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Inhibition of nitric oxide synthase (NOS) aggravates *Staphylococcus aureus* septicaemia and septic arthritis

E. SAKINIENE, T. BREMELL & A. TARKOWSKI *Department of Rheumatology, University of Gothenburg, Gothenburg, Sweden*

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SUMMARY

The aim of this study was to assess the role of NO and its metabolites in bacterial arthritis. The murine model of haematogenously acquired septic arthritis was used. Swiss mice treated with NOS inhibitors (N^G-monomethyl-L-arginine or N^ω-nitro-L-arginine methyl ester) were injected intravenously with toxic shock syndrome toxin-1 (TSST-1) producing *Staphylococcus aureus* LS-1. Arthritis was evaluated clinically and histopathologically. Serum cytokine levels, bacterial isolates and intracellular capacity of macrophages to kill bacteria were also analysed. The frequency of arthritis in mice treated with NOS inhibitors was three to four-fold higher than that in non-treated controls (75% *versus* 20%). The severity of arthritis, expressed as mean arthritic index, was 1·4 and 0·4, respectively. Cartilage and/or bone destruction occurred in 63% of NOS inhibitor-treated mice, but only in 10% of controls. Also, the cumulative septicaemia-induced mortality was clearly higher in mice treated with NOS inhibitors compared with non-treated controls. Intracellular killing capacity of the peritoneal macrophages, treated *in vitro* with NOS inhibitors, was decreased. Thus, 24 h after bacterial inoculation peritoneal macrophages pretreated with NOS inhibitors killed more than 10 times less bacteria than the control ones ($P < 0{\cdot}01$). We conclude that NOS inhibitors aggravate *S. aureus* arthritis, possibly by inducing impairment of the intracellular killing capacity of macrophages.

Keywords infectious arthritis nitric oxide N^G-monomethyl-L-arginine

INTRODUCTION

Bacterial arthritis is a severe, rapidly progressing erosive disease with high morbidity and mortality [1]. *Staphylococcus aureus* is the most common bacterium causing this ailment [1].

We have recently developed a murine model of haematogenously spread *S. aureus* arthritis [2]. Using this model we have demonstrated that the host immune response is responsible for most of the sequels as a consequence of infection. More specifically, CD4 T lymphocytes contribute to the disease, since depletion of either CD4 T cells [3] or $\alpha\beta$ T cell receptor (TCR)-expressing cells [4] ameliorates the disease outcome. The activation and clonal *in situ* expansion of T cells [3,5] is due to superantigenic properties of the exotoxins produced by *S. aureus* [6]. Macrophages play an important role in the process of joint destruction. For example, macrophage-derived cytokines such as IL-1β and tumour necrosis factor-alpha (TNF-α), found during *S. aureus* infection in the joint tissue [7], contribute to bone and cartilage degradation [8]. In contrast, polymorphonuclear cells are important in the defence mechanism during *S. aureus* infection ([9] and Verdrengh, unpublished results).

NO has both beneficial and harmful effects in inflammation: e.g. it contributes to the cytotoxic properties of activated macrophages [10] and to the bactericidal capacity of neutrophils [11], it is involved in causing tissue injury [12], and it modulates T cell proliferation [13,14]. However, the role of NO in arthritis is largely unknown. In contrast, NOS inhibitors were reported to suppress adjuvant and streptococcal cell wall-induced arthritis [15–17], yet had no effect on borrelial arthritis [18].

The aim of the present study was to evaluate the effect of NOS inhibitors on the course of septic superantigen-mediated arthritis.

Correspondence: Egidija Sakiniene, Department of Rheumatology, Guldhedsgatan 10A, S-413 46 Gothenburg, Sweden.

MATERIALS AND METHODS

Mice

Male and female 5–7-week-old pathogen-free Swiss mice, originally purchased from ALAB (Stockholm, Sweden), were used. Mice were bred and maintained in the animal facility of the Department of Clinical Immunology, University of Gothenburg. The mice were housed up to 10 in each cage, under standard conditions of

temperature and light. They were fed standard laboratory chow and water *ad libitum*.

Bacterial strain, culture conditions and injection of mice

Staphylococcus aureus strain LS-1, originally isolated from a swollen joint of a spontaneously arthritic NZB/W mouse [19], was cultured on blood agar for 24 h, then reincubated on blood agar for another 24 h. Bacteria were kept frozen at −20°C, in PBS (0·13 M sodium chloride, 10 mM sodium phosphate, pH 7·4) containing 5% bovine serum albumin (BSA) and 10% dimethylsulfoxide (C_2H_6OS), until use. Before the experiment was started, the bacterial solution was thawed, washed in PBS twice, and diluted in PBS to achieve the desired concentration of bacteria. Mice were injected intravenously into the tail vein with an optimal or suboptimal arthritogenic dose of *S. aureus* LS-1 in 0·2 ml of PBS, as previously described [2]. Viable counts in the leftover solution were determined to ascertain the number of bacteria injected.

Clinical evaluation of arthritis

All the mice were followed individually and arthritis was evaluated. Limbs were inspected on days 0, 3, 7, 10 after bacterial inoculation, by two observers, one of them always being blinded. Arthritis was defined as visible erythema and/or swelling of joint. To evaluate the intensity of arthritis, a clinical scoring (arthritic index) was carried out using a system where macroscopic inspection yielded a score of 0–3 points for each limb (0 point, no swelling or erythema; 1 point, mild swelling and/or erythema; 2 points, moderate swelling and erythema; 3 points, marked swelling and erythema) [20]. The overall condition was evaluated by assessment of weight, general appearance, alertness and skin abnormalities. Mortality was registered.

