INFECTION AND IMMUNITY, July 1997, p. 2932–2940 Vol. 65, No. 7
0019-9567/97/$04.00+0

Induction of Nitric Oxide Synthesis and Xanthine Oxidase and Their Roles in the Antimicrobial Mechanism against *Salmonella typhimurium* Infection in Mice

KAZUO UMEZAWA,[1] TAKAAKI AKAIKE,[1] SHIGEMOTO FUJII,[1] MORITAKA SUGA,[2] KEISUKE SETOGUCHI,[2] ATSUSHI OZAWA,[3] AND HIROSHI MAEDA[1]*

Departments of Microbiology[1] and Internal Medicine I,[2] Kumamoto University School of Medicine, Kumamoto 860, and Department of Infectious Diseases, Tokai University School of Medicine, Kanagawa 259-11,[3] Japan

Received 22 November 1996/Returned for modification 5 March 1997/Accepted 31 March 1997

The role of superoxide anion (O_2^-) and nitric oxide (NO) in the host defense mechanism against *Salmonella typhimurium* (LT-2) was examined by focusing on xanthine oxidase (XO) as an O_2^--generating system and on inducible NO synthase (iNOS). When ICR mice were infected with a 0.1 50% lethal dose (2×10^5 CFU) of *S. typhimurium*, bacterial growth in the liver reached a peak value 3 days after infection ($10^{4.32}$ CFU/g of liver) and decreased thereafter. XO activity in the liver became maximum at 7 days after infection; the value was 34.6 ± 1.4 mU/g of liver at 7 days (compared with 11.0 ± 1.3 mU/g of liver before infection). The time profile of NO production in the liver as determined by electron spin resonance spectroscopy was consistent with that of XO activity. Histological examination of infected liver showed the formation of multiple microabscesses with granulomatous lesions consisting of polymorphonuclear cells and mononuclear cells, and iNOS-expressing cells were localized in the confined areas of the microabscesses. When XO inhibitors such as allopurinol and 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) were administered to the infected mice, the mortality of the mice was significantly increased (10 of 21 and 11 of 20 for the allopurinol- and AHPP-treated groups, respectively, versus 2 of 20 for control mice), and bacterial growth was significantly enhanced. A similar exacerbation of the infection was obtained with N^{ω}-monomethyl-L-arginine (L-NMMA) treatment of the mice. Of considerable importance is that granuloma formation in the liver was poorly developed by treatment with either XO inhibitors or L-NMMA. These results suggest that XO and NO play an important role in the antimicrobial mechanism against *S. typhimurium* in mice.

It is well known that oxygen free radicals such as superoxide anion (O_2^-) are produced as a host defense response and have antimicrobial effects on intruding bacteria (4, 15). Further, in recent years, nitric oxide (NO), which was first described as an endothelium-derived relaxation factor (32, 34), also became known to play an important role in the host defense against a variety of microbes (12, 16, 22, 23, 28). There is increasing evidence that O_2^- interacts with NO (5, 12, 20, 30, 36), and their reaction product peroxynitrite ($ONOO^-$) not only exhibits cytotoxic as well as pathogenic effects (3, 7, 36) but also functions as an inflammatory mediator (9, 17, 27, 38).

Typhoid fever is a major infectious disease affecting the human population worldwide. The clinical symptoms of typhoid fever are caused mainly by *Salmonella typhi*, but other *Salmonella* species, including *Salmonella paratyphi* A and B and, on rare occasions, even *Salmonella typhimurium*, can be causative agents (24). These *Salmonella* species are gram-negative, motile, facultative intracellular bacilli, and invasion of and multiplication within the mononuclear phagocytic cells in the liver, spleen, lymph nodes, and Peyer's patches are the hallmark of typhoid fever. To obtain a better understanding of the pathogenesis of typhoid fever, it seems crucial to elucidate the host reaction mechanism against *Salmonella*, with a focus on defense-oriented molecules such as oxygen radicals and NO. *S. typhimurium* infection in mice has been analyzed with regard to antimicrobial and pathological events in host defense (10, 37).

In our previous study, generation of O_2^- from neutrophils and macrophages in livers of mice infected with *S. typhimurium* was unequivocally identified by using ex vivo chemiluminescence; O_2^- generation correlated well with extension of granulomatous lesions in the infected liver (48). Specifically, suppression of O_2^- generation by treatment with superoxide dismutase (SOD) in vivo resulted in a decrease in the lesion area in the liver and a simultaneous acceleration of bacterial growth in the liver. This result suggests the possible involvement of O_2^- in the host defense mechanism against *S. typhimurium* infection. In the present study, the role of O_2^- and NO in this defense mechanism was further investigated, focusing on the function of xanthine oxidase (XO) as an O_2^--generating system and on NO produced from the inducible isoform of NO synthase (iNOS) in the liver.

MATERIALS AND METHODS

Animals. Six-week-old male ICR mice (specific-pathogen-free grade), purchased from SLC Japan Inc. (Shizuoka, Japan), were used in this study. All experiments were carried out according to the guidelines in the Laboratory Protocol of Animal Handling, Kumamoto University School of Medicine.

Bacteria. *S. typhimurium* (LT-2 strain) was used throughout the experiments. The number of bacteria was determined by the colony formation assay as described previously (48). Briefly, serially diluted liver homogenates were plated on deoxycholate-hydrogen sulfate-lactose agar (Nissui, Tokyo, Japan) plates, and colonies were counted after incubation for 18 h at 37°C.

Production of *S. typhimurium* infection in mice. *S. typhimurium* in 0.2 ml of 0.01 M sodium phosphate-buffered 0.15 M saline (PBS) (pH 7.4) was given intraperitoneally (i.p.) to the ICR mice at a dose of either 2×10^6 or 2×10^5 CFU/mouse (1.0 or 0.1 50% lethal dose [LD_{50}], respectively). At various times after inoculation with *S. typhimurium*, bacterial growth, induction of both XO

* Corresponding author. Mailing address: Department of Microbiology, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860, Japan. Phone: 81-96-373-5098. Fax: 81-96-362-8362. E-mail: msmaedah@gpo.kumamoto-u.ac.jp.

and xanthine dehydrogenase (XD), and NO biosynthesis in mouse liver, as well as the survival rate of the mice, were examined as described below.

XO and XD assay. Both XO and XD activities were assayed by a fluorometric method as described earlier (2, 6). After the mice were exanguinated by cutting of the right ventricle under ether anesthesia followed by perfusion of the liver via the portal vein with 20 ml of PBS (pH 7.4) containing 10 U of heparin, the liver was homogenized on ice with ice-cold 50 mM potassium phosphate buffer (pH 7.6) containing 0.1 mM EDTA (Dojindo Laboratories, Kumamoto, Japan), 10 μg of leupeptin (Institute of Microbial Chemistry, Tokyo, Japan) per ml, 100 μg of phenylmethylsulfonyl fluoride (Wako Pure Chemical, Osaka, Japan) per ml, 1.0 mM dithiothreitol (Wako), 10 μg of soybean trypsin inhibitor (Kunitz type; Fuji Seiyu Co., Osaka, Japan) per ml, and 0.32 M sucrose. A Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) was used. After the homogenates were centrifuged at 100,000 × *g* at 4°C for 1 h, the resultant supernatants were dialyzed against PBS (pH 7.4) for 18 h at 4°C. The supernatants of the liver homogenates were then subjected to a fluorometric assay for XO and XD activities with pterine as a substrate. Isoxanthopterin, a reaction product, was measured by use of a spectrofluorometer (model 650-40; Hitachi Ltd., Tokyo, Japan), with excitation at 345 nm and emission at 390 nm. The assay mixture consisted of 0.1 ml of sample solution and 0.9 ml of 50 mM potassium phosphate buffer (pH 7.6) containing pterine (final concentration, 10 μM), and the reaction was allowed to proceed at 37°C for 60 min. To measure the combined XO plus XD activity, this reaction was carried out in the presence of 10 μM methylene blue, which replaces NAD^+ as an electron acceptor. To confirm the specificity of XO and XD activities, allopurinol was added to the reaction mixture to give a final concentration of 10 μM so as to inhibit XO and XD activities, followed by incubation at 37°C for 30 min before addition of pterine. Both XO and XD activities in the liver are expressed as units per gram of liver tissue; 1.0 U is defined as 1.0 μmol of isoxanthopterin produced per min (6).

