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An Anti-CD3 Monoclonal Antibody Protects Mice against a Lethal Infection with *Listeria monocytogenes* through Induction of Endogenous Cytokines

AKIO NAKANE,* MUNENORI OKAMOTO, MISAKO ASANO, MASASHI KOHANAWA, AND TOMONORI MINAGAWA

Department of Microbiology, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo 060, Japan

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Mice were protected against a lethal infection with *Listeria monocytogenes* when treated with low doses of an anti-CD3 monoclonal antibody (MAb). Injection of anti-CD3 MAb induced rapid production of endogenous tumor necrosis factor (TNF) in the spleens and endogenous gamma interferon (IFN-γ) in the bloodstreams and spleens of mice. Administration of anti-Thy1.2 MAb or a combination of anti-CD4 MAb and anti-CD8 MAb resulted in suppression of anti-CD3 MAb-induced endogenous cytokine production and antilisterial resistance. Alternatively, in vivo depletion of anti-CD3 MAb-induced TNF and IFN-γ by the simultaneous administration of antibodies against TNF and IFN-γ suppressed anti-CD3 MAb-induced antilisterial resistance. Moreover, injection of anti-complement receptor type 3 (Mac-1, CD11b) resulted in inhibition of anti-CD3 MAb-induced antilisterial resistance. These results suggest that the preventive effect of anti-CD3 MAb might be due to activation of phagocytes by TNF and IFN-γ induced by stimulating $CD4^+$ T cells and $CD8^+$ T cells with the MAb. Furthermore, treatment with anti-CD3 MAb did not inhibit establishment of acquired resistance against secondary infection with *L. monocytogenes*.

Complete elimination of *Listeria monocytogenes*, a facultative intracellular pathogen, from the tissues of infected animals is performed by macrophages activated by T-cell-dependent mechanisms (23, 30). Production of endogenous cytokines involving tumor necrosis factor (TNF), gamma interferon (IFN-γ), interleukin-1 (IL-1), IL-6, and colony-stimulating factors has been reported for *L. monocytogenes*-infected mice (3, 14–16, 25, 29). Studies on in vivo administration of anticytokine antibodies demonstrated that TNF, IFN-γ, IL-1, and macrophage–colony-stimulating factor are involved in host resistance to *L. monocytogenes* infection (2, 11, 13, 24, 25, 31). These studies suggest that TNF and IFN-γ are crucial in antilisterial resistance.

The hamster monoclonal antibody (MAb) 145-2C11 that specifically recognizes the ε chain of the mouse CD3 molecule is a potent immunosuppressive agent (17, 22). However, anti-CD3 MAb also has properties of potently activating T cells (18). It was reported that in vivo administration of low doses of the MAb prevented lethal Sendai virus infection and malignant tumor growth in mice (8, 19). Moreover, high levels of endogenous cytokines involving TNF, IFN-γ, IL-2, IL-3, and IL-6 were released in the circulation of mice soon after injection (1, 10). In this report, we provide evidence that a single injection of low doses of anti-CD3 MAb can rescue mice from a lethal infection with *L. monocytogenes* and that the protective effect is dependent on endogenous TNF and IFN-γ induced by anti-CD3 MAb.

MATERIALS AND METHODS

Mice. Female ddY outbred mice and female C57BL/6 mice (age, 5 weeks; SLC, Hamamatsu, Shizuoka, Japan) were used.

Bacteria. *L. monocytogenes* 1b 1684 cells were prepared as described previously (26). The concentration of washed cells was adjusted spectrophotometrically at 550 nm. Mice were infected intravenously with 0.2 ml of a solution containing 5×10^5 CFU (2.5 50% lethal doses) of viable *L. monocytogenes* cells in 0.01 M phosphate-buffered saline (PBS; pH 7.4).

Anti-CD3 MAb treatment. Hybridoma line 145-2C11 cells (anti-CD3, hamster immunoglobulin G) (22) were injected into pristane-primed CD-1 *nu/nu* mice. The MAb in the ascites fluid was partially purified by 50% $(NH_4)_2SO_4$ precipitation. 145-2C11 cells were kindly provided by H. Yagita and K. Okumura, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. Mice were injected intravenously with a single 1-μg dose of anti-CD3 MAb or normal hamster globulin in pyrogen-free saline (0.2-ml total injected volume) 24 h before *L. monocytogenes* infection. Normal hamster globulin was prepared and partially purified by $(NH_4)_2SO_4$ precipitation from the pooled sera of Syrian hamsters (SLC).

