

Role of Nitric Oxide in Experimental Obliterative Bronchiolitis (Chronic Rejection) in the Rat

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Abstract

The role of nitric oxide in obliterative bronchiolitis development, i.e., chronic rejection, was investigated in the heterotopic rat tracheal allograft model. An increase in the intra-graft inducible nitric oxide synthase (iNOS) mRNA and mononuclear inflammatory cell iNOS immunoreactivity was demonstrated during progressive loss of respiratory epithelium and airway occlusion in nontreated allografts compared to syngeneic grafts. In nontreated allografts, however, intra-graft nitric oxide production was decreased, most likely because of loss of iNOS epithelial expression. Treatment with aminoguanidine, a preferential inhibitor of inducible nitric oxide synthase, was associated with enhanced proliferation of α -smooth muscle actin immunoreactive cells and the intensity of obliterative bronchiolitis early after transplantation. Aminoguanidine treatment did not affect iNOS mRNA synthesis or intra-graft nitric oxide production, but decreased iNOS immunoreactivity in smooth muscle cells. Treatment with L-arginine, a precursor of nitric oxide, significantly reduced obliterative changes. L-arginine supplementation enhanced intra-graft iNOS mRNA synthesis and iNOS immunoreactivity in capillary endothelial and smooth muscle cells as well as intra-graft nitric oxide production. Immunohistochemical analysis of allografts showed that neither iNOS inhibition nor supplementation of the nitric oxide pathway affected the number of graft-infiltrating CD4⁺ and CD8⁺ T cells, ED1⁺ and ED3⁺ macrophages, immune activation with expression of IL-2R or MHC class II, or production of macrophage or Th1 cytokines. In contrast, L-arginine treatment was associated with increased staining for Th2 cytokines IL-4 and IL-10. In conclusion, this study demonstrates that nitric oxide has a protective role in obliterative bronchiolitis development in this model, and suggests that nitric oxide either directly or indirectly inhibits smooth muscle cell proliferation and modulates im-

mune response towards Th2 cytokines. (*J. Clin. Invest.* 1997. 100:2984–2994.) Key words: inducible nitric oxide synthase • L-arginine • aminoguanidine • transplantation • lung

Introduction

Despite recent advances in understanding chronic rejection, the regulatory and effector mechanisms underlying the disease process remain incompletely known (1). Observations in a heterotopic rat tracheal allograft model demonstrate that recruitment of CD4⁺ and CD8⁺ T cells and macrophages into airway wall induces local cytokine and growth factor production leading to epithelial damage, proliferation of smooth muscle cells, and gradual occlusion of airway lumen (2–4), i.e., histological changes similar to those observed in human lung allografts with obliterative bronchiolitis (OB)[†] (5).

Nitric oxide (NO) has a significant role in several physiological and pathological conditions such as vasorelaxation, neurotransmission, platelet and leukocyte adhesion, and host defense mechanisms against bacteria and viruses (6). NO is generated from L-arginine by the enzyme NO synthase (NOS) (7). NOS can be either constitutively expressed (cNOS) or induced by cytokines and LPS (iNOS) (7). cNOS has been localized in vascular endothelium (eNOS), platelets, and neurons (nNOS) of the central nervous system (7). cNOS produces small quantities of NO with activity regulated by intracellular calcium/calmodulin, and is involved in a variety of normal physiological functions. iNOS can produce large amounts of NO, and is calcium-independent. iNOS is expressed in macrophages, vascular smooth muscle cells, endothelial cells, mesangial cells, and airway epithelium (7–9). In contrast to many other organs, NO is continuously produced by iNOS in normal airway epithelium, and regulates bronchomotor tone (10, 11). In addition, NO may regulate several inflammatory responses in the lung such as development of asthma and granulomatous inflammation (12–14).

During chronic rejection, there is a major release of proinflammatory molecules by infiltrating activated T cells and macrophages that participate in the immune reaction to foreign MHC and other antigens present in endothelial, epithelial, and other cells of the transplanted organ. Cytokines derived from activated macrophages, i.e., TNF- α and IL-1 β , and those derived from Th1 cells, i.e., IL-2 and IFN- γ , promote iNOS expression (8). Recently induction of iNOS (15) and production of NO (16) during acute allograft rejection was demonstrated,

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; cNOS, constitutive nitric oxide synthase; eNOS, cNOS localized in the vascular endothelium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; nNOS, cNOS localized in neurons; NO, nitric oxide; OB, obliterative bronchiolitis; RT, reverse transcriptase.

and it was shown that treatment with aminoguanidine, a preferential inhibitor of iNOS (17), significantly attenuated pathogenesis of acute cardiac and lung allograft rejection (18, 19). This study was undertaken to investigate whether iNOS is induced during experimental OB in rat tracheal allografts, and to investigate the biological role of NO by supplementation of NO pathway by L-arginine, or by inhibition of the iNOS pathway by aminoguanidine in this model.