Treatment

N^G-monomethyl-L-arginine, monoacetate salt ($C_7H_{16}N_4O_2{\cdot}CH$-COOH) (KeLab, Gothenburg, Sweden) was injected intraperitoneally at the dosage of 0·4 mg/g body weight every 24 h. N^{ω}-nitro-L-arginine methyl ester ($C_7H_{15}N_5O_4{\cdot}HCl$) (KeLab) was injected intraperitoneally at the dosage of 0·1 mg/g body weight every 24 h.

Bacteriological examination

Samples for bacterial analysis of joints were obtained using charcoaled sticks, after dissection of talocrural and radiocarpal joints, and transferred to 5% horse blood agar. The culture was considered positive if >20 *S. aureus* colonies were present after 48 h of incubation at 37°C [19]. Spleens, kidneys and livers were aseptically removed, homogenized for 20 min in Colworth Stomacher 80 homogenizer (AJ Seward, London, UK) at 4°C, diluted in nutrient culture medium and inoculated on horse blood agar in serial dilutions to estimate the bacterial growth in each organ. Also samples of heparinized blood were inoculated on horse blood agar. All bacterial isolates were tested for catalase and coagulase activity.

Haematological analysis

Leucocytes were counted in Bürker's chamber or with the help of Sysmex F-300 (Toa Medical Electronics, Kobe, Japan) microcell counter. The differential count was estimated examining blood smears stained by Giemsa technique.

Serological analysis

IL-6 assay. Cell line B13.29, which is dependent on IL-6 for growth, has been previously described [21]. For IL-6 determinations, the more sensitive subclone B9 was used [22,23]. B9 cells were harvested from tissue culture flasks, seeded into microtitre plates (Nunc, Roskilde, Denmark) at the concentration of 5000 cells/well, and cultured in Iscove's medium supplemented with 5×10^{-5} M 2-mercaptoethanol (2-ME), 5% fetal calf serum (FCS; Seralab, Crawley Down, UK), penicillin 100 U/ml, and streptomycin 100 mg/ml, and serum samples were added. ^{3}H-thymidine was added after 68 h of culturing, and the cells were harvested 4 h later. The samples were tested in two-fold dilutions and compared with a recombinant mouse IL-6 standard (Genzyme, Cambridge, MA) [24]. B9 cells were previously shown not to react with several recombinant cytokines, including IL-1α, IL-1β, IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and interferon-gamma (IFN-γ). There was only weak reactivity with IL-4 [23]. Addition of anti-IL-6 antibody reduced proliferative response of B9 cells to IL-6 by at least 95% [25].

Immunoglobulins. Serum levels of total IgG1, IgG2a, IgG3 and IgM were measured by the radial immunodiffusion technique, as previously described [26,27]. Antisera and immunoglobulin standards specific for IgG1, IgG2a, IgG3 and IgM were purchased from Sigma (St Louis, MO).

Anti-ssDNA antibodies. Levels in serum of antibodies to denatured single-stranded DNA (ssDNA) were measured by an ELISA with methylated BSA (10 μg/ml) to precoat wells and 50 μg/ml of heat-denatured (boiled for 20 min and then cooled rapidly on ice) calf thymus DNA (Sigma) as coating. All sera were serially diluted in PBS–BSA 0·5% and incubated in wells. To measure the level and class specificity of anti-ssDNA antibodies bound to the solid phase, affinity-purified and biotinylated $F(ab')_2$ fragments of goat anti-mouse IgG and IgM (Jackson Labs, West Grove, PA), diluted 1:3000 in PBS–Tween 20, were added, followed by 0·5 μg/ml of avidin-horseradish peroxidase (HRP; Sigma) and 2·5 μg/ml of the enzyme substrate 2,2-azino-bis-(-3-ethylbenzothiazoline sulfonic acid) (Sigma) in citrate buffer pH 4·2, containing 0·0075% H_2O_2. The absorbance at 405 nm was measured in a Titertek Multiscan photometer (Flow Labs, McLean, VA). All optical density (OD) values were converted to antigen-specific arbitrary units with calibration curves based on the OD values obtained from serial dilutions of a reference pool of sera. The calibration curves were constructed with a computer program based on weighted logit-log models [28,29].

Antibodies to cell walls of S. aureus *LS-1*. Levels in serum of IgG and IgM antibodies to *S. aureus* cell wall constituents were estimated by the same ELISA as that described above, with the exception that the wells were precoated with 50 mg/ml of poly-L-lysine (Sigma) followed by incubation with whole, formalin-treated (4%, 20 min) *S. aureus* LS-1 cells (10^8/ml) instead of denatured ssDNA. The concentration of bacteria was chosen after checkerboard titration (results not shown).

Anti-toxic shock syndrome toxin-1 antibodies. Levels in serum of IgG and IgM antibodies to toxic shock syndrome toxin-1 (TSST-1) were estimated by an ELISA using 0·5 μg/ml of highly purified TSST-1 (Toxin Technology, Sarasota, FL) as a solid-phase coating.

IFN-γ. Levels of serum IFN-γ were measured by ELISA using 2 μg/ml of purified anti-mouse IFN-γ MoAb (PharMingen, San Diego, CA) in sodium bicarbonate pH 9·6 for coating. All sera were serially diluted in PBS–BSA 0·5% and incubated in wells. Biotinylated anti-mouse IFN-γ (2 μg/ml; PharMingen) was added to measure the level of IFN-γ bound to solid phase. This procedure was followed by stepwise addition of extravidin alkaline

phosphatase (Sigma). The enzyme substrate was then added, and absorbance was measured in a Titertek Multiscan photometer (Flow Labs) at 405 nm. The samples were tested in two-fold dilutions and compared with recombinant mouse IFN-γ standard (Genzyme) [30].

Determination of nitrates in sera. Serum nitrate was determined with a stable isotope ($Na^{15}NO_3$) dilution assay, utilizing positive ion/chemical ionization gas chromatography/mass spectrometry, after conversion of endogenous and labelled nitrate in the samples to nitrotoluene, as previously described in detail [31].

Analysis of the phagocytic activity of leucocytes

Freshly obtained heparinized whole blood was vortexed and aliquoted on the bottom of a 5-ml tube. Pre-cooled FITC-conjugated bacteria were added (1×10^9/ml) and incubated for 20 min at 37°C. Ice-cold quenching solution was then added to remove cell surface-bound FITC. The erythrocytes were lysed and leucocyte membranes solubilized to permit detection of intracellular FITC-bacterial deposits (Pharma, Heidelberg, Germany). Measurements were performed with FACScan (Becton Dickinson, San Jose, CA).

Histopathological examination

Histological examination was performed after routine fixation, decalcification and paraffin embedding. Tissue sections stained with haematoxylin and eosin from upper extremities (elbow, wrist, carpal joints, fingers) and lower extremities (knee, ankle, tarsal joints and toes) were prepared. The joints were examined with regard to synovial hypertrophy, defined as synovial membrane thickness of more than two cell layers [32], pannus formation (synovial tissue overlaying joint cartilage), cartilage and subchondral bone destruction. In addition, infiltration of inflammatory cells to the extra-articular space and types of invading cells were evaluated. All slides were coded.

Immunization procedure and registration of DTH reactions

Mice were sensitized by epicutaneous application of 150 μl of absolute ethanol/acetone solution containing 3% OXA (4-ethoxymethylene-2-phenyloxazolone) on the shaved thorax and abdomen. Seven days after the sensitization, right ears of all mice were challenged on both sides by topical application of 30 μl of 1% of OXA dissolved in olive oil. Ear thickness was measured before and 24 h after challenge using an Oditest spring calliper (Kröplin, Schlüchtern, Germany) [33]. Challenges and measurements were performed under light anaesthesia. The intensity of DTH was expressed as increase of ear thickness in mm.

Olive oil-induced inflammation

Inflammation was induced by injection of 50 μl olive oil intradermally in the hind foot pad. This inflammatory response is granulocyte-mediated but T cell-independent [34,35]. Foot pad thickness was measured before and 24 h after the injection using an Oditest spring calliper [35]. Injection and measurements were performed under light anaesthesia. The inflammatory response was expressed as the increase of foot pad thickness in mm.

Intracellular killing activity of intraperitoneal macrophages

The intracellular killing activity of i.p. macrophages was tested by the modification of a previously described method by Lissner *et al*. [36]. Peritoneal macrophages were recovered by injecting 3 ml ice cold medium (Iscove's, 10% FCS, 1% gentamycin) into the peritoneal cavity of sacrificed mice, and aspirating the medium after 2 min of vigorous massage. The cells were adjusted to the concentration of 2×10^6/ml medium (Iscove's, 10% FCS, 1% gentamycin), seeded in 200-ml volumes into 24-well plates (Nunc) and incubated at room temperature 90 min. Afterwards, 500 μl medium were added to each well, and the cells were incubated for 4 h at 37°C. The medium was then removed and 500 μl of medium without any antibiotics were added to the cells. After incubation overnight at 37°C the cells were washed once with Iscove's, and 500 μl of *S. aureus* LS-1 suspension were added at a concentration of 2×10^6 bacteria/ml for 50 min. Then the cells were washed three times with Iscove's to remove bacteria which were not ingested, and the macrophages were analysed at three different time points, starting directly after bacterial incubation, and 4 h and 24 h later. To those macrophages which were further incubated for 4 h and 24 h, Iscove's with 5% FCS and minimal inhibitory concentration of gentamycin for *S. aureus* (4 μg/ml) was added to avoid the extracellular replication of bacteria. The macrophages were lysed with distilled water for 20 min, and the lysate, diluted 1:1, 1:10, 1:100 and 1:1000, was cultured on 5% horse blood agar plates. The plates were incubated for 24 h, and the number of bacterial colonies counted.

Proliferation and differentiation of spleen mononuclear cells

Spleens were teased with forceps and passed twice through the nylon mesh. The cells were suspended in PBS and centrifuged at 515 ***g*** for 5 min. The pelleted cells were resuspended for 10 min in Tris-buffered 0·83% ammonium chloride to lyse erythrocytes. After two washings in PBS, the mononuclear cells were counted using a microcell counter Sysmex F-300. Spleen mononuclear cells were incubated at 2×10^6/ml in Iscove's complete medium (GIBCO, Paisley, UK) with either formalin-killed *S. aureus* strain LS-1 (10^7 cells/ml), 10 μg/ml of purified TSST-1 (Toxin Technology), or 2·5 μg/ml of concanavalin A (Con A; ICN Biochemicals, Cleveland, OH). ^{3}H-thymidine was added after 30 h of culturing, and the cells were harvested 18 h later. The results are expressed as counts of radioactive decay of incorporated ^{3}H-thymidine per minute. Also, supernatants were then collected for the determination of IFN-γ 24 h and 48 h after incubation.

Statistical analysis

The differences between parametric and non-parametric values in all treatment groups were tested for significance by use of the two-tailed Student's *t*-test and the Wilcoxon signed rank test, respectively. Differences between groups regarding the occurrence of arthritis, mortality rate, incidence of positive joint, spleen and kidney bacteriological cultures were analysed by the χ^2 test with the Yates' correction. Results are presented as means $\pm$ s.e.m. $P \leqslant 0{\cdot}05$ was considered statistically significant.

RESULTS

Effects of NOS inhibitor administration on the clinical course of arthritis

Female Swiss mice (7 weeks old) were injected into the tail vein with 0·2 ml of bacterial solution in PBS, containing a suboptimal arthritogenic dose of *S. aureus* LS-1 (6×10^6 colony-forming units (CFU)/mouse) [2]. Mice were subdivided into two groups, 10 in every group. The treatment with L-NMMA was started 2 h before the inoculation of bacteria. The control group received no treatment. On days 0, 3, 7 and 10 all mice were weighed, their general condition was evaluated, and they were examined for clinical signs of arthritis.

On day 10 after inoculation of bacteria, blood was obtained for haematological and serological analysis, nitrate assay, and mice were killed by cervical dislocation. Kidneys and spleens were taken for bacteriological examination. Bacteriological samples from left front and hind limbs were obtained, right front and hind limbs were used for histological examination.

Already on day 3 after inoculation with a suboptimal dose (6×10^6 CFU/mouse) of bacteria the frequency of arthritis in L-NMMA-treated mice was two-fold higher than in controls (40% *versus* 20%). This difference became even more pronounced 7 days after inoculation of bacteria, when 75% of mice treated with L-NMMA developed clinical symptoms of arthritis, compared with 20% in the control group. The severity of arthritis, expressed as mean arthritic index, was 1·4 and 0·4 respectively (Fig. 1a,b). Also, treatment with L-NMMA affected the septicaemia-induced mortality. Of mice treated with L-NMMA, 20% died, while all mice in the control group survived.

In the next set of experiments we used a higher (optimal) dose of bacteria ($1{\cdot}4\times10^7$ CFU/mouse), otherwise the experimental protocol was similar. On day 3 after inoculation with *S. aureus* the prevalence of clinical arthritis in the L-NMMA-treated group

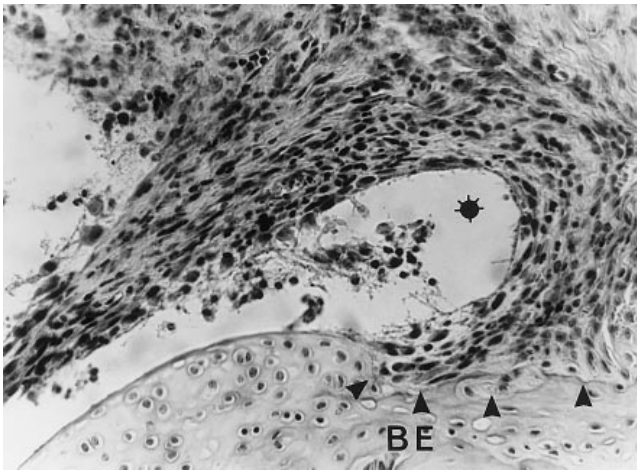

Fig. 2. Micrograph showing tarsal joint of an L-NMMA-treated 9-week-old female Swiss mouse 10 days after inoculation with *Staphylococcus aureus*. Marked synovial hypertrophy is visible and there is bone erosion (BE) at the cartilage–synovium junction. There are also polymorphonuclear cells in the joint cavity, which is marked with an asterisk. (Mag. ×32.)

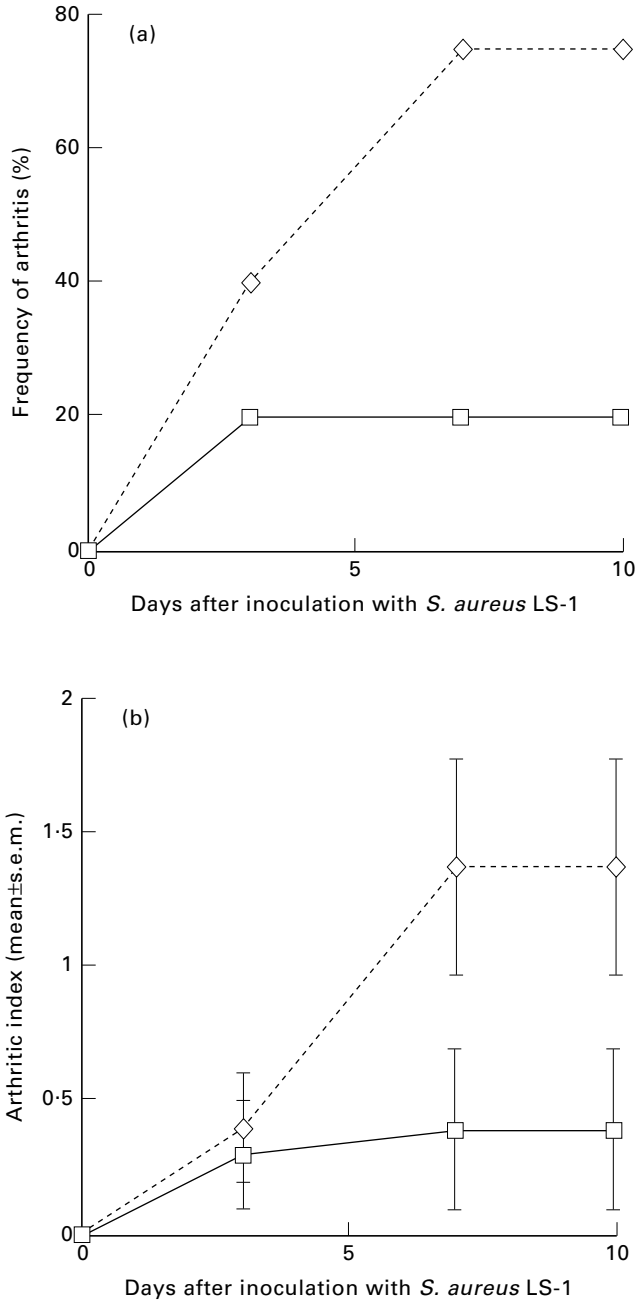

Fig. 1. Effect of L-NMMA treatment on the prevalence (a) and severity (b) of arthritis ($n=8$–10 per group) in mice inoculated with a suboptimal dose of *Staphylococcus aureus*. ◇, L-NMMA-treated; □, controls.

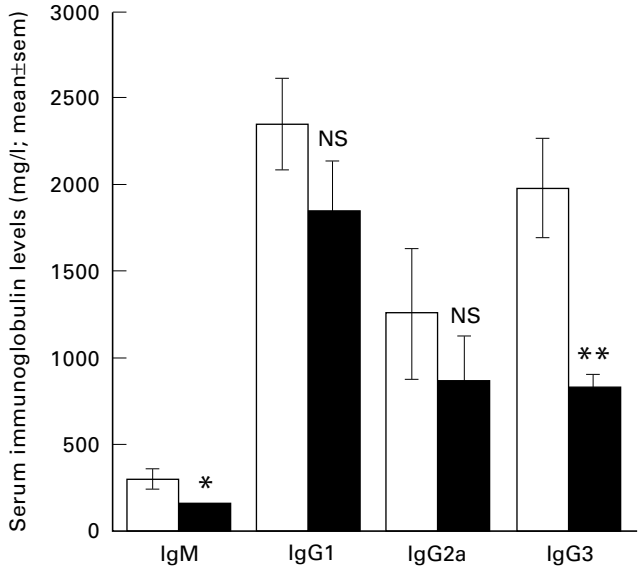

Fig. 3. Effect of L-NMMA on serum levels of IgG1, IgG2a, IgG3 and IgM (mg/*l*; mean ± s.e.m.) in Swiss mice 10 days after inoculation with a suboptimal arthritogenic dose of *Staphylococcus aureus* strain LS-1 ($n=8$–10 per group). The determination of immunoglobulin levels was performed by a single radial immunodiffusion technique. $^*P<0{\cdot}05$; $^{**}P<0{\cdot}01$; NS, not significant difference. □, Controls; ■, L-NMMA-treated.

was two-fold higher than in the control group (50% *versus* 22%). L-NMMA had an immense effect on the mortality rate: 7 days after inoculation of bacteria 80% of animals in the L-NMMA-treated group died, compared with 30% in the control group.

Similar results were obtained using another NOS inhibitor, L-NAME. Twenty days after inoculation of bacteria all mice in the L-NAME-treated group had symptoms of arthritis, compared with 67% in the control group. The mortality rate was 54% and 31%, respectively.

Histopathological findings

Of mice inoculated with the suboptimal dose of bacteria in the L-NMMA-treated group, 63% displayed synovitis, whereas it occurred only in 30% of control animals. The prevalence of severe joint engagement, i.e. cartilage and/or bone destruction, was found in 63% of L-NMMA-treated mice, but only in 10% of controls (Fig. 2). There were no evident differences between the groups with respect to the proportions of the polymorphonuclear *versus* mononuclear cells in the synovial tissue.

Effect of NOS inhibitor on the clearance of bacteria from the tissues of mice infected with arthritogenic dose of S. aureus *LS-1*

Thirty-one 7-week-old female Swiss mice were subdivided into two groups. One group of mice was treated with L-NMMA (0·4 mg/g body weight intraperitoneally every 24 h). The controls received no treatment. All mice were inoculated with approximately 1×10^7 CFU/mouse of *S. aureus* LS-1. The mice were killed by cervical dislocation at the intervals of 4, 24, 48 h and 7 days after inoculation of bacteria (three to four from each group at every time point), and their blood, spleens, kidneys, livers and isolates from left radiocarpal and talocrural joints were obtained for bacteriological analysis. Right radiocarpal and talocrural joints were used for histological examination. Blood was obtained for differential count as well. Two out of four mice treated with L-NMMA harboured *S. aureus* in the joints 48 h after inoculation of bacteria, while bacteria were not isolated from the joints of the control animals. Seven days after inoculation of bacteria none of the controls but three out of four L-NMMA-treated animals harboured bacteria in the joints. There were no differences in the number of isolated bacteria from blood, liver, spleen or kidneys at any time point between the groups.

Serological and haematological manifestations

Treatment with both L-NMMA and L-NAME slightly reduced serum immunoglobulin levels of both M and G classes (Figs 3 and 4) in infected mice. The levels of specific antibodies to *S. aureus* whole cells, TSST-1 and ssDNA were modestly affected by the treatment (data not shown). To exclude the possible immunosuppressive effect of NOS inhibition we administered L-NAME to non-infected mice for 20 days, then determined the amount of serum immunoglobulins, and observed that IgM, IgG1 and IgG3 increased rather than decreased as a consequence of treatment (Fig. 4).

IFN-γ is a Th1 cytokine of importance in the pathogenesis of septic arthritis [7]. The serum levels of this cytokine were, however, essentially unaltered in the L-NMMA-treated group compared with controls (882 ± 146 mU/ml *versus* 609 ± 314 mU/ml) in infected animals. Serum levels of IL-6, a proinflammatory cytokine, were significantly decreased in L-NMMA-treated animals during the infection (Fig. 5).

Staphylococcus aureus infection leads to splenomegaly and leucocytosis. Administration of L-NMMA significantly decreased spleen weight ($P \leqslant 0{\cdot}01$). The total number of leucocytes in the

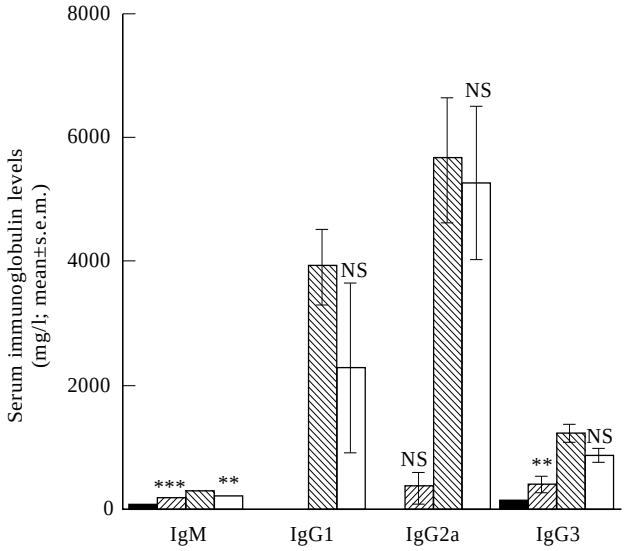

Fig. 4. Serum levels of IgG1, IgG2a, IgG3 and IgM (mg/*l*; mean ± s.e.m.) 20 days after the start of the experiment in untreated Swiss mice not receiving bacterial inoculum (■), in mice not receiving bacterial inoculum, but treated with L-NAME (▨), in untreated mice inoculated with an arthritogenic dose of *Staphylococcus aureus* (▧), and in L-NAME-treated mice inoculated with *S. aureus* (□) ($n = 6$–12 per group). $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; NS, not significant difference, groups of mice not treated with L-NAME are compared with those receiving L-NAME.

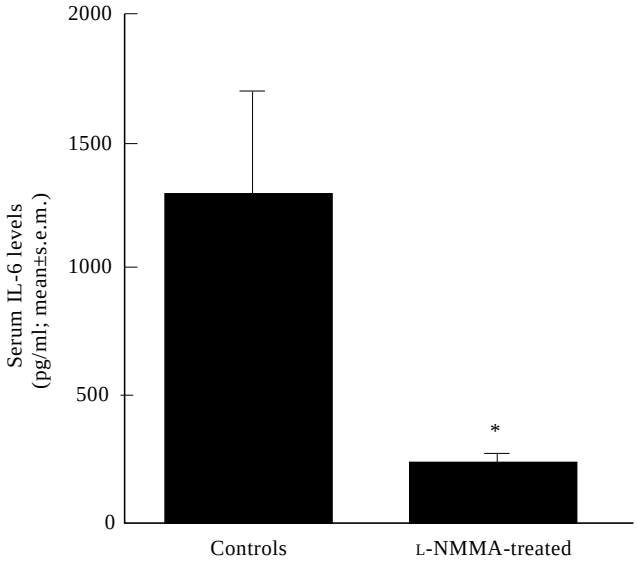

Fig. 5. Effect of L-NMMA on serum IL-6 levels (pg/ml; mean ± s.e.m.) in Swiss mice 10 days after inoculation with a suboptimal arthritogenic dose of *Staphylococcus aureus* strain LS-1 ($n = 8$–10 per group). The determination of IL-6 was performed by a bioassay using an IL-6-dependent B9 cell clone. $^{*}P < 0{\cdot}05$.

peripheral blood was also decreased in this group due to the decreased number of polymorphonuclear cells. L-NMMA treatment of non-infected mice had no significant effect either on the total number of leucocytes, or on their differential count.

Effect of L-NMMA on the phagocytosis and intracellular killing capacity

Sixteen male 7-week-old Swiss mice were subdivided into two groups. One group of mice was treated with L-NMMA (0·4 mg/g body weight intraperitoneally every 24 h). Controls received no treatment. Mice were bled from the tail vein, and the phagocytic activity of leucocytes was determined.

Twenty-four male 7-week-old Swiss mice were subdivided into four groups. Two groups of mice were treated with L-NMMA (0·4 mg/g body weight intraperitoneally every 24 h) for 3 days. Controls received no treatment. Afterwards all mice were killed, their i.p. macrophages obtained for determination of intracellular killing activity. The cells from the mice from one control and one *in vivo* L-NMMA-pretreated group were treated with L-NMMA *in vitro*, adjusting its concentration to 1 mM in all applied medias.

There were no differences in the number of peripheral blood phagocytic cells nor in their capacity to phagocytize bacteria between the groups. Forty-three percent of peripheral leucocytes had phagocytic activity in the L-NMMA-treated group compared with 38% in controls. The uptake of bacteria per cell, expressed in mean fluorescence intensity (MFI) ± s.e.m. was 3213 ± 475 and 2905 ± 332, respectively. The phagocytic activity of i.p. macrophages in L-NMMA-treated animals was not different from that in the control group. However, intracellular killing capacity of the peritoneal macrophages, treated *in vitro* with L-NMMA, was decreased. Indeed, 24 h after inoculation of bacteria, L-NMMA-treated peritoneal macrophages killed more than 10 times less bacteria than the control ones ($P < 0{\cdot}01$) (Fig. 6).

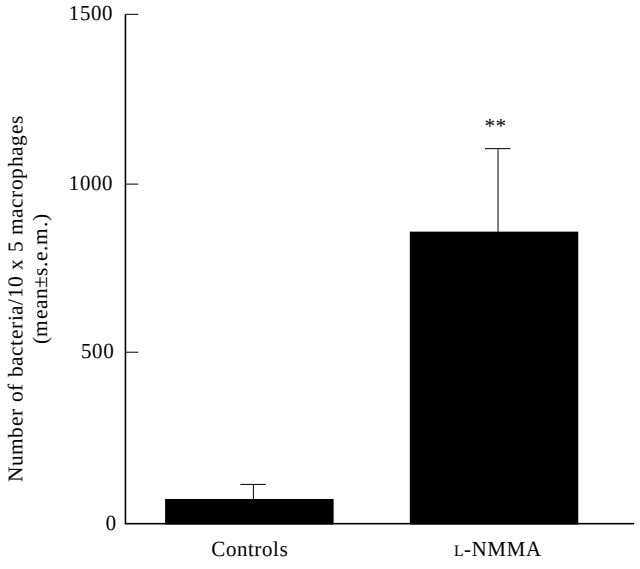

Fig. 6. Decreased macrophage intracellular killing capacity of *Staphylococcus aureus*. Murine intraperitoneal macrophages were *in vitro* treated with L-NMMA and incubated with bacteria for 24 h (number of bacteria $\times 10^5$/macrophages; mean ± s.e.m.). $^{**}P < 0{\cdot}01$.

Effect of L-NMMA on the T cell-dependent and granulocyte-dependent inflammatory responses

DTH reaction is an antigen-specific and class II-restricted T cell-mediated immune response [37]. Since *S. aureus* sepsis and septic arthritis are also T cell-dependent, we decided to assess the outcome of decreased NO production in a non-bacterial model of T cell-dependent reactions. Thirty-one 5-week-old female Swiss mice were subdivided into two groups. One group of mice was treated with L-NMMA (0·4 mg/g body weight intraperitoneally every 24 h). Controls received no treatment. Two days after the treatment was started, all mice were immunized with OXA. After the period of 7 days they were boosted, and after 24 h DTH reaction was evaluated. Treatment with L-NMMA profoundly reduced the

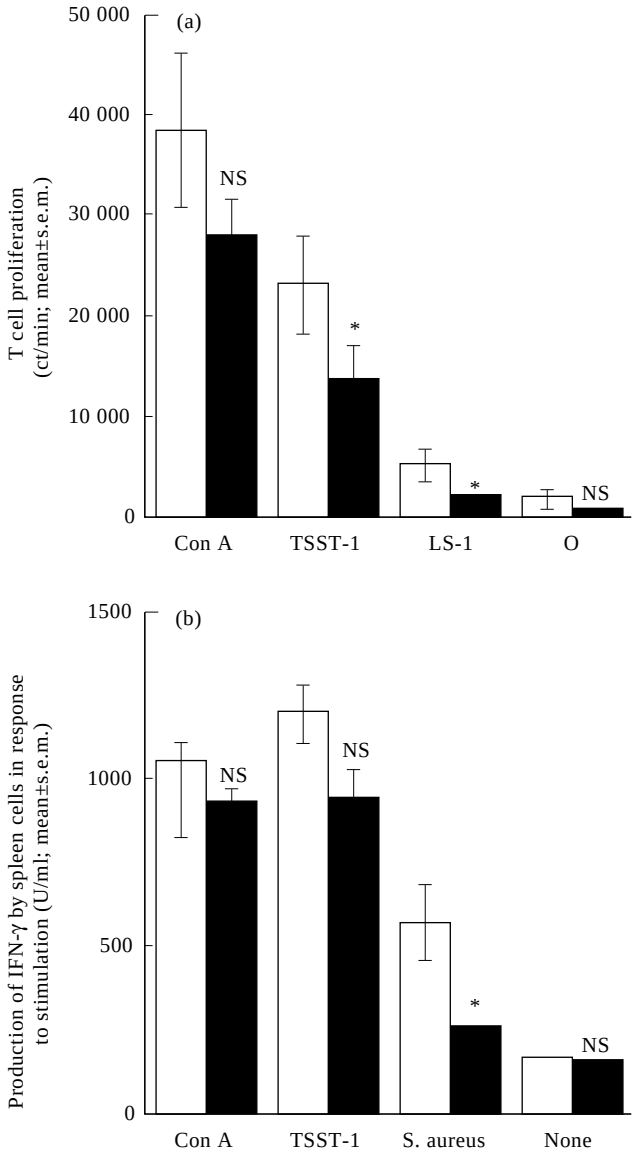

Fig. 7. Effect of pretreatment with L-NMMA on T lymphocyte proliferation (a) and production of IFN-γ (U/ml) (b) in response to stimulation with concanavalin A, toxic shock syndrome toxin-1 (TSST-1) and *Staphylococcus aureus* strain LS-1 cells ($n = 8$ per group). Proliferative *in vitro* responses are recorded as incorporation of ^{3}H-thymidine. Results are expressed in ct/min (mean ± s.e.m.). □, Controls; ■, L-NMMA-treated.