Measurement of NO generation in vivo. NO generation in mouse liver was determined by measuring the NO-diethyldithiocarbamate (DETC)-Fe^{2+} complex, which is formed de novo after administration of DETC and $FeSO_4$; electron spin resonance (ESR) spectroscopy was performed as described previously (3, 11). Specifically, the mouse was injected subcutaneously with DETC at 400 mg/kg and $FeSO_4$ at 40 mg/kg ($FeSO_4$ was dissolved in a 50-mg/ml sodium citrate solution); separate sites for injection of DETC and the $FeSO_4$ solution were used to prevent precipitation of the iron complex. Thirty minutes after DETC-$FeSO_4$ administration, the mouse liver was perfused with 20 ml of PBS (pH 7.4) containing 10 U of heparin via the portal vein. The perfused liver was resected and cut into small pieces, which were then transferred to the ESR sample tube. The sample in the tube was immediately frozened in liquid nitrogen, and the NO-DETC-Fe^{2+} complex produced was quantified by using ESR spectroscopy (Bruker Instrument, Inc., Rheinstetten, Germany) at 110 K. Moreover, NO-hemoglobin generated in mouse blood was measured by using ESR spectroscopy at 110K without treatment with the DETC-Fe^{2+} complex, as described recently (3, 11).

Identification of iNOS mRNA expression in the liver. Induction of iNOS mRNA in liver infected with *S. typhimurium* was examined by reverse transcriptase PCR (RT-PCR) and Southern blot analyses as described previously (3, 11). After the mouse liver was removed and washed three times with PBS, total RNA was extracted with Trizol (Gibco BRL, Gaithersburg, Md.). Briefly, 0.3 μg of template RNA and 50 pmol of each oligonucleotide primer were used for each PCR assay. Simultaneously, expression of glyceraldehyde-3-phosphate dehydrogenase was tested as a standard mRNA expressed in the liver. The nucleotide sequences of the primers for RT-PCR and reaction conditions for the RT-PCR have been described elsewhere (11). A cDNA probe for murine iNOS was used for Southern blotting after RT-PCR analyses, as presented earlier (3, 11).

Treatment of *Salmonella*-infected mice with XO or NOS inhibitors. The effect of inhibition of XO or NOS on *S. typhimurium* infection in mice was investigated by treatment of mice with XO inhibitors such as allopurinol and 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) (Aldrich Chemical) or N^{ω}-monomethyl-L-arginine (L-NMMA) (provided by I. Sakuma and R. G. Kilbourn). Allopurinol and AHPP were given orally to the *Salmonella*-infected mice at 1.0 mg/mouse in 0.2 ml of 0.25 M NaOH every day on days 3 to 7 after inoculation of the bacteria. *Salmonella*-infected mice given oral doses of 0.2 ml of 0.25 M NaOH served as the vehicle-treated control. In a separate experiment, infected animals were injected i.p. with 2.0 mg of L-NMMA (in 0.2 ml of PBS, pH 7.4) every day during the same time periods as for the XO inhibitor treatment. The control group for the L-NMMA treatment was given 0.2 ml of PBS i.p.

Histopathology and immunohistochemistry. For histological study, liver tissues were fixed in 10% buffered neutral formalin solution, embedded in paraffin, and cut into 3-μm-thick sections. The sections were stained with Masson trichrome stain, and sections of liver untreated or treated with either XO inhibitors or L-NMMA were observed for histopathological changes.

Similarly, liver tissues with or without use of XO inhibitors or L-NMMA treatment were subjected to immunohistochemical analysis for iNOS expression as reported recently (38). Briefly, tissues were fixed in 2% periodate-lysine-paraformaldehyde fixative at 4°C for 4 h. After 12 h of washing with PBS containing 10, 15, and 20% sucrose plus 10% glycerol, tissues were embedded in tissue-embedding medium (Miles, Elkhart, Ind.), frozen in liquid nitrogen, and cut into 6-μm-thick sections by use of a cryostat. Sections were stained by the indirect immunoperoxidase method, with a specific antibody for murine iNOS (Wako Pure Chemical) as a primary antibody and were visualized by the reaction with the substrate 3,3′-diaminobenzidine (Wako). In addition, formation of nitrotyrosine in liver tissue was analyzed by use of a specific antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, N.Y.), as described recently (3, 38), in the same manner as for iNOS expression.

Statistical analysis. All data are expressed as means ± standard errors of the means (SEM). Statistical differences among each experimental group were determined by the Mann-Whitney U test or by Fisher's exact probability test.

RESULTS

Bacterial growth in the liver. When ICR mice were infected with 2×10^5 CFU of *S. typhimurium* (0.1 LD_{50}), the number of bacteria in the liver increased up to $10^{4.32 \pm 0.69}$ and $10^{3.99 \pm 0.50}$ CFU/g of liver at 3 and 7 days after infection, respectively, and then decreased thereafter (Fig. 1A). At 21 days after infection, the bacteria became undetectable in our colony formation assay (less than 10^2 CFU/g of liver). A similar trend was observed in the group infected with 2×10^6 CFU of *S. typhimurium* (1.0 LD_{50}), but the bacterial yield was almost 10-fold higher than that in the 0.1 LD_{50}-infected group throughout the time course of infection, and even on day 21 a significant amount of bacteria ($10^{3.13 \pm 0.08}$ CFU/g of liver) was detected in mouse liver (Fig. 1B).

Induction of XO activity, NO production, and iNOS in vivo. In a group infected with 0.1 LD_{50} of *Salmonella*, XO activity reached a maximum at 7 days after infection (34.60 ± 1.43 mU/g of liver) and decreased thereafter to a level at 21 days after infection that was similar to the level before infection (12.67 ± 2.54 mU/g of liver versus 11.01 ± 1.33 mU/g of liver before infection) (Fig. 2A). The percentages of XO activity as related to total activity [XO/(XO + XD)] were 67, 70, 53, and 46% at 3, 7, 14, and 21 days after infection (0.1 LD_{50}), respectively; the corresponding percentage for the uninfected control was 55%. The increase in XO plus XD activity in the 1.0 LD_{50}-infected mice was similar to that in the 0.1 LD_{50}-infected group (Fig. 2B). The percentages of XO activity in the 1.0 LD_{50}-infected group, however, were larger than those in the 0.1 LD_{50}-infected group: 78, 77, 100, and 96% at 3, 7, 14, and 21 days after infection, respectively.

The time profile of NO production in the liver was consistent with that of XO activity, as assessed by ESR spectroscopic study of production of the NO-DETC-Fe^{2+} complex (Fig. 3A). The amount of NO generated in the liver was elevated at 3 and 7 days after infection (0.1 LD_{50}) and decreased thereafter to reach the level seen before infection at 21 days after infection. Without treatment with the DETC-Fe^{2+} complex, a similar time profile was observed for NO-hemoglobin generation in the blood of mice infected with 0.1 LD_{50} of *S. typhimurium* (Fig. 3B). NO-hemoglobin generation began to increase at 3 days after infection. The highest level of NO-hemoglobin in the blood was observed at 7 days after infection, and the level was considerably reduced at 21 days after infection. In the 1.0 LD_{50}-infected group, NO production was induced after the bacterial infection in a same time-dependent manner as in the 0.1 LD_{50}-infected animals, except that the NO level was higher than that in the 0.1 LD_{50}-infected group throughout the time course of the infection (Fig. 3). In addition, generation of NO-hemoglobin in the blood of the 1.0 LD_{50}-infected mice was sustained for a longer time than that in the 0.1 LD_{50} group and showed the maximum value at 14 days after infection (Fig. 3B).

