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# Involvement of Nitric Oxide in the Pathogenesis of Cyclophosphamide-Induced Hemorrhagic Cystitis

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***The involvement of nitric oxide (NO) and the potential modulation of NO synthase (NOS) activity by platelet-activating factor were investigated in a rat model of cyclophosphamide-induced hemorrhagic cystitis. Male Wistar rats received a single intraperitoneal injection of cyclophosphamide, and cystitis was evaluated 6, 12, 24, 48, and 72 hours later by determining the changes in bladder wet weight and plasma protein extravasation and the macro- and microscopic morphological alterations. In addition, NOS activity and NADPH-diaphorase histochemistry were studied in bladder tissues. Normal bladders showed extensive NADPH-diaphorase staining and a high level of constitutive NOS whereas the activity of inducible NOS was almost undetectable. Cyclophosphamide dose- and time-dependently increased the bladder wet weight and bladder plasma protein extravasation. These events were accompanied at a microscopic level by urothelial necrosis, sloughing, ulceration, hemorrhage, and leukocyte infiltration. Cyclophosphamide also increased the levels of inducible NOS but reduced those of constitutive NOS. The NOS inhibitors L-N$^G$-nitroarginine methyl ester and L-N$^G$-nitroarginine significantly reduced the cyclophosphamide-induced plasma protein extravasation and urothelial damage. This reduction was completely reversed by L-arginine but not by D-arginine. The administration of the platelet-activating factor antagonist BN 52021 decreased the cyclophosphamide-induced plasma protein extravasation as well as the rise in inducible NOS activity but had no effect on the fall in constitutive NOS activity. These results suggest that endogenous NO participates in the urothelial damage and in the inflammatory events leading to cyclophosphamide-induced hemorrhagic cystitis. Platelet-activating factor also seems to be involved in the pathogenesis of this condition, possibly by inducing NOS. (Am J Pathol 1997, 150:247–256)***

Since the introduction of cyclophosphamide in clinical therapeutics in 1958, hemorrhagic cystitis has been recognized as a common and distressing complication that limits its use.[1,2] The incidence of this side effect varies from 2 to 40% in patients on long-term, low-dose treatment with cyclophosphamide whereas it can be as high as 75% in patients receiving a high intravenous dose.[3,4]

Although it has been proposed that urothelial damage occurs through direct contact with acrolein, a urotoxic metabolite of cyclophosphamide that causes edema, necrosis, ulceration, hemorrhage, leukocyte infiltration, and neovascularization, the endogenous inflammatory mediators involved in bladder damage still remain unknown.[5]

Increasing evidence indicates that nitric oxide (NO) is involved in acute and chronic inflammation. NO is an inorganic, free radical gas released during the metabolism of L-arginine by a family of isoenzymes called NO synthases (NOSs).[6] Two of these enzymes are constitutively expressed (cNOS)

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whereas a third (iNOS) can be induced by immunological stimuli such as interferon-γ, interleukin-1, and tumor necrosis factor.[7] The NO released by cNOS acts as an important signaling molecule in the cardiovascular and nervous systems whereas that generated by the iNOS over long periods by cells of the immune system has been shown to be cytostatic/cytotoxic for tumors and other cells and for a variety of microorganisms.[7–9] The administration of NOS inhibitors reduces the severity of the inflammatory response to carrageenin[10] and to Freund's adjuvant[11] in rats, whereas L-arginine enhances them. Immune-complex-induced vascular injury in rat lungs and in the dermal vasculature can also be attenuated by NOS inhibitors.[12] Clinically, the colonic mucosa of patients with ulcerative colitis has an increased NO synthesis,[13] and NOS inhibitors have been shown to ameliorate experimentally induced chronic ileitis.[14] In addition, nitrite concentrations in plasma and synovial fluid are increased in patients with rheumatoid arthritis and osteoarthritis.[15]

The aim of the present study was to investigate the involvement of NO in the pathogenesis of cyclophosphamide-induced hemorrhagic cystitis in rats. In addition, as platelet-activating factor (PAF) is an important inflammatory mediator[16,17] and has been proposed to participate in the induction of NOS in several different tissues,[18] we also examined the effect of a PAF antagonist in the regulation of NOS activity in this model of cystitis.

## Materials and Methods

### Animals

Male Wistar rats weighing 180 to 200 g were housed in temperature-controlled rooms and received water and food *ad libitum* until used.

### Drugs

Cyclophosphamide (Enduxan) was from Abbott (São Paulo, Brazil), BN 52021 was from the Institute Pasteur and L-[U-$^{14}$C]arginine (0.05 μCi, ~1 μmol/L) was from Amersham (Little Chalfont, UK). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

### Induction and Quantification of Cystitis

Cystitis was induced by the intraperitoneal injection of cyclophosphamide (50, 100, and 200 mg/kg). At different times thereafter, the animals were killed, and their bladders were removed by careful dissection, emptied of urine, and evaluated for the different parameters described below.