Methods

Heterotopic rat tracheal transplantation model and study design. Specific pathogen-free, 2–3-mo-old inbred male DA (AG-B4, RT1^a) and WF (AG-B2, RT1^b) rats weighing 200–300 g (Laboratory Animal Center, University of Helsinki, Helsinki, Finland) were used. Allogeneic heterotopic tracheal transplantations were performed as described (3, 4) from DA to WF rats, and syngeneic controls from DA to DA rats. Four groups were formed: (a) nontreated syngeneic controls, (b) nontreated allogeneic controls, (c) allografts treated with aminoguanidine, a preferential inhibitor of iNOS (17), and (d) allografts supplemented with an NO precursor, L-arginine (7). The grafts were removed 3, 10, and 30 d after transplantation to determine histological changes, relative intragraft iNOS mRNA, protein expression, and nitrite/nitrate production. In addition, a detailed immunohistochemical analysis was performed to localize iNOS production and to examine how NO may modulate inflammatory cell infiltration and cytokine production in the airway wall of tracheal transplants.

Aminoguanidine and L-arginine administration. Aminoguanidine-treated allografts received aminoguanidine hemisulfate (Sigma Chemical Co., St. Louis, MO) by injection of 400 mg/kg/d i.p. in three doses (100 mg/kg at 9:00 AM, 100 mg/kg at 1:00 PM, and 200 mg/kg at 5:00 PM) in 0.9% NaCl in a concentration of 100 mg/ml, previously shown to decrease serum NO concentrations to 30% of normal in rats (18). L-arginine (L-arginine hydroxychloride; Sigma Chemical Co.) was given orally at a concentration of 2.25% dissolved in tap water (available ad libidum), a solution previously shown to increase plasma L-arginine levels twofold (20).

Histological evaluation. The grafted trachea was excised, embedded in Tissue-Tek (Miles Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -70°C until used. For histological evaluation, frozen sections were stained with Mayer's hematoxylin-eosin. The histological changes in respiratory epithelium were evaluated as a percentage of normal respiratory, abnormal cuboidal and squamous epithelium, or loss of epithelium. Luminal occlusion was evaluated as a reduction of luminal area using an ocular grid. All the analyses were done in a blinded review by two observers, and the scores of these two observers were highly correlated ($r^2 = 0.95$). The degree of airway wall inflammation, the cell types of the fibroproliferative lesion, and the number of proliferating inflammatory and myofibroblastic cells were analyzed using immunohistochemistry.

Single immunostaining. Serial frozen sections (4–6 μm) were air-dried on silane-coated slides, fixed in acetone at -20°C for 20 min, and stored at -20°C until used. Before immunostaining, the slides were refixed with chloroform and then air-dried. After incubation with appropriate 1.5% nonimmune serum (Vector Laboratories, Inc., Burlingame, CA) for mAbs or pAbs, frozen sections were incubated with a mouse mAb at room temperature for 30 to 60 min, or with a rabbit or goat pAb at $+4^{\circ}\text{C}$ for 12 h. The primary Abs were diluted in PBS with 1% BSA and appropriate 3% nonimmune serum. With intervening washes in Tris-buffered saline, the slides were immersed in bionylated horse anti-mouse, goat anti-rabbit, or rabbit anti-goat rat-absorbed antibodies at room temperature for 30 min followed by avidin-bionylated horseradish complex (Vectastain Elite ABC Kit; Vector Laboratories) in PBS at room temperature for 30 min. The reaction was revealed by chromogen 3-amino-9-ethylcarbazole (AEC; Sigma Chemical Co.) containing 0.1% hydrogen peroxidase, yielding

a brown-red reaction product. The specimens were counterstained with hematoxylin and coverslips were aquamounted (Aquamount; BDH Chemicals Ltd., Poole, England).

