The role of gamma interferon in acquired host resistance against *Staphylococcus aureus* infection in mice

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Abstract

We investigated the expression of an acquired host resistance against *Staphylococcus aureus* infection in mice. When C57BL/6 mice were immunized with viable *S. aureus* and challenged with *S. aureus* eight weeks later, the elimination of *S. aureus* from the spleen and liver was enhanced in the immunized mice compared with the non-immunized mice. When gamma interferon (IFN-$\gamma^{-/-}$) mice were immunized and challenged, the bacterial numbers in the organs of immunized mice were comparable to those in the nonimmunized mice, suggesting that IFN-γ plays a critical role in an acquired host resistance against *S. aureus* infection. IFN-$\gamma^{-/-}$ mice produced the lower level of anti-*S. aureus* immunoglobulin M (IgM) and IgG2a antibodies compared with C57BL/6 mice. To elucidate the role of IFN-γ produced during a challenge with *S. aureus*, a single injection of anti-IFN-γ monoclonal antibody to mice was carried out 1 h before challenge. An acquired resistance against *S. aureus* infection was inhibited by injecting with anti-IFN-γ monoclonal antibody. However, anti-IFN-γ monoclonal antibody treatment failed to modulate anti-*S. aureus* IgM, IgG1 or IgG2a responses in these animals. These results demonstrated that IFN-γ is required for an acquired resistance against *S. aureus* infection in mice. However, IFN-γ induced during the challenge failed to affect the secondary antibody responses.

Introduction

Staphylococcus aureus is a major cause of community- and hospital-acquired skin, respiratory, endovascular, soft tissue, bone and joint infections (Lowy, 1998). Multiple-drug-resistant *S. aureus* has increased as a result of the use of antibiotics (Sieradzki *et al.*, 1999). To develop new strategies of prevention and therapy for multiple-drug-resistant *S. aureus* infection, elucidation of the pathogenesis of staphylococcal disease should be required.

Antibodies are reportedly involved in host defense against *S. aureus* infection. Previous studies reported that antibodies to capsular polysaccharides from *S. aureus* protected the animals from *S. aureus* infection (Fattom *et al.*, 1996; Lee *et al.*, 1988, 1997), and that immunization of staphylococcal enterotoxin. A provided protection against *S. aureus* sepsis (Nilsson *et al.*, 1999). In contrast, antibodies to protein A or teichoic acid derived from *S. aureus* were not protective in animal models of *S. aureus* infection (Greenberg D *et al.*, 1987; Greenberg *et al.*, 1989). Our recent studies demonstrated that antibodies to staphylococcal enterotoxin C and toxic shock syndrome toxin-1 were contributed to the protection mice from multiple-drug-resistant *S. aureus* infection (Hu *et al.*, 2003, 2005).

In addition to humoral immunity, cell-mediated immunity is also required for resistance against *S. aureus* infection (Nakane *et al.*, 1995; Zhao *et al.*, 1995; Verdrengh & Tarkowski, 1997, 2000; Sasaki *et al.*, 2000; Gomez *et al.*, 2002). Staphylococci and their products are capable of strongly inducing various cytokines and are capable of activating cellular immunity. Cytokines including interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-10 and IL-18 reportedly regulate host resistance against *S. aureus* infection (Nakane *et al.*, 1995; Zhao *et al.*, 1995; Verdrengh & Tarkowski, 1997; Sasaki *et al.*, 2000; Gomez *et al.*, 2002). IFN-γ-deficient mice, IFN-γ receptor-deficient mice and IL-4-deficient C57BL/6 mice increased survival rates of *S. aureus* infection compared with that of wild-type mice, whereas the development of septicemia by *S. aureus* infection was inhibited in IL-18-deficient mice (Zhao *et al.*, 1995; Hultgren *et al.*, 1999; Wei *et al.*, 1999; Sasaki *et al.*, 2000). Alternatively, IL-4-deficient 129Sv mice displayed high mortality compared with wild-type mice, and IFN-γ receptor-deficient mice and IL-18-deficient mice developed

significantly more severe septic arthritis as a result of *S. aureus* infection (Zhao *et al.*, 1995; Hultgren *et al.*, 1999; Wei *et al.*, 1999). These results indicated that cytokines have beneficial or harmful roles in *S. aureus* infection, depending on the stage of the disease, the state of the host immune response and the genetic background. Neutrophils and macrophages are reportedly principal effecter cells in host resistance against *S. aureus* infection (Verdrengh & Tarkowski, 1997, 2000).