#### *Measurement of Vesical Edema*

Vesical edema was quantified either by an increase in bladder wet weight[1,5] or by the determination of vesical vascular permeability.[19] The bladder wet weights (in milligrams) are reported as the mean ± SEM/100 g of body weight. Vesical vascular permeability was quantified by the Evans blue dye extravasation technique. Evans blue (25 mg/kg) was administered intravenously *via* the penial venous sinus 1 hour before the animals were sacrificed. The bladders were then dissected and placed in glass tubes containing 1.5 ml of formamide, after which they were incubated at 56°C overnight. The optical density of the extracted dye was estimated at 600 nm, and the results are reported as the mean ± SEM (in micrograms) of Evans blue per bladder.

#### *Assay of NOS Activity*

NOS activity was evaluated using a citrulline production assay.[20,21] Bladders from control and cyclophosphamide-treated (100 mg/kg) animals were homogenized in appropriate buffer (50 mmol/L Tris/HCl, pH 7.4, containing 3.2 mmol/L sucrose, 1 mmol/L dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin) at 4°C. The homogenate was centrifuged at 10,000 × *g* for 20 minutes at 4°C, and the NOS activity in the supernatant fractions was assayed for the conversion of [$^{14}$C]L-arginine into [$^{14}$C]L-citrulline. The assay buffer (pH 7.2) contained 50 mmol/L $KH_2PO_4$, 1 mmol/L $MgCl_2$, 0.2 mmol/L $CaCl_2$, 50 mmol/L valine, 20 μmol/L L-citrulline, 20 μmol/L L-arginine, 1 mmol/L dithiothreitol, 100 μmol/L NADPH, 3 μmol/L tetrahydrobiopterin, 3 μmol/L flavin adenine dinucleotide, 3 μmol/L flavin mononucleotide, and 0.05 μCi of L-[U-$^{14}$C]arginine. After a 20-minute incubation at 37°C, the reaction was terminated by removing the substrate through the addition of a 1:1 mixture (v/v) of Milli-Q water/Dowex-AG50W (200–400, 8% cross-linked, $Na^+$T-form). The activity of the $Ca^{2+}$-dependent enzyme was determined as the difference between the [U-$^{14}$C]arginine to [U-$^{14}$C]citrulline conversion in control samples and in samples containing EGTA (3 mmol/L). The activity of the $Ca^{2+}$-independent enzyme was determined from the difference between samples containing EGTA (3 mmol/L) and samples containing the NOS inhibitor L-NMMA (1 mmol/L). The protein content of the frac-

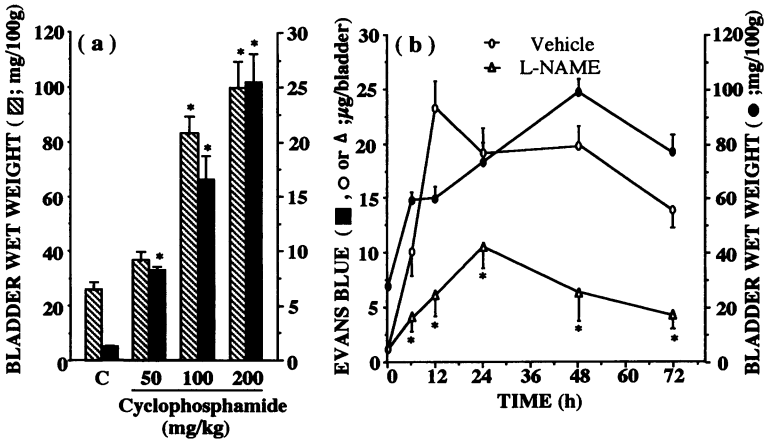


Figure 1. *L-NAME inhibits the cyclophosphamide-induced time-dependent increase in wet weight and plasma protein extravasation in rat bladders.* a: *Bladder wet weight* (hatched bars) *and plasma protein extravasation* (solid bars) *in normal animals (C, control) and in those treated 48 hours earlier with cyclophosphamide (50, 100, and 200 mg/kg).* b: *Plasma protein extravasation* (○) *and bladder wet weight* (●) *in animals treated 6, 12, 24, 48, or 72 hours earlier with cyclophosphamide (100 mg/kg); △, plasma protein extravasation induced by cyclophosphamide (100 mg/kg) in L-NAME-treated (20 mg/kg) animals 6, 12, 24, 48, and 72 hours after the induction of cystitis. The results are reported as the mean ± SEM ($n \geq 6$). *P < 0.05 compared with the respective control (C and vehicle) by ANOVA, Fisher's F-test.*

tions was determined by the Coomassie Blue binding method (Pierce Chemical, Rockford, IL). NOS activity was expressed as picomoles of citrulline per milligram of protein per minute.

