Br. J. Pharmacol. (1993), **110**, 701–706

Modulation of adjuvant arthritis by endogenous nitric oxide

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1 The role of endogenous nitric oxide (NO) in adjuvant arthritis in Lewis rats has been studied by use of L-arginine, the amino acid from which NO is synthesized, and N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase. Prolonged modulation (35 days) of the L-arginine: NO pathway in rats was achieved by dissolving test compounds in the drinking water (L-arginine: 3, 10 and 30 mg ml^{-1}; L-NAME: 0.1, 1 and 10 mg ml^{-1}).

2 Arthritis was exacerbated by L-arginine and suppressed by L-NAME in a dose-related fashion. Combined treatment with L-NAME (1 mg ml^{-1}) and L-arginine (30 mg ml^{-1}) did not modify the arthritis.

3 Reduced weight gain, which is a feature of adjuvant arthritis, was modified by these compounds so that L-arginine reduced weight gain whereas L-NAME increased weight gain compared with that in control animals.

4 D-Arginine (30 mg ml^{-1}), N^G-nitro-D-arginine methyl ester (D-NAME: 1 mg ml^{-1}) and L-lysine (30 mg ml^{-1}), an amino acid not involved in the generation of NO, were without effect on either arthritis or body weight gain.

5. Antigen-stimulated proliferation of T-lymphocytes as well as generation of nitrite (NO_2^-) and release of acid phosphatase from macrophages were all enhanced in L-arginine-treated arthritic rats and reduced in L-NAME-treated animals.

6 These results suggest that endogenous NO modulates adjuvant arthritis, possibly by interfering with the activation of T-lymphocytes and/or macrophages.

Keywords: Adjuvant arthritis; L-arginine; lymphocyte proliferation; macrophage activation; N^G-nitro-L-arginine methyl ester

Introduction

Adjuvant-induced arthritis in the rat is an experimental immunopathy that is thought to share many features with human rheumatoid arthritis and, as such, is one of the most widely used models for studying the anti-inflammatory/antirheumatic properties of compounds (for review see Billingham & Davies, 1979). Chronic polyarthritis is induced in the rat by intradermal injection of an oil emulsion of dead mycobacteria, usually *M. tuberculosis*.

Although the aetiology and pathogenesis of adjuvant arthritis are not yet fully understood, there is substantial evidence that immunological mechanisms are involved and that T-lymphocytes play a major role (for review see Cohen, 1991). L-Arginine exerts a trophic effect on the thymus and improves host immunity. Thus it has been shown that L-arginine enhances the lymphocyte cell count of the thymic gland, blastogenesis of these lymphocytes in response to mitogens, skin allograft rejection and tumour regression (for review see Barbul, 1986).

The biosynthesis of nitric oxide (NO) from L-arginine is a pathway for the regulation of cell function and communication (for review see Moncada *et al.*, 1991). In all cell types so far studied, NO is generated following oxidation and cleavage of the terminal nitrogen atom(s) of L-arginine by an enzyme, the NO synthase. At least two types of NO synthase have been identified so far. The enzyme found in endothelial cells and brain is constitutive, Ca^{2+}-dependent and releases picomolar amounts of NO for a short period following receptor stimulation. In contrast, the enzyme found in the macrophage is induced following stimulation with cytokines or endotoxin, is Ca^{2+}-independent, and releases nanomolar amounts of NO for a long period.

Nitric oxide produced by the constitutive enzyme modulates various functions, such as vascular tone and neurotransmission in the central nervous system, via activation of the soluble guanylate cyclase. The production of NO by the inducible enzyme has been identified as a major mechanism of the cytostatic/cytotoxic action of activated macrophages on target cells. This occurs as a result of combination of NO with iron-sulphur centres in key enzymes of the respiratory cycle and of the pathway for DNA synthesis in the target cells (Hibbs *et al.*, 1990).

The NO synthase is inhibited *in vitro* and *in vivo* by some analogues of L-arginine (Rees *et al.*, 1990; McCall *et al.*, 1991). The identification of these inhibitors has provided an important pharmacological tool for investigating the relevance of NO in biological processes.