Our previous study indicated that *S. aureus* infection induced T-helper 2 (Th2) responses, and that IL-4 and IL-10 might play a protective role through the regulation of IFN-γ in *S. aureus* infection and that IFN-γ-deficient mice were resistant to *S. aureus* infection compared with wild-type mice (Sasaki *et al.*, 2000). Therefore we were interested in the role of IFN-γ in an acquired host resistance against *S. aureus* infection in mice. In this study, we demonstrate that IFN-γ is important for the expression of an acquired resistance against *S. aureus* infection.

Materials and methods

Animals

C57BL/6 mice and IFN-γ-deficient mice (IFN-$\gamma^{-/-}$ mice) on a C57BL/6 × Sv129 background (Tagawa *et al.*, 1997), 8 to 10 weeks old, were used. C57BL/6 mice were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The animals were maintained under the specific-pathogen-free condition in the Institute for Animal Experiment, Hirosaki University School of Medicine. This study was carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

Bacteria

Staphylococcus aureus 834 and *Listeria monocytogenes* 1b 1684 were prepared as described previously (Nakane *et al.*, 1996; Sasaki *et al.*, 2000). *S. aureus* 834 is a clinical isolate and that produces toxic shock syndrome toxin-1 and staphylococcal enterotoxin C. In each experiment, *S. aureus* was cultured on tryptic soy agar (Difco Laboratories, Detroit, MI) for 24 h at 37 °C, inoculated into tryptic soy broth (Difco Laboratories) and incubated for another 15 h. The organisms were collected by centrifugation and resuspended in 0.01 M phosphate-buffered saline (PBS; pH 7.4). The concentration of resuspended cells was adjusted spectrophotometrically at 550 nm. *Listeria monocytogenes* grown in tryptic soy broth were dispensed and stored at −80 °C until use (Nakane *et al.*, 1996). Mice were infected with 10^6 or 10^7 CFU of *S. aureus* in PBS for immunization, and reinfected with 10^7 CFU of *S. aureus* in PBS 8 weeks later for challenge. In some experiments, *S. aureus* immunize mice were challenged with 5×10^4 CFU of *L. monocytogenes*.

Determination of the numbers of viable bacterial cells in the organs

The spleens and livers of infected animals were homogenized in PBS with a Dounce grinder (Asahi Glass Co., Tokyo, Japan). The numbers of viable *S. aureus* or *L. monocytogenes* were established by plating serial 10-fold dilutions of organ homogenates in PBS. Briefly, 0.1 mL of serially diluted organ homogenates were inoculated into tryptic soy agar plates and incubated at 37 °C. Colonies were routinely counted 18 to 24 h later.

In vivo depletion of endogenous cytokines

A hybridoma cell line secreting monoclonal antibody (mAb) against mouse IFN-γ [R4-6A2, rat immunoglobulin GI (IgG1)] was used. mAb found in the ascites fluid was partially purified by $(NH_4)_2SO_4$ precipitation (Nakane *et al.*, 1995). The mice were given a single intravenous injection of 1 mg of anti-IFN-γ mAb or normal rat globulin (NRG) as a control for the mAb 1 h before infection. NRG was prepared as described previously (Nakane *et al.*, 1995). All *in vivo* effects of mAb and NRG described were verified by the use of reagents tested by the *Limulus* amoebocyte lysate assay to contain < 0.1 ng per injected dose.

Cytokine assay

The IFN-γ assay was carried out by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (Nakane *et al.*, 1995). Purified rat antimouse IFN-γ mAb produced by hybridoma R4-6A2 and rabbit antirecombinant mouse IFN-γ sera were used for IFN-γ ELISA. All ELISAs were run with recombinant mouse IFN-γ produced and purified by Genentech Inc. (San Francisco, CA).