Antibodies used. Details of the Abs and their use in immunohistochemistry were described previously unless otherwise specified (3). Abs were directed against rat macrophages (ED1; Serotec Ltd., Oxford, United Kingdom), activated macrophages (ED3; Serotec Ltd.), CD4 (W3/25; Sera-Lab, Sussex, United Kingdom), and CD8 (OX8; Sera-Lab) subsets. Immune activation was determined using mouse mAbs to MHC class II common determinant (OX6; Sera-Lab) and IL-2R (PharMingen, San Diego, CA); mouse mAbs to IFN- γ (CY-047; Innogenetics, Zwijndrecht, Belgium), TNF- α (CY-051; Innogenetics), and IL-4 (MRC OX-81; Serotec Ltd.); rabbit pAbs to IL-1 α (IP-110) and IL-1 β (LP-712; Genzyme Corp., Cambridge, MA); and goat IgG pAbs to IL-2 (SC-1786; Santa Cruz Biotechnology, Santa Cruz, CA) and IL-10 (AB-417-NA; R&D Systems, Minneapolis, MN). NOS was localized using rabbit IgG pAbs to mouse and rat iNOS (NOS2, SC-650; Santa Cruz Biotechnology) and cNOS (NOS3, SC-654; Santa Cruz Biotechnology).

Double immunostaining. To localize iNOS expression on macrophages, endothelial cells, and smooth muscle cells, double staining was applied on representative frozen sections. After staining for iNOS using the peroxidase ABC method described above (yielding a brown-red reaction product), tracheal segments were washed in Tris-buffered saline, and avidin-biotin complex from the first step was blocked by incubating the sections with an excess of avidin and biotin (Avidin/Biotin Blocking Kit; Vector Laboratories, Inc.). After application of antibodies to macrophages (ED1), endothelial cells (RECA 1; Serotec Ltd.), or α -smooth muscle cell actin (1A4; Biomakor, Rehovot, Israel) at room temperature for 30 min, sections were incubated with bionylated horse anti-mouse rat-absorbed antibodies at room temperature for 30 min, followed by incubation with alkaline phosphatase avidin-biotin complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc.) and visualized by Vector blue, an alkaline phosphate substrate kit (Vector Laboratories, Inc.) that produced a blue reaction. Double-stained sections were not counterstained.

Specificity controls of immunostaining. Controls were performed using the same immunoglobulin concentrations of species and isotype-matched antibodies: mouse monoclonal IgG1 antibody (catalog no. X931; Dako Corp., Carpinteria, CA) and rabbit polyclonal immunoglobulin fraction (catalog no. X936; Dako Corp.) for mAbs and pAbs, respectively. Additional specificity controls for iNOS, cNOS, TNF- α , IL-1 α , IL-1 β , and IFN- γ stainings involved use of a working dilution of the polyclonal antibody after overnight incubation with a 10 to 20-molar excess of recombinant cytokine (Genzyme Corp.) or peptide (Santa Cruz Biotechnology). None of these control stainings showed any immunoreactivity.

Quantitation of immunohistochemistry. The immunohistochemical analysis was done in a blind review by two observers, and the score assigned was determined by consensus. The intensity of the staining was scored from 0 to 3 as follows: 0, no visible staining; 1, few cells with faint staining; 2, moderate intensity with multifocal staining; and 3, intense diffuse staining of the cells analyzed. Positive staining for IL was scored as the number of positive cells in cross-section.

In vivo labeling for cell proliferation. All recipients were injected with 400 μl i.v. of a concentrated solution of bromodeoxyuridine (BrdU; Zymed Laboratories, Inc., San Francisco, CA) 3 h before death. Cell proliferation in frozen sections was revealed by an IgG1 mouse mAb to BrdU 1:20 (M744; Dako A/S, Glostrup, Denmark) and a Vectastain Elite ABC Kit (Vector Laboratories) method as described above. Before staining, the frozen sections were fixed with buffered formalin for 15 min. After a 10-min wash in PBS, the sections were microwave-treated with 500 W in 0.1 M citrate buffer, pH 6, for 5 min to break the double-stranded DNA, followed by a 10-min wash in PBS. Cell proliferation was measured by counting the number of labeled nuclei in tracheal cross-sections.

Reverse transcriptase (RT) PCR. Relative iNOS gene transcript levels were measured with semiquantitative RT-PCR after normal-

ization against levels of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA isolation was extracted from fresh trachea tissue using a guanidine-isothiocyanate preparation as described (21, 22). The total RNA yields were estimated by spectrophotometry, and were confirmed by 1% agarose/TBE gel electrophoresis.