In this study we have investigated the effect of L-arginine and of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, on adjuvant arthritis in the rat. We have also studied the effects of D-arginine, N^G-nitro-D-arginine methyl ester (D-NAME) and L-lysine, an amino acid not involved in the biosynthesis of NO. The possible involvement of the L-arginine: NO pathway in T-lymphocyte proliferation and macrophage activation in arthritic rats was also studied, since it has been shown that murine T-lymphocyte clones express the inducible NO synthase and generate NO following stimulation with cytokines (Kirk *et al.*, 1990).

Some of these results were presented at the meeting of The British Pharmacological Society held in Cambridge on 5–7th January, 1993.

Methods

Animals

Male Lewis rats (Nossan, Italy) weighing 160–180 g were used for this study. Animals were housed in propylene cages with food and water *ad libitum*. The light cycle was automatically controlled (on 07 h 00 min; off 19 h 00 min) and the room temperature thermostatically regulated to 22 ± 1°C. Prior to the experiments animals were housed in these conditions for 6–8 days to become acclimatized.

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Treatments

Prolonged modulation of NO production in Lewis rats was achieved by spontaneous ingestion of test compounds dissolved in drinking water. The water intake of a Lewis rat was found in pilot experiments to be 26–28 ml day^{-1}. When the tap water was replaced with the solution of test compounds a transient (3–4 days) reduction (23–24 ml day^{-1}) in water intake was observed, after which it returned to normal values.

Animals were divided into several groups. Two groups were given tap water: one of these did not receive any treatment and was used for recording weight gain and other parameters in normal animals during the time of the experiments (naive rats), the other served as control of the adjuvant arthritis (control rats). Other groups were given solutions of test compounds in various concentrations or combinations (see Results). In preliminary experiments it was established that Lewis rats given solutions of test compounds at the concentrations described, gained weight at the same rate as rats given tap water to drink.

Arthritis

Four days after the start of treatment, adjuvant arthritis was induced in all rats (except the naive group) by a single intradermal injection (0.1 ml) into the right foot pad of 0.3 mg heat-killed *M. tuberculosis* in Freund's incomplete adjuvant.

The magnitude of the inflammatory response was evaluated by measuring the volume of the contralateral (non-injected) hind paw (secondary lesion). The paw volume was determined by plethysmometry (Basile, Italy) immediately after immunization and every 7 days for a period of 35 days. Weight gain, a further parameter of severity of arthritis (Newbold, 1963), was also recorded on these days. Eight animals were used per group for measurement of arthritis and weight gain.

Splenocyte and macrophage collection

Three groups of 15 animals were used for these studies; one served as a control group, the others were treated with either L-arginine (30 mg ml^{-1}) or L-NAME (1 mg ml^{-1}). Three rats from each group were killed at weekly intervals, i.e. on day 7, 14, 21, 28, 35. On the same days three naive rats were also killed in order to evaluate the splenocyte and macrophage activities (see below) in non-arthritic rats. The spleens were removed and single cell suspensions were prepared in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 25 mM HEPES, penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% foetal calf serum (complete medium).

Lymphocytes of each spleen were separately isolated from cell suspension by density-gradient centrifugation using Ficoll-Paque and then resuspended (10^6 cells ml^{-1}) in complete medium. Cell viability, assessed by the trypan blue dye exclusion method, was 93–95%.

At the same time as removal of the spleen, peritoneal cells were also collected by washing the cavity with 8 ml sterile phosphate-buffered saline (PBS, pH 7.2) containing heparin (50 u ml^{-1}). Cells were washed twice and plated onto 35 mm Petri dishes (4–5 × 10^6 cells per dish) containing complete medium (see above). After 3 h at 37°C in 5% CO_2 humidified air, nonadherent cells were removed by washing with sterile PBS containing 1 mM EDTA.

Macrophages (80–85% of total cells) were removed from the culture dishes by vigorous pipetting, centrifuged (300 *g* for 10 min) and resuspended in complete medium at a concentration of 10^6 cells ml^{-1}. Macrophage viability (95–98%) was determined by exclusion of trypan blue.

T-lymphocyte proliferation

T-lymphocyte proliferation assays were carried out in triplicate and performed in 96-microwell plates. Cells (10^5 cells per well) were stimulated with *M. tuberculosis* (final concentration 5 μg ml^{-1}) and incubated for 7 days at 37° in 5% CO_2 humidified air. One μCi of [^{3}H]-thymidine (47 Ci mmol^{-1}) was then added to each well. After a 6 h incubation at 37°C, cultures were harvested on glass fibre strips and [^{3}H]-thymidine incorporation was measured in a beta counter. The data are expressed as mean total c.p.m. ± s.e.mean.

