are primarily eliminated by nonspecific cellular and/or humoral mechanisms. However, in contrast to this scheme, it has been shown that specific T lymphocytes may also be important in immunity to extracellular bacteria such as *Bacteroides fragilis* and *B. gingivalis* (13, 41), *P. aeruginosa* (47, 62), and *S. typhimurium*, although the role of T lymphocytes in immunity to *S. typhimurium* still is unclear and is controversially discussed (37, 42, 56, 57, 60). The data described in this report indicate that *Y. enterocolitica* might be a further candidate demonstrating that specific T lymphocytes play an important role during infection with and may be required for the elimination of extracellularly located bacteria. Considering the host response to *Y. enterocolitica* and to the other bacterial species mentioned above, the model of immunity to bacterial infections described previously (23) will probably have to be revised with a more differentiated separation of categories of bacteria.

This study was our first approach to characterize the cellular immune response to *Y. enterocolitica*. We described for the first time the generation and partial characterization of T-cell clones specific for *Y. enterocolitica* and provided evidence toward the understanding of the role of T lymphocytes during infection with *Y. enterocolitica*. Further experiments are required to elucidate (i) the role of plasmid-encoded antigens expressed at 37°C, (ii) the role of macrophages and cytokines (e.g., IFN-γ) for elimination of the invading microorganism, and (iii) the influence of the route of infection on the immune response.

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