To identify the optimum PCR conditions for accurate measurement of each gene, we established the logarithmic assay range with respect to cycle number and starting template concentration against different dilutions of cDNA. 1 μ g of total RNA was reverse-transcribed to cDNA in a 20- μ l volume of master mix: 1 $\times$ RT buffer (Promega Corp., Madison, WI), 300 mM dNTP (GeneAmp; Perkin-Elmer Corp., Norwalk, CT), 50 pmol oligo-dT primer (p[dT]₁₅ primer; Boehringer-Mannheim, Mannheim, Germany), 20 U RNasin (RNasin Ribonuclease Inhibitor; Promega Corp.), and 100 U reverse transcriptase (M-MLV-RTase Reverse Transcriptase, RNase H Minus; Promega Corp.) at +37°C for 90 min. 1 μ l of cDNA reaction mixture was added to 37.4 μ l of water and supplemented with 5 μ l 10 $\times$ PCR buffer I (GeneAmp; Perkin Elmer Corp.), 10 nmol dNTP (GeneAmp; Perkin Elmer Corp.), 1 μ Ci α [³²P]dATP, 3000 Ci/mmol (Amersham Intl., Buckinghamshire, England), 10 pmol iNOS sense primer (5'-CTT TGT GCG GAG TGT CAG TGG-3' [GenBank accession no. D12520]), 10 pmol iNOS antisense primer (5'-TTC TTC CTG ATA GAG GTG GTCC-3'), and 2.5 U AmpliTaq Gold (Perkin Elmer Corp.). The samples were heated to 95°C for 10 min, and then cycled 37 times (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) using the 96-well PCR apparatus (GenAmp PCR System 9600 apparatus; Perkin Elmer Corp.). The relative amount of GAPDH mRNA was analyzed by a similar protocol using GAPDH sense (5'-GTC TTC ACC ACC ATG GAG AAG GCT-3' [GenBank accession no. M17701]) and GAPDH antisense (5'-TGT AGC CCA GGA TGC CCT TTA GTG-3') primers. The PCR protocol for GAPDH was as follows: 95°C for 10 min, and then amplification for 33 cycles (94°C, 1 min, 57°C, 1 min, 72°C, 2 min). Gene transcript levels for all samples for a single gene were amplified simultaneously, and each PCR analysis was completed in triplicate. For each experiment, the negative controls for which water was used instead of cDNA or reverse transcriptase was omitted during cDNA synthesis. The PCR samples were electrophoresed through 2% agarose. Subsequently, the gel was dried (LKB 2003 Slab Gel Dryer; LKB, Bromma, Sweden), exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan), and the gels were quantified using a Fuji BAS1500 phosphorimager. The mean values of

the three determinations were used for final analysis, and the normalized iNOS mRNA levels were derived by dividing the mean of iNOS mRNA with the mean of GAPDH mRNA for each tissue sample.

Spectrometric determination of intragraft nitrites and nitrates. Intra-graft NO production was determined as stable metabolite nitrites and nitrates (NO_x). Tracheas were snap-frozen and stored in -70°C. Homogenization was done in ultrapure water (MilliQ; Millipore Corp., Bedford, MA) with a Douche homogenizer at +4°C. The crude homogenate was centrifuged (4000 g). To prevent interference of proteins and hemegroups, the supernatant was filtrated through Centricon filters (Amicon Inc., Beverly, MA), exclusive size 10.000 D, and the rest of the proteins in the filtrate were precipitated in 1:20 of 20% ZnSO₄. The supernatants were divided for two assays: one for measurement of NO₂⁻ + NO₃⁻ and for the measurement of NO₂⁻. NO₂⁻ was measured with the Griess reaction, and nitrate reductase (EC 1.6.6.2, *Aspergillus* species; Boehringer Mannheim) was used to reduce the NO₃⁻ to NO₂⁻ used in the Griess reaction (23, 24). All determinations were performed in duplicate, and the results are given in nmol/mg of tissue. The detection limit of the assay was 0.25 μ M, and absorbance in 540 nm was measured.