MAbs against other cell surface molecules. Hybridoma cell lines GK1.5 (anti-CD4, rat immunoglobulin G2b) (5), 53-6.72 (anti-CD8, rat immunoglobulin G2a) (21), 30-H12 (anti-Thy1.2, rat immunoglobulin G2b) (21), PK-136 (anti-NK1.1, rat immunoglobulin G2a) (20), and 5C6 (anti-complement receptor type 3 [CR3, Mac-1, CD11b], immunoglobulin G2b) (32) were used. 30-H12 cells and PK-136 cells were purchased from the American Type Culture Collection, Rockville, Md. 5C6 cells were kindly donated by H. Rosen, Merck Sharp & Dohme Research Laboratories, Rahway, N.J. The MAbs in the ascites fluid were partially purified by 50% $(NH_4)_2SO_4$ precipitation. Mice were given intravenously a single 400-μg injection of anti-CD4 MAb, anti-CD8 MAb, anti-Thy 1.2 MAb, or anti-NK1.1 MAb on day −3 of *L. monocytogenes* infection. A single 500-μg intravenous injection of anti-CR3 MAb was carried out 2 h before infection. Normal rat globulin was injected as a control for

* Corresponding author.

the rat MAbs. Normal rat globulin was prepared as described previously (28). In the schedules and the doses, the MAbs could deplete the corresponding cells or block the functions of the corresponding cells as described previously (12, 20, 28, 33).

In vivo depletion of endogenous IFN-γ and TNF. To deplete endogenous IFN-γ in vivo, we gave each mouse a single intravenous injection of 1 mg of rat anti-mouse IFN-γ MAb (in 0.2 ml of pyrogen-free saline), partially purified by $(NH_4)_2SO_4$ precipitation, 2 h before *L. monocytogenes* infection as described previously (25). Normal rat globulin was injected as a control. To deplete endogenous TNF in vivo, we gave each mouse a single 400-μg intravenous injection of rabbit antibodies against recombinant mouse TNF-α (rMuTNF-α) in the same manner as that used for the anti-IFN-γ MAb (24, 25). Rabbit anti-mouse TNF-α antibody, which was partially purified by $(NH_4)_2SO_4$ precipitation, was kindly provided by N. Tsuruoka, Suntory Institute for Biomedical Research, Osaka, Japan. Normal rabbit globulin was injected as a control.

Tests for endotoxin contamination. All in vivo effects of antibodies or normal globulins described were verified by using reagents tested by the *Limulus* amoebocyte lysate assay to contain <0.1 ng per injected dose.

Preparation of organ extracts. The spleens or livers were suspended in RPMI 1640 medium containing 1% (wt/vol) 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; Wako Pure Chemical Co., Kyoto, Japan), and 10% (wt/vol) homogenates were prepared with a Dounce grinder and then clarified by centrifuging at 2,000 × *g* for 20 min. The organ extracts were stored at −70°C until cytokine assays were performed.

IFN-γ assay. The IFN-γ assay was carried out by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (27). Purified rat anti-mouse IFN-γ MAb produced by hybridoma R4-6A2 (35) and rabbit anti-rMuIFN-γ serum (27) were used for the ELISA. All ELISAs were run with rMuIFN-γ produced and purified by Genentech, Inc., San Francisco, Calif.

TNF assay. The TNF assay was carried out by using a double-sandwich ELISA as described previously (29). Purified hamster anti-rMuTNF-α MAb (Genzyme Co., Boston, Mass.) and rabbit anti-rMuTNF-α globulin (24) were used for the ELISA. All ELISAs were run with rMuTNF-α (Genzyme).

Determination of numbers of viable *L. monocytogenes* cells in the organs. The numbers of viable *L. monocytogenes* cells in the spleens of the infected animals were established by plating serial 10-fold dilutions of organ homogenates in PBS on tryptic soy agar (Difco Laboratories, Detroit, Mich.). Colonies were routinely counted 18 to 24 h later.

