INFECTION AND IMMUNITY, June 1994, p. 2590–2599
0019-9567/94/$04.00+0

Vol. 62, No. 6

Immune Responses to *Yersinia enterocolitica* in Susceptible BALB/c and Resistant C57BL/6 Mice: an Essential Role for Gamma Interferon

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Received 27 December 1993/Returned for modification 15 February 1994/Accepted 24 March 1994

Susceptibility of mice to infection with *Yersinia enterocolitica* has been shown to be related to neither the *Ity* locus encoding for resistance to *Salmonella typhimurium* and other pathogens nor the *H-2* locus. Recent studies in our laboratory have demonstrated that T-cell-mediated immune responses are required for overcoming primary *Yersinia* infection. In the present study, we investigated the course of infection with *Y. enterocolitica* and the resulting immune responses in *Yersinia*-susceptible BALB/c and *Yersinia*-resistant C57BL/6 mice. In the early phase of infection, the clearance of the pathogen was comparable in both strains of mice, suggesting similar mechanisms of innate resistance. Splenic T cells from *Yersinia*-infected C57BL/6 mice exhibited marked proliferative responses and produced gamma interferon (IFN-γ) upon exposure to heat-killed yersiniae. By contrast, the *Yersinia*-specific T-cell response in BALB/c mice was weak, and IFN-γ production could not be detected before day 21 postinfection. T cells isolated from C57BL/6 mice 7 days after infection mediated immunity to *Y. enterocolitica* but those from BALB/c mice did not, while at 21 days postinfection T cells from both strains mediated protection. Neutralization of IFN-γ abrogated resistance to yersiniae in C57BL/6 mice but to a far smaller extent in BALB/c mice. Administration of recombinant IFN-γ or anti-interleukin-4 antibodies rendered BALB/c mice resistant to yersiniae, whereas this treatment did not significantly affect the course of the infection in C57BL/6 mice. These results indicate that the cellular immune response, in particular the production of IFN-γ by *Yersinia*-specific T cells, is associated with resistance of mice to *Y. enterocolitica*.

Yersinia enterocolitica is a gram-negative rod-shaped bacterium which is enteropathogenic for humans and rodents (9, 10, 17). Certain mouse strains (BALB/c, C3H/HeN, BALB/b, DBA/2, Swiss, and SWR) are highly susceptible to *Y. enterocolitica* infection (intravenous [i.v.] median lethal dose [LD_{50}], 2×10^2 to 6×10^2), while others, e.g., C57BL/6 mice, are resistant (LD_{50}, 2×10^5) (13). Resistance to *Y. enterocolitica* is related to neither the *Ity* locus encoding for resistance to *Salmonella typhimurium* and other intracellular pathogens nor the *H-2* locus (14). Although a tentative linkage of resistance with the *ES-1* locus (encoding for an esterase) has been described, resistance of mice to *Y. enterocolitica* appears to be multigenic (14).

In our previous studies, we demonstrated that T-cell-dependent cellular immune responses are involved in and required for overcoming *Y. enterocolitica* infection in mice (1, 3, 4). Moreover, we have shown that the adoptive transfer of *Yersinia*-specific Th1-like $CD4^+$ T-cell lines and clones as well as $CD8^+$ clones can mediate immunity against *Y. enterocolitica* (3). Furthermore, resistance to *Y. enterocolitica* could be abrogated by in vivo neutralization of gamma interferon (IFN-γ) and/or tumor necrosis factor alpha (TNF-α) prior to or during *Yersinia* infection, suggesting that T-cell-activated macrophages are involved in the protective host response against *Y. enterocolitica* (2).

In experimental murine leishmaniasis, C57BL/6 mice are resistant to infection (healer mice) while BALB/c mice are highly susceptible to infection (nonhealer mice) (28, 30). Susceptibility to *Leishmania major* is thought to be related to the balance between T helper cell subsets (Th1 and Th2 [29]) during the infection (28, 30). $CD4^+$ T cells from infected healer mice express high levels of IFN-γ mRNA but no detectable quantities of interleukin-4 (IL-4) mRNA (19, 20), whereas $CD4^+$ T cells from nonhealer mice express high levels of IL-4 mRNA but very little IFN-γ mRNA (19, 20). Hence, IFN-γ-producing Th1 cells promote control of leishmaniae, whereas IL-4-producing Th2 cells promote disease progression.

In an attempt to analyze the relationship between T-cell responses and susceptibility to *Y. enterocolitica*, we compared the course of *Y. enterocolitica* infection and the immune responses in *Yersinia*-susceptible BALB/c and *Yersinia*-resistant C57BL/6 mice. Our results indicate that after *Yersinia* infection, T cells from C57BL/6 mice but not BALB/c mice produce significant quantities of IFN-γ. Production of IFN-γ by *Yersinia*-specific T cells correlated with their protective capacity in adoptive transfer experiments. Moreover, administration of either recombinant IFN-γ or anti-IL-4 antibodies rendered BALB/c mice resistant to infection. The data presented here suggest that the strength of *Yersinia*-specific IFN-γ-producing T cells may be related to the resistance of mice to *Y. enterocolitica*. The putative roles of Th1 and Th2 cells in yersiniosis remain to be elucidated.

MATERIALS AND METHODS

Mice. Female 6- to 8-weeks-old C57BL/6 and BALB/c mice were purchased from Charles River Wiga (Sulzfeld, Germany)

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and kept under specific-pathogen-free conditions (positive-pressure cabinet).

Infection of animals. Freshly thawed, plasmid-harboring *Y. enterocolitica* WA serotype O:8 organisms (18) suspended in 0.1 ml of sterile phosphate-buffered saline (PBS; pH 7.4) were used for i.v. infection as described previously (3, 4). The median lethal dose of i.v. administered *Y. enterocolitica* for C57BL/6 and BALB/c mice was determined by the method of Reed and Muench (34). The actual numbers of bacteria administered were determined by plating serial dilutions of the inoculum on Mueller-Hinton agar and counting CFU after an incubation period of 36 h at 26°C. In kinetic studies, five mice per group were killed by carbon dioxide asphyxiation on days 1, 3, 7, 10, 15, and 21 after infection with a sublethal inoculum (0.5 to 0.1 LD_{50}). Blood from each mouse was taken, and serum samples were prepared according to standard methods. The spleens, livers, and lungs were aseptically removed, weighed, and homogenized in 5 ml of PBS containing 0.1% Tergitol TMN 10 (Fluka, Buchs, Switzerland) and 0.1% bovine serum albumin (Merck, Darmstadt, Germany). Then, duplicates of 0.1 ml of serial dilutions of the homogenates were plated on Mueller-Hinton agar (3, 4). The limit of detectable CFU was 25 ($\log_{10} 25 = 1.4$). All experiments were repeated at least two times and revealed comparable results.