Statistical analyses. All data are expressed as mean $\pm$ SEM. For multiple group comparisons of small sample size nonparametric, Kruskal-Wallis (Statview 512+ program; Brain Power Inc., Calabasas, CA) one-way analysis by ranks was used. The rank sums obtained with the Kruskal-Wallis test were then used for the Dunn test at significance levels of 5 and 1% (Medstat; Astra Group A/S, Copenhagen, Denmark). In groups with normal distribution and standard variance (F-test), one-factor ANOVA and Fisher PLSD-test were used at significance levels of 5 and 1%. $P < 0.05$ was regarded as statistically significant.

Results

Histology. The morphological changes of respiratory epithelium and the degree of luminal occlusion of tracheal grafts at different time points are summarized in Fig. 1. 3 d after transplantation, the respiratory epithelium of syngeneic grafts was slightly damaged, probably due to ischemic injury before revascularization of the graft. 30 d after transplantation, the respiratory epithelium of syngeneic grafts was fully recovered,

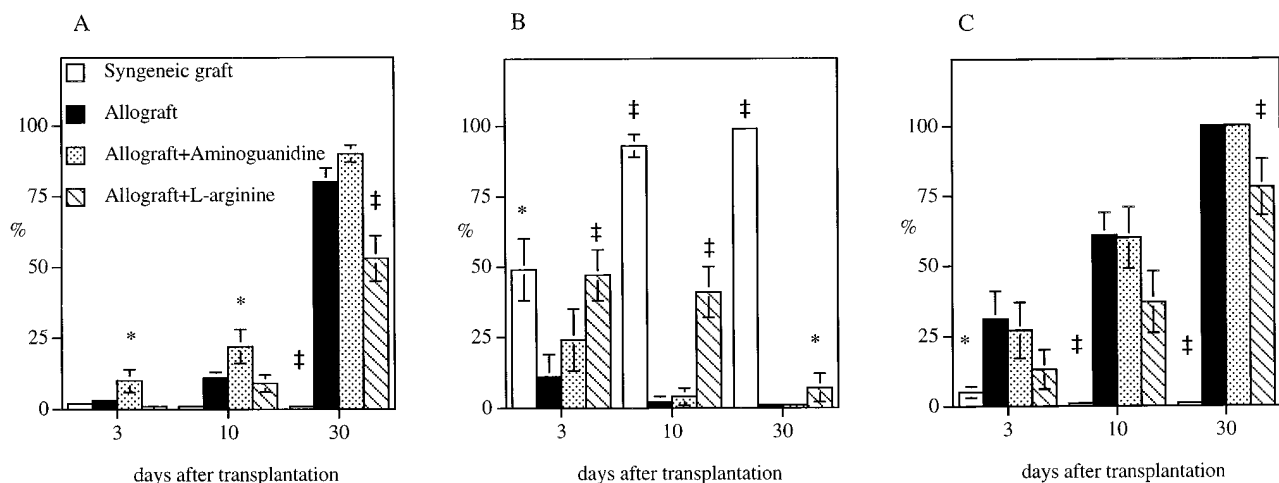
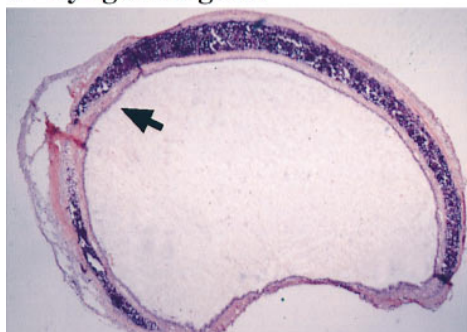
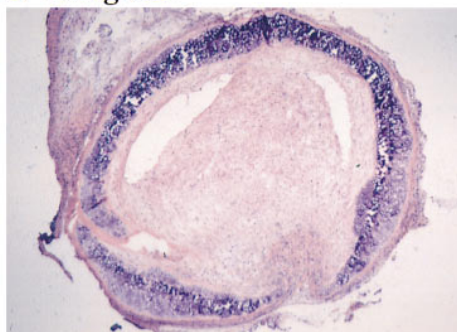


Figure 1. Histological features of syngeneic grafts, nontreated, aminoguanidine-treated, and L-arginine-treated allografts at various time points. (A) The degree of luminal occlusion, (B) percentage of airway lumen covered with normal respiratory epithelium, and (C) percentage of epithelial necrosis. Data is expressed as mean $\pm$ SEM. One-factor ANOVA and Fisher PLSD-test were used to evaluate significancies among the four groups. * $P < 0.05$, ‡ $P < 0.01$ when compared with nontreated allografts.