Statistical evaluation of the data. Data were expressed as means ± standard deviations, and the Wilcoxon rank sum test was used to determine the significance of the differences of bacterial counts in the organs or the cytokine titers between control and experimental groups. The generalized Wilcoxon test was used to determine the significance of differences in survival rate. Each experiment was repeated at least twice and accepted as valid only when trials showed similar results.

RESULTS

Effect of anti-CD3 MAb on lethal infection with *L. monocytogenes*. Mice were injected intravenously with different doses of anti-CD3 MAb or normal hamster globulin 24 h before infection with a lethal dose of *L. monocytogenes* cells (5×10^5 CFU [2.5 50% lethal doses]). The number of mortalities in each group was observed for 10 days (Fig. 1). All normal hamster globulin-treated mice died within 5 days after infection. More than half of the mice, which had received 0.1 to 10 μg of anti-CD3 MAb per mouse, survived. The injection of a 1-μg dose of the MAb resulted in complete protection against the lethal infection. However, none of the mice survived when they received 100 μg of the MAb.

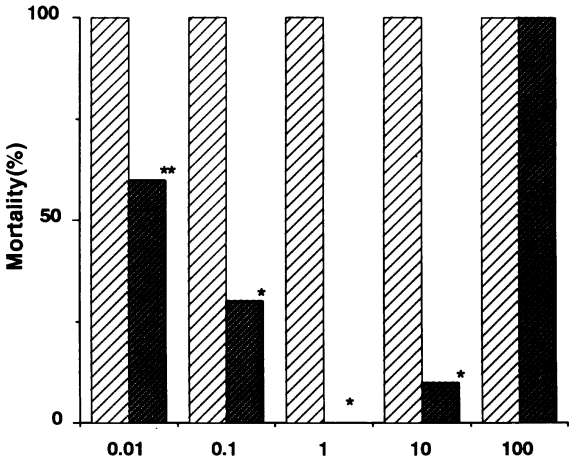

FIG. 1. Effect of anti-CD3 MAb on lethal infection with *L. monocytogenes*. ddY mice were injected intravenously with different doses of anti-CD3 MAb (■) or normal hamster globulin (▨) 24 h before infection with 2.5 50% lethal doses of *L. monocytogenes*. The survival was observed until day 10 of infection. Each point represents a group of 10 mice from two experiments. Single and double asterisks indicate significant differences from the value for the normal hamster globulin-treated group at $P < 0.01$ and $P < 0.05$, respectively.

Effect of timing of anti-CD3 MAb administration on the protection against lethal infection. A single injection of 1 μg of anti-CD3 MAb or normal hamster globulin per mouse was given at different stages of infection, and the number of mortalities in each group was observed for 10 days (Fig. 2). Mortalities were reduced in anti-CD3 MAb-treated mice when the mice were injected with the MAb on day −7 to day 0 of infection. Complete protection was observed when the MAb was injected on day −1 (−24 h) of infection. In contrast, no protective effect of anti-CD3 MAb was shown when the MAb was injected after infection.

Effect of anti-CD3 MAb on multiplication of *L. monocytogenes* cells in the organs of mice. Mice were injected with anti-CD3 MAb or normal hamster globulin (each, 1 μg per mouse) 24 h before infection with 2.5 50% lethal doses of *L. monocytogenes* cells, and the number of bacterial cells in the spleens and livers of the mice was determined after infection (Fig. 3). Explosive growth of bacteria occurred in both organs of normal hamster globulin-injected mice, and all of them died on day 4 or 5 of infection. In anti-CD3 MAb-injected mice, in contrast, the number of bacterial cells in the spleens and livers increased until day 3 of infection, thereafter decreased, and were finally eliminated from both organs.

Induction of endogenous TNF and IFN-γ by anti-CD3 MAb in uninfected mice. Before the use of *L. monocytogenes*-infected mice, we investigated the in vivo induction of