MAbs. The monoclonal antibodies (MAbs) used in this study were anti-αβ-T-cell receptor (H57-597; Pharmingen), anti-γδ-T-cell receptor (GL3), anti-CD4 (GK1.5 [Becton Dickinson] and YTS 191), anti-CD8 (53-6.7 [Becton Dickinson] and YTS 169), anti-CD3 (145 2C11), anti-CD11a/CD18 ([LFA-1] FD 18.5), anti-CD25 ([IL-2 receptor, p55 subunit, Tac antigen] PC 61), anti-CD44 ([Pgp-1] IM 7.8), anti-murine IFN-γ (R4-6A2 and AN18.17.24; purified DB-1 provided by P. van der Meide), anti-IL-4 (11B11 and BVD6 24G2), and anti-IL-5 (TRFK4 and TRFK5). Antibodies were purified from hybridoma supernatants by protein G-Sepharose 4 Fast Flow (Pharmacia) and fast performance liquid chromatography (Pharmacia) and then coupled to NHS-biotin (Sigma) or fluorescein isothiocyanate (FITC; Sigma) by standard methods (11).

In vivo administration of antibodies and cytokines. In various experiments, the course of infection was modulated by intraperitoneal administration of (i) polyclonal sheep anti-murine TNF-α antibodies (5×10^4 neutralizing units [2, 24] injected either on days 0 and 1 postinfection [p.i.] or days 3 and 4 p.i.), (ii) anti-IFN-γ MAb (DB-1; 300 μg on days 0 and 1 or days 3 and 4 p.i.), (iii) anti-IL-4 MAb (11B11; 5 mg on day 0 or 3 p.i.), and (iii) recombinant murine IFN-γ (kindly provided by G. Adolf, Bender, Vienna; 3×10^4 U on day 0 or 3 p.i.) as reported previously (2, 24). Control animals were injected intraperitoneally with the appropriate amount of either normal sheep serum (Sigma) or rat immunoglobulin G (IgG; Dianova).

Preparation of splenic T cells. Single-cell suspensions of the spleens were prepared, and 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 Click/RPMI 1640 cell culture medium (Biochrom) supplemented with 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5×10^{-5} M 2-mercaptoethanol, 10 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% heat-inactivated fetal calf serum (Biochrom). T cells were enriched by passage through a nylon-wool column (22). The eluted nonadherent cell fraction contained 85 to 95% $CD3^+$ T cells.

Proliferation assay. Purified splenic T cells (5×10^4 per well) were cultured in the presence of 2×10^5 irradiated (3,500 rads) syngeneic nonimmune splenic mononuclear cells and antigen in 0.2 ml of cell culture medium in round-bottom microculture plates (Nunc). As antigens, we used heat-killed (1 h at 60°C) *Y. enterocolitica* WA-P serotype O:8 (HKY), *Listeria monocytogenes* (serotype 1/2a, strain EGD), and *S. typhimurium* (clinical isolate 2943) at a concentration of 10 μg/ml (3, 4). After 3 days, cultures were pulsed with 0.5 μCi (18.5 kBq per well) of [^{3}H]thymidine (Dupont, NEN Research Products) per well for 16 h. [^{3}H]thymidine uptake was measured in a liquid scintillation counter (Betaplate; Pharmacia LKB).

Cytokine assays. For determination of cytokine production, 5×10^5 T cells were cultured in the presence of 2×10^6 irradiated antigen-presenting cells (APC) and 10 μg of HKY per ml or 3 μg of concanavalin A (ConA; Pharmacia) per ml in 2 ml of cell culture medium in 12-well macroculture plates (Costar, Cambridge, Mass.). After 24 and 48 h, supernatants were collected and used in the cytokine assays.

(i) IFN-γ. IFN-γ levels were determined by capture enzyme-linked immunosorbent assay (ELISA) as described recently (3, 4, 40). Briefly, ELISA microtiter plates (Greiner) were coated with anti-IFN-γ MAb (AN-18.17.24). After blocking of nonspecific binding sites, supernatants were added to the wells and incubated overnight. After several wash steps, biotin-labeled anti-IFN-γ MAb (R4-6A2) was added. Finally, an avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP kit; Camon) was added. For signal development *p*-nitrophenyl phosphate disodium (pNPP; Sigma) was added, and the optical density was determined at wavelengths of 405 and 490 nm with an ELISA reader. The levels of IFN-γ from T-cell culture supernatants were finally determined from the straight-line portion of the standard curve by using recombinant murine IFN-γ (kindly provided by G. Adolf).

(ii) IL-2. Supernatants (0.1 ml) were added to 50 μl of medium containing 1.5×10^4 cells of a CTLL cell line (IL-2 and IL-4 sensitive) in flat-bottom microtiter plates (Nunc). Twenty-four hours later, 10 μl of an MTT solution [(3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] at a concentration of 5 μg/ml of PBS was added to each well to determine survival of CTLL cells (16). Three to 4 h later, 100 μl of acid-isopropanol (40 ml of 1 N HCl in 100 ml 2-isopropanol) was added to dissolve the crystals. Optical density was measured at 570 nm. Wells containing serial dilutions of recombinant mouse IL-2 (a kind gift from R. Lissner, Biotest) served as reference values for estimating the actual IL-2 production of T-cell cultures in units per milliliter.

(iii) IL-4. IL-4 production was estimated in parallel to the determination of IL-2 by the addition of neutralizing anti-IL-4 MAb (supernatant of hybridoma 11B11) to the CTLL cultures. Ten percent of the 11B11 supernatant neutralized 200 U of IL-4 per ml of medium. As described for IL-2, serial dilutions of either recombinant murine IL-4 (Pharmingen) or recombinant IL-4 plus recombinant IL-2 were added to the cultures to estimate IL-4 production of T cells in units per milliliter. To confirm the data, we performed in parallel an IL-4-specific ELISA as described for IFN-γ including the anti-IL-4 MAbs 11B11 (biotin labeled) and BVD6 24G2.