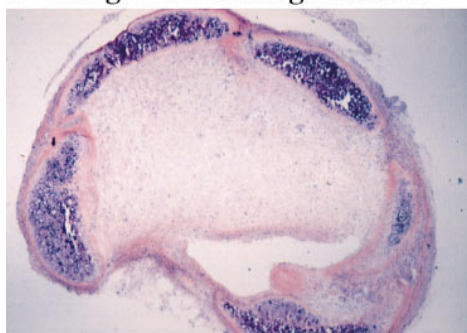
A Syngeneic graft



B Allograft



C Allograft+Aminoguanidine



D Allograft+L-arginine

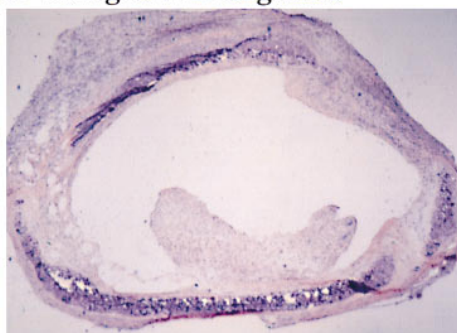


Figure 2. Photomicrographs of (A) syngeneic graft, (B) nontreated, (C) aminoguanidine-treated, and (D) L-arginine-treated allografts 30 d after transplantation. Syngeneic grafts were filled with mucus, and the airway was covered with normal respiratory epithelium (arrow). In nontreated and aminoguanidine-treated allografts, the airway lumen was severely compromised by dense myofibroproliferative lesions (B and C). L-arginine treatment significantly ameliorated development of the obliterative lesion. Mayer's haematoxylin & eosin staining, 40× original.

and the grafts were filled with mucous, a sign of normal epithelium function. In syngeneic grafts, no myofibroproliferation occurred, and overall histology resembled that of nontransplanted tracheas (Fig. 2 A). In nontreated allografts, there was a progressive loss of respiratory epithelium. Airway wall inflammation peaked 10 d after transplantation, and subsided thereafter. In nontreated allografts, the luminal surface was reduced by myofibroproliferative lesion already at 10 d, and at

30 d intense myofibroproliferation nearly totally occluded the lumen (Fig. 2 B). Aminoguanidine treatment significantly accelerated myofibroproliferative lesion development, compromising the luminal area already at 3 and 10 d compared to nontreated allografts ($P < 0.05$). Thereafter, the difference leveled off as the tracheal lumen was nearly totally occluded in both groups at 30 d (Figs. 1 and 2 C). L-arginine treatment significantly delayed epithelial destruction, and significantly down-

Table 1. Expression of iNOS in Normal Tracheas, Syngeneic Grafts, Nontreated, Aminoguanidine-, and L-arginine-treated Allografts

Days after transplantation	Group	n	Epithelial cells	Myofibroproliferative lesion	Capillary endothelial cells	Mononuclear inflammatory cells	Smooth muscle cells
0	Normal DA trachea	6	0.5±0		0±0	0.2±0.1	0.1±0.1
3	Syngeneic graft	10	0.1±0.1		0.1±0.1	0.3±0.1	0.1±0.1
	Allograft	12	0.3±0.3		0	0.8±0.1	0.1±0.1
	Allograft + aminoguanidine	12	1.1±0.2		0.7±0.1	1.2±0.2	0.5±0.2
	Allograft + L-arginine	12	1.0±0.1		1.4±0.1 [‡]	1.2±0.1	0.9±0.1 [‡]
10	Syngeneic graft	10	2.2±0.3		0.9±0.2	0.6±0.1*	0.5±0.1
	Allograft	10	1.3±0.3		0.8±0.1	1.2±0.2	0.6±0.1
	Allograft + aminoguanidine	15	1.0±0.2		0.4±0.1	0.8±0.1	0.1±0.0 [‡]
	Allograft + L-arginine	15	1.9±0.3		0.9±0.2	1.4±0.2	0.5±0.1
30	Syngeneic graft	10	0.6±0.2		0.8±0.2	0.3±0.1	0.8±0.1
	Allograft	14		0.2±0.1	0.6±0.1	0.5±0.1	0.4±0.1
	Allograft + aminoguanidine	14		0.3±0.1	0.9±0.1	0.7±0.1	0.6±0.1
	Allograft + L-arginine	12		0.7±0.2	0.9±0.2	0.8±0.1	0.6±0.1

In allogeneic groups (allograft, allograft + L-arginine, and allograft + aminoguanidine) at 3 and 10 d, only some of the grafts had preserved epithelium where iNOS expression could be scored. Myofibroproliferative lesion was scored only at 30 d, as at earlier time-points it was not observed constantly for scoring. Values are given as mean±SEM. * $P < 0.05$, and [‡] $P < 0.01$ when compared with nontreated allografts (Kruskal-Wallis and Dunn tests).