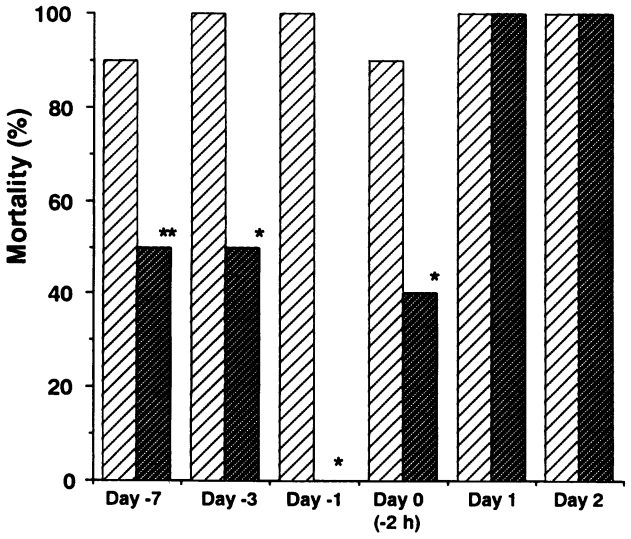

FIG. 2. Effect of timing of administration of anti-CD3 MAb on lethal infection with *L. monocytogenes*. ddY mice were injected with anti-CD3 MAb (▩) or normal hamster globulin (▨) (each, 1 μg per mouse) at the indicated day after infection. The survival was observed until day 10 of infection. Each result represents a group of 10 mice from two experiments. Single and double asterisks indicate significant differences from the value for the normal hamster globulin-treated group at $P < 0.01$ and $P < 0.05$, respectively.

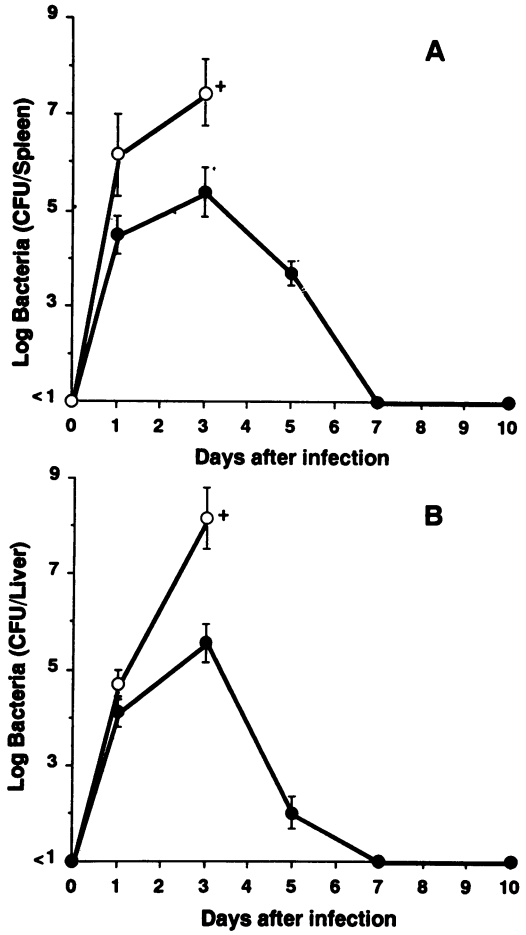

FIG. 3. Growth of *L. monocytogenes* cells in the spleens (A) and livers (B) of mice which received normal hamster globulin (○) or anti-CD3 MAb (●). ddY mice were injected intravenously with either normal hamster globulin or anti-CD3 MAb (1 μg per mouse) 24 h before infection. Each point represents the mean ± standard deviation for a group of five mice.

cytokines by anti-CD3 MAb in uninfected mice. Mice were injected intravenously with 1 μg of anti-CD3 MAb. TNF and IFN-γ in the bloodstreams and spleens were monitored at various times (Fig. 4). IFN-γ appeared at 1 h and then peaked at 6 h in the bloodstreams and spleens. TNF production peaked at 1 or 2 h after injection in the spleens, whereas no TNF was detectable in any of the serum samples. On the other hand, no cytokine induction was observed after injection of normal hamster globulin (data not shown).

T-cell subsets which are responsible for cytokine production and the protective effect induced by anti-CD3 MAb. To deplete the corresponding cells in vivo, mice were injected intravenously with normal rat globulin, anti-CD4 MAb, anti-CD8 MAb, anti-Thy1.2 MAb, or anti-NK1.1 MAb (each, 400 μg per mouse) 3 days before cytokine induction by anti-CD3 MAb. The sera from mice 6 h after injection and the spleen extracts from mice 2 h after injection of anti-CD3 MAb were assayed for IFN-γ and TNF, respectively (Fig. 5). Production of neither IFN-γ nor TNF was significantly affected in mice which had been injected with anti-CD4 MAb, anti-CD8 MAb, or anti-NK1.1 MAb ($P > 0.05$). In contrast, IFN-γ production was completely suppressed in mice which had received anti-Thy1.2 MAb or a combination of anti-CD4 MAb and anti-CD8 MAb ($P < 0.01$). Although TNF production was significantly but not completely inhibited in mice which had been injected with a combination of anti-CD4 MAb and anti-CD8 MAb ($P < 0.01$), injection of anti-Thy1.2 MAb completely blocked it ($P < 0.01$).

Mice which had received MAbs against different cell surface molecules were infected with a lethal dose of *L. monocytogenes* cells 24 h after injection of anti-CD3 MAb, and the number of mortalities in each group was observed for 10 days (Fig. 6). Survival rates of anti-CD4 MAb-, anti-CD8 MAb-, or anti-NK1.1 MAb-injected mice were comparable to that of the normal rat globulin-treated group. Moreover, depletion of $Thy1^+$ cells or both $CD4^+$ cells and $CD8^+$ cells resulted in complete abrogation of the protective effect of anti-CD3 MAb against the lethal infection ($P < 0.01$).

Effect of antibodies against TNF and IFN-γ on the protective effect of anti-CD3 MAb. Mice were injected intravenously with rat anti-mouse IFN-γ MAb, rabbit anti-mouse TNF-α immunoglobulin, or both (each, 400 μg per mouse) 2 h before injection of anti-CD3 MAb. Normal rat globulin, normal rabbit globulin, or both were injected into mice as controls. These animals were infected with a lethal dose of *L. monocytogenes* cells 24 h after anti-CD3 MAb treatment, and the number of mortalities in each group was observed for 10 days (Fig. 7). All mice which had been injected with normal globulins or anti-IFN-γ MAb survived, whereas half of the anti-TNF-α immunoglobulin-treated mice died. Moreover, the simultaneous administration of both antibodies resulted in complete abrogation of the protective effect of anti-CD3 MAb on the lethal infection.

Both endogenous TNF and IFN-γ produced during *L. monocytogenes* infection are crucial in host defense against

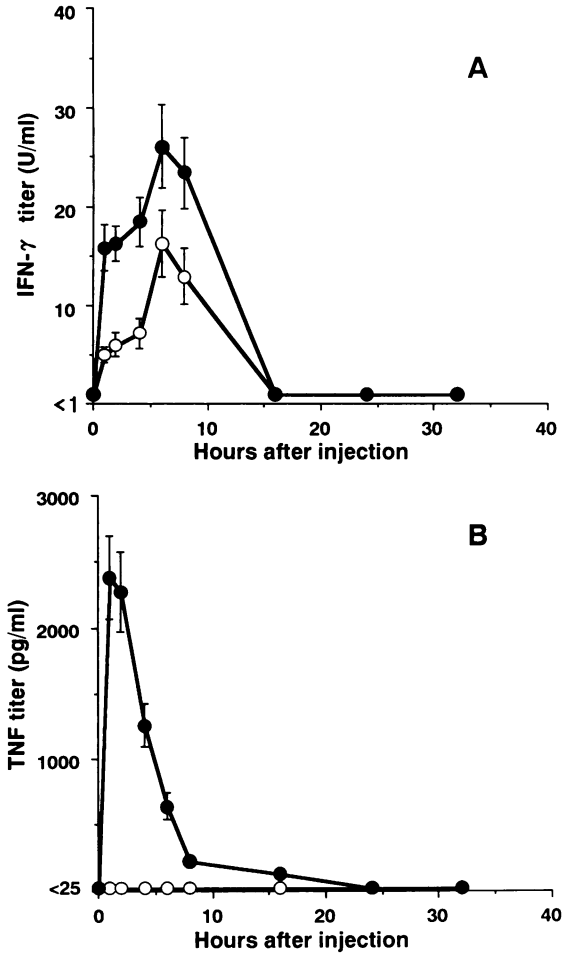

FIG. 4. Induction of endogenous IFN-γ (A) and TNF (B) in the bloodstreams (○) and spleens (●) of uninfected ddY mice by intravenous injection of anti-CD3 MAb (1 μg per mouse). Each point represents the mean ± standard deviation for a group of five mice.

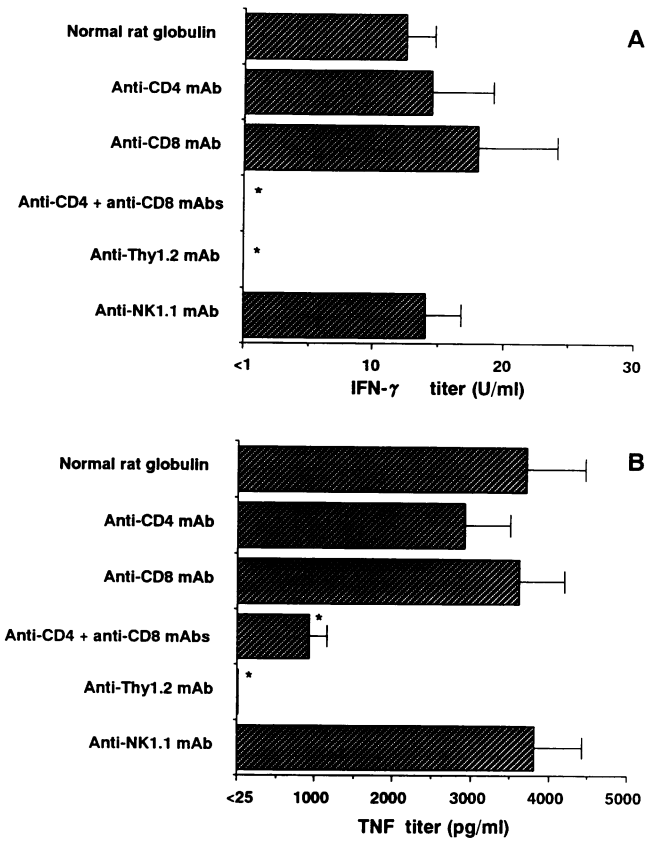

FIG. 5. Effect of in vivo administration of MAbs against T-cell subsets and NK cells on endogenous cytokine production induced by anti-CD3 MAb. Uninfected C57BL/6 mice were injected intravenously with the MAbs (each, 400 μg per mouse) 3 days before cytokine induction by anti-CD3 MAb. The serum samples for the IFN-γ assay were taken 6 h after injection and the spleens for the TNF assay were taken 2 h after injection of anti-CD3 MAb. Each result represents the mean ± standard deviation for a group of five mice. An asterisk indicates a significant difference from the value for the normal rat globulin-treated group at $P < 0.01$.

the sublethal infection (2, 13, 14, 24, 25). Therefore, we investigated the possibility that the suppressive effect of anticytokine antibodies might be due to depletion of the cytokines produced by *L. monocytogenes* cells after infection. Mice were injected with anti-IFN-γ MAb, anti-TNF-α immunoglobulin, or both (each, 400 μg per mouse; 10 mice per group), and they were infected with 0.1 50% lethal dose of *L. monocytogenes* cells 2 h later. Lethal infection resulted in none of the mice during the 10 days after infection (data not shown).

Participation of phagocytes on augmented host resistance by anti-CD3 MAb. Mice which had received anti-CD3 MAb were divided into two groups (10 mice per group). They were injected with normal rat globulin or anti-CR3 MAb 2 h before *L. monocytogenes* infection, and the number of mortalities in each group was observed for 10 days. All mice survived in the normal rat globulin-treated group, whereas no mice survived in the anti-CR3 MAb-treated group (data not shown).

Acquirement of immunity in anti-CD3 MAb-treated mice. To investigate whether anti-CD3 MAb-pretreated mice which had escaped a lethal infection with *L. monocytogenes* could acquire antilisterial immunity to the secondary infection, they were reinfected intravenously with 5 50% lethal doses of *L. monocytogenes* cells, and the number of bacterial cells in the spleens and livers was determined 2 days later (Table 1). As a negative control, nonimmunized mice were challenged with the same dose of bacteria. As a positive control, mice which had been immunized with 0.1 50% lethal dose of *L. monocytogenes* cells without anti-CD3 MAb pretreatment were also reinfected. The growth of bacteria in the organs of both the anti-CD3 MAb-treated mice and the immunized controls was significantly inhibited compared with that of the nonimmunized controls ($P < 0.01$).

DISCUSSION

The studies presented here demonstrate that mice pretreated with low doses of anti-CD3 MAb acquired resistance against a lethal infection with *L. monocytogenes* and that the effect depended on TNF and IFN-γ induced by stimulating both $CD4^+$ T cells and $CD8^+$ T cells with anti-CD3 MAb.

Anti-CD3 MAb-induced protection against a lethal infection with *L. monocytogenes* depended on the timing of the MAb administration (Fig. 2). Anti-CD3 MAb showed a preventive effect but not a therapeutic effect. In vivo admin-

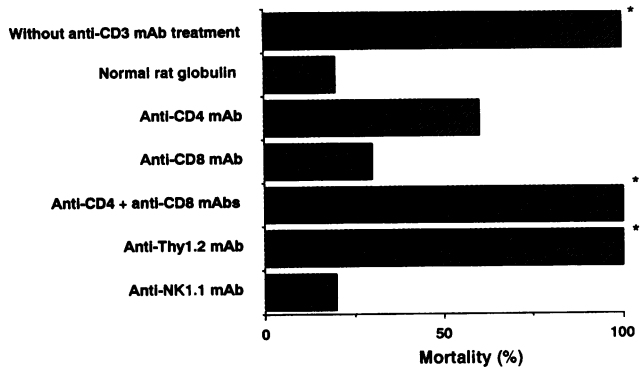

FIG. 6. Effect of in vivo administration of MAbs against T-cell subsets and NK cells on the protective effect of anti-CD3 MAb on lethal infection with *L. monocytogenes*. C57BL/6 mice were pretreated with MAbs as described in the legend to Fig. 5. They were infected with *L. monocytogenes* 24 h after injection of anti-CD3 MAb. The survival was observed until day 10 of infection. Each result represents a group of 10 mice from two experiments. An asterisk indicates a significant difference from the value for the normal rat globulin-treated group at $P < 0.01$.

istration of anti-CD3 MAb was described to activate T cells (8, 18). However, the effect depended on the antibody concentrations. The injection of low doses of anti-CD3 MAb could induce antitumor immunity and antimicrobial immunity (8, 19), whereas anti-CD3 MAb at high concentrations caused immunosuppression, morbidity, and even mortality (1, 10, 18). In this study, an injection of a 1-μg dose of anti-CD3 MAb provided the greatest protection, and either lower doses or higher doses of the MAb were less effective. Especially, a 100-μg dose of the MAb provided no protection (Fig. 1).

The injection of anti-CD3 MAb induced rapid production of endogenous TNF and IFN-γ (Fig. 4). The result coincided with that reported previously (1, 10). IFN-γ production was affected by treatment with neither anti-CD4 MAb nor anti-

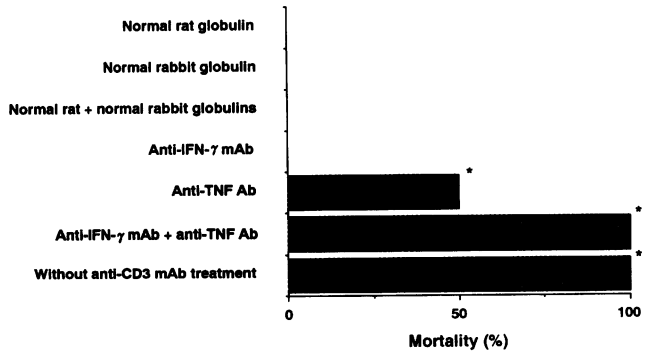

FIG. 7. Effect of in vivo administration of antibodies against IFN-γ and TNF on the protective effect of anti-CD3 MAb on lethal infection with *L. monocytogenes*. ddY mice were injected intravenously with rat anti-IFN-γ MAb, rabbit anti-TNF-α antibody, or the corresponding normal globulins (each, 400 μg per mouse) 2 h before injection of anti-CD3 MAb. They were infected with *L. monocytogenes* 24 h after injection of anti-CD3 MAb. The survival was observed until day 10 of infection. Each result represents a group of 10 mice from two experiments. Symbol: *, significant difference from the value for the corresponding globulin-treated group at $P < 0.01$.