### *Histochemistry of NADPH-Diaphorase*

For the histochemical demonstration of NADPH-diaphorase activity, bladders were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 hours and subsequently cryopreserved in a 15% sucrose solution for 24 hours. The bladders were then frozen in liquid nitrogen, and 12- to 16-μm sections were cut. For NADPH-diaphorase staining, sections were incubated with 1 mmol/L β-NADPH and 0.5 mmol/L nitroblue tetrazolium in 50 mmol/L Tris/HCl (pH 8.0) containing 0.2% Triton X-100 for 60 minutes at 37°C in a humid chamber.[22]

### *Macroscopic and Microscopic Evaluation of the Bladder*

Bladders were excised, freed from the surrounding connective tissue, and examined grossly for edema and hemorrhage. Histological examination was performed by a pathologist in a single-blind fashion. Edema, bleeding, and histological changes were evaluated according to the criteria of Gray et al[23] as follows. Edema was considered severe (3+) when fluid was seen externally and internally in the walls of the bladder, moderate (2+) when the edema was confined to the internal mucosa, mild (1+) between normal and moderate, and none (0) as normal. Hemorrhage was scored as follows: 3+, intravesical clots; 2+, mucosal hematomas; 1+, telangiectasia or dilatation of the bladder vessel; 0, normal. Histopathology was scored as follows: 0, normal epithelium and absence of inflammatory cell infiltration and

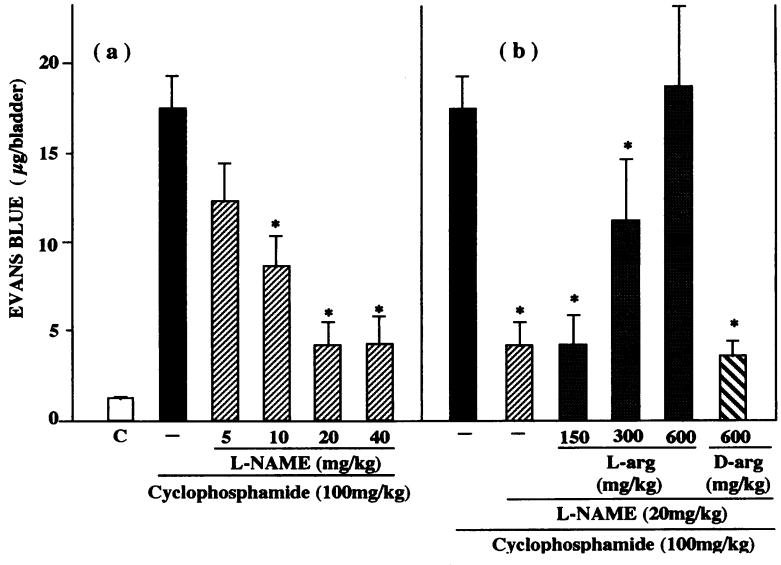


Figure 2. *L-Arginine reverses the dose-dependent inhibitory effect of L-NAME on cyclophosphamide-induced plasma protein extravasation in rat bladders.* a: *Cyclophosphamide-induced (100 mg/kg) plasma protein extravasation 12 hours after the induction of cystitis in vehicle (−) and L-NAME-treated animals (5, 10, 20, or 40 mg/kg).* b: *Effect of L-arginine (150, 300, and 600 mg/kg) and D-arginine (600 mg/kg) on the inhibition by L-NAME (20 mg/kg) of the cyclophosphamide-induced plasma protein 12 hours after the induction of cystitis. The results are reported as the mean ± SEM ($n \geq 6$). *P < 0.05 compared with group treated with cyclophosphamide alone by ANOVA, Fisher's F-test.*

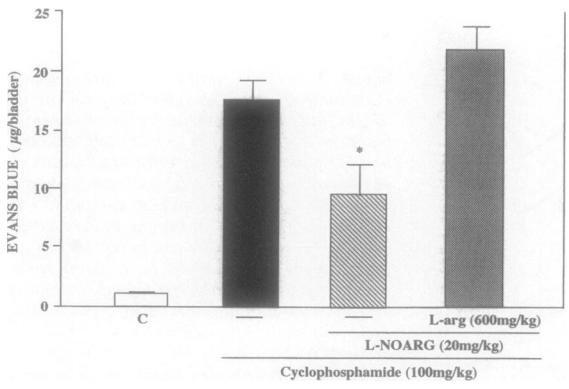


**Figure 3.** *L-Arginine reverses the inhibitory effect of L-NOARG on the cyclophosphamide-induced plasma protein extravasation in rat bladders. The* **bars** *show the increase in plasma protein extravasation induced by cyclophosphamide (100 mg/kg) in vehicle (−), L-NOARG (20 mg/kg) alone, and L-NOARG plus L-arginine-treated (600 mg/kg) animals. The results are reported as the mean ± SEM ($n \geq 6$). *P < 0.05 compared with group treated with cyclophosphamide alone by ANOVA, Fisher's* F*-test.*

ulceration; 1, mild changes involving diminished epithelial cells, flattening with submucosal edema, mild hemorrhage, and few ulcerations; 2, severe changes including mucosal erosion, inflammatory cell infiltration, fibrin deposition, hemorrhage, and multiple ulcerations.