(iv) IL-5. An ELISA was performed as described above. The TRFK4 MAb was coupled to biotin, and the TRFK5 MAb was used purified. Recombinant murine IL-5 (Dianova) was used in controls.

Flow cytometry studies. Splenic mononuclear cells or T cells (10^5) were suspended in PBS containing 2% fetal calf serum and stained with the biotin-labeled or FITC-conjugated MAbs described above. As a second-step reagent for biotin-labeled antibodies, we used Streptavidine-Phycoerythrine (Becton Dickinson). Labeling procedures were carried out at 4°C.

FITC-conjugated rat IgG of the appropriate isotypes (Pharmingen) or an FITC-conjugated irrelevant rat MAb (provided by W. Bohne) was used in controls. From each sample, 5,000 to 10,000 cells were analyzed in a FACScan (Becton Dickinson). Dead cells were excluded from analysis by gating out propidium iodide (Sigma)-positive cells in parallel samples.

***Yersinia*-specific ELISA.** *Yersinia*-specific IgM and IgG, including the IgG subtypes IgG1, IgG2a, IgG2b, and IgG3, antibodies in sera of infected mice were measured by ELISA (25, 43). Briefly, whole cells of *Y. enterocolitica* serotype O:8, strain WA-P, were used as the antigen. The sera were diluted 1:500 in PBS containing 0.5% Tween 20 (Merck) and 1% fetal calf serum (Biochrom). As secondary antibodies, we used anti-mouse IgG-, IgG1-, IgG2a-, IgG2b-, IgG3-, or IgM-alkaline phosphatase conjugates (Dianova). pNPP was used as a substrate, and the optical density was determined at 405 and 490 nm. Five serum samples from control mice (noninfected C57BL/6 and BALB/c mice) were tested as negative controls to obtain cutoff values. The cutoff was defined as being the mean of negative-control values plus two standard deviations.

Adoptive transfer experiments. Seven and 21 days after i.v. sublethal (0.1 LD_{50}) infection with *Y. enterocolitica*, C57BL/6 and BALB/c mice were killed and splenic T cells were prepared as described above. T cells of C57BL/6 or BALB/c mice were pooled, and 10^7 T cells were resuspended in a volume of 0.2 ml of PBS and injected i.v. into the lateral tail veins of either C57BL/6 or BALB/c mice. Control mice received either 10^7 nonimmune splenic T cells or PBS. Cell viability was determined by trypan blue exclusion. Purity of the transferred T cells was determined by fluorescence-activated cell sorter analysis. One day later, C57BL/6 and BALB/c mice were challenged i.v. with a lethal dose (5 to 10 times the LD_{50} for the respective mouse strain) of *Y. enterocolitica*. Three and 6 days after infection, mice were killed, and the spleens, livers, and lungs were removed and homogenized. The extent of protection was determined by plating homogenates of these organs and counting CFU. Protection (log protection) was assessed by subtracting the mean CFU value (log_{10} CFU per organ) for the test groups from the mean CFU value (log_{10} CFU per organ) for the control group.

Statistics. The data shown represent the results of one of three separately performed experiments, which gave comparable results. The values given are the means for five mice per group ± the standard deviation. The statistical significance of the differences of the means between the experimental groups was determined by the unpaired Student *t* test. *P* values of <0.05 were considered statistically significant.

RESULTS

Course of *Y. enterocolitica* infection in resistant C57BL/6 and susceptible BALB/c mice. The LD_{50} of i.v. infection with *Y. enterocolita* O:8 was 2×10^4 for C57BL/6 mice (resistant) and 5×10^2 for BALB/c mice (susceptible). To compare the course of the infection, both mouse strains were infected in parallel with an inoculum of 4×10^2 (sublethal for both), 4×10^3 (lethal for BALB/c), and 4×10^4 (lethal for both) *Y. enterocolitica* cells, and the numbers of bacteria were determined in the spleen, liver, and lungs on days 1, 3, 7, 10, 15, and 21 p.i. The weight of spleens in C57BL/6 mice increased significantly after sublethal ($P = 0.014$) and lethal ($P = 0.001$) infection, whereas BALB/c mice developed only little splenomegaly (Fig. 1). After infection with 4×10^2 (sublethal) and 4×10^4 (lethal for both) bacteria, no significant differences in splenic and hepatic CFU were found between C57BL/6 and BALB/c mice. After infection with 4×10^3 bacteria, CFU were comparable on days 1 and 3 p.i. However, by day 7 p.i., C57BL/6 mice cleared the pathogen from the spleen and liver whereas BALB/c mice did not. While C57BL/6 mice showed only little involvement of the lungs after sublethal infection, BALB/c mice exhibited high CFU in the lungs ($P = 0.001$) even after a sublethal inoculum (4×10^2). Thus, BALB/c mice are more susceptible to *Yersinia* infection than C57BL/6 mice, possibly because of the pronounced involvement of the lungs.