Macrophage activation

Macrophages were plated in 24 well culture plates at a concentration of 2.5 × 10^5 cells ml^{-1} and incubated for 24 h. Aliquots of the medium were then collected for assay of nitrite (NO_2^-), acid phosphatase (AP) and lactic dehydrogenase (LDH). Each assay was carried out in triplicate.

Nitrite levels were measured with Griess reagent as previously described (Di Rosa *et al.*, 1990) and results were expressed as nmol of NO_2^- generated by 10^6 cells in 24 h.

AP activity was evaluated with β-glycerophosphate as a substrate and measuring the amount of inorganic P released in 30 min by 100 μl of medium, according to the method of Gianetto & De Duve (1955). Results are expressed as μmol of inorganic P liberated in 30 min by the enzyme released in 24 h by 10^6 cells.

LDH activity was assayed by measuring the amount of pyruvic acid reduced to lactic acid by 100 μl of medium in 30 min, according to the method of Cabaud & Wroblewski (1958). Results are expressed as μmol of pyruvic acid transformed in 30 min by the enzyme released in 24 h by 10^6 cells.

Statistics

Data are expressed as the mean ± s.e.mean; statistical analysis of the data was performed using a Pharm/PCS computer programme. Means were compared by Student's test for unpaired data.

Drugs

M. tuberculosis and PBS were obtained from Difco. All reagents for cell culture except foetal calf serum (Flow Labs) were from Gibco. [^{3}H]-thymidine was obtained from Amersham and Ficoll-Paque from Pharmacia. D-NAME was synthesized by Bachem (Switzerland) and all other chemicals were purchased from Sigma.

Results

Arthritis

In each group of rats ($n = 8$) a single intradermal injection of adjuvant to the right hind paw resulted in a gradual increase in volume of the left paw (secondary lesion), which reached a peak value 21 days after immunization (Figure 1). In the rats given L-arginine (30 mg ml^{-1}) arthritis was exacerbated throughout the time course of the development of the arthropathy while in aminals receiving L-NAME (1 mg ml^{-1}) the process was greatly suppressed (Figure 1).

Rats given L-arginine had a dose-related increase in paw swelling throughout the time course of the arthritis (Figure 2). The greatest response (+44%, $P<0.01$) was observed in rats drinking 30 mg ml^{-1} L-arginine. Rats given L-NAME showed a dose-related reduction in paw swelling throughout the time course of the arthritic reaction (Figure 2). The greatest reduction (−50%, $P<0.01$) was observed in rats drinking 10 mg ml^{-1} L-NAME. Rats given a combination of L-arginine (30 mg ml^{-1}) and L-NAME (1 mg ml^{-1}) had a foot volume increase that was slightly, but not significantly,

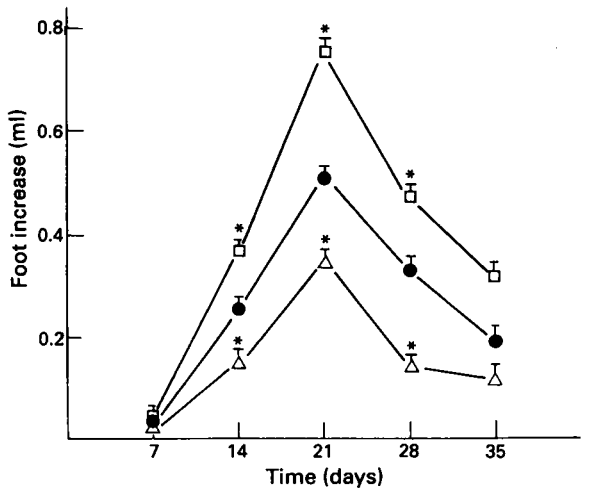

Figure 1 Effect of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on adjuvant arthritis (secondary lesion). The control group (●) was given tap water. Other groups were given L-arginine 30 mg ml^{-1} (□) or L-NAME 1 mg ml^{-1} (△) dissolved in drinking water. Data are expressed as mean ± s.e.mean from 8 animals. *$P<0.01$ vs control group.

above the control value (Figure 2). The arthritis occurring at day 21 in rats given D-arginine (30 mg ml^{-1}), D-NAME (1 mg ml^{-1}) and L-lysine (30 mg ml^{-1}) was similar to that observed in control rats.