Furthermore, induction of iNOS in the liver after *Salmonella* infection was examined by RT-PCR and Southern blot analysis. As demonstrated in Fig. 4, iNOS mRNA expression was clearly observed in mouse liver at 3 and 7 days after infection (0.1 LD_{50}). The time course of iNOS mRNA expression was

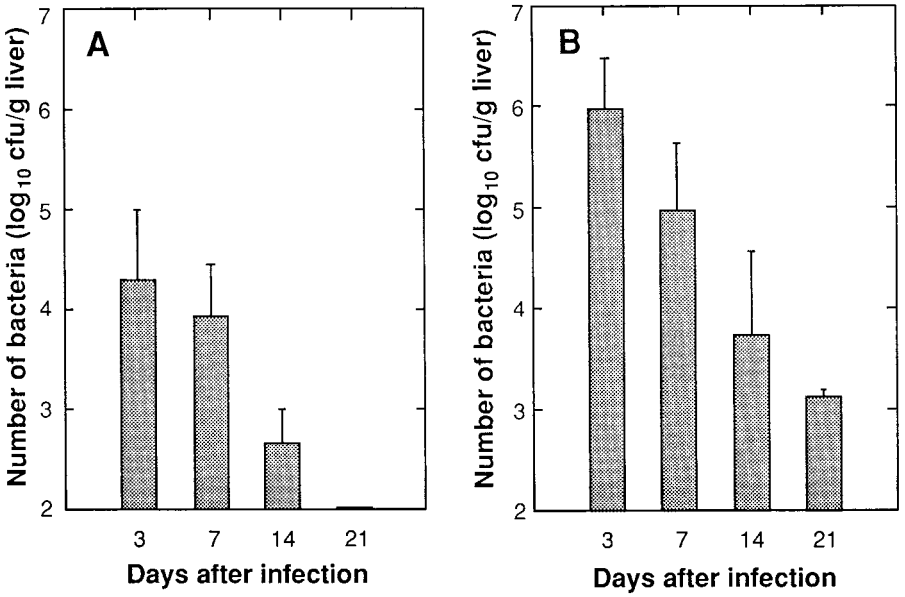

FIG. 1. Bacterial growth in livers of mice infected with *S. typhimurium*. Mice were infected with 2×10^5 CFU of *S. typhimurium* (0.1 LD_{50}) (A) or with 2×10^6 CFU of the bacteria (1.0 LD) (B). Columns and error bars indicate means ± SEM ($n = 4$). The number of bacteria was determined by the colony-forming assay. See text for details.

nearly consistent with that of NO production as assessed by ESR spectroscopy (Fig. 3).

The host's defense responses assessed by induction of XO and XD activity and NO production (Fig. 2 and 3) were correlated with the bacterial growth in the livers for the groups infected with 0.1 LD_{50} and 1.0 LD_{50} of bacteria (Fig. 1). Although the level of total activity of XO and XD induced in the *Salmonella*-infected liver appears to be within the same range in the two groups, there is a great difference in the ratio of XO to XD between them, indicating that the conversion of XD to XO occurs more effectively in the group infected with 1.0 LD_{50} than in that infected with 0.1 LD_{50}. Furthermore, a similar difference in the magnitude of the host's response against *Salmonella* infection was observed in the production of NO as revealed by ESR measurement of various NO adducts generated in the liver and the blood.

Effect of XO and NOS inhibitors on survival rate of mice and bacterial growth in vivo. When specific inhibitors of XO such as allopurinol and AHPP were administered to *Salmonella*-infected mice (0.1 LD_{50}), the survival of the mice was significantly reduced: 10 of 21 and 11 of 20 (deaths with respect to the total number of mice, in allopurinol- and AHPP-treated

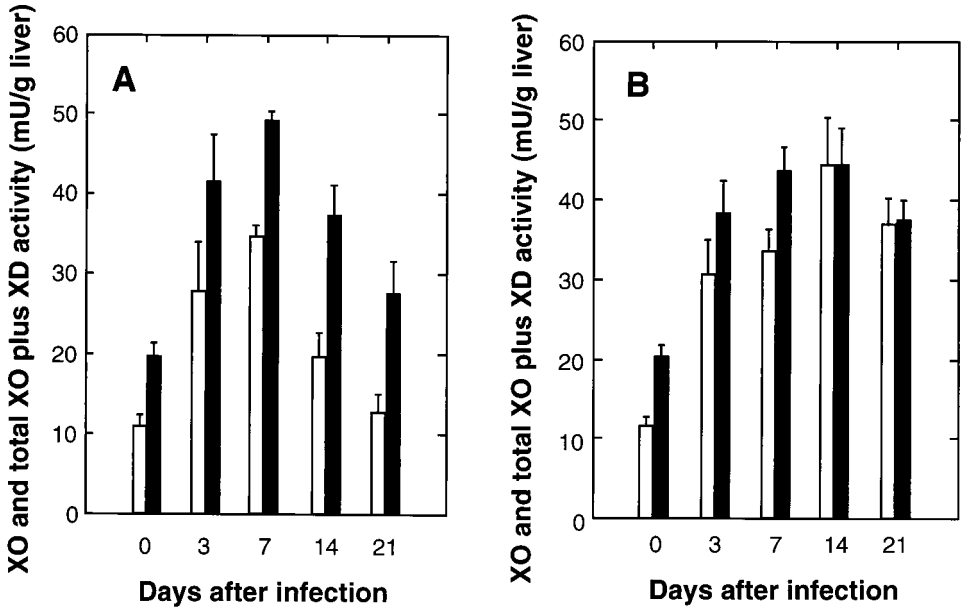

FIG. 2. XO and total XO-plus-XD activities in the liver during *S. typhimurium* infection. Mice were infected with 2×10^5 CFU of *S. typhimurium* (0.1 LD_{50}) (A) or with 2×10^6 CFU of the bacteria (1.0 LD_{50}) (B). Open columns, XO activity; closed columns, total (XO and XD) activity. Data are means ± SEM ($n = 7$). XO and XO-plus-XD activities were determined fluorometrically in the presence or absence of 9 μM methylene blue. See text for details.