Preparation of staphylococcal cell components

Staphylococcus aureus cellular protein and cell wall fraction were prepared as follows: *S. aureus* 834 grown in tryptic soy broth was washed three times with PBS and resuspended in nonpyrogenic double-distilled water to make a 20% cell suspension [wet w/v (weight in volume)]. The cells were sonically disrupted with a sonic oscillator (TAITEC Co., Tokyo, Japan; 150 W, 20 kHz) for 20 min by ice-chilling and were centrifuged at 14 500 ***g*** for 60 min at 4 °C. The supernatant fluid obtained was sterilized by filtration through a Millipore filter (Millipore, Billerica, MA; pore size 0.2 nm). The precipitate by differential centrifugation was washed three times with nonpyrogenic double distilled water and autoclaved. All preparations were carried out under aseptic conditions. *S. aureus* cellular protein and cell wall fraction were stored at –80 °C.

Quantitation of *Staphylococcus aureus*-specific antibodies

The levels of IgM, IgG1 and IgG2a to *S. aureus* in mouse sera were measured by ELISAs as described previously (Hu *et al.*, 2003). A 96-well microplate was coated with 250 ng of *S. aureus* cellular protein in PBS containing 2.5 μg protein mL^{-1}. After blocking the plate with PBS containing 10% Blockace (Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan), serum specimen diluted 1 : 100 with PBS containing 10% Blockace was added to each well. To detect the anti-*S. aureus* antibodies, horseradish peroxidase-conjugated with goat antimouse IgM, IgG1, or IgG2a antibody (Southern Biotechnology Associates Inc., Birmingham, AL) were used. Positive control sera were prepared from *S. aureus* infected mice that were boosted intraperitoneally with 0.5 mg of *S. aureus* cellular protein on days 28 and 35 postinfection. On day 42 postinfection, mice were sacrificed and pooled sera arbitrarily assigned *S. aureus*-specific IgM, IgG1 and IgG2a titers of 10 U mL^{-1}, respectively, were included in each assay as a standard.

Preparation of sera and organ extracts for cytokine assays

Mice were injected intravenously with 100 μg of *S. aureus* cell wall fraction and were sacrificed 2 or 4 h postinjection. The spleens and livers of animals were homogenized in RPMI 1640 medium containing 1% (w/v) 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfate (CHAPS; Wako Pure Chemical Co., Osaka, Japan) as described previously (Sasaki *et al.*, 2000), and then clarified by centrifuging at 2000 ***g*** for 20 min. The organ extracts and sera were stored at –80 °C until cytokine assays were performed.

Statistical evaluation of the data

Data were expressed as the median, together with the range, and the Mann–Whitney nonparametric test was employed to detect the differences among the groups. IFN-γ titers were expressed as mean ± standard deviation.

Results

Expression of an acquired host resistance against *Staphylococcus aureus* infection in C57BL/6 mice and IFN-$\gamma^{-/-}$ mice

To evaluate the expression of an acquired host resistance against *Staphylococcus aureus* infection, C57BL/6 mice were immunized with 10^7 CFU of *S. aureus*, and the mice were challenged with 10^7 CFU of *S. aureus* after 8 weeks of immunization, and the numbers of *S. aureus* in the spleens and livers were determined on day 3 of the challenge (Fig. 1a). The numbers of *S. aureus* in the spleens and livers were significantly lower in the immunized mice than those in the