Weight gain

In all groups of rats ($n = 8$) the average weight gain was related to the severity of arthritis. At the time of the peak foot volume increase (day 21) the average weight gain of naive rats was 73 ± 4 g whereas control arthritic rats gained only 51 ± 3 g ($P<0.01$ vs naive). The effects of the various test compounds on weight gain mirrored their effect on the severity of the arthritis so that on day 21 weight gain was significantly reduced compared with the control group in those rats drinking L-arginine while it was significantly greater in those drinking L-NAME. The effects of L-arginine and L-NAME on weight gain were dose-related (Figure 3).

In contrast, in rats given D-arginine, D-NAME and L-lysine the weight gain was not significantly different from that of the control group, neither was the weight increase of rats given a combination of L-NAME (1 mg ml^{-1}) and L-arginine (30 mg ml^{-1} Figure 3).

Proliferation of T-lymphocytes

The proliferation of T-lymphocytes stimulated with *M. tuberculosis* (5 μg ml^{-1}) was evaluated at weekly intervals in three rats from the control, the L-arginine (30 mg ml^{-1})- and L-NAME (1 mg ml^{-1})-treated groups. In each group the proliferative response observed at day 7 increased up until day 21 (peak response) and then decreased to approximately the initial value (day 7) at day 35 (Figure 4). Thus, in each group the time course of the proliferative response paralleled the development of arthritis. The proliferation of T-lymphocytes taken from L-arginine-treated animals was always greater than that observed in those from control rats. The difference between the two groups was significant ($P<0.01$) at days 14, 21, 28 and 35. The proliferation of T-lymphocytes collected from L-NAME-treated rats was always less than that in cells from control rats and was significantly reduced ($P<0.01$) on days 14, 21 and 28 (Figure 4). The incorporation of [^{3}H]-thymidine in stimulated T-lymphocytes from naive rats ranged from 13,296 to 15,019 c.p.m. ($n = 15$) (Figure 4).

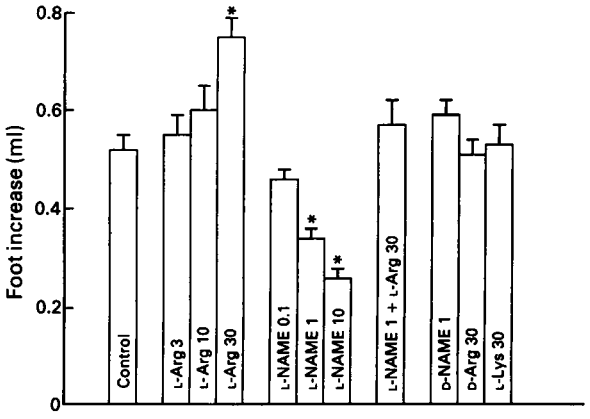

Figure 2 Adjuvant arthritis (secondary lesion) on day 21 post adjuvant challenge. The control group was given tap water and the other groups were given solutions of test compounds at the concentrations (mg ml^{-1}) shown inside the columns. L-Arg and D-Arg: L- and D-arginine; L-NAME and D-NAME: N^G-nitro-L-arginine methyl ester and N^G-nitro-D-arginine methyl ester; L-Lys = L-lysine. Data are expressed as mean ± s.e.mean from 8 animals. *$P<0.01$ vs control group.

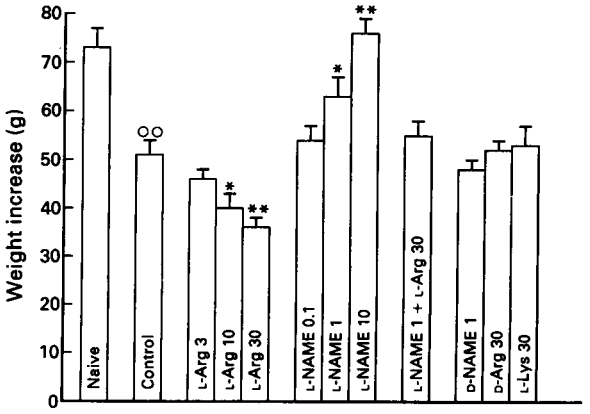

Figure 3 Increase in body weight on day 21 post adjuvant challenge. The naive and control groups were given tap water and the other groups were given solutions of test compounds at the concentrations (mg ml^{-1}) shown inside the columns: compounds as in Figure 2. Data are expressed as mean ± s.e.mean from 8 animals. $^{oo}P<0.01$ vs naive group; **$P<0.01$; *$P<0.05$ vs control group.