## *Effect of L-N$^G$-Nitroarginine Methyl Ester (L-NAME), L-N$^G$-Nitroarginine (L-NOARG), and BN 52021 on Cyclophosphamide-Induced Hemorrhagic Cystitis*

### *Effect of L-N$^G$-Nitroarginine Methyl Ester (L-NAME)*

The animals received the NOS inhibitor L-NAME (5, 10, 20, and 40 mg/kg, intraperitoneal (i.p.)) 30 minutes before and 6, 24, and 30 hours after an injection of cyclophosphamide (100 mg/kg, i.p.). Their bladders were then analyzed 6, 12, 24, and 48 hours after cyclophosphamide injection, as described above. In another set of experiments, L-arginine (150, 300, or 600 mg/kg, i.p.) or D-arginine (600 mg/kg, i.p.) was administered simultaneously with L-NAME (20 mg/kg), and the cystitis was analyzed 12 hours after cyclophosphamide injection.

### *Effect of L-N$^G$-Nitroarginine (L-NOARG)*

The rats were injected with another NOS inhibitor, L-NOARG (20 mg/kg, i.p.) 30 minutes before and 6 hours after cyclophosphamide (100 mg/kg, i.p.). L-Arginine (600 mg/kg, i.p.) was also administered to a group of animals simultaneously with L-NOARG. The bladders were analyzed 12 hours after the induction of cystitis.

### *Effect of BN 52021*

The animals were treated with BN 52021 (10 mg/kg, subcutaneous) 30 minutes before and 6, 24, and 30 hours after cyclophosphamide (100 mg/kg, i.p.), and their bladders were analyzed 6, 12, and 48 hours after cyclophosphamide administration. The NOS activity in the bladders of one group of these animals was determined 12 hours after cyclophosphamide, as described above.

## *Statistical Analysis*

The results are expressed as the mean ± SEM. Statistical significance ($P < 0.05$) was assessed by analysis of variance (ANOVA) followed by Fisher's test. The morphological data were analyzed by the nonparametric Mann-Whitney *U*-test.

## *Results*

Cyclophosphamide (50 to 200 mg/kg, i.p.) dose-dependently increased the bladder wet weight and Evans blue extravasation in rats (Figure 1a) but did not alter the bladder dry weight (not shown). The increases in bladder wet weight increase and Evans blue extravasation observed with cyclophosphamide (100 mg/kg) were time dependent (Figure 1b). Figure 1b also shows that the Evans blue extravasation was significantly inhibited by L-NAME. The latter also inhibited the bladder wet weight increase measured 48 hours after cyclophosphamide administration (data not shown).

The inhibitory effect of L-NAME (5 to 40 mg/kg) on Evans blue extravasation was dose dependent and was reversed by the simultaneous administration of

**Figure 4.** *Histological analysis of representative bladder walls in cross section.* **A:** *Bladder of a control rat.* **B:** *Cystitis in a rat treated 12 hours previously with cyclophosphamide. The epithelium is thin and sometimes completely denuded with fibrin deposition and areas of ulcerations being present. Edema and leukocyte infiltration can also be seen.* **C:** *Cystitis induced by cyclophosphamide in an L-NAME-treated rat. The epithelium is more preserved, the edema and leukocyte infiltration are attenuated, and ulceration is not present.* **D:** *The cystitis induced by cyclophosphamide in an L-NAME plus L-arginine-treated rat. The epithelium is completely denuded with more ulcerations and mucosal erosions. Severe edema and several clots are present. H&E; magnification, ×40.*