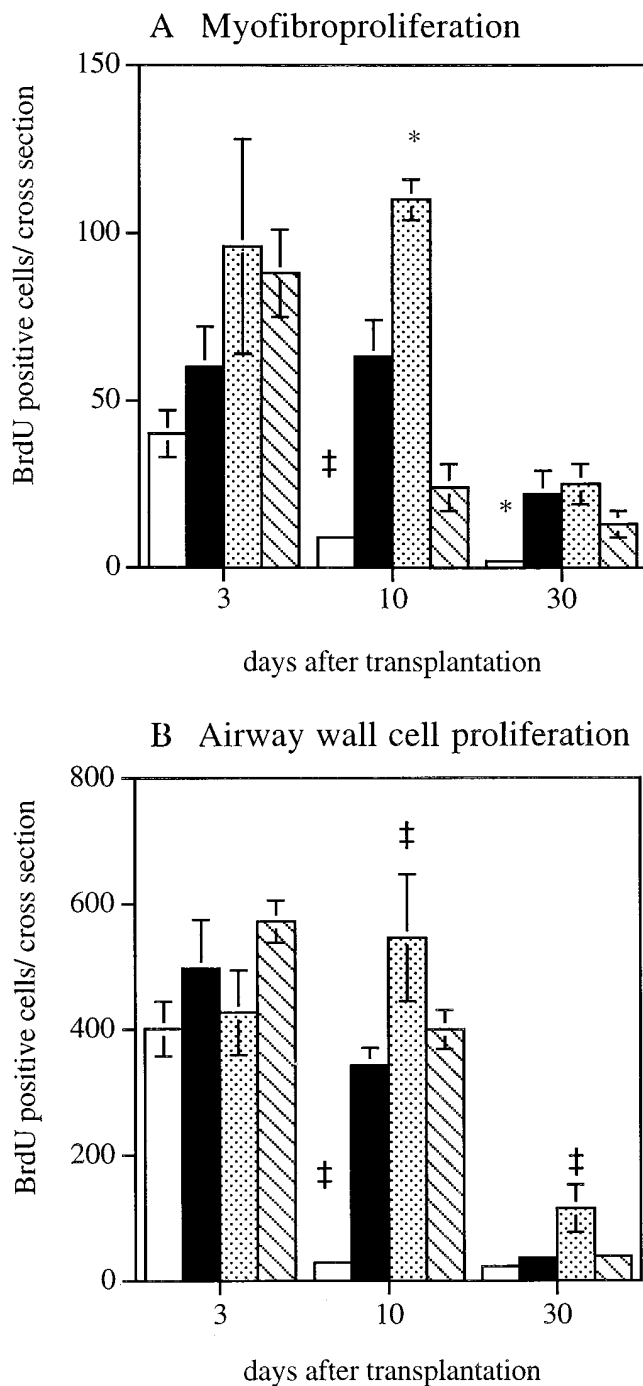


Figure 3. Myofibroproliferation (A) and airway wall cell proliferation (B) in syngeneic grafts, nontreated, aminoguanidine-treated, and L-arginine-treated allografts at various time points. The number of BrdU-positive cells per cross section was counted. Data is expressed as mean $\pm$ SEM. One-factor ANOVA and Fisher PLSD-test were used to evaluate significancies among the four groups. * $P < 0.05$, ‡ $P < 0.01$ when compared with nontreated allografts. White bars, syngeneic graft; black bars, allograft; stippled bars, allograft + aminoguanidine; striped bars, allograft + L-arginine.

regulated the development of myofibroproliferative lesion at 30 d when compared to nontreated allografts ($P < 0.01$) (Figs. 1 and 2 D).