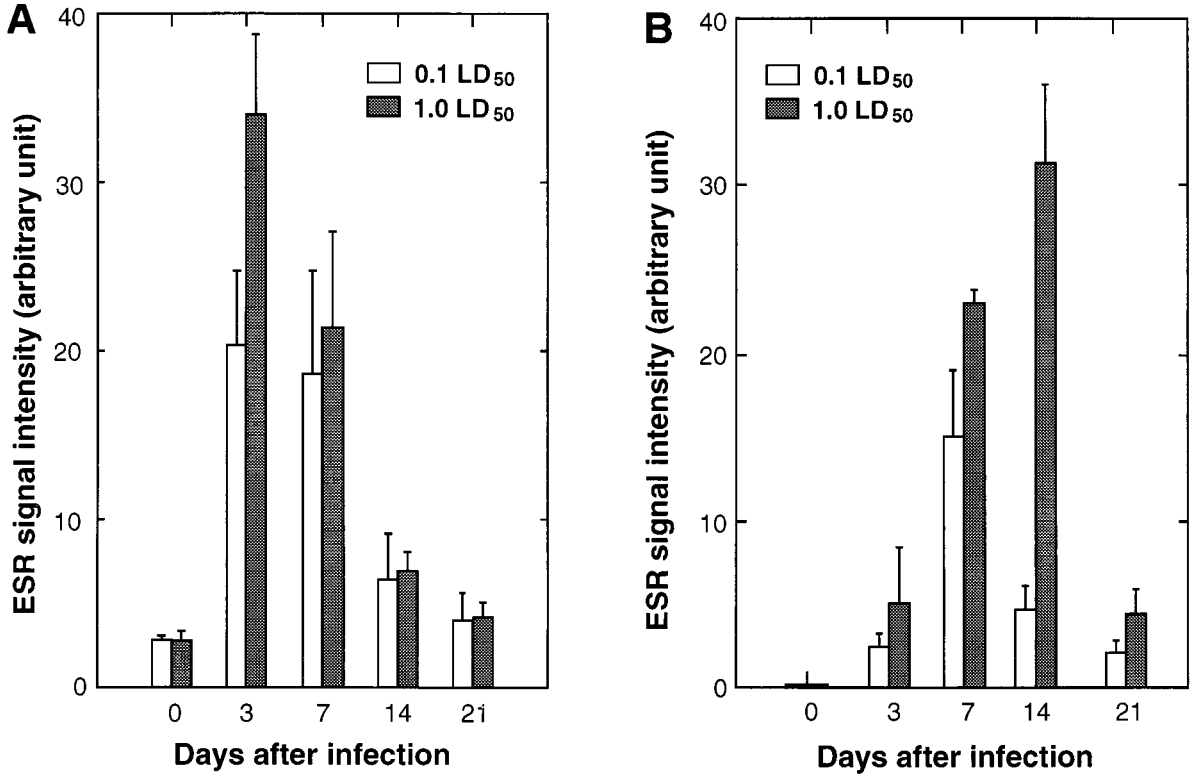

FIG. 3. NO generation in blood and liver in mice infected with *S. typhimurium* (0.1 and 1.0 LD_{50}). NO generation was determined by measuring the amounts of NO-DETC-Fe^{2+} complex produced in the liver (A) and NO-hemoglobin produced in the blood (B) by using ESR spectroscopy at 110 K. Data are means ± SEM (n = 4). See text for details.

groups, respectively), compared with 2 of 20 for the vehicle-treated control group ($P < 0.05$ between the control and XO inhibitor-treated groups by Fisher's exact probability test) (Fig. 5). The numbers of bacteria in the liver at day 7 after infection were significantly increased by treatment with allopurinol or AHPP ($10^{5.83 \pm 0.22}$ and $10^{5.80 \pm 0.23}$ CFU/g of liver, respectively, versus $10^{4.22 \pm 0.33}$ CFU/g of liver in the control group; $P < 0.05$ by the Mann-Whitney U test) (Fig. 6A). However, the amount of NO-hemoglobin generated in the blood was not appreciably affected by treatment with XO inhibitors (Fig. 6B). Increases in mortality and bacterial growth were also observed after L-NMMA treatment of the *Salmonella*-infected mice; the mortality was 10 of 22 and the bacterial yield was $10^{5.47 \pm 0.35}$

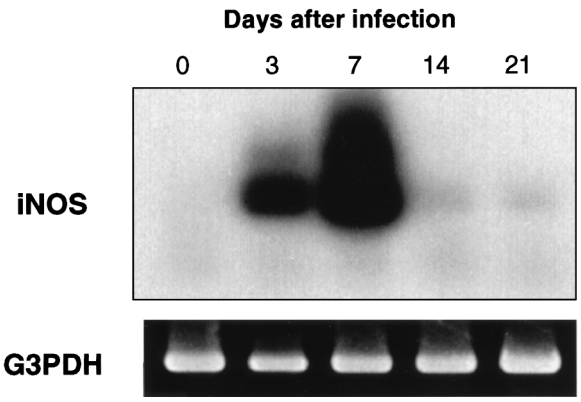

FIG. 4. iNOS mRNA expression in the livers of mice infected with 0.1 LD_{50} of *S. typhimurium*. RT-PCR and Southern blot analyses were used to identify the iNOS mRNA expressed in mouse livers at various times after infection. RT-PCR was used for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA in the liver as the control, and the DNA band was visualized by ethidium bromide staining after agarose electrophoresis. See text for details.

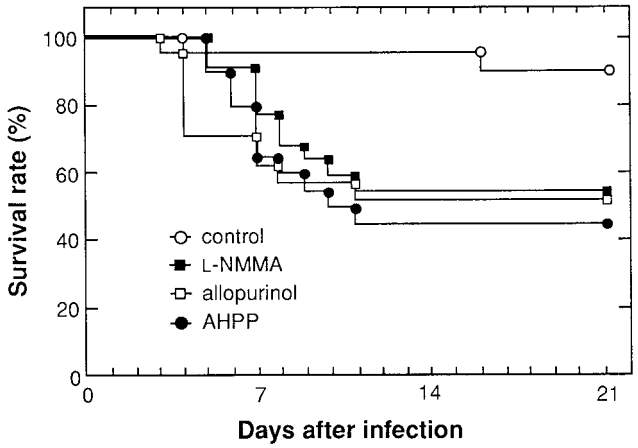

FIG. 5. Effect of allopurinol, AHPP, and L-NMMA on the survival rate of mice infected with *S. typhimurium* (0.1 LD_{50}). Allopurinol (n = 21) and AHPP (n = 20) were administered orally at a dose of 1.0 mg/day per mouse. L-NMMA (2.0 mg/day/mouse) (n = 22) was given i.p. Treatment was carried out every day with each solution, starting 3 days after infection and continuing until 6 days after infection. Animals treated with only vehicle (either 0.2 ml of 0.25 M NaOH orally or 0.2 ml of PBS i.p.) served as controls (n = 20 for each control group). Similar time courses for survival rates were observed for the two different vehicle-treated groups; thus, the survival rate for the NaOH-treated group is shown as the representative control. See text for details.

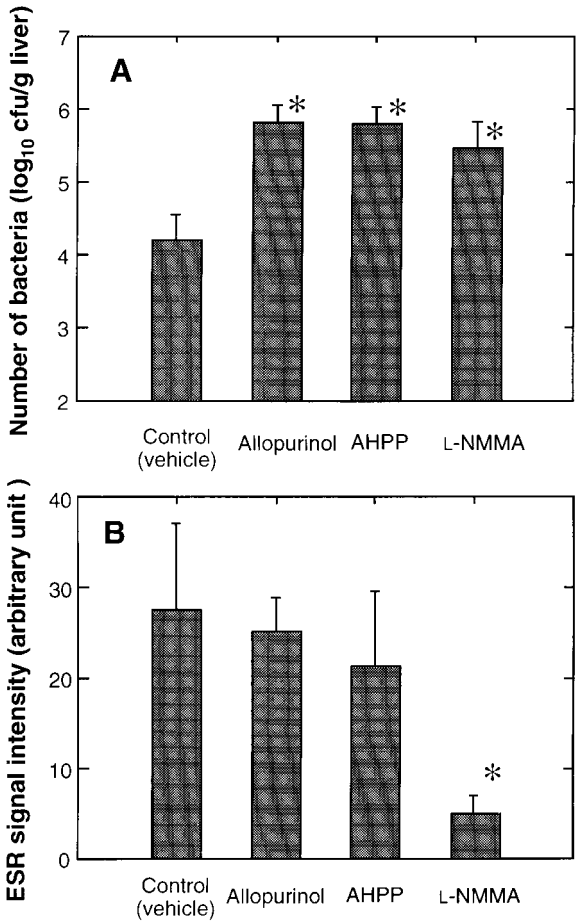

FIG. 6. Effect of allopurinol, AHPP, and L-NMMA on bacterial growth in livers (A) and on NO-hemoglobin generation in the blood (B) of mice infected with *S. typhimurium*. Mice were treated with allopurinol, AHPP, or L-NMMA from day 3 to day 6 after infection (0.1 LD_{50}) every day in the same manner as described for Fig. 5, and liver tissue was obtained at 7 days after infection. The number of bacteria was quantified by use of the colony-forming assay as for Fig. 1. NO-hemoglobin was measured in mouse blood obtained at 7 days after infection. The control (vehicle) group was given 0.2 ml of 0.25 M NaOH orally. Data are means ± SEM ($n = 7$). *, $P < 0.05$ versus the control group by the Mann-Whitney U test. See text for details.