Phenotypes of splenic T lymphocytes in C57BL/6 and BALB/c mice after *Y. enterocolitica* infection. At various intervals after infection, T cells were isolated from the spleens of C57BL/6 and BALB/c mice. For flow cytometry studies, spleen cells were stained with the MAbs listed in Materials and Methods and analyzed in a FACScan. We found a slight increase in $CD3^+$ $\alpha\beta TCR^+$ T cells after infection in both strains of mice. To estimate the extent of activation of CD4 and CD8 T cells during infection, these cells were stained in parallel with anti-CD11a/CD18 (LFA-1), anti-CD44 (Pgp-1), and anti-CD25 (IL-2 receptor, Tac antigen). In Fig. 2, representative stainings of splenic mononuclear cells from BALB/c and C57BL/6 mice on day 7 p.i. are depicted. After the infection, we found only few $CD25^+$ T cells (data not shown). A significant portion of T cells were $CD44^+$, and nearly all cells were $CD11a/CD18^+$. However, we observed a marked increase in CD4 and CD8 T cells which expressed high levels of CD11a/CD18 and CD44 (designated $CD11a/CD18^{hi}$ and $CD44^{hi}$) (Fig. 2). Comparison of the relative number of $CD4^+$ $CD11a/CD18^{hi}$) and $CD8^+$ $CD11a/CD18^{hi}$ in both strains of mice revealed an earlier increase of these cells in the spleens of C57BL/6 mice compared with BALB/c mice (Fig. 2b) and a peak on day 15 p.i. with 30% $CD4^+$ $CD11a/CD18^{hi}$ and 45% $CD8^+$ $CD11a/CD18^{hi}$ in both strains of mice. The relative number of $CD4^+$ $CD44^{hi}$ and $CD8^+$ $CD44^{hi}$ cells increased and peaked on day 7 p.i. in both strains of mice (data not shown). Thus, the different susceptibilities of BALB/c and C57BL/6 mice to *Yersinia* infection were paralleled by differences in the kinetics of the increase of both $CD4^+$ $CD11a/CD18^{hi}$ and $CD8^+$ $CD11a/CD18^{hi}$ cells. The significance of this observation will have to be further elucidated.

Proliferative response of T cells. Splenic T cells were isolated from C57BL/6 and BALB/c mice at various intervals after *Yersinia* infection and stimulated with either ConA or antigen (HKY, heat-killed *S. typhimurium*, or heat-killed *L. monocytogenes*) in the presence of irradiated feeder cells. The proliferative responses to ConA were comparable in both strains of mice (data not shown). However, stimulation with specific antigen (HKY) revealed striking differences (Fig. 3). While T cells from C57BL/6 mice exhibited a significant *Yersinia*-specific proliferative response at days 3 and 7 p.i., with a peak on day 10 p.i., T cells from BALB/c mice developed only a weak *Yersinia*-specific proliferative response. The nonspecific or cross-reactive response of T cells to heat-killed *S. typhimurium* was similar in both strains, whereas heat-killed *L. monocytogenes* did not induce any significant T cell response. Hence, the strength of the *Yersinia*-specific proliferative T-cell response was associated with resistance to infection.

Cytokine production by *Yersinia*-specific T cells. To determine the cytokine production by T cells, the supernatants of the T-cell cultures described above were collected and tested in cytokine assays. Using the IL-2-dependent CTLL cells, we detected only small and insignificant quantities of IL-2 in supernatants of HKY- or ConA-stimulated T cells from both strains of mice (data not shown), possibly because of consumption of IL-2 by the proliferating T cells. Since this cell line is also sensitive to IL-4, we performed parallel assays with anti-IL-4 MAbs. We obtained similar results with anti-IL-4

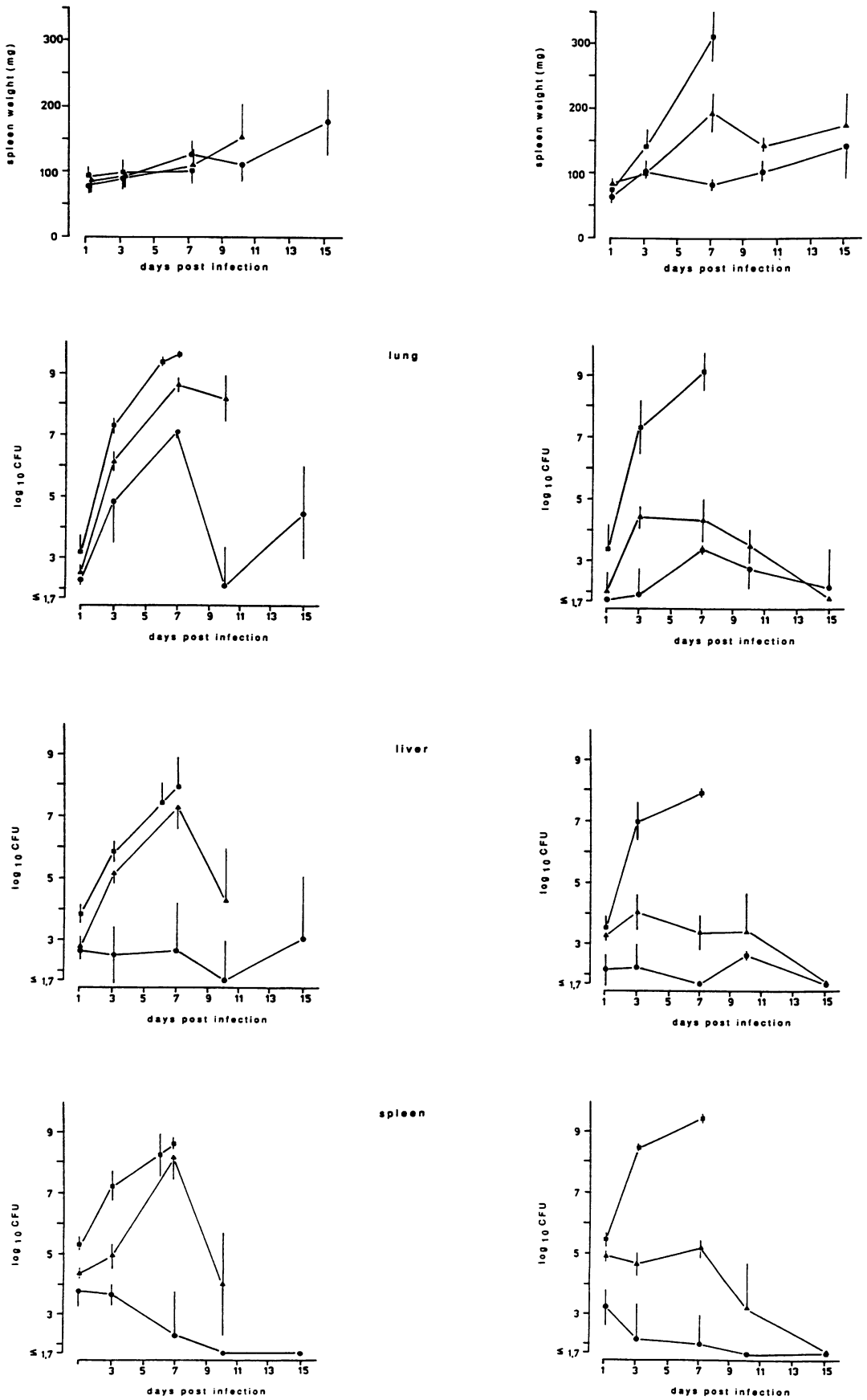

FIG. 1. Spleen weight and number of bacteria (expressed as $\log_{10}$ CFU) in spleens, livers, and lungs of BALB/c (left column) and C57BL/6 (right column) mice after i.v. infection with 4×10^2 (●), 4×10^3 (▲), and 4×10^4 (■) *Y. enterocolitica* WA-P serotype O:8 organisms. Results are the means ± standard deviations for five animals.

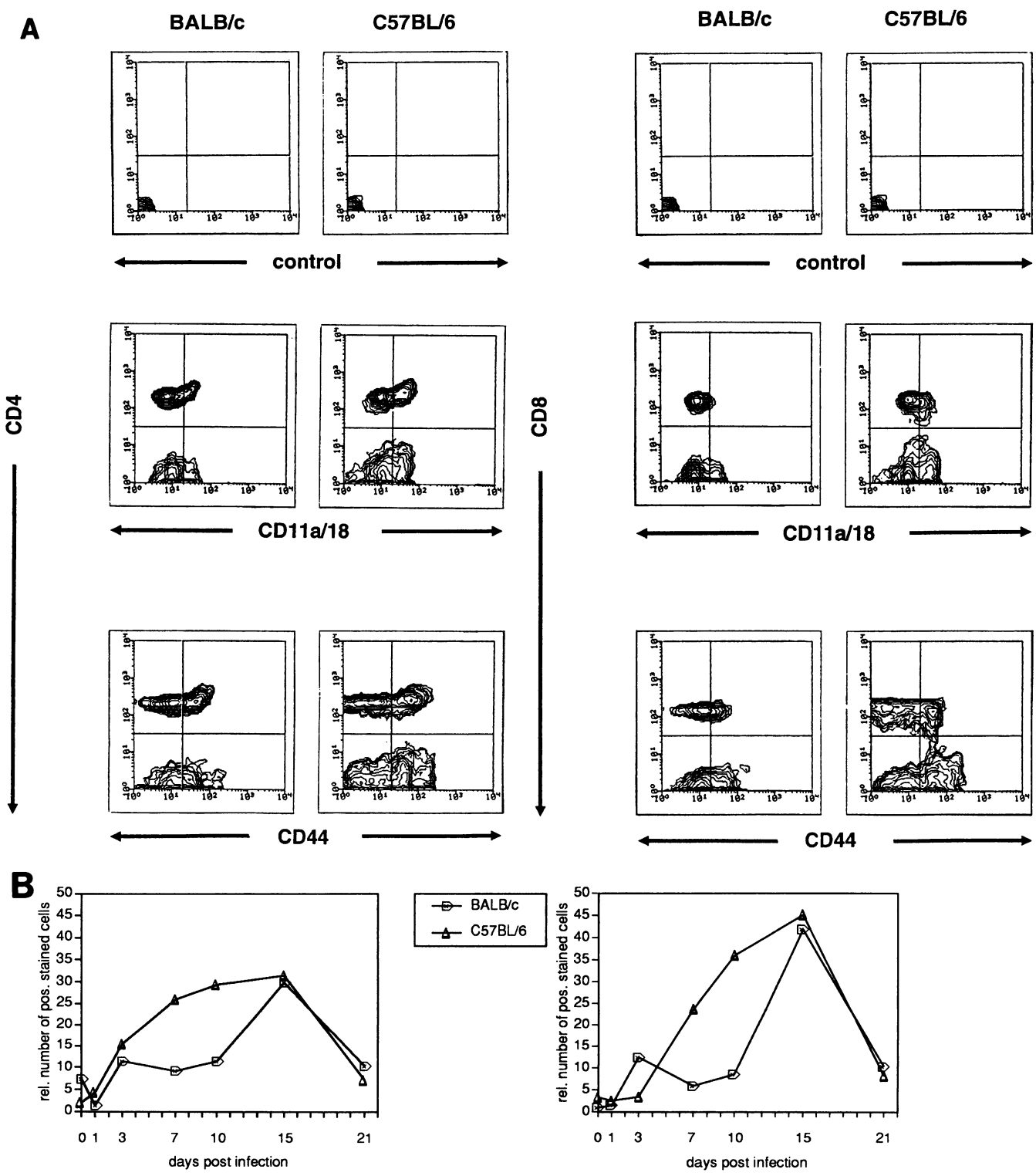

FIG. 2. Fluorocytometric analysis of spleen cells of C57BL/6 and BALB/c mice 7 days after infection with sublethal dose (0.1 LD_{50}) of *Y. enterocolitica*. (A) Double fluorescence staining of cells with anti-CD4 or anti-CD8 and with anti-CD11a/CD18 (LFA-1) or anti-CD44 (Pgp-1). The CD4 and CD8 T cells in the right upper quadrant were designated CD11a/$CD18^{hi}$ and $CD44^{hi}$. (B) Relative number of CD4 CD11a/$CD18^{hi}$ (left) and CD8 CD11a/$CD18^{hi}$ (right) splenic T cells at various intervals after *Yersinia* infection.

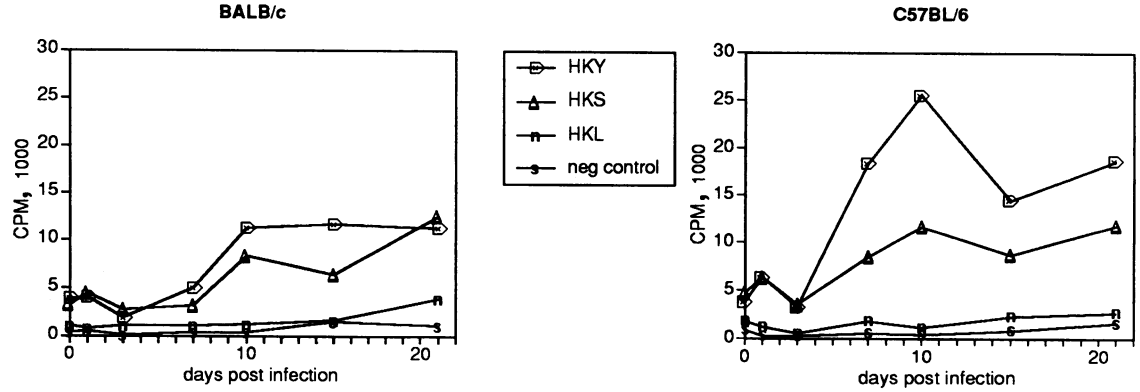

FIG. 3. Proliferative response of splenic T cells of C57BL/6 and BALB/c mice after infection with *Y. enterocolitica*. Triplicates of 5×10^4 T cells were stimulated with 10 μg of HKY, heat-killed *S. typhimurium* (HKS), or heat-killed *L. monocytogenes* (HKL) per ml in the presence of 2×10^5 irradiated syngeneic splenic feeder cells. Results are the means for five animals.

MAbs, and therefore, we can exclude significant quantities of IL-4 (<2 U/ml) in the supernatants. These results were confirmed by an IL-4-specific ELISA. Likewise, we did not detect IL-5 (<5 U/ml) in the supernatants of T-cell cultures from both strains of mice (data not shown). However, determination of IFN-γ revealed striking differences between C57BL/6 and BALB/c mice. While *Yersinia*-specific T cells from C57BL/6 mice produced significant quantities of IFN-γ by days 3, 7, and 10 p.i., we found no IFN-γ in supernatants of T cells from BALB/c mice ($P = 0.001$ to 0.009 for the differences between C57BL/6 and BALB/c at the various time points) (Fig. 4). Only on day 21 p.i. were small quantities of IFN-γ detectable in BALB/c T-cell culture supernatants. By contrast, supernatants from ConA-stimulated T cells from both C57BL/6 and BALB/c mice contained comparable quantities of IFN-γ (data not shown). These data suggest different frequencies of *Yersinia*-specific IFN-γ-producing T cells in both strains of mice and provide evidence that production of IFN-γ by *Yersinia*-specific T cells is correlated with the resistance of mice to the infection.

Production of *Yersinia*-specific antibodies. We showed recently that the production of *Yersinia*-specific antibodies is strictly T-cell dependent (4). Since we found a distinct *Yersinia*-specific T-cell response in C57BL/6 and BALB/c mice, we measured the levels of *Yersinia*-specific IgM and IgG, including IgG1, IgG2a, IgG2b and IgG3, in sera of infected mice. However, production of *Yersinia*-specific antibodies, including IgG1 and IgG2a, was comparable in both strains of mice (data not shown). Only on day 21 p.i. did we find higher levels of *Yersinia*-specific IgG2b antibodies in sera of C57BL/6 mice. Thus, the *Yersinia*-specific IgG antibody responses including the subclasses IgG1 and IgG2a in resistant and susceptible mice are comparable and do not reflect a different *Yersinia*-specific T-cell response.

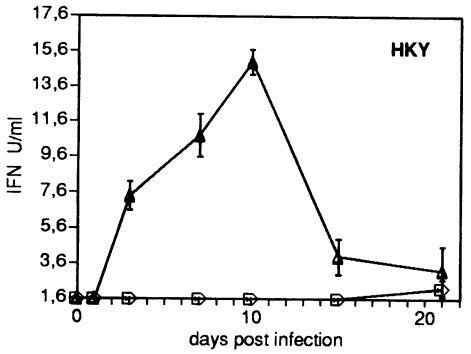

FIG. 4. IFN-γ production by splenic T cells of C57BL/6 (△) and BALB/c (□) mice after infection with *Y. enterocolitica*. T cells were stimulated with 10 μg of HKY per ml in the presence of irradiated feeder cells in macroculture wells. Supernatants were harvested 24 h later and used in an IFN-γ-specific ELISA (see Materials and Methods). The optical density values revealed in the IFN-γ ELISA are expressed as units of IFN-γ per milliliter according to the linear portion of the standard curve. Results are the means ± standard deviations for five animals.

In vivo modulation of cytokines. The fact that *Yersinia*-specific T cells from C57BL/6 mice only and not those from BALB/c mice produced IFN-γ in vitro raised the question of whether this difference may be of relevance in vivo. To address this question, we administered either neutralizing polyclonal anti-TNF-α antibodies, anti-IFN-γ MAb, or recombinant IFN-γ prior to or during the infection in both strains of mice. As has been reported previously, anti-TNF-α antibodies abrogated resistance to yersiniae (Fig. 5A). This was also true for BALB/c mice ($P = 0.006$). Anti-IFN-γ treatment abrogated resistance to yersiniae in resistant C57BL/6 mice ($P < 0.02$) but only marginally modulated the course of infection in susceptible BALB/c mice (Fig. 5B). Consequently, administration of recombinant IFN-γ did not modulate the infection in C57BL/6 mice but rendered BALB/c mice resistant to a normally lethal *Yersinia* inoculum ($P < 0.005$) (Fig. 5C). Hence, these results are consistent with the finding that *Yersinia*-specific T cells from C57BL/6 mice only produced IFN-γ in vitro, while those from BALB/c mice did not. Although we did not detect IL-4 in the supernatants of *Yersinia*-specific T cells, we tested the impact of neutralizing anti-IL-4 antibodies on the course of infection. While administration of anti-IL-4 antibodies did not affect the course of infection in C57BL/6 mice, this treatment rendered BALB/c mice resistant to a lethal *Yersinia* infection ($P < 0.001$) (Fig. 5D). These results suggest that IFN-γ is related to resistance against yersiniae whereas IL-4 is related to susceptibility.