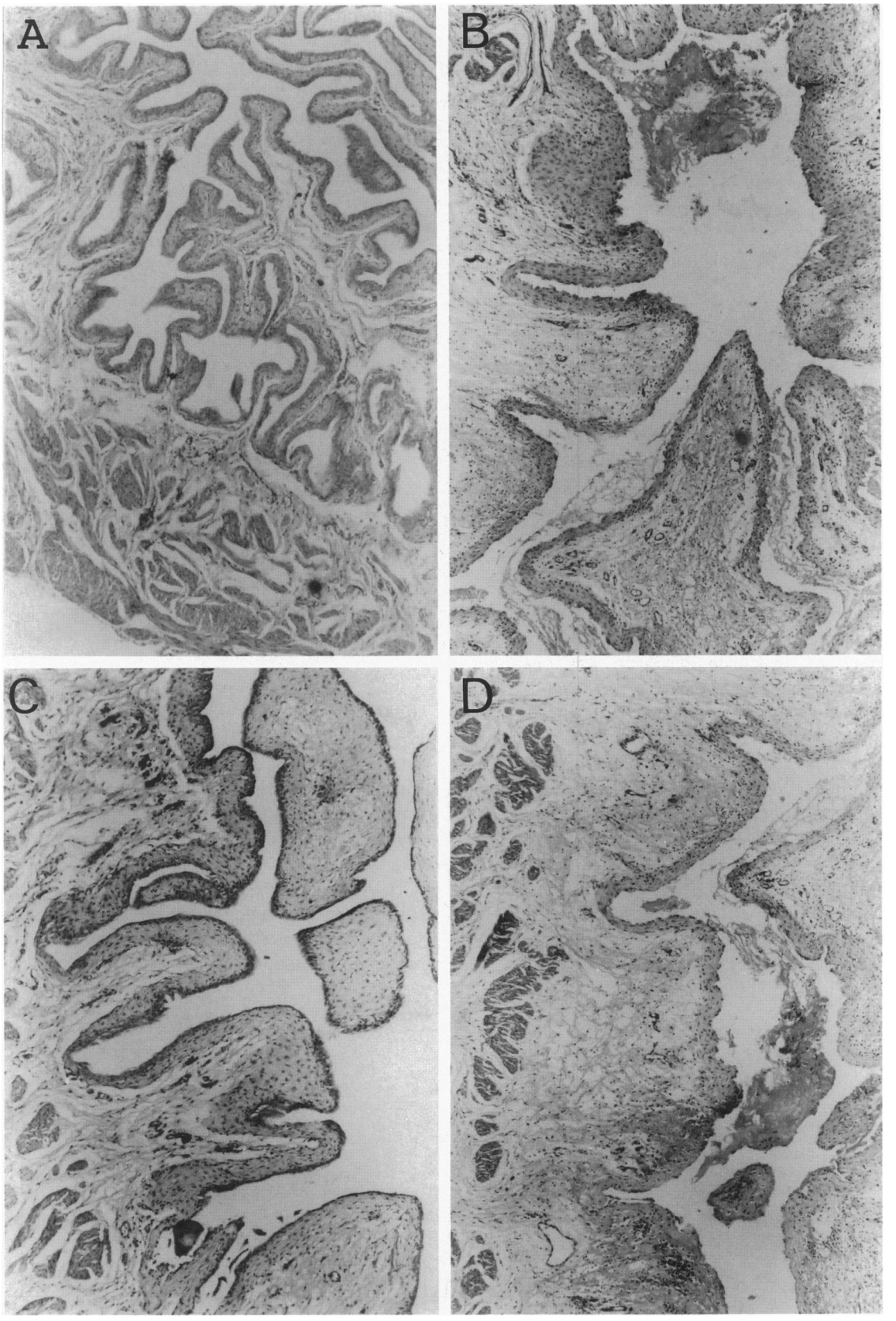
A
B
C
D

Table 1. *Median Values (and Ranges) of the Macroscopic and Microscopic Scores (Gray's Scores) for Cyclophosphamide-Induced Hemorrhagic Cystitis after 12 Hours in Animals Treated with Saline, L-NAME (20 mg/kg), and L-NAME (20 mg/kg) plus L-arginine (600 mg/kg)*

| Morphological analysis | Treatment | | |
|---|---|---|---|
| | Saline | L-NAME | L-NAME plus L-arginine |
| Microscopic | 2 (1–2) | 1 (0–1)* | 2 (2–2) |
| Macroscopic | 2 (2–3) | 1 (0–1)* | 2 (2–3) |

*Mann-Whitney *U* test; $P < 0.05$.

L-arginine but not of D-arginine (Figure 2). Similarly, L-NOARG (20 mg/kg) significantly reduced the increase in plasma protein extravasation induced by cyclophosphamide after 12 hours. This reduction was completely reverted when L-arginine (600 mg/kg) was given simultaneously with L-NOARG (Figure 3).

Cyclophosphamide induced severe cystitis characterized macroscopically by the presence of intravesical clots, mucosal hematomas, and severe edema. Microscopically, extensive mucosal erosion with ulceration, fibrin deposition, hemorrhage, edema, and leukocyte infiltration was observed. These alterations were reduced by L-NAME but could be reinstated by L-arginine (Figure 4 and Table 1). Interestingly, in the animals that received L-arginine, the cyclophosphamide-induced cystitis tended to be more severe.