TABLE 1. Acquired antilisterial resistance in mice which had received anti-CD3 MAb before primary infection

Anti-CD3 MAb[a]	Primary infection[a] (LD_{50}/mouse)	Secondary infection[a] (LD_{50}/mouse)	Log no. of bacteria (CFU/organ)[b]	
			Spleen	Liver
−	None	5	7.74 ± 0.33	7.99 ± 0.50
−	0.1	5	5.03 ± 0.37[c]	4.26 ± 0.42[c]
+	2.5	5	4.55 ± 0.25[c]	4.40 ± 0.13[c]

[a] ddY mice were injected with anti-CD3 MAb or not 2 h before primary infection. They were reinfected on day 28 of infection. LD_{50}, 50% lethal dose.
[b] The number of *L. monocytogenes* cells in the organs was determined 2 days after secondary infection. Each result represents the mean ± standard deviation for a group of four mice.
[c] Significantly different from the value for nonimmunized group ($P < 0.01$).

CD8 MAb but was completely suppressed in mice injected with both MAbs (Fig. 5), suggesting that both $CD4^+$ T cells and $CD8^+$ T cells are responsible for anti-CD3-induced IFN-γ production. In contrast, TNF production was significantly but not completely suppressed in both $CD4^+$ and $CD8^+$ cell-depleted mice, whereas the production was completely inhibited in anti-Thy1.2 MAb-injected mice (Fig. 5), suggesting that mainly $CD4^+$ T cells and $CD8^+$ T cells and partially $Thy1^+$ $CD4^-$ $CD8^-$ cells are responsible for anti-CD3 MAb-induced TNF production.

TNF and IFN-γ are described to be crucial in antilisterial resistance (2, 13, 24, 25). We estimated the role of anti-CD3 MAb-induced TNF and IFN-γ by in vivo administration of antibodies against these cytokines. The injection of a combination of antibodies against TNF and IFN-γ before anti-CD3 treatment resulted in the suppression of the preventive effect of anti-CD3 MAb (Fig. 7). Endogenous TNF and IFN-γ were also produced in the spleens and livers of anti-CD3 MAb-injected mice in parallel with bacterial growth in these organs (data not shown). Therefore, it is possible that suppression of the preventive effect by anticytokine antibodies might be due to depletion of the cytokines produced by *L. monocytogenes* cells after infection. However, the possibility was unlikely because mice never died when treated with antibodies against TNF and IFN-γ at the concentrations used herein in a sublethal infection with *L. monocytogenes*. These results suggest that both TNF and IFN-γ induced by anti-CD3 MAb are crucial in the MAb-induced antilisterial resistance. It coincided with the result that anti-Thy1.2 MAb-treated mice or both anti-CD4 and anti-CD8 MAb-treated mice, in which production of TNF and IFN-γ induced by anti-CD3 MAb was suppressed, lapsed into lethal infection without expressing anti-CD3 MAb-induced antilisterial resistance (Fig. 6).

A possible role of TNF and IFN-γ in antilisterial resistance is described to be activation of macrophages (2, 24). In the early stage of *L. monocytogenes* infection, the roles of neutrophils and NK cells as well as macrophages have been reported (4, 6). In this study, pretreatment of mice with anti-CD3 MAb, which inhibits the recruitment of myelomonocytic cells into inflammatory foci (32, 33), resulted in suppression of anti-CD3 MAb-induced antilisterial resistance (Fig. 7), whereas pretreatment of mice with anti-NK1.1 MAb did not affect it (Fig. 6). These results suggest that activation of neutrophils and macrophages but not NK cells by TNF and IFN-γ induced by anti-CD3 MAb might be the main mechanism of the MAb-induced antilisterial resistance.

In previous reports (1, 9, 10), injection of anti-CD3 MAb

induced sickness involving hypothermia, hypomotility, and prostrating diarrhea. Moreover, the toxicity correlated with serum TNF titers, and it was caused by TNF. In this study, the low dose of anti-CD3 MAb induced endogenous TNF in the spleens but not in the circulation (Fig. 4), and it did not induce any symptoms in mice. Moreover, the MAb did not affect establishment of the acquired resistance against secondary infection with *L. monocytogenes* (Table 1). The results presented here suggest that administration of a low dose of anti-CD3 MAb might be a useful tool for prevention of severe infections with intracellular pathogens.

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