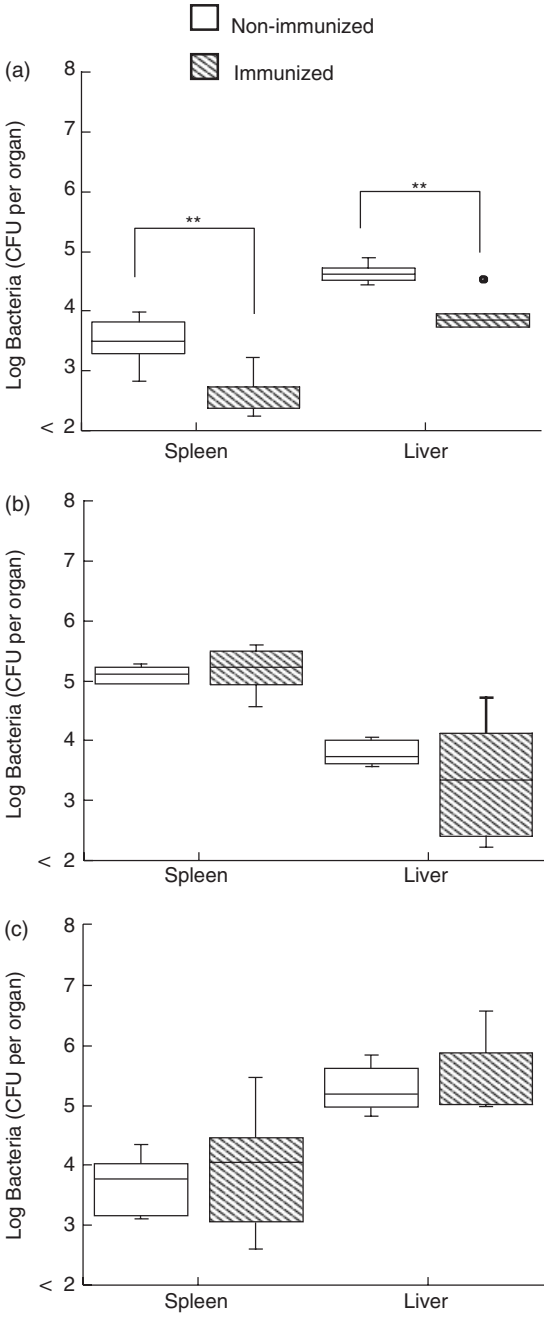

Fig. 1. Acquired host resistance against *Staphylococcus aureus* infection in C57BL/6 mice and gamma interferon (IFN-$\gamma^{-/-}$) mice. C57BL/6 mice (a,b) or IFN-$\gamma^{-/-}$ mice (c) were immunized with 10^7 CFU of *S. aureus*. After 8 weeks of immunization, mice were challenged with 10^7 CFU of *S. aureus* (a,c), or challenged with 5×10^4 CFU of *Listeria monocytogenes* (b). Bacterial numbers in the spleens and livers were determined on day 3 of the challenge of *S. aureus* and on day 2 of challenge of *L. monocytogenes*. For nonimmunized controls, age-matched naive mice were challenged with the same doses of bacteria. Data are expressed as the median together with the range of a group of six mice from two independent experiments. A double asterisk indicates a significant difference from the value for nonimmunized control group ($P < 0.01$).