Adoptive transfer of splenic T cells. In order to compare the protective properties of *Yersinia*-specific splenic T cells from C57BL/6 and BALB/c mice, these cells were isolated and transferred to naive animals (10^7 T cells per mouse). T cells isolated on 7 and 21 days p.i. from C57BL/6 mice mediated

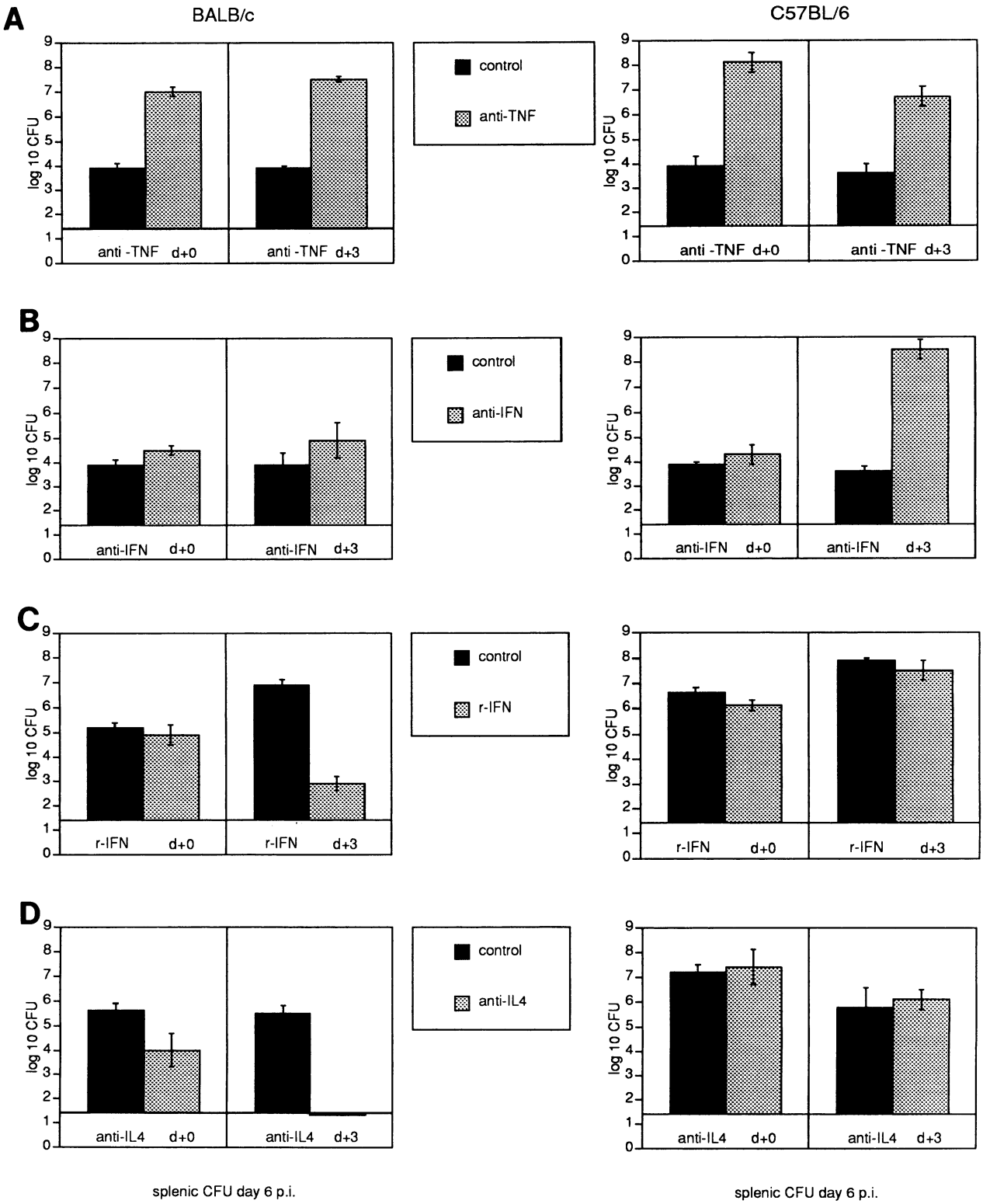

FIG. 5. Effects of in vivo administration of anti-TNF-α (A), anti-IFN-γ (B), recombinant IFN-γ (C), and anti-IL-4 (D) antibodies on clearance of *Y. enterocolitica* from spleens of sublethally (0.1 LD_{50} [A and B]) or lethally (10 LD_{50} [C and D]) infected BALB/c and C57BL/6 mice. Recombinant IFN-γ (r-IFN) and neutralizing antibodies were administered on either days 0 and 1 (d + 0) (left) or days 3 and 4 (d + 3) (right) after infection. The numbers of bacteria (CFU) in spleens were determined on days 3 and 6 p.i. Results are the means ± standard deviations for five animals.

significant protection against a lethal *Yersinia* infection (log protection, 3.5 and 3.7, respectively; $P = 0.04$; data not shown). In contrast, T cells isolated on day 7 p.i. from BALB/c mice did not mediate significant protection (log protection, 0.7). These results support the hypothesis that the presence of IFN-γ-producing *Yersinia*-specific T cells correlates with resistance against *Yersinia* infection. However, T cells isolated on day 21 p.i. from BALB/c mice mediated protection (log protection, 4.3; $P < 0.001$) comparable to that of T cells from C57BL/6 mice.

DISCUSSION

The aim of this study was the characterization of the immune responses of different mouse strains against *Y. enterocolitica* in order to improve the understanding of the different susceptibilities of mouse strains to this pathogen. The salient findings presented in this paper are that, first, the innate mechanisms of resistance to yersiniae seem to be similar in resistant and susceptible strains of mice. Second, after *Yersinia* infection, T cells from *Yersinia*-resistant C57BL/6 mice produced IFN-γ upon exposure to antigen (HKY), while T cells from *Yersinia*-susceptible BALB/c mice did not. Third, while administration of either recombinant IFN-γ or anti-IL-4 antibodies rendered BALB/c mice more resistant to *Y. enterocolitica*, this treatment did not affect bacterial clearance in a priori resistant C57BL/6 mice. Consequently, anti-IFN-γ treatment abrogated resistance to yersiniae in C57BL/6 mice but not, or to a far smaller extent, in BALB/c mice.

$CD4^+$ T helper cells segregate into two subsets, designated Th1 and Th2, with distinct functions and can be classified according to their distinct cytokine production patterns (6, 29, 33). It has been suggested that Th1 cells, which characteristically produce IFN-γ and IL-2, provide help for cell-mediated immune responses while Th2 cells, which typically produce IL-4, IL-5, and IL-10, mediate humoral immune responses (6, 33). Hence, the former T-cell type should be involved in the host response against intracellular pathogens, while the latter one may dominate in defense against extracellular pathogens. Furthermore, a Th1 response is thought to be associated with localization or cure of infectious diseases whereas a Th2 response can be associated with chronic or progressive diseases. In murine leishmaniasis, the different susceptibilities of various mouse strains are related to the T helper cell subset being activated and expanded during infection (28, 30). Thus, resistant mice develop a Th1 response and produce high levels of IFN-γ while susceptible mice develop a Th2 response and produce IL-4 (19, 20).