NOS activity in the normal bladder was mainly of the cNOS (calcium-dependent) isoform (>95%). Cyclophosphamide administration increased significantly the activity of iNOS (calcium-independent), which was already evident 6 hours after injection and remained elevated for up to 48 hours (Figure 5B). In contrast, cNOS decreased over a similar time course in the presence of cyclophosphamide (Figure 5A). Consistent with these findings, intense NADPH diaphorase staining was observed in the urothelium of control rats (Figure 6, A and B). This activity decreased dramatically 12 hours after cyclophosphamide administration and coincided with an intense sloughing of the urothelium (Figure 6, C and D), which persisted up to 48 hours after treatment with cyclophosphamide (data not shown). Numerous NADPH-diaphorase-positive cells were also observed in the lamina propria within 12 hours (Figure 6D) and were still present 48 hours after the induction of cystitis (data not shown).

The cyclophosphamide-induced increase in plasma protein extravasation at 12 and 48 hours after its administration was significantly reduced by treatment of the animals with the PAF antagonist BN 52021 (Figure 7A). This treatment also significantly inhibited the cyclophosphamide-induced bladder wet weight gain measured 48 hours after the induction of cystitis (data not shown). The increase in bladder iNOS activity caused by cyclophosphamide was significantly reduced by BN 52021 (Figure 7C). Interestingly, BN 52021 did not significantly modify the alteration in cNOS activity induced by cyclophosphamide (Figure 7B).

## Discussion

In the present paper, we have shown that endogenous NO is involved in the inflammatory events leading to hemorrhagic cystitis after the administration of cyclophosphamide, as demonstrated by the following observations: 1) two NOS inhibitors, L-NAME and L-NOARG, dose-dependently inhibited the cyclophosphamide-induced increase in plasma protein extravasation and bladder wet weight; this inhibition could be reversed by L-arginine; 2) NOS inhibition by

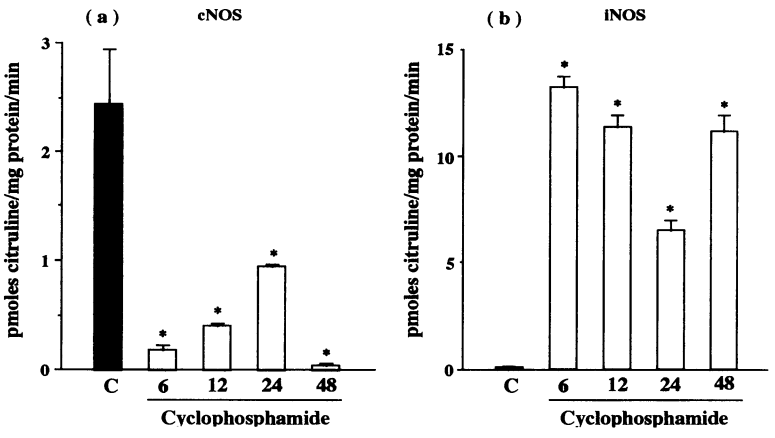


Figure 5. *Time course of NOS activity in cyclophosphamide-induced hemorrhagic cystitis. The* bars *represent the cNOS* (a) *and iNOS* (b) *activities in the bladders of rats treated 6, 12, 24, and 48 hours previously with cyclophosphamide (100 mg/kg). NOS activities were assayed by a citrulline production assay. The first bar in both panels (C) represents the cNOS and iNOS activities in naive animals, respectively. The results are reported as the means ± SEM ($n = 6$). *$P < 0.05$ compared with the naive animals (C) by ANOVA, Fisher's* F*-test.*