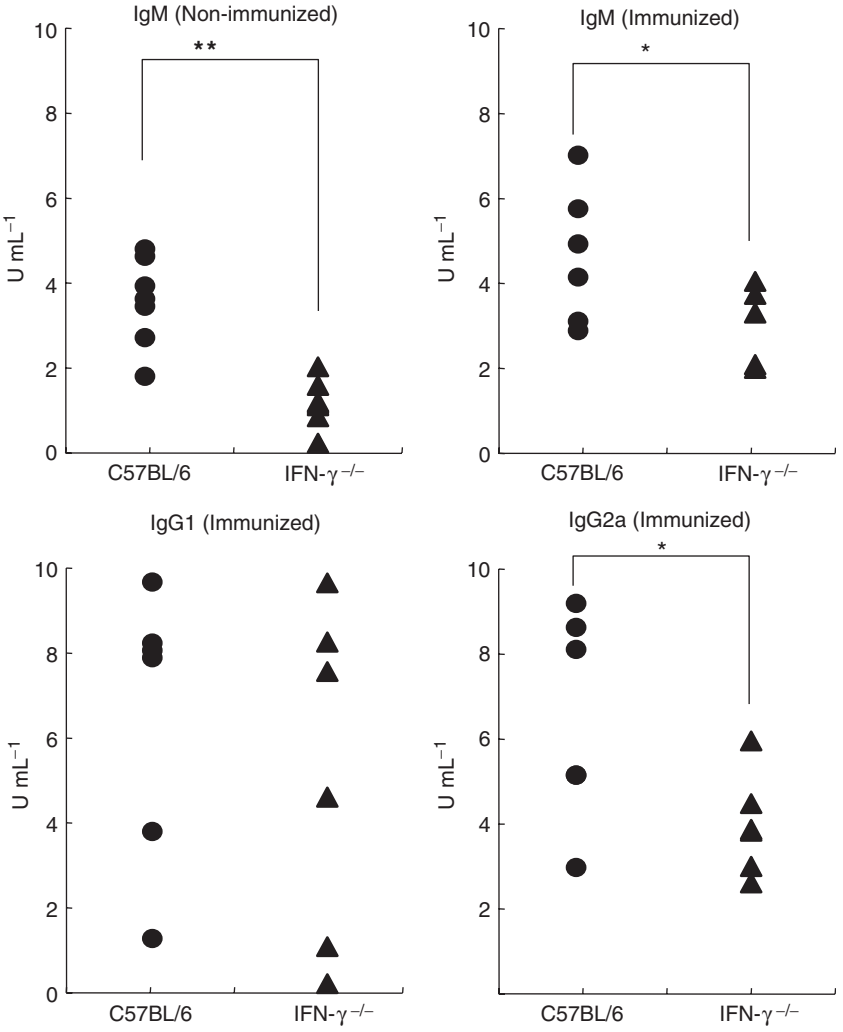

Fig. 2. Anti-*Staphylococcus aureus* antibody production in the sera obtained from *Staphylococcus aureus* infected gamma interferon (IFN-$\gamma^{-/-}$) mice. Sera of immunized or nonimmunized C57BL/6 mice or IFN-$\gamma^{-/-}$ mice were obtained on day 3 of *S. aureus* challenge. Anti-*S. aureus* immunoglobulin M (IgM), IgG1 and IgG2a in the sera were detected by enzyme-linked immunosorbent assays. Each point represents the value of individual animals of a group of six mice from two independent experiments. Single and double asterisks indicate significant differences from the value for nonimmunized control group at $P < 0.05$ and at $P < 0.01$, respectively.

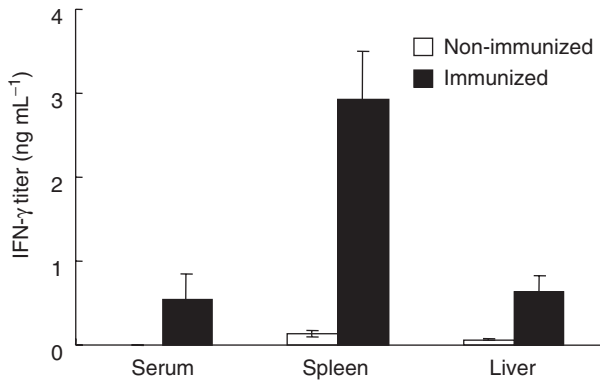

Fig. 3. Antigen-specific gamma interferon (IFN-γ) induction in the sera and organs obtained from *Staphylococcus aureus*-immunized mice. The sera, spleens and livers of nonimmunized mice or immunized mice were obtained at 4 h after injection of *Staphylococcus aureus* cell wall fraction. Data are expressed as the mean and standard deviation for a group of four mice. The results were reproduced in three repeated experiments.

nonimmunized mice ($P < 0.01$). To exclude the possibility that nonspecific activation of host resistance induced by the immunization may still remain 8 weeks later, we estimated whether the enhancement of host resistance against infection with an unrelated bacterium, *Listeria monocytogenes*, would be observed in *S. aureus* immunized mice. The *S. aureus* immunize C57BL/6 mice were infected with 5×10^4 CFU of *L. monocytogenes* and the numbers of *Listeria monocytogenes* in the spleens and livers were determined on day 2 of infection (Fig 1b). The numbers of *L. monocytogenes* in the spleens and livers of immunized mice was comparable to those of nonimmunized mice. Moreover, to elucidate the role of in IFN-γ in an acquired resistance against *S. aureus* infection, IFN-$\gamma^{-/-}$ mice were immunized and challenged at the same schedule. The numbers of *S. aureus* in the spleens and livers of IFN-$\gamma^{-/-}$ immunized mice after challenge were comparable to those in nonimmunized IFN-$\gamma^{-/-}$ mice (Fig. 1c).