CFU/g of liver at day 7 after infection ($P < 0.05$ for bacterial yield and mortality between control and L-NMMA-treated groups) (Fig. 5 and 6A). L-NMMA markedly suppressed production of NO-hemoglobin in the blood in *Salmonella*-infected mice (Fig. 6B).

Effect of XO and NOS inhibitors on formation of pathological lesions in livers of mice infected with *S. typhimurium*. Histological examination of liver obtained at day 7 after infection (0.1 LD_{50} of *S. typhimurium*) revealed multiple lesions of nodular microabscesses or granulomatous lesions, which were composed of degenerated hepatocytes, infiltrated polymorphonuclear (PMN) cells, and macrophages (Fig. 7A and D). No significant pathological change was found in hepatocytes surrounding the lesion (Fig. 7A). In contrast, the granulomatous lesion induced by *Salmonella* infection was poorly developed, and degenerated hepatocytes were seen throughout the liver in L-NMMA- and allopurinol-treated groups (Fig. 7B, C, and E) and also in AHPP-treated mice (data not shown). Immunohistochemistry with an anti-iNOS antibody showed that iNOS-positive cells, which appeared to be macrophages and PMN cells, were localized mostly in the confined areas of the microabscesses in the *Salmonella*-infected liver (Fig. 8A, 8, and D). However, infiltration of iNOS-positive cells in microabscesses was remarkably reduced by treatment with either XO inhibitors or L-NMMA, and these cells were rather diffusely distributed in the liver tissue (Fig. 8E and F).

Formation of nitrotyrosine in *Salmonella*-infected livers. By immunohistochemical study with an antinitrotyrosine antibody, generation of nitrotyrosine in liver tissue was investigated. As shown in Fig. 9, strong staining with the antibody was evident in infected liver. However, in contrast to the localization of iNOS-positive cells, nitrotyrosine formation was observed in the periphery of the granuloma formed in the liver.

DISCUSSION

In our previous work, it was suggested that O_2^- is an important antimicrobial molecular species in *S. typhimurium*-infected mice (48). Our present study revealed that XO is responsible for the O_2^--dependent host defense mechanism. It is of considerable importance that excessive production of NO by iNOS expressed in mouse liver was also critically involved in the antimicrobial mechanism against *S. typhimurium*. This is the first detailed report showing that both O_2^- and NO are major effector molecules in the host defense against *S. typhimurium* in vivo.

There is evidence that XO and XD activity is expressed in a variety of cells, including endothelial cells (13, 24), hepatocytes (41, 45, 46), fibroblasts (14), and neutrophils and macrophages (18, 35, 47). Also, it has been reported that XO (or XD) is upregulated by stimulation with lipopolysaccharide and proinflammatory cytokines, e.g., gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (13, 14, 45), which are induced in infections with various viruses and bacteria, such as influenza virus (2), cytomegalovirus (21), and *S. typhimurium* (44). In the present experiments, the increase in XO and XD in *Salmonella*-infected liver was clear. As described previously, O_2^- generated from XO plays a pathogenic role in the pathogenesis of influenza virus and cytomegalovirus infections in mice. In contrast, inhibition of XO by treatment with either allopurinol or AHPP resulted in increased mortality of mice infected with *S. typhimurium* together with enhancement of bacterial growth in the *Salmonella*-infected liver. Enhancement of mortality by allopurinol treatment was reported in infection with *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* in mice (46). We recently found that AHPP is a more specific and more potent XO inhibitor than allopurinol, and even in vivo it effectively suppressed O_2^- generation dependent on XO (31). Therefore, the results obtained in the present study suggest that XO, via production of O_2^-, exerts a protective effect against invasion of *Salmonella* in vivo.

Formation of NO has also been demonstrated in a wide variety of cells, including vascular endothelial cells, neuronal cells, PMN cells, bronchial epithelial cells, hepatocytes, and activated macrophages (1, 16, 32, 34, 38, 40). There are three different isoforms of NOS, i.e., two types of constitutive enzymes in neuronal and endothelial cells (29, 34) and iNOS, which is induced by lipopolysaccharide and various proinflammatory cytokines such as IFN-γ and TNF-α and produces an excessive amount of NO (39, 40). NO produced excessively, in particular by activated macrophages, has been shown to function as a cytotoxic or cytostatic molecule and inhibits the growth of a diverse array of infectious agents (12, 16, 22, 23, 28). On the other hand, inhibition of NO biosynthesis improves the pathogenesis of influenza virus-induced pneumonia in mice