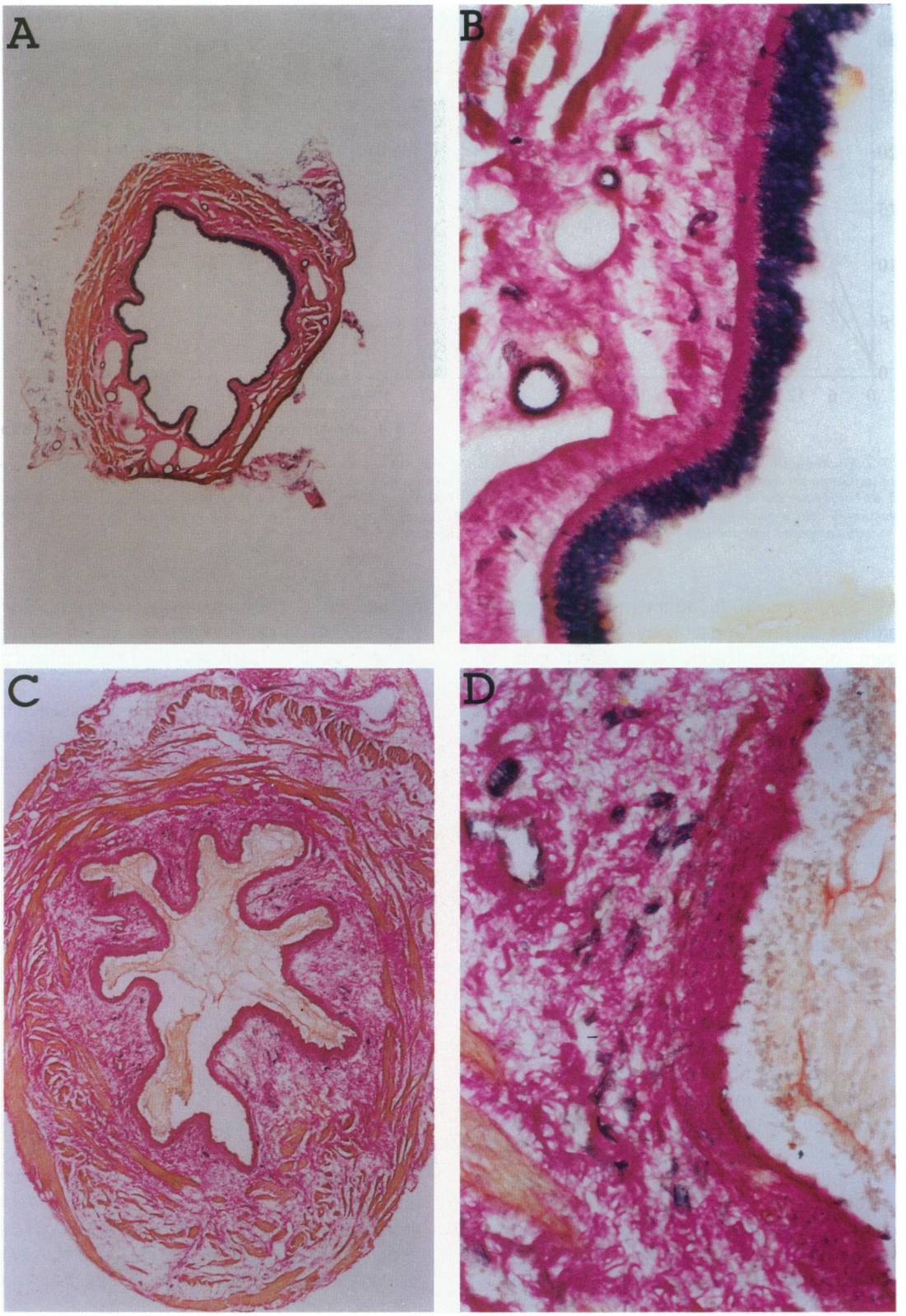


Figure 6. *Histochemical localization of NADPH-diaphorase/NOS-positive structures in the urinary bladder of control rats* (A *and* B) *and in rats 12 hours after the administration of cyclophosphamide* (C *and* D). *In bladders from control rats, the urothelium is strongly positive for NADPH-diaphorase whereas in the bladders from cyclophosphamide-treated rats a complete sloughing of the urothelium is observed and NADPH-diaphorase-positive cells are now observed in the lamina propria. Magnification,* ×*6.25* (A *and* C) *and* ×*50* (B *and* D).