DTH responses ($0{\cdot}08 \pm 0{\cdot}01$ mm *versus* $0{\cdot}13 \pm 0{\cdot}01$ mm, $P \leqslant 0{\cdot}01$) in non-infected mice.

Polymorphonuclear cell-dependent but T cell-independent inflammation was induced by i.d. injection of olive oil into footpad [34]. Non-infected animals, pretreated with L-NMMA, exhibited significantly higher inflammatory response: the footpad thickness increased by $1{\cdot}8 \pm 0{\cdot}1$ mm, compared with $1{\cdot}5 \pm 0{\cdot}1$ mm in the control group ($P \leqslant 0{\cdot}05$).

In order to check the efficiency of L-NMMA in inhibiting NO synthase, the serum levels of NO_3^-, a stable metabolite of NO, were determined and proved to be significantly decreased in mice treated with L-NMMA compared with controls ($70{\cdot}1 \pm 7{\cdot}5$ *versus* $135{\cdot}2 \pm 24{\cdot}6$ μM/*l*; $P \leqslant 0{\cdot}05$).

Effect of L-NMMA on T cell proliferation and differentiation in response to S. aureus *LS-1, TSST-1 and Con A*

L-NMMA moderately reduced spleen T cell proliferation *in vitro* and the production of IFN-γ in response to *S. aureus* LS-1 whole, formalin-fixed cells, TSST-1 and Con A stimulation (Fig. 7a,b).

To investigate the effect of NOS inhibition *in vitro*, we added various amounts (10 μM, 50 μM, 150 μM, 200 μM and 300 μM) of L-NMMA or 1 mM L-arginine to the medium and assessed proliferative responses according to the above protocol. However, we did not detect any clear cut effect of either L-NMMA or L-arginine on the proliferative response of T cells.

DISCUSSION

In the present study we used a murine model of haematogenously acquired *S. aureus* arthritis to evaluate the effect of NOS inhibition on the progression of bacterial arthritis and outcome of septicaemia.

We observed that administration of NOS inhibitors increased the prevalence and severity of *S. aureus* arthritis. These results were not affected by the dose of inoculated bacteria, nor by the choice of NOS inhibitor (L-NMMA or L-NAME). Histological examination of joints supported our clinical findings: synovitis and cartilage and/or bone destruction were more common in the NOS inhibitor-treated group compared with control animals. The septicaemia-induced mortality was also increased in the group of animals receiving NOS inhibitor. The main reason for this outcome might very well be the deficient clearance of bacteria in L-NMMA-treated mice. In this respect, NO plays a crucial role in the microbicidal activity of macrophages [38,39]. In our study NOS inhibitors significantly decreased the capacity of i.p. macrophages to kill phagocytized *S. aureus*. In addition, NOS inhibition down-regulated the number of polymorphonuclear cells, a major cell type providing protection against *S. aureus* ([9], Verdrengh, unpublished results). Altogether, these properties of L-NMMA and L-NAME aggravated septic arthritis.

In contrast, there have been several reports showing the beneficial effect of NOS inhibitors on the course of autoimmune arthritis [15–17,40]. This could be due to the down-regulation of polyclonal B cell activation as well as T cell activities [41,42]. In this context, B [30] and T cells [3,4] contribute to the progression of septic arthritis. Such effects were, however, not observed when naive (i.e. not infected) mice received NOS inhibitors. In contrast, we even observed increased production of IgM and IgG3 in mice receiving L-NAME. Also, L-NMMA-treated naive mice displayed a vivid granulocyte-dependent inflammation compared with untreated control animals. These findings can be explained by the fact that NO plays a modulatory role in inflammation and immunity [42,43]. For example, NOS inhibitors down-regulate proinflammatory cytokines [14]. Indeed, L-NMMA treatment significantly reduced the levels of IL-6 in infected mice. The probable existence of a regulatory loop by which NO inhibits the production of IFN-γ which induces its own synthesis [44] explains why serum IFN-γ levels were slightly but not significantly increased in the animals receiving NOS inhibitor.

Our previous reports [3,4] have shown that T lymphocytes contribute to the course of bacterial arthritis. Regulation of lymphocyte proliferation by suppressor macrophages is mediated by products of the NOS pathway [45]. There are data suggesting that reactive nitrogen oxide released from the activated macrophages is involved in the down-regulation of proliferative responses of T cells [46]. The macrophages were described to mediate the feedback control of T cell activation by releasing NO and thus down-regulating their proliferation [41,47]. We hypothesize that inhibition of NOS disturbed this mechanism, resulting in increased T cell proliferation, and thus worsened the course of the disease. However, NO has a dual effect on T cell activity. On one hand high concentrations of NO inhibit T cell proliferation [48]. On the other hand, NO is necessary for the proliferation of T cells [49], explaining why the spleen cells from mice pretreated with L-NMMA showed decreased proliferative response. In this respect, down-regulation of DTH reaction does not contradict earlier described results, as it was performed in naive, non-infected mice.

We conclude that nitric oxide inhibitors impair the intracellular killing capacity of macrophages and disturb the feedback mechanism of T cell activation, thereby aggravating *S. aureus* arthritis and sepsis-induced mortality.

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