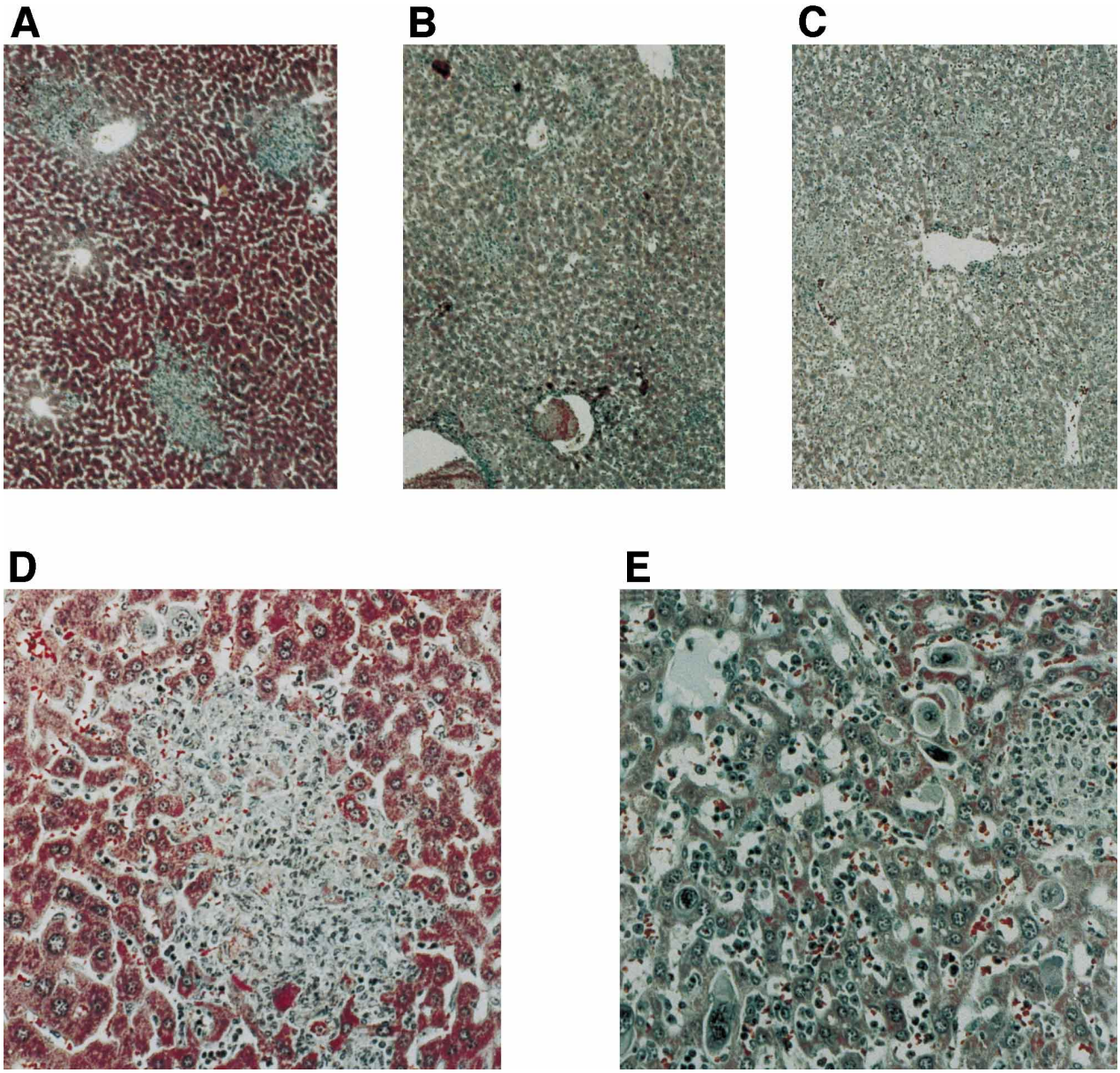

FIG. 7. Effect of XO and NOS inhibitors on formation of pathological lesions in livers of mice infected with *S. typhimurium*. Liver obtained at 7 days after infection (0.1 LD_{50}) was fixed with 10% formaldehyde, and sections were stained with Masson trichrome stain. Treatment with XO inhibitors or L-NMMA was performed as described for Fig. 5. (A and D) Control group; (B) L-NMMA-treated group; (C and E) allopurinol-treated group. Magnifications, ×48 (A to C) and ×180 (D and E). See text for details.

(3). Moreover, overproduction of NO has been implicated in the pathogenesis of sepsis; of carcinogenesis induced by parasites, viruses, or bacteria (*Helicobacter pylori*); and of cerebral malaria (3, 22, 33, 52). Thus, overproduction of NO may be detrimental to hosts in some microbial infections. Identification of the role of NO in protection against microbial infections will therefore provide insight into the delicate balance of host-microbe interaction.

The present result for *S. typhimurium* infection in mice clearly illustrates the protective effect of NO produced during murine salmonellosis. The beneficial qualities of the cell-mediated immune effector mechanism have been shown in this salmonellosis model, involving cytokine signaling, particularly that dependent on Th1-related IFN-γ production (43). Because, as mentioned earlier, NO production and upregulation of XO and XD are mediated through IFN-γ and TNF-α, the host defense action against *Salmonella* can be exerted via production of both NO and O_2^- at the ultimate step in the cytokine signaling pathway.

It is now well documented that NO per se is not a potent bactericidal molecular species (7, 8, 19, 51). The most effective cytotoxic effect of NO appears to be via reaction with O_2^- (8, 19, 30, 36). NO reacts with O_2^- (with a diffusion-limited rate constant of 6.7×10^9 M^{-1} s^{-1}) to yield peroxynitrite ($ONOO^-$) (5, 20, 30). Peroxynitrite generated in the tissue can be assessed by immunohistochemical detection of nitrotyrosine in tissue (3, 26, 42). Although it has been reported that nitrotyrosine can be formed in a peroxynitrite-independent chemi-

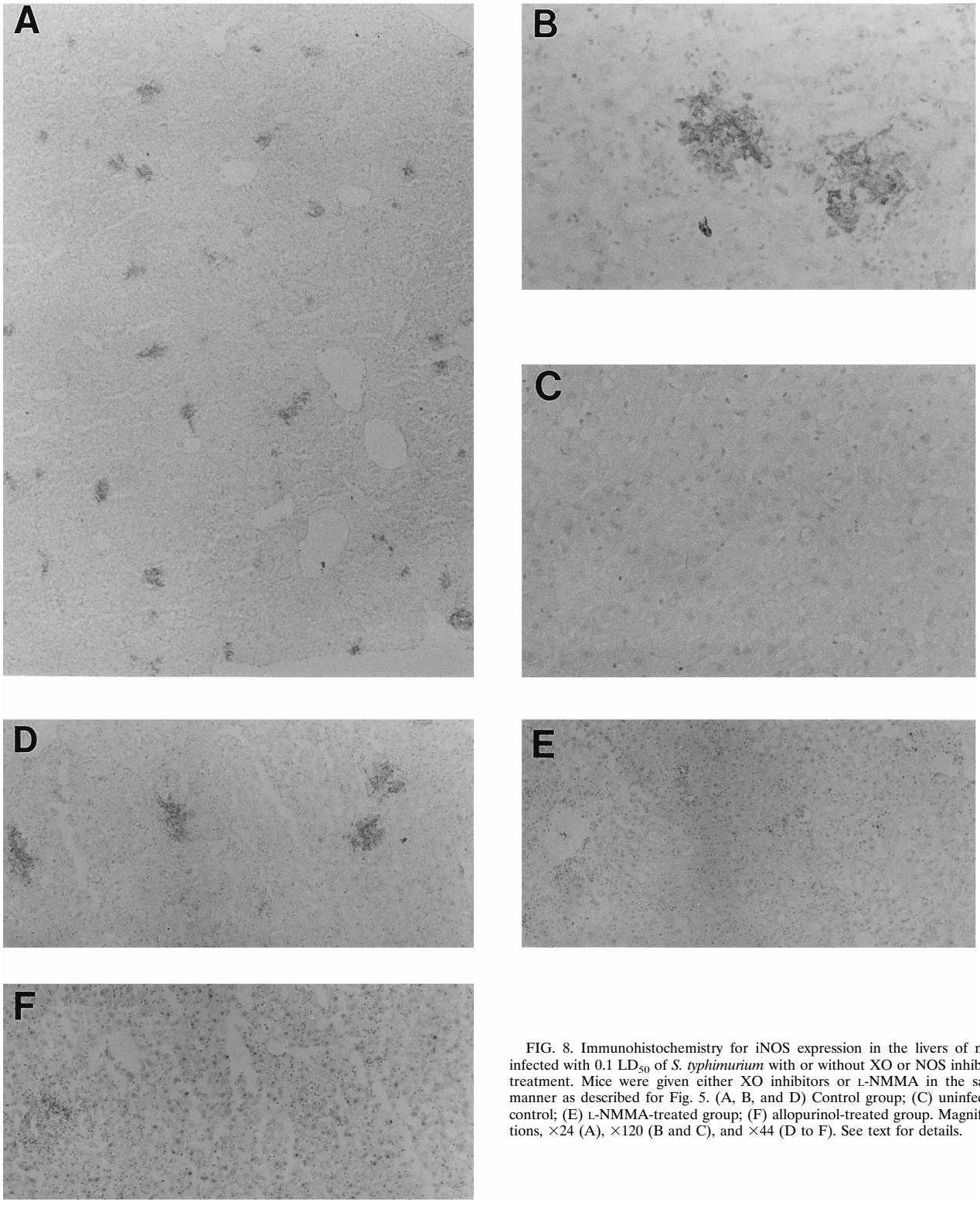

FIG. 8. Immunohistochemistry for iNOS expression in the livers of mice infected with 0.1 LD_{50} of *S. typhimurium* with or without XO or NOS inhibitor treatment. Mice were given either XO inhibitors or L-NMMA in the same manner as described for Fig. 5. (A, B, and D) Control group; (C) uninfected control; (E) L-NMMA-treated group; (F) allopurinol-treated group. Magnifications, ×24 (A), ×120 (B and C), and ×44 (D to F). See text for details.