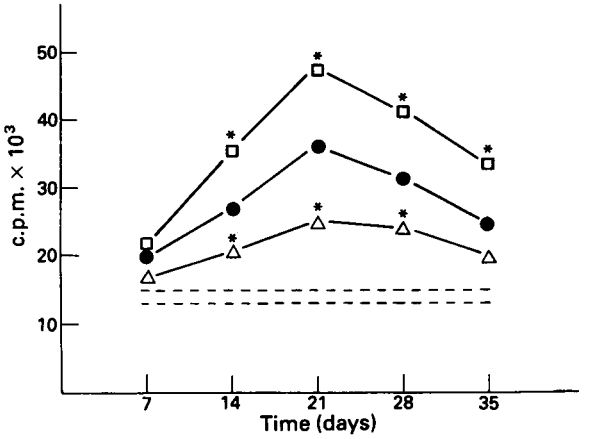

Figure 4 Effect of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on antigen-stimulated [^{3}H]-thymidine incorporation in spleen lymphocytes collected at different times from arthritic rats. The control group (●) was given tap water and the other groups were given L-arginine 30 mg ml^{-1} (□) or L-NAME 1 mg ml^{-1} (△) dissolved in drinking water. Each point represents the mean c.p.m. product by 10^5 cells separately collected from 3 rats. Standard errors were always less than 5% of the respective means and are not shown because they are covered by the symbols. The [^{3}H]-thymidine incorporation in cells obtained from naive rats ($n = 15$) lay within the dotted lines. *$P<0.01$ vs control group.

Macrophage activation

During the development of the arthritis, peritoneal macrophages were collected at weekly intervals from those rats used for evaluation of T-lymphocyte proliferation (see above). Macrophages were incubated in complete medium for 24 h in order to evalue NO_2^- production, as well as the release of both AP and LDH. These biochemical parameters were also evaluated in macrophages from naive rats.

Nitrite production These results are shown in Figure 5. In the control group the NO_2^- production increased from day 7 to day 14 and day 21. Thereafter the NO_2^- production declined on day 28 and was further reduced on day 35. Macrophages from the L-arginine-treated group produced larger amounts of NO_2^-, although the time course of NO_2^- generation followed a pattern similar to that exhibited by the cells from the control group. In contrast, NO_2^- production by macrophages from the L-NAME-treated group was greatly decreased when compared to that of the control group. Furthermore, in this group the time course of NO_2^- production did not have the clear bell shape observed in the other two groups. The difference between the control group and those treated with L-arginine or L-NAME was significant ($P<0.01$) at each time. The amount of NO_2^- produced by macrophages from naive rats ranged from 8.8 to 10.5 ($n = 15$) (Figure 5).

Acid phosphatase These results are shown in Figure 6. In the macrophages from the control group the release of AP gradually increased from day 7 to day 14 and day 21. Thereafter the release of AP declined on day 28 and was further reduced on day 35.

The AP release from the macrophages collected from the L-arginine-treated group was greater than that occurring in the cells from the control rats at each time interval. The difference between the two groups was significant ($P<0.05$) on day 21. Macrophages from L-NAME-treated rats always released smaller amounts of AP than the respective control cells. The difference between the two groups was significant on days 21 ($P<0.01$) and 28 ($P<0.05$). The AP release from naive rat macrophages ranged from 45 to 52 ($n = 15$) (Figure 6).

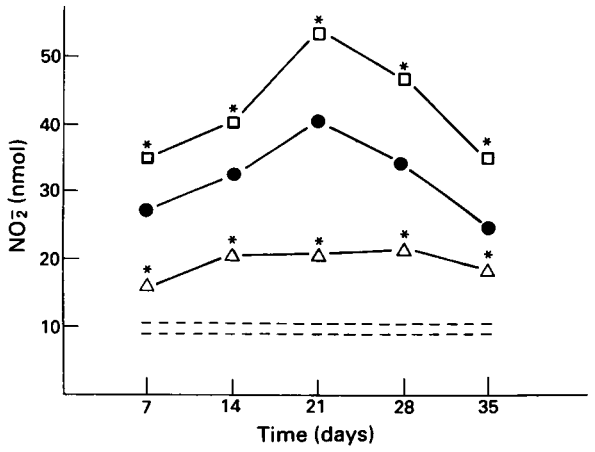

Figure 5 Effect of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on nitrite (NO_2^-) generation by peritoneal macrophages collected at different times from arthritic rats. The control group (●) was given tap water and the other groups were given L-arginine 30 mg ml^{-1} (□) or L-NAME 1 mg ml^{-1} (△) dissolved in drinking water. Each point represents the mean value of NO_2^- (nmol produced in 24 h by 10^6 cells separately collected from 3 rats). Standard errors were always less than 5% of the respective means and are not shown because they are covered by the symbols. The production of NO_2^- by cells from naive rats ($n = 15$) lay within the dotted lines. *$P<0.01$ vs control group.

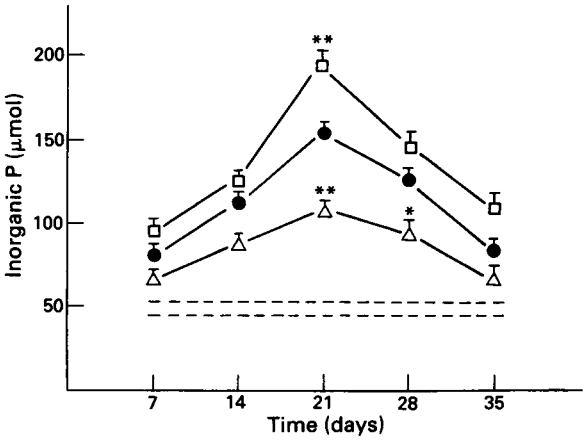

Figure 6 Effect of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on the release of acid phosphatase from peritoneal macrophages collected at different times from arthritic rats. The control group (●) was given tap water and the other groups were given L-arginine 30 mg ml^{-1} (□) or L-NAME 1 mg ml^{-1} (△) dissolved in drinking water. Each point represents the mean value ± s.e.mean of inorganic P (μmol) liberated in 30 min by the enzyme released in 24 h by 10^6 cells separately collected from 3 rats. The acid phosphatase released by cells from naive rats ($n = 15$) lay within the dotted lines. **$P<0.01$; *$P<0.05$ vs control group.

Lactic dehydrogenase The release of LDH (expressed as μmol of pyruvic acid transformed in 30 min by the enzyme released by 10^6 cells in 24 h) from macrophages of the three groups was virtually the same at any time. Thus it ranged from 67.2 to 69.2 ($n = 15$) in macrophages from control rats, from 66.4 to 69.6 ($n = 15$) in cells from the L-arginine-treated group and from 67.6 to 70.4 ($n = 15$) in the L-NAME-treated group. These values were virtually identical to the LDH release by macrophages from naive rats, which ranged from 65.2 to 67.8 ($n = 15$).

Discussion

Our results show that the L-arginine:NO pathway plays a role in adjuvant arthritis induced in the rat. Thus, ingestion of L-arginine, but not D-arginine or L-lysine, dose-dependently exacerbated the arthritis, which was reduced by L-NAME and unaffected by D-NAME. A combination of L-arginine and L-NAME led to an arthritic process similar to that in control animals.

Adjuvant arthritis is an experimental immunopathy which involves a T-lymphocyte-mediated delayed hypersensitivity reaction (Cohen, 1991). The disease is transferable to naive recipient rats by inoculation of specific T cells from treated animals and the proliferative response of these cells correlates with the incidence and severity of the arthritis (Holoshitz *et al.*, 1982; 1984).

Throughout the time course of the syndrome, T-lymphocytes from L-arginine-treated rats exhibited an enhanced proliferative response, whereas this was markedly depressed in L-NAME-treated animals. Furthermore, macrophages from L-arginine-treated rats generated greater amounts of NO_2^- and released higher levels of AP than those from the control group, whereas both NO_2^- generation and AP release were significantly reduced in cells from L-NAME-treated rats. The cytoplasmic enzyme LDH remained unaltered in macrophages during the development of arthritis, showing that lysosomal enzyme release was selective and not due to non-specific cell damage. Thus, manipulation of the L-arginine:NO pathway in the whole animal results in cellular changes which parallel the severity of the arthritis.

It has been shown that L-arginine improves host immunity, since it augments the number of thymic lymphocytes and the

response of T cells to mitogens, as well as enhancing tumour regression (Barbul *et al.*, 1980; 1985; Reynolds *et al.*, 1987). Furthermore, dietary supplements of L-arginine increase the activity of both natural-killer and lymphokine-activated-killer cells in healthy volunteers (Park *et al.*, 1991) and enhance development and interleukin (IL)-2 receptor expression in cytotoxic T-lymphocytes of mice (Reynolds *et al.*, 1988). It is therefore possible that increased lymphocyte proliferation leads to enhanced macrophage activation and thus an amplification of the general immunological response. The role of the L-arginine:NO pathway in lymphocytes is at present unclear. However, it has recently been observed that stimulated murine T-lymphocytes generate NO following induction of NO synthase (Kirk *et al.*, 1990).

The interaction of T cells with macrophages results in the production by both types of cells of several cytokines including IL-1, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) which have been implicated in immune arthritis and are known to play a role in macrophage activation (Cooper *et al.*, 1988; Hom *et al.*, 1988; Thorbecke *et al.*, 1992). It has been shown that the leishmanicidal activity of macrophages correlates with their ability to express NO synthase following activation with IFN-γ or TNF-α (Liew *et al.*, 1991). Macrophage-derived NO is not only cytotoxic for invading micro-organisms and tumours, but may also produce host tissue damage (Kolb *et al.*, 1991; Kroncke *et al.*, 1991). Furthermore, cytokines might lead to induction of NO synthase in tissues other than the macrophages, including endothelial cells and chondrocytes, all of which may contribute to the general inflammatory response.

Although we did not measure cytokine levels in our experiments, it is interesting that the weight gain of arthritic rats given L-arginine was significantly lower than in control animals while L-NAME caused a significant and dose-dependent increase in weight gain. It is well known that TNF-α or cachectin is closely linked to the loss in weight occurring in animals suffering from chronic inflammation and/or infection (Beutler & Cerami, 1989). The effects of L-arginine and L-NAME on weight gain, which appear to be correlated to their respective pro- and anti-inflammatory actions, may possibly involve a modulatory role of these agents on the formation and/or the effects of TNF-α in arthritic rats.

In vitro inhibition of the L-arginine:NO pathway by L-NMMA has been reported to cause an increase in T cell proliferation induced by mitogens or alloantigens, suggesting an inhibitory role for NO in these models of T cell activation (Hoffman *et al.*, 1990; Albina *et al.*, 1991). The reasons for the apparent discrepancy between these *in vitro* and our *in vivo* findings and those described above, which indicate an enhancing role for NO in the immune system, need to be reconciled.

Although our results point towards an effect of the different treatments in the cellular immunological mechanism, it is important to recognize that L-NAME induces increases in blood pressure and reduction in blood flow in different organs (Gardiner *et al.*, 1990). Furthermore, L-arginine may increase vasodilatation and vascular permeability at the inflammatory site, as we have recently shown in some models of acute inflammation (Ialenti *et al.*, 1992). These vascular effects may also influence the development of the arthritic process in a way which remains to be determined.

Glucocorticoids inhibit the induction of NO synthase in macrophages, thus part of the anti-inflammatory and immunosuppressive actions of these steroids is due to this inhibition (Di Rosa *et al.*, 1990). It may be that the well-known suppressive effect of glucocorticoids in adjuvant arthritis may depend, at least in part, on a similar mechanism, i.e. the inhibition of the induction of NO synthase in T-lymphocytes and/or macrophages. It is also possible that selective inhibitors of the inducible NO synthase(s) that is expressed in stimulated macrophages and T-lymphocytes may represent a new class of antirheumatic-immunosuppressive agent.

The authors wish to thank Annie Higgs for her assistance in the preparation of the manuscript.

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(Received January 4, 1993
Revised May 21, 1993
Accepted June 15, 1993)