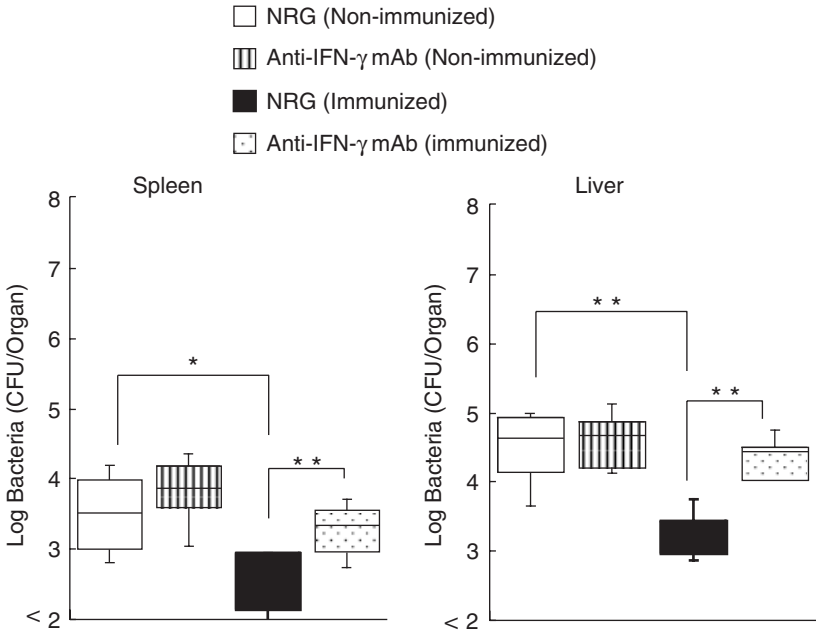

Fig. 4. Effect of *in vivo* administration of monoclonal antibodies (mAb) against gamma interferon (IFN-γ) before the secondary challenge on an acquired host resistance to *Staphylococcus aureus* infection. Mice were immunized with 10^6 CFU of *S. aureus*. After 8 weeks of immunization, mice were injected with normal rat globulin (NRG) or anti-IFN-γ mAb 1 h before challenge with 10^7 CFU of *S. aureus*. For nonimmunized controls, age-matched naive mice were injected with NRG or anti-IFN-γ mAb 1 h before challenge of *S. aureus*. Data are expressed as the median together with the range of a group of eight mice from two independent experiments. Single and double asterisks indicate significant differences of values between nonimmunized groups and immunized groups at $P < 0.05$ and $P < 0.01$, respectively.

Anti-*Staphylococcus aureus* antibody production in the sera obtained from *Staphylococcus aureus* infected C57BL/6 mice and IFN-$\gamma^{-/-}$ mice

To estimate antibody responses to *S. aureus* in immunized mice, sera were obtained on day 3 of the challenge from nonimmunized and immunized groups of C57BL/6 mice and IFN-$\gamma^{-/-}$ mice, and anti-*S. aureus* IgM, IgG1, and IgG2a titers were determined (Fig. 2). In the nonimmunized mice, anti-*S. aureus* IgM production in IFN-$\gamma^{-/-}$ mice was significantly lower than that in C57BL/6 mice ($P < 0.01$). Neither anti-*S. aureus* IgG1 nor IgG2a was detected in the nonimmunized mice (data not shown). In the immunized mice, IFN-$\gamma^{-/-}$ mice produced low levels of anti-*S. aureus* IgM and IgG2a compared with C57BL/6 mice ($P < 0.05$), whereas anti-*S. aureus* IgG1 production in C57BL/6 mice was comparable to that of IFN-$\gamma^{-/-}$ mice.

Induction of IFN-γ in the sera and organs obtained from *Staphylococcus aureus*-immunized mice

In our previous studies (Nakane *et al.*, 1995; Sasaki *et al.*, 2000), we demonstrated that IFN-γ is induced in the spleens of *S. aureus* infected mice. Therefore, we verified the antigen-specific induction of IFN-γ in the immunized mice. Mice were immunized with 10^6 CFU of *S. aureus*, and mice were injected with *S. aureus* cell wall fraction intravenously after 8 weeks of immunization. The production of IFN-γ in the sera, spleens and livers of mice was determined 2 or 4 h later (Fig. 3). The marginal level of IFN-γ production was observed in the sera and organs of nonimmunized mice 4 h postinjection. In contrast, IFN- γ was detected in the sera, spleens and livers of immunized mice at 4 h postinjection. No IFN-γ was detected in the organs at 2 h.