Y. enterocolitica is predominantly located extracellularly in infected host tissues (15, 27, 37, 39). In contrast to intracellular pathogens such as *L. monocytogenes*, both specific antibodies and specific T cells transfer immunity against yersiniae to naive animals (3, 4, 43). Similar to the findings in experimental leishmaniasis (28, 30), certain strains of mice are *Yersinia* resistant (e.g., C57BL/6) while others (e.g., BALB/c) are *Yersinia* susceptible (13, 14). The basis of the different susceptibilities of mice to *Y. enterocolitica* has not yet been understood. Previous studies have failed to reveal clear-cut linkage of resistance with certain gene loci and suggested that resistance against yersiniae might be controlled multigenically (13, 14).

Recent studies have demonstrated that T cells are involved in and required for control of a primary *Yersinia* infection (1, 3, 4). Therefore, in the present study we wanted to investigate whether evidence exists for different roles of T cells during infection in *Yersinia*-resistant and -susceptible mice. The results of this paper suggest that T cells from C57BL/6 mice produce IFN-γ after *Yersinia* infection while T cells from BALB/c mice do not. However, the types of T cells (Th1 $CD4^+$ and $CD8^+$ or $CD4^-$ and $CD8^-$) which do actually contribute to IFN-γ production after *Yersinia* infection remain to be investigated. Moreover, it may well be that cells other than T cells, e.g., NK cells, are a source of IFN-γ in yersiniosis. Nevertheless, IFN-γ, probably via activation of macrophages, may promote a protective host response against yersiniae. By contrast, T cells from BALB/c mice, at least during the early phase of *Yersinia* infection, lack detectable IFN-γ. However, although anti-IL-4 MAb treatment rendered BALB/c mice resistant to yersiniae, suggesting a detrimental effect of IL-4 in yersiniosis, we did not observe production of IL-4 or IL-5 in supernatants of T-cell cultures from BALB/c mice in vitro. Hence, there is no evidence for a *Yersinia*-induced Th2 response in BALB/c mice, and we cannot exclude that, for example, mast cells may contribute to IL-4 production. Thus, the data are consistent with a model in which IFN-γ-producing T cells from BALB/c mice are less responsive to *Yersinia* infection rather than one in which a Th2-like response accounts for susceptibility. On the other hand, there is evidence that different levels of soluble IL-4 receptor in BALB/c and C57BL/6 mice may account for different roles of IL-4 in these mouse strains (5). Finally, it may well be that in our in vitro system, HKY may have induced IL-12 and thus stimulated Th1 cells, as has been recently shown in vivo (42) and for an in vitro model including stimulation with heat-killed *L. monocytogenes* (21).

Furthermore, evidence is missing for a Th2-directed switch of *Yersinia*-specific IgG in sera of BALB/c mice. Thus, in sera of both strains of mice, we found comparable levels of *Yersinia*-specific IgG1 (promoted by Th2 cells) and IgG2a (promoted by Th1 cells) antibodies. Similar observations have been reported for the leishmania model (23). Hence, in this study there was no association of IL-4 or serum IgE production (expansion of Th2 cells) with disease progression in susceptible mice, although production of IFN-γ by spleen cells (expansion of Th1 cells) correlated directly with the reduction of the parasites in the liver (23). In our model, by day 21 p.i. minute IFN-γ production of *Yersinia*-specific T cells from BALB/c mice was observed and correlated with significant protection against *Y. enterocolitica* after adoptive transfer of these cells into naive mice. Therefore, we assume that in addition to IFN-γ, other cytokines and other T-cell-mediated functions must be involved in protection against *Y. enterocolitica*. Because of the fact that both specific T cells and specific antibodies of susceptible BALB/c mice can transfer immunity against yersiniae, it seems unlikely that the different susceptibilities might be related to different receptor repertoires of *Yersinia*-specific T cells of C57BL/6 and BALB/c mice. This assumption is further supported by the findings (i) that a single antigen can underlie both Th1 and Th2 responses (35) and (ii) that vaccination rendered leishmania-susceptible BALB/c mice resistant by induction of a Th1-cell-mediated immunity (7).

A further important observation of this study was that C57BL/6 mice developed significant splenomegaly after *Yersinia* infection while BALB/c mice did not. In a recent study we have shown that splenomegaly correlates with the recruitment of $CD11b/CD18^+$ phagocytes and NK cells after the infection and that this phenomenon is T-cell independent (4). Hence, it may well be that susceptibility of BALB/c mice to yersiniae is partially caused by a delayed recruitment of putative effector cells such as phagocytes.

Adhesion molecules such as CD11a/CD18 and CD44 are involved in certain functions of T cells (41), including their

protective properties in vivo (36). Analysis of the phenotypes of T cells in BALB/c and C57BL/6 mice revealed that the number of CD11a/CD18^{hi} and CD44^{hi} T cells increased after *Yersinia* infection, the former particularly in C57BL/6 mice. However, the significance of this observation remains to be elucidated.

Taken together, our data suggest that IL-4, as has been reported for other murine infection models, including those for listeriosis (12), *Francisella tularensis* (26), trichinelliasis (32), schistosomiasis (31), and leishmaniasis (8, 38), as well as human leprosy (44), might suppress T-cell responses which are associated with IFN-γ production and protective cell-mediated immunity against microorganisms. On the other hand, both IFN-γ and IL-4 can be produced by cells other than T cells. Thus, future studies, including limiting dilution and ELISPOT assays, will need to elucidate the regulation of the T-cell responses during yersiniosis in more detail, including the roles of IL-10 and IL-12.

ACKNOWLEDGMENTS

We thank G. Adolf for recombinant IFN-γ and Sonja Preger for expert technical assistance.

This work was supported by a grant of the Bundesministerium für Forschung und Technologie (BMFT).

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