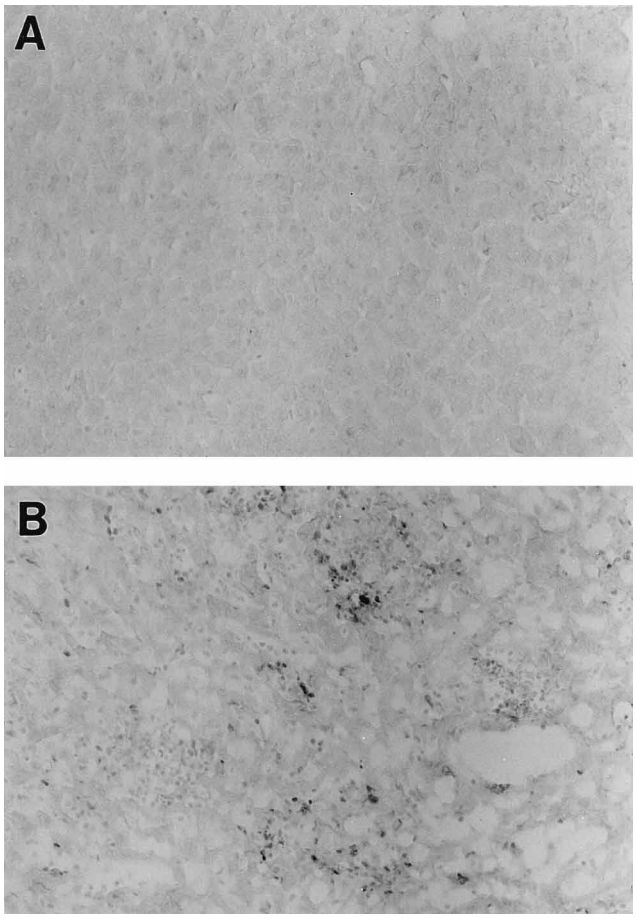

FIG. 9. Formation of nitrotyrosine in a *Salmonella*-infected liver lesion as determined by immunohistochemistry with an antinitrotyrosine antibody. (A) Uninfected control group; (B) 7 days after infection (0.1 LD_{50}). Magnification, ×130. See text for details.

cal reaction, such as the NO_2^--H_2O_2-peroxidase pathway (50), peroxynitrite appears to be one of the possible contributors to nitrotyrosine formation in vivo, in particular when excessive amounts of both NO and O_2^- are produced simultaneously at similar concentrations (30, 36). This notion seems to be further supported by the demonstration by Pryor's group that the nitration reaction by peroxynitrite can be enormously potentiated by carbon dioxide, which is abundant in biological systems (49). Our immunohistochemical study with antinitrotyrosine antibody showed intense staining in livers of mice infected with *S. typhimurium*. Furthermore, in view of our observation that inhibition of either NO or O_2^- results in impairment of the antimicrobial effect in murine salmonellosis (present results and reference 48), it is most reasonable that peroxynitrite generated in mouse liver has an antibacterial effect in *Salmonella* infection in mice.

The histopathological examination with Masson trichrome stain and the immunohistochemical assay with anti-iNOS antibody showed that treatment of the *Salmonella*-infected mice with L-NMMA or XO inhibitors significantly reduced formation of granulomatous lesions in the liver. A similar result was obtained in our previous study with a polymer-conjugated SOD, in which SOD administered to the *Salmonella*-infected mice resulted in poor development of the granulomatous changes and in enhancement of bacterial growth in the liver (48). These data may indicate that both NO and O_2^-, possibly through formation of peroxynitrite, mediate recruitment of inflammatory cells such as PMN cells and monocyte-derived macrophages to the infectious foci, resulting in containment of the intruding bacteria in the restricted area with formation of localized microabscesess and granulomatous lesions. This interpretation is supported by our recent report that inhibition of NO production by NOS inhibitors in silica- and zymosan-induced pulmonary granulomatosis in rats decreased inflammatory cell infiltration in lung tissues and reduced induction of chemokine macrophage chemoattractant protein-1 (38). These results may suggest that NO and its oxidized metabolites such as peroxynitrite function as inflammatory mediators in microbial infections. In this context, the NO-O_2^- interaction in the microcirculation has been well delineated by Kubes et al. (17, 27), in that a delicate balance between NO and O_2^- affects the various inflammatory responses such as vascular permeability and leukocyte adhesion to the endothelial cells in the microvasculature. Therefore, better understanding of a novel aspect of NO in the inflammatory response of the host will provide new insights into the role of NO and oxygen radicals in the host defense mechanism and pathogenesis of various types of infectious diseases.

In conclusion, from our present results it is apparent that O_2^- generated through XO induction and NO production by iNOS has a potent antimicrobial effect against *S. typhimurium* in murine salmonellosis, and this protective effect is likely to be mediated through formation of peroxynitrite rather than O_2^- or NO per se.

ACKNOWLEDGMENTS

This work is supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan.

We thank J. Gandy for editing and R. Yoshimoto for preparing the manuscript. Thanks are also due to late H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan) for kindly providing leupeptin.

REFERENCES

1. **Adamson, G. M., and R. E. Billings.** 1993. Cytokine toxicity and induction of NO synthase activity in cultured mouse hepatocytes. Toxicol. Appl. Pharmacol. **119:**100–107.
2. **Akaike, T., M. Ando, T. Doi, S. Ijiri, S. Araki, and H. Maeda.** 1990. Dependence on O_2^- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. J. Clin. Invest. **85:**739–745.
3. **Akaike, T., Y. Noguchi, S. Ijiri, K. Setoguchi, M. Suga, Y. M. Zheng, B. Dietzschold, and H. Maeda.** 1996. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. Proc. Natl. Acad. Sci. USA **93:**2448–2453.
4. **Badway, J. A., and M. L. Karnovsky.** 1980. Active oxygen species and the functions of phagocytic leukocytes. Annu. Rev. Biochem. **49:**695–726.
5. **Beckman, J. S., T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman.** 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA **87:**1620–1624.
6. **Beckman, J. S., D. A. Dale, J. D. Pearson, P. A. Marshall, and B. A. Beckman.** 1989. A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. Free Radical Res. Commun. **6:**607–615.
7. **Brunelli, L., J. P. Crow, and J. S. Beckman.** 1995. The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. Arch. Biochem. Biophys. **316:**327–334.
8. **Castro, L., R. Marianela, and R. Radi.** 1994. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J. Biol. Chem. **269:** 29409–29415.
9. **Clancy, R. M., and S. B. Abramson.** 1995. Nitric oxide: a novel mediator of inflammation. Proc. Soc. Exp. Biol. Med. **210:**93–101.
10. **Collins, F. M., G. B. Mackaness, and R. V. Blanden.** 1966. Infection-immunity in experimental salmonellosis. J. Exp. Med. **124:**601–619.
11. **Doi, K., T. Akaike, H. Horie, Y. Noguchi, S. Fujii, T. Beppu, M. Ogawa, and H. Maeda.** 1996. Excessive production of nitric oxide in rat solid tumor and its implication in rapid tumor growth. Cancer **77:**1598–1604.
12. **Doi, T., M. Ando, T. Akaike, M. Suga, K. Sato, and H. Maeda.** 1993. Resistance to nitric oxide in *Mycobacterium avium* complex and its implication to pathogenesis. Infect. Immun. **61:**1980–1989.

13. **Dupont, G. P., T. P. Huecksteadt, B. C. Marshall, U. S. Ryan, J. R. Michael, and J. R. Hoidal.** 1992. Regulation of xanthine dehydrogenase and xanthine oxidase activity and gene expression in cultured rat pulmonary endothelial cells. J. Clin. Invest. **89:**197–202.
14. **Falciani, F., P. Ghezzi, M. Terao, G. Cazzaniga, and E. Garattini.** 1992. Interferons induce xanthine dehydrogenase gene expression in L929 cells. Biochem. J. **285:**1001–1008.
15. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
16. **Granger, D. L., J. B. Hibbs, Jr., J. R. Perfect, and D. T. Durack.** 1988. Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. J. Clin. Invest. **81:**1129–1136.
17. **Granger, D. N., and P. Kubes.** 1994. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. J. Leukocyte Biol. **55:** 662–675.
18. **Grum, C. M., T. J. Gross, C. H. Mody, and G. Sitrin.** 1990. Expression of xanthine oxidase activity by murine leukocytes. J. Lab. Clin. Med. **116:**211–218.
19. **Hausladen, A., and I. Fridovich.** 1994. Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. J. Biol. Chem. **269:**29405–29408.
20. **Huie, R. E., and S. Padmaja.** 1993. Reaction of NO with $O_2^{\cdot -}$. Free Radical Res. Commun. **18:**195–199.
21. **Ikeda, T., K. Shimokata, T. Daikoku, T. Fukatsu, Y. Tsutsui, and Y. Nishiyama.** 1992. Pathogenesis of cytomegalovirus-associated pneumonitis in ICR mice: possible involvement of superoxide radicals. Arch. Virol. **127:**11–24.
22. **James, S. L.** 1995. Role of nitric oxide in parasitic infections. Microbiol. Rev. **59:**533–547.
23. **James, S. L., and J. Glaven.** 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. J. Immunol. **143:**4208–4212.
24. **Jarasch, E.-D., C. Grund, G. Bruder, H. W. Heid, T. W. Keenan, and W. W. Franke.** 1981. Localization of xanthine oxidase in mammary-gland epithelium and capillary endothelium. Cell **25:**67–82.
25. **Keusch, G. T.** 1986. Typhoid fever, p. 1189–1195. *In* A. I. Braude, E. D. Charles, and J. Fierer (ed.), Infectious diseases and medical microbiology, 2nd ed. W. B. Saunders Company, Philadelphia, Pa.
26. **Kooy, N., J. Royall, Y. Ye, D. Kelly, and J. S. Beckman.** 1995. Evidence for in vivo peroxynitrite production in human acute lung injury. Am. J. Respir. Crit. Care Med. **151:**1250–1254.
27. **Kubes, P.** 1995. Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. Microcirculation **2:**235–244.
28. **Liew, F. Y., S. Millott, C. Parkinson, R. M. J. Palmer, and S. Moncada.** 1990. Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. J. Immunol. **144:**4794–4797.
29. **Lowenstein, C. J., and S. H. Snyder.** 1992. Nitric oxide, a novel biologic messenger. Cell **70:**705–707.
30. **Miles, A. M., D. S. Bohle, P. A. Glassbrenner, B. Hansert, D. A. Wink, and M. B. Grisham.** 1996. Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. J. Biol. Chem. **271:**40–47.
31. **Miyamoto, Y., T. Akaike, M. Yoshida, S. Goto, H. Horie, and H. Maeda.** 1996. Potentiation of nitric oxide-mediated vasorelaxation by xanthine oxidase inhibitors. Proc. Soc. Exp. Biol. Med. **211:**366–373.
32. **Nathan, C.** 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. **6:**3051–3064.
33. **Ohshima, H., and H. Bartsch.** 1994. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutant. Res. **305:**253–264.
34. **Palmer, R. M. J., A. G. Ferrige, and S. Moncada.** 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature **327:**524–526.
35. **Rinaldo, J. E., M. Clark, J. Parinello, and V. L. Shepherd.** 1994. Nitric oxide inactivates xanthine dehydrogenase and xanthine oxidase in interferon-γ-stimulated macrophages. Am. J. Respir. Cell Mol. Biol. **11:**625–630.
36. **Rubbo, H., V. Darley-Usmar, and B. A. Freeman.** 1996. Nitric oxide regulation of tissue free radical injury. Chem. Res. Toxicol. **9:**809–820.
37. **Salgers, A. A., and D. D. Whitt.** 1994. *Salmonella* infections, p. 229–243. *In* A. A. Salgers and D. D. Whitt (ed.), Bacterial pathogenesis: a molecular approach. ASM Press, Washington, D.C.
38. **Setoguchi, K., M. Takeya, T. Akaike, M. Suga, R. Hattori, H. Maeda, M. Ando, and K. Takahashi.** 1996. Expression of inducible nitric oxide synthase and its involvement in pulmonary granulomatous inflammation of rats. Am. J. Pathol. **149:**2005–2022.
39. **Sheffler, L. A., D. A. Wink, G. Melillo, and G. W. Cox.** 1995. Exogenous nitric oxide regulates IFN-gamma plus lipopolysaccharide-induced nitric oxide synthase expression in mouse macrophages. J. Immunol. **155:**886–894.
40. **Stueher, D. J.** 1992. Mammalian nitric oxide synthases. Adv. Enzymol. Relat. Areas Mol. Biol. **65:**287–346.
41. **Suematsu, M., H. Suzuki, H. Ishii, S. Kato, T. Yanagisawa, H. Asako, M. Suzuki, and M. Tsuchiya.** 1992. Early midzonal oxidative stress preceding cell death in hypoperfused rat liver. Gastroenterology **103:**994–1001.
42. **Szabo, C., A. L. Salzman, and H. Ichiropoulos.** 1995. Endotoxin triggers the expression of an inducible isoform of nitric oxide synthase and the formation of peroxinitrite in the rat aorta in vivo. FEBS Lett. **363:**235–238.
43. **Sztein, M. B., S. S. Wasserman, C. O. Tacket, R. Edelman, D. Hone, A. A. Lindberg, and M. M. Levine.** 1994. Cytokine production patterns and lymphoproliferative responses in volunteers orally immunized with attenuated vaccine strains of *Salmonella typhi*. J. Infect. Dis. **170:**1508–1517.
44. **Takahashi, M., T. Ushijima, and Y. Ozaki.** 1988. Changes in hepatic superoxide dismutase and xanthine oxidase activity in mice infected with *Salmonella typhimurium* and *Pseudomonas aeruginosa*. J. Med. Microbiol. **26:**281–284.
45. **Terao, M., G. Cazzaniga, P. Ghezzi, M. Bianchi, F. Falciani, P. Perani, and E. Garattini.** 1992. Molecular cloning of cDNA coding for mouse liver xanthine dehydrogenase. Biochem. J. **283:**863–870.
46. **Tubaro, E., B. Lotti, G. Cavallo, C. Croce, and G. Borelli.** 1980. Liver xanthine oxidase in mice in three pathological models. Biochem. Pharmacol. **29:**1939–1943.
47. **Tubaro, E., B. Lotti, C. Santiangeli, and G. Cavallo.** 1980. Xanthine oxidase increase in polymorphonuclear leukocytes and macrophages in mice in three pathological situations. Biochem. Pharmacol. **29:**1945–1948.
48. **Umezawa, K., N. Ohnishi, K. Tanaka, S. Kamiya, Y. Koga, H. Nakazawa, and A. Ozawa.** 1995. Granulation in livers of mice infected with *Salmonella typhimurium* is caused by superoxide released from host phagocytes. Infect. Immun. **63:**4402–4408.
49. **Uppu, R. M., G. L. Squadrito, and W. A. Pryor.** 1996. Acceleration of peroxynitrite oxidations by carbon dioxide. Arch. Biochem. Biophys. **327:** 335–343.
50. **van der Vilet, A., J. P. Eiserich, B. Halliwell, and C. E. Cross.** 1996. Phenolic nitration via myeloperoxidase-catalyzed oxidation of nitrite. An alternative *in vivo* mechanism of protein nitration?, abstr. C-37, p. 98. *In* Abstracts of the 2nd International Conference on Biochemistry and Molecular Biology of Nitric Oxide 1996. UCLA, Los Angeles, Calif.
51. **Yoshida, K., T. Akaike, T. Doi, K. Sato, S. Ijiri, M. Suga, M. Ando, and H. Maeda.** 1993. Pronounced enhancement of NO-dependent antimicrobial action by an NO-oxidizing agent, imidazolineoxyl *N*-oxide. Infect. Immun. **61:**3552–3555.
52. **Yoshida, M., T. Akaike, Y. Wada, K. Sato, K. Ikeda, S. Ueda, and H. Maeda.** 1994. Therapeutic effects of imidazolineoxyl *N*-oxide against nitric oxide-scavenging activity. Biochem. Biophys. Res. Commun. **202:**923–930.

Editor: J. R. McGhee