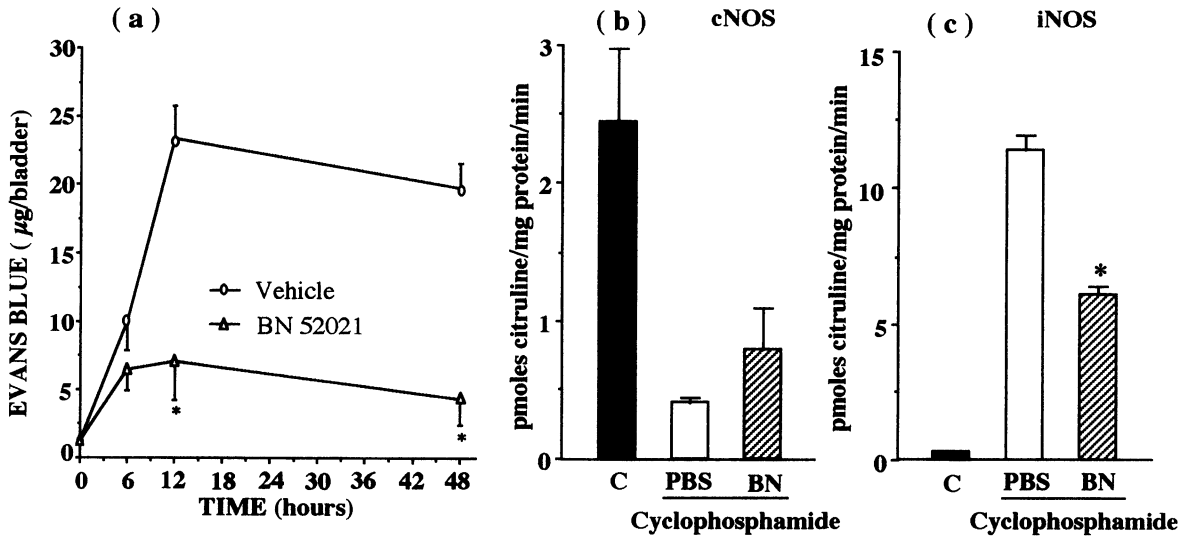


Figure 7. *Inhibitory effect of BN 52021 on plasma protein extravasation and iNOS activity in cyclophosphamide-induced hemorrhagic cystitis.* a: *Increase in plasma protein extravasation induced by cyclophosphamide (100 mg/kg) in vehicle (○) and by BN-52021 (10 mg/kg; △) in animals 6, 12 and 48 hours after the induction of cystitis.* b *and* c: *cNOS and iNOS activities, respectively, in the cytosolic fraction of bladders 12 hours after the induction of cystitis by cyclophosphamide in vehicle (PBS) and BN 52021 (BN)-treated rats. The results are reported as the means ± SEM (n = 6).* $\Psi < 0.05$ *compared with the PBS-treated animals (C) by ANOVA, Fisher's* F-*test.*

L-NAME significantly reduced the mucosal damage, hemorrhage, edema, and leukocyte infiltration in the bladders of cyclophosphamide-treated rats; 3) NADPH-diaphorase histochemistry showed that NOS-containing cells present in the urothelium of control rats disappeared in cyclophosphamide-treated animals and that positive cells, most likely immune cells, appeared in the lamina propria of the inflamed mucosa; 4) cyclophosphamide markedly increased calcium-independent NOS activity in the bladder with a time course similar to that of the histopathological alterations observed. At the same time, the activity of calcium-dependent NOS decreased, possibly reflecting the urothelial sloughing observed in the histopathological analysis.

The participation of NO in cyclophosphamide-induced cystitis is consistent with previous reports showing that NO is cytotoxic to different cells[7–9] and that it participates in the inflammatory events. L-NMMA has been reported to dose-dependently reduce the cell damage observed in several pathological conditions, such as endotoxic shock.[24] In addition, both L-NMMA and L-NAME diminished the edema formation induced by substance P,[25] by carrageenin and dextran,[10] and by BK, 5-hydroxytryptamine, and polycations.[26,27] This latter effect of NOS inhibitors could be reversed by the co-administration of a vasodilator agent such as iloprost (a $PGI_2$ analogue), suggesting that the anti-edema effect of L-NAME is a consequence of the inhibition of blood flow. However, experiments performed in the hamster cheek pouch microvasculature have shown that L-NAME can also reduce the increase in vascular permeability in response to bradykinin and adenosine-5′-diphosphate,[28] suggesting that NO by itself is able to modulate vascular permeability. It has been recently demonstrated that iNOS-deficient mice generated by gene knockout have a reduced inflammatory response to carrageenin.[29]

Our data further indicated that PAF could be one of the inflammatory mediators contributing to the activation of the L-arginine-NO pathway. The involvement of PAF and its modulation of NOS activity in cyclophosphamide-induced hemorrhagic cystitis is suggested by the observations that 1) pretreating the rats with the PAF antagonist BN 52021 significantly reduced the cyclophosphamide-induced plasma protein extravasation and the increase in bladder wet weight and that 2) BN 52021 reduced iNOS activity. Interestingly, BN 52021 did not prevent the decrease in cNOS activity when compared with the bladders of rats treated with cyclophosphamide alone. This finding indicates that PAF may participate in the inflammatory response after urothelial damage has occurred and is consistent with the urothelial sloughing observed in the BN-52021-treated animals. The reduction of iNOS activity in the BN-52021-treated rats suggests that PAF may be involved in the recruitment of inflammatory cells containing iNOS (based on the diaphorase staining) or in the induction of NOS in the already emigrated cells. This hypothesis is strengthened by evidence that

PAF may contribute to the induction of NOS in inflammatory cells.[18]

We have recently shown that in addition to PAF and NO, the inflammatory cytokines tumor necrosis factor-$\alpha$ and interleukin-1 are also important in the appearance of cyclophosphamide-induced cystitis.[19] As these cytokines are able to induce the NOS in inflammatory cells,[7,8] it is likely that they also contribute to the induction of NO production in the bladder after cyclophosphamide administration.

In conclusion, our results suggest that endogenous NO is involved in urothelial damage and in the inflammatory events leading to hemorrhagic cystitis after cyclophosphamide administration. The induction of NOS in the inflammed bladder appears to require the action of PAF.

## *Acknowledgments*

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