Effect of *in vivo* administration of anti-IFN-γ mAb before challenge on an acquired host resistance to *Staphylococcus aureus* infection

Finally, to determine the role of endogenous IFN-γ induced during a challenge with *S. aureus* in the acquired resistance, the immunized mice were injected with anti-IFN-γ mAb 1 h before challenging with 10^7 CFU of *S. aureus*, and the numbers of *S. aureus* in the spleens and livers were estimated 3 days later. No significant differences in the numbers of *S. aureus* were observed in the spleens and livers of nonimmunized mice between the NRG-injected group and the anti-IFN-γ mAb-injected group (Fig. 4). The numbers of *S. aureus* in the spleens ($P < 0.05$) and livers ($P < 0.01$) of immunized mice were significantly lower than those of nonimmunized mice in the NRG-pretreated group. In the immunized mice, the numbers of *S. aureus* in the organs of NRG-pretreated mice were significantly lower than those of anti-IFN-γ mAb-injected mice ($P < 0.01$). Despite the lack of acquired host resistance in anti-IFN-γ mAb-pretreated immunized mice, there was no significant difference in the production of anti-*S. aureus* IgM, IgG1, or IgG2a between NRG-pretreated and anti-IFN-γ mAb-pretreated immunized mice (Fig. 5). Anti-*S. aureus* IgM titers in the nonimmunized mice were comparable between the anti-IFN-γ mAb-injected group and the NRG-injected group.

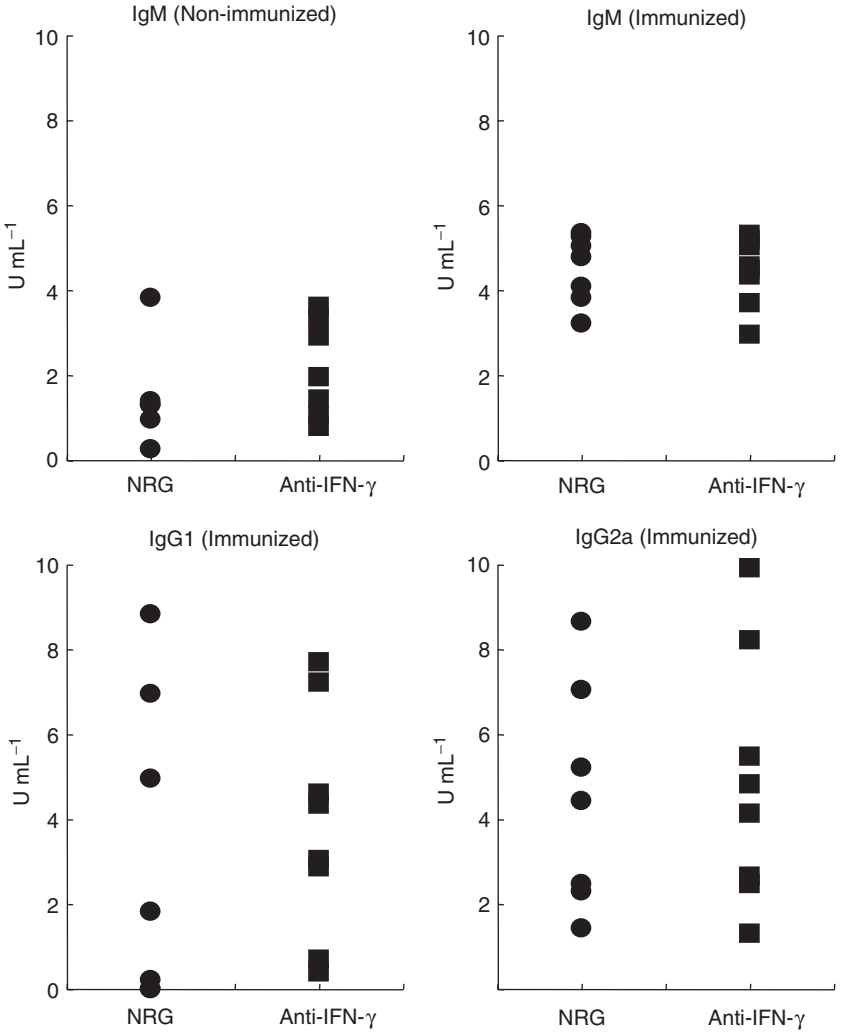

Fig. 5. Effect of *in vivo* administration of monoclonal antibodies (mAb) against gamma interferon (IFN-γ) before a secondary challenge on anti-*Staphylococcus aureus* antibody production. Sera of normal rat globulin (NRG)-pretreated or anti-IFN-γ mAb-pretreated immunized mice were obtained on day 3 of *S. aureus* challenge. Anti-*S. aureus* immunoglobulin M (IgM), IgG1 and IgG2a in the sera were detected by enzyme-linked immunosorbent assays. For nonimmunized controls, age-matched naive mice were injected with NRG or anti-IFN-γ mAb 1 h before challenge. Each point represents the value of individual animals of a group of eight mice from two independent experiments.

Discussion

Our present study demonstrated that IFN-γ is involved in an acquired resistance against *Staphylococcus aureus* infection, but that IFN-γ might not be involved in secondary anti-*S. aureus* antibody responses.

When C57BL/6 mice were immunized with *S. aureus*, a significant decrease in the bacterial growth was observed in the spleens and livers of mice immunized with *S. aureus* (Figs 1a, 3 and 4). These results suggest that immunization with *S. aureus* can induce an acquired resistance against *S. aureus* infection. The challenge was carried out at 8 weeks after primary infection of *S aureus*. To exclude the possibility that the enhanced resistance against *S. aureus* in the immunized mice is nonspecific, the immunized mice were challenged with an unrelated bacterium, *Listeria monocytogenes*, instead of *S. aureus*. However, *S. aureus* immunized mice showed no enhanced resistance to *L. monocytogenes* (Fig. 1b), suggesting that the enhanced resistance in the immunized mice might be specific for *S. aureus*.

Our previous study (Sasaki *et al.*, 2000) demonstrated that IFN-γ plays a detrimental role in host resistance against a primary infection with *S. aureus* because an increase in survival rates, a decrease in bacterial numbers in the organs and an amelioration of histological abnormalities in the organs were observed in IFN-$\gamma^{-/-}$ mice compared with those in IFN-$\gamma^{+/+}$ mice. Alternatively, Zhao & Tarkowski (1995) and Zhao *et al.* (1995) reported that IFN-γ is involved in host resistance against primary *S. aureus* infection depending on the state of host response. In this study, endogenous IFN-γ production was observed in the sera, spleens and livers at 4 h after injection of the cell wall fraction of *S. aureus* (Fig. 3). Moreover, the immunized effect on the bacterial elimination from the spleens and livers was diminished in IFN-$\gamma^{-/-}$ mice (Fig. 1c). These results suggested that IFN-γ is involved in an acquired host resistance against *S. aureus* infection.

It is possible that IFN-γ might be involved in both induction and expression of an acquired resistance against *S. aureus* infection. However, the role of IFN-γ is indistinguishable either time point in IFN-$\gamma^{-/-}$ mice. Therefore, we addressed the implication of IFN-γ induced by a challenge with *S. aureus* in an acquired resistance against *S. aureus* infection. A single injection of anti-IFN-γ mAb to mice was carried out 1 h before a challenge with *S. aureus* to neutralize IFN-γ induced after the challenge. The numbers of *S. aureus* in the spleen and liver of anti-IFN-γ mAb-treated mice were significantly increased compared with those in NRG-treated mice (Fig. 4). The results indicated that an acquired host resistance against *S. aureus* infection was inhibited in mice that had been injected with anti-IFN-γ mAb before challenge, suggesting that IFN-γ induced during the challenge may be involved in the expression of an acquired resistance against *S. aureus* infection.

IFN-γ is involved in the regulation of not only cell-mediated immunity but also of Ig class switch (Lin & Chen, 1993). IFN-γ promotes B cells into IgG2a-secreting cell (Banchereau *et al.*, 1994a, b; Lin & Chen, 1993). The present result showed that the lower level of anti-*S. aureus* IgG2a production was observed in IFN-$\gamma^{-/-}$ mice compared with those in wild-type mice (Fig. 2). Moreover, anti-*S. aureus* IgM production in the *S. aureus* immunize group and the nonimmunized group of IFN-$\gamma^{-/-}$ mice was also reduced compared with that in C57BL/6 mice (Fig. 2). These results suggest that IFN-γ is involved in anti-*S. aureus* IgM and IgG2a production. To investigate further that the temporary presence of IFN-γ is required for anti-*S. aureus* Ig production in the secondary antibody responses, anti-*S. aureus* Ig responses were estimated in C57BL/6 mice in which anti-IFN-γ mAb had been injected before the challenge (Fig. 5). No significant effect was observed when anti-IFN-γ mAb had been injected before the challenge, suggesting that the presence of IFN-γ is required for anti-*S. aureus* IgM and IgG2a production in the primary response.

Taken together, our present study demonstrated that IFN-γ is required for the expression of an acquired resistance against *S. aureus* infection in mice. However, IFN-γ induced during the challenge failed to affect the secondary Ig responses.

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