In vivo cell proliferation. Cell proliferation in the airway

wall and myofibroproliferative lesion of tracheal grafts was determined by BrdU immunoreactivity (Fig. 3). 3 d after transplantation, airway wall cell proliferation was prominent in both syngeneic grafts and allografts. In syngeneic grafts, the proliferative response ceased thereafter. In nontreated allografts, the airway wall cell proliferation peaked at 10 d, whereas intense fibroproliferation continued up to 30 d resulting in total occlusion of the tracheal lumen as compared to syngeneic grafts. In aminoguanidine-treated allografts, the proliferation of airway wall cells, mainly smooth muscle cells and mononuclear inflammatory cells, was significantly enhanced at days 10 and 30 compared to other allogeneic groups. Aminoguanidine treatment was further associated with upregulation of fibroproliferation 10 d after transplantation, but not thereafter. L-arginine treatment reduced fibroproliferation at 10 and 30 d after transplantation, resulting in markedly diminished luminal occlusion, but had no effect on airway wall cell proliferation.

NOS protein expression. Immunohistochemistry and double stainings revealed iNOS expression in macrophages, smooth muscle cells, capillary endothelial cells, and epithelium (Fig. 4). Immunohistochemical analysis of normal nontransplanted tracheas localized weak iNOS expression in respiratory epithelium (Table I, Fig. 4 A). In syngeneic grafts, induction of epithelial iNOS expression was observed at 10 d, coinciding with recovery of respiratory epithelium of the grafts (Fig. 4 B). In nontreated allografts, intensity of epithelial iNOS expression was downregulated compared to syngeneic grafts, reflecting ongoing damage of epithelial cells during the alloimmune response (Fig. 4 C). Inflammatory cell iNOS expression was somewhat upregulated (Fig. 4 F). Aminoguanidine treatment did not affect the intensity of epithelial iNOS expression, but reduced the expression of capillary endothelial, ED1+ mononuclear inflammatory cell and smooth muscle cell ($P < 0.01$) iNOS expression at 10 d compared to nontreated allografts (Table I). L-arginine treatment significantly enhanced capillary endothelial and smooth muscle cell iNOS expression early after transplantation (Table I). In normal tracheas, weak cNOS expression was recorded in capillary endothelium, and in a few inflammatory cells. Neither transplantation, aminoguanidine, or L-arginine treatment had any effect on cNOS expression (data not shown).

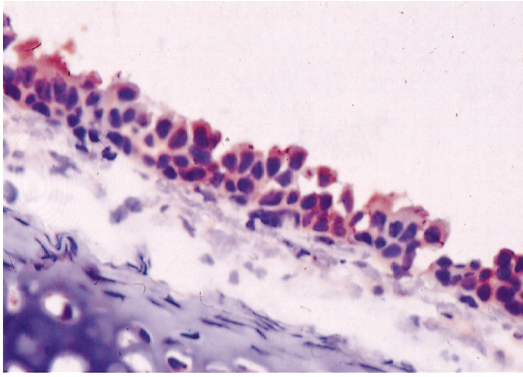
Production of nitrites and nitrates. Spectrometric analysis and Griess-reaction were used to determine the intragraft NO production as nitrites and nitrates (NO_x) (Table II). In a syngeneic group, intragraft NO_x production was comparable to

Table II. Intragraft Nitric Oxide Production

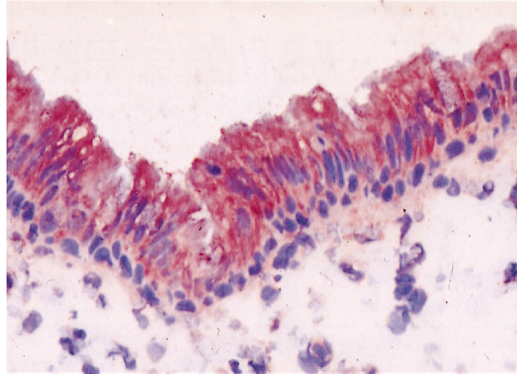
Days after transplantation	n	Normal DA trachea	Syngeneic graft	Allograft + aminoguanidine	Allograft + L-arginine
0	5	61 $\pm$ 4			
3	5		61 $\pm$ 2*	25 $\pm$ 3	32 $\pm$ 6
10	5		90 $\pm$ 18*	16 $\pm$ 3	38 $\pm$ 6
30	5		64 $\pm$ 28	61 $\pm$ 8	68 $\pm$ 5
					99 $\pm$ 17

Intragraft nitric oxