Protective Role of Nitric Oxide in *Staphylococcus aureus* Infection in Mice

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This study was carried out to determine the role of nitric oxide (NO) in *Staphylococcus aureus* infection in mice. NO production in spleen cell cultures was induced by heat-killed *S. aureus*. Expression of mRNA of the inducible isoenzyme of NO synthase (iNOS) was induced in the spleens and kidneys of *S. aureus*-infected mice. When mice were treated with monoclonal antibodies (MAbs) against tumor necrosis factor alpha (TNF-α) or gamma interferon (IFN-γ) before *S. aureus* infection, the induction of iNOS mRNA expression in the kidneys was inhibited. These MAbs also inhibited NO production in spleen cell cultures stimulated with heat-killed *S. aureus*. NO production in the spleen cell cultures and levels of urinary nitrate plus nitrite were suppressed by treatment with aminoguanidine (AG), a selective inhibitor of iNOS. The survival rates of AG-treated mice were significantly decreased by either lethal or sublethal *S. aureus* infections. However, an effect of AG administration on bacterial growth was not observed in the spleens and kidneys of mice during either type of infection. Production of TNF-α and IFN-γ was not affected by AG treatment in vitro and in vivo. These results suggest that NO plays an important role in protection from lethality by the infection, but the protective role of NO in host resistance against *S. aureus* infection was not proved. Moreover, our results show that TNF-α and IFN-γ regulate NO production while NO may not be involved in the regulation of the production of these cytokines during *S. aureus* infection.

Nitric oxide (NO) and other reactive nitrogen intermediates derived from arginine have been shown to play a critical role in diverse functions, including the regulation of blood pressure and flow (18, 30), neurotransmission (9), and immune responses, including some of the effecter functions of macrophages, such as cytotoxicity toward tumor cells and microorganisms (20, 28, 29). NO-dependent functions of macrophages are known to be regulated by the inducible form of NO synthase (iNOS). NO produced by iNOS of macrophages has been believed to play an important role in host defense against intracellular pathogens (1–3, 12, 16, 17, 21, 23, 24, 33, 34). However, repeated analyses have revealed variations among different experimental setups and analyzed species. For example, recent studies showed that iNOS is not involved in protection against infection by some bacteria, such as *Listeria monocytogenes* (6, 32). Similarly, it was believed that NO induced by iNOS might be important in the pathogenesis of endotoxin-induced shock because this compound mediates profound hypotension that is refractory to vasoconstrictor therapy (18). However, by using iNOS gene knockout mice, iNOS was shown to be involved in only a limited number of endotoxin-induced shock cases (22, 23). Alternatively, NO is reportedly protective against endotoxin-induced shock (35) as well as shock induced by a product of *Staphylococcus aureus*, staphylococcal enterotoxin B (SEB) (7). Proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) induce the transcription of iNOS (19), and in vivo studies have shown that these cytokines play a major role in upregulating NO production during microbial infections (10, 13). Our previous study (27) showed that TNF-α plays a protective role but that IFN-γ plays a detrimental role in *S. aureus* infection in mice. Moreover, Zhao and Tarkowski (37) reported that IFN-γ plays a protective role in the early phase of *S. aureus* infection but is eventually harmful. The present study was designed to investigate the role of NO in *S. aureus* infection and reciprocal regulation of NO and cytokines such as TNF-α and IFN-γ. This report documents that administration of aminoguanidine (AG), a potent and selective inhibitor of iNOS (5), showed no significant effect on the growth of *S. aureus* in the spleens and kidneys of the infected mice but led to increased mortality of the mice. Moreover, we report that the induction of iNOS in spleens and kidneys of *S. aureus*-infected mice was upregulated by TNF-α and IFN-γ but that NO might not be involved in the regulation of the production of these cytokines.

### MATERIALS AND METHODS

**Mice.** Female ddY outbred mice (age 5 to 9 weeks; obtained from SLC, Hamamatsu, Shizuoka, Japan) were used.

**Bacteria.** *S. aureus* 834 was prepared as described previously (27). In each experiment, bacteria were cultured on tryptic soy agar (Difco Laboratories, Detroit, Mich.) for 24 h at 37°C, inoculated into tryptic soy broth (Difco), and incubated for another 15 h. The organisms were collected by centrifugation and resuspended in 0.85% NaCl. The concentration of resuspended cells was adjusted spectrophotometrically at 550 nm. Mice were infected intravenously with 0.2 ml of a solution containing 10⁷ (0.25 50% lethal dose) or 10⁸ (2.5 50% lethal doses) of viable *S. aureus* cells in saline. For in vitro studies, an *S. aureus* cell suspension (10⁹ cells/ml in saline) which had been boiled for 10 min was used as heat-killed *S. aureus*.

**Determination of numbers of viable *S. aureus* cells in organs.** The spleens and kidneys of infected animals were homogenized in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 1% (wt/vol) 3-cholamidopropyl-dimethylammonio)-1-propanesulfate (CHAPS, Wako Pure Chemical Co., Osaka, Japan) with a Dounce grinder (27). Numbers of viable *S. aureus* cells were established by plating serial 10-fold dilutions of organ homogenates or whole blood in 0.01 M phosphate-buffered saline (pH 7.4) on tryptic soy agar. Colonies were routinely counted 24 h later.

**Inhibitor administration.** The administration of a 1% (wt/vol) solution of AG hemisulfate (Sigma Chemical Co., St. Louis, Mo.) in sterile drinking water was...
started from 7 to 0 days before infection and was continued throughout the study (3). The control mice were given drug-free drinking water.

**Spleen cell cultures.** Mouse spleens were removed aseptically, and spleen cells were squeezed. The cell suspension was filtered through stainless steel mesh (size 100), and red thromocytes were lysed with 0.85% NH₄Cl and then washed three times with RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum, 0.075% sodium bicarbonate, and 2 mM L-glutamine. The washed cells, in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.075% sodium bicarbonate, 2 mM L-glutamine, 200 U of penicillin G per ml, and 200 μg of streptomycin per ml, were cultured at 10⁷/well in 24-well tissue culture plates (Greiner, Frickenhausen, Germany) with 10⁴ cells of heat-killed *S. aureus* per well at 37°C in a humidified 5% CO₂ incubator. The culture supernatant was harvested at 24 and 48 h of incubation and stored at -80°C until the nitrite and cytokine assays were performed.

**Measurement of nitrite in culture supernatants.** The nitrite concentration in the culture supernatant was assayed in a 96-well microplate (Nunc, Roskilde, Denmark) by mixing 100 μl of culture supernatant with 100 μl of Griess reagent (11). The Δ₅₄₀ was measured 10 min later, and the concentration was determined by referring to a standard curve for 1 to 35 μM sodium nitrite.

**Urinary nitrate-plus-nitrite assay.** Levels of nitrate plus nitrite in urine were measured as described previously (2, 11, 32). Urine was diluted 10- or 50-fold with 0.02 M Tris-HCl buffer (pH 7.6). Nitrate was reduced to nitrite by addition of 0.14 U of *Aspergillus* spp. nitrate reductase (Sigma) per ml at room temperature for 3 h in the presence of 0.5 mM B-NAPDH (Nakarai Chemical Co., Kyoto, Japan). Nitrite concentrations were then determined by mixing treated urine with Griess reagent, as described above. Values were corrected for efficiency of conversion of nitrate to nitrite by measuring the conversion of standard concentrations of nitrate to nitrite.

**Reverse transcription-PCR.** Total RNA was isolated from pieces of spleens and kidneys (0.05 g each) by a guanidium thiocyanate-phenol-chloroform single-step method (4). Preparation of cDNA by reverse transcription was performed in the following way. Total RNA, as described above (1 μg in a volume of less than 10 μl), was mixed with 4 μl of reverse transcription buffer (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.), 4 μl of 1.25 mM deoxynucleoside triphosphates (Pharmacia Biotechnology AB, Uppsala, Sweden), 0.5 μl of random primer (Takara Shuzo Co., Otsu, Shiga, Japan), and distilled water to make the final volume 19 μl. The mixture was overlaid with 100 μl of mineral oil (Aldrich Chemical Co., Milwaukee, Wis.) and heated at 80°C for 5 min. After cooling the overlaid mixture on ice, 1 μl (200 U) of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) was added. The mixture was incubated at 37°C for 60 min and then heated at 85°C for 5 min.

**PCR amplification** was performed with a Program Temp Control System (PC-700; ASTEC, Inc., Fukuoka, Japan) as reported previously (26). Briefly, the reaction mixture consisted of 20 μl of sample cDNA, 8 μl of PCR amplification buffer (Gibco-BRL), 4 μl of 1.25 mM deoxynucleoside triphosphates, 1 μl of 20 μM 5’ and 3’ primers, 0.5 μl (2.5 U) of Taq DNA polymerase (Gibco-BRL), and 47 μl of distilled water to make the final volume 80.5 μl. The following oligonucleotides were used: for iNOS, 5'-ATGGCTGGCCCTGGAAGTTTC-3' and 5'-TACTTTGGAGTCATTGC-3' for TNF-α, 5'-CCGCAAGCGTTTCTCTTGAGAATCTCAGTG-3' for TNF-β, 5'-CTACTTGGGATCTGGC-3' and 5'-CAATCTGAGGCTCAGTAAG-3' for IFN-γ, 5'-AGGCGTCTGACTGAACTCAGATTGTAG-3' and 5'-ATGGCTTGCCCCTGGAAGTTTC-3' for IFN-α, 5'-GGCAGGTCGGATTTG-3' and 5'-AGCACATACGTACGACGAC-3'. These primers were made to our specifications by Hokkaido System Science Co., Sapporo, Japan. The predicted sizes of amplified products for iNOS, TNF-α, TNF-β, and IFN-γ were 317, 308, 244, and 277 bp, respectively. The PCR cycle was run under the following conditions: DNA denaturation at 93°C for 29 s, primer annealing at 55°C for 29 s, and DNA extension at 72°C for 2 min. After 30 cycles of amplification, the reaction was terminated.

**Agarose gel electrophoresis.** The PCR products were analyzed by agarose gel electrophoresis in a horizontal 4.0% agarose gel (NuSand Bioproducts, Rockland, Maine) in 1X Tris-acetate-EDTA (TAE) buffer supplemented with 0.005% ethidium bromide for DNA staining. Undiluted PCR product (6 μl) and 1 μl of bromophen blue were applied to each well. Gels were run in 1X TAE buffer at 100 V for 50 min. The PCR products were visualized and photographed on a UV transilluminator (Fotodyne Inc., New Berlin, Wis.).

**In vivo depletion of endogenous cytokines.** Hybridoma cell lines secreting monoclonal antibodies (MAbs) against mouse IFN-γ (R4-6A2; rat immunoglobulin G1) and mouse TNF-α (MP6-XT22; rat immunoglobulin G1) were used. MAbs found in the ascites fluid were partially purified by (NH₄)₂SO₄ precipitation (27). The mice were given single intravenous injections of 1 mg of anti-TNF-α or anti-IFN-γ MAb 1 h before infection (27). Normal rat globulin (NRC) was injected as a control for the Mabs. NRG was prepared as described previously (27). All in vivo effects of MAbs and NRG described herein were verified by referring to a standard curve for 1 to 35 μg of recombinant mouse IFN-γ produced and purified by Genentech, Inc., South San Francisco, Calif.

**TNF assay.** TNF determinations were made by double-sandwich enzyme-linked immunosorbent assay (ELISA), as described previously (27). Purified rat anti-mouse IFN-γ MAb produced by hybridoma R4-6A2 and rabbit anti-recombinant mouse IFN-γ serum (Genzyme) were used for the ELISA. All ELISAs were run with recombinant mouse IFN-γ produced and purified by Genentech, Inc., South San Francisco, Calif.

**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 in the organ bacterial counts, nitrite concentrations, or cytokine titers between the control and experimental groups. The generalized Wilcoxon test was used to determine the significance of differences in survival rates. Each experiment was repeated at least three times and accepted as valid only when the trials showed similar results.

**RESULTS**

**Induction of iNOS mRNA expression and NO production during *S. aureus* infection.** Mice were infected with 2.5 50% lethal doses of *S. aureus* cells, and iNOS mRNA expression in the spleen and kidneys was investigated by reverse transcription-PCR (Fig. 1). Neither organ of uninfected mice expressed iNOS mRNA, whereas the transcripts were already detected in the spleens and kidneys of mice at 3 h after *S. aureus* infection, and expression was also observed 24 and 72 h later (data not shown). Next, we assessed the induction of NO production by *S. aureus*, nitrite concentrations in the supernatants of uninfected-mouse spleen cell cultures exposed to heat-killed *S. aureus* were determined after 24, 48, and 72 h of incubation (Fig. 2). Concentrations of NO were higher in heat-killed *S. aureus*-stimulated cultures than in unstimulated cultures at 48 and 72 h of incubation (*P < 0.01*).

**Effect of in vivo administration of MAbs against TNF-α and IFN-γ on iNOS mRNA expression in organs of *S. aureus*-infected mice.** TNF-α and IFN-γ are known to be involved in the induction of iNOS (19). We investigated the regulation of NO production by these cytokines. First, the kinetics of in vivo induction of TNF-α, IFN-γ, and iNOS mRNA after *S. aureus* infection were investigated. iNOS mRNA expression in the organs was determined in parallel with expression of TNF-α and IFN-γ mRNA (Fig. 1). Next, we investigated the in vivo effect of MAbs against TNF-α and IFN-γ on iNOS mRNA expression. One milligram of anti-TNF-α MAb, anti-IFN-γ MAb, or NRG was injected into mice 1 h before induction of a lethal *S. aureus* infection, and iNOS mRNA expression in the spleens and kidneys was investigated 24 h after infection (Fig. 1).
was significantly decreased compared with that of the controls were given sterilized drinking water containing 1% AG, while production was markedly inhibited (1 or 10 mM AG (Fig. 4), heat-killed in vivo. When mouse spleen cells were cultured in the presence of effect of AG on NO production was determined in vitro and in impact on blood pressure (5). In preliminary experiments, the potent and selective inhibitor of iNOS which has a minimal (108 cells/ml). Nitrite concentrations in the supernatant fluids were determined at various times during cultivation. Each result represents the mean ± standard deviation of data from two experiments. An asterisk indicates a significant difference for the unstimulated specimens at a P value of <0.05.

3). Pretreatment with neither MAb affected iNOS mRNA expression in the spleen, while expression in the kidneys was suppressed by administration of anti-TNF-α or anti-IFN-γ MAb.

Effect of MAb against TNF-α and IFN-γ on NO production in spleen cell cultures stimulated with S. aureus. In the above-described experiments, no effect of MAb against TNF-α or IFN-γ on iNOS mRNA expression in the spleen was observed. We are unable to evaluate the difference in iNOS mRNA levels if these MAbs inhibited expression incompletely, because the reverse transcription-PCR assay used in this study is not quantitative. Therefore, we investigated the effect of these MAb against NO production induced by heat-killed S. aureus in spleen cell cultures (Table 1). NO production was significantly inhibited in the presence of anti-TNF-α or anti-IFN-γ MAb (P < 0.01). However, the combination of both MAb showed no synergistic effect.

Inhibition of NO production by AG. AG is reportedly a potent and selective inhibitor of iNOS which has a minimal impact on blood pressure (5). In preliminary experiments, the effect of AG on NO production was determined in vitro and in vivo. When mouse spleen cells were cultured in the presence of 1 or 10 mM AG (Fig. 4), heat-killed S. aureus-induced NO production was markedly inhibited (P < 0.05). Next, test mice were given sterilized drinking water containing 1% AG, while the control animals received drug-free drinking water. The urinary nitrite-platinum-nitrate concentration of AG-treated mice was significantly decreased compared with that of the controls (mean ± standard deviation, 201 ± 25 μM versus 555 ± 102 μM; P < 0.01).

Effect of AG on lethal and sublethal infections with S. aureus. To investigate the role of NO in S. aureus infection in vivo, mice were given sterilized drinking water containing 1% AG until the experiment was halted. Mice were divided into four groups; the first three groups were given AG beginning on days −7, −3, day 0 of S. aureus infection, respectively, while mice in the fourth group were given drug-free water during the entire experiment. Uninfected mice never died, and a decrease in body weight was never observed when drinking water containing 1% AG was given continuously. In mice with lethal infections, all of the animals treated with drug-free water died within 10 days while the survival period was significantly shortened in mice that were given AG from day −7 to 3 on (P < 0.01) (Fig. 5A). When mice were infected with 107 CFU of S. aureus cells, which is equivalent to 0.25 50% lethal dose, 80% of the control mice survived, while survival rates of AG-treated mice were significantly decreased even when AG treatment was begun on day −7 (P < 0.01), day −3 (P < 0.05), or day 0 (P < 0.05) (Fig. 5B).

Effect of AG on resistance to lethal and sublethal infections with S. aureus. We investigated whether the decrease in survival rates of AG-treated mice might be due to inhibition by the drug of host resistance to S. aureus infection. Mice received sterilized drinking water containing 1% AG beginning on day

![FIG. 2. NO production in spleen cell cultures stimulated with S. aureus. Spleen cells (10^9 cells/ml) obtained from naive mice were cultured with (filled bars) or without (hatched bars) heat-killed S. aureus (10^9 cells/ml). Nitrite concentrations in the supernatant fluids were determined at various times during cultivation. Each result represents the mean ± standard deviation of data from two experiments. An asterisk indicates a significant difference for the unstimulated specimens at a P value of <0.05.](http://iai.asm.org/)

![FIG. 4. Effect of AG on NO production in spleen cell cultures stimulated with S. aureus. Spleen cells obtained from naive mice were stimulated with heat-killed S. aureus in the presence of 1 mM (open bars) or 10 mM (filled bars) AG. The control cultures contained no AG (hatched bars). Each result represents the mean ± standard deviation of data from two experiments. An asterisk indicates a significant difference for the unstimulated specimens at a P value of <0.05.](http://iai.asm.org/)
of infection with a lethal dose of \textit{S. aureus} cells, and the bacterial numbers in the blood, spleens, and kidneys of mice were determined on days 1 and 3 after infection (Fig. 6). There were no differences in the numbers of bacteria in the blood, spleens, and kidneys between AG-treated mice and the control animals. Similarly, on days 2 and 7 after infection with a sub-lethal dose of \textit{S. aureus} cells, the numbers of bacterial cells in the blood and organs of AG-treated mice were comparable to those of animals receiving the drug-free water (Fig. 7).

**Effect of AG on \textit{S. aureus}-induced IFN-\gamma and TNF-\alpha production.** NO reportedly inhibits IFN-\gamma production by T helper 1 cells (33). Therefore, we investigated the regulation of NO in IFN-\gamma and TNF-\alpha production induced by \textit{S. aureus}. Mouse spleen cells were incubated with heat-killed \textit{S. aureus} in the presence or absence of AG, and the titers of IFN-\gamma and TNF-\alpha in the culture supernatants were determined 24 and 48 h later (Table 2). The production of neither cytokine was significantly inhibited by 1 or 10 mM AG. Next, the in vivo effect of AG on endogenous IFN-\gamma and TNF-\alpha production induced by \textit{S. aureus} infection was investigated. Mice were given sterilized drinking water containing 1% AG starting on day −3 of the lethal infection, and the expression of IFN-\gamma and TNF-\alpha mRNA in the spleens and kidneys was determined by reverse transcription-PCR at 24 h postinfection. The induction of these cytokine mRNAs was observed in AG-treated mice as well as in the controls (data not shown). Moreover, the levels in the spleens and kidneys of these animals were determined by ELISA. No significant effect of AG on TNF-\alpha concentrations in these organs was observed (data not shown), while IFN-\gamma was not detected in either sample.

**DISCUSSION**

Our present study demonstrated that NO production by iNOS, which is induced by \textit{S. aureus} infection, is important in the protection of mice against death from \textit{S. aureus} infection.

When mice were infected with a lethal dose of \textit{S. aureus} cells, the expression of iNOS mRNA in the spleens and kidneys was already induced at 3 h postinfection and persisted thereafter (Fig. 1), indicating that iNOS expression is induced immediately after \textit{S. aureus} infection. The induction of NO production by \textit{S. aureus} was confirmed in spleen cell cultures stimulated with heat-killed \textit{S. aureus} (Fig. 2). TNF-\alpha and IFN-\gamma play an important role in the induction of iNOS expression and upregulation of NO production (10, 13, 19). With regard to the regulation of NO production in vivo, it was reported that splenic iNOS mRNA expression and serum NO\textsubscript{3−2} levels were partially reduced by administration of MAbs against TNF-\alpha or IFN-\gamma and completely suppressed by treatment with both MAbs (15, 36) and that urinary NO\textsubscript{3−2} production was completely inhibited by administration of anti-TNF-\alpha MAb as well as anti-IFN-\gamma MAb (13). In this study, the alternative administration of anti-TNF-\alpha or anti-IFN-\gamma MAb resulted in suppression of iNOS mRNA expression in the kid-
neys (Fig. 3). Although an obvious inhibition of iNOS mRNA expression by the administration of MAbs against TNF-α or IFN-γ was not observed in the spleen (Fig. 3), NO production in heat-killed S. aureus-stimulated spleen cell cultures was significantly inhibited by either MAb (Table 1). These results suggest that both cytokines are required for the induction of NO production in the spleen and kidneys.

NO production is one of the principle mechanisms of macrophage cytotoxicity to pathogens, especially to intracellular organisms (29). However, the roles of NO induced by iNOS in microbial infections are diverse. Deterioration of the host defense against infections with Mycobacterium tuberculosis and Leishmania major has been reported in iNOS gene knockout mice (24, 34), while the elimination of L. monocytogenes from hosts is independent of NO (6, 32). Moreover, the protective roles of NO are reportedly diverse for different tissues in Toxoplasma gondii infection (31) and for genetically susceptible and resistant mouse strains in Legionella pneumophila infection (14). In this study, we estimated the role of NO by treatment with AG (5), which is reportedly a selective inhibitor, in vivo and in vitro. NO production was significantly inhibited in vivo and in vitro (Fig. 4) by the drug. The survival period in AG-treated mice with lethal S. aureus infections (Fig. 5A) was significantly shortened, and the survival rates were significantly decreased in AG-treated mice during sublethal infection (Fig. 5B), suggesting that NO might play a beneficial role in S. aureus infection. We presumed that NO is important in host defense against S. aureus infection. However, no significant effect on bacterial growth in the organs was observed in AG-treated mice with either lethal or sublethal infections (Fig. 6 and 7). These results suggest that the modulation of bacterial growth in the organs may not be involved in a protective role of NO.

Our present study showed that NO might play a protective role in S. aureus infection. However, we could not show a role for NO in the elimination of bacteria from the organs. It is presumable that NO is involved in protection from death in the infected hosts. Florquin et al. (7) demonstrated that NO is protective against SEB-induced shock; almost all of the mice coinjected with N-nitro-L-arginine methyl ester, an NOS inhibitor, and SEB died, whereas no lethality occurred in mice injected with SEB alone. Several observations indicated that both TNF-α and IFN-γ are critically involved in the pathogenesis of SEB-induced shock (7, 8, 25). Florquin et al. reported that NO could downregulate TNF-α and IFN-γ production and that the vasoactive properties of NO, as well as its ability to inhibit platelet aggregation and adhesion, might be important in counteracting the prothrombotic properties of TNF-α and IFN-γ (7). The S. aureus strain used in the present study produces SEC and toxic shock syndrome toxin 1, both of which act as superantigens. Therefore, it is possible that superantigen-induced shock might have resulted in the death of S. aureus-infected mice in this study. However, the production of TNF-α, which was protective in either lethal or sublethal S. aureus infections in our previous study (27), and of IFN-γ, which is involved in the pathogenesis of S. aureus infection (27, 37), was not modulated by AG treatment (Table 2). These results indicate that superantigen-induced shock might not have been the main agent of death from S. aureus infection in the present study and that the protective mechanism of NO might not be completely elucidated by the regulation of TNF-α and IFN-γ production.

Staphylococci, including S. aureus, are a major source of morbidity and mortality in medical facilities. Our present study showed that treatments involving suppression of iNOS induction and NO production might be unsuitable in cases of severe S. aureus infection as well as staphylococcal superantigen-induced shock.

<table>
<thead>
<tr>
<th>Time of cultivation (h)</th>
<th>Treatment</th>
<th>Conc. (ng/ml) of IFN-γ</th>
<th>Conc. (ng/ml) of TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>None</td>
<td>11.8 ± 3.0</td>
<td>105.2 ± 25.9</td>
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<tr>
<td></td>
<td>1 mM AG</td>
<td>9.0 ± 1.7</td>
<td>124.5 ± 19.1</td>
</tr>
<tr>
<td></td>
<td>10 mM AG</td>
<td>8.5 ± 2.4</td>
<td>115.0 ± 28.7</td>
</tr>
<tr>
<td>48</td>
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<td>8.5 ± 2.4</td>
<td>93.5 ± 24.0</td>
</tr>
<tr>
<td></td>
<td>1 mM AG</td>
<td>8.4 ± 2.0</td>
<td>109.3 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>10 mM AG</td>
<td>6.8 ± 2.8</td>
<td>100.2 ± 35.0</td>
</tr>
</tbody>
</table>

*Spleen cells were cultured with heat-killed S. aureus in the absence or presence of AG at 37°C for 24 or 48 h.*

*The concentrations of TNF-α and IFN-γ in each culture supernatant were determined. Each result represents the mean ± standard deviation of data for a group of four separate samples. The results were reproduced in three repeated experiments. The concentrations of the cytokines in AG-treated samples were not significantly different from those of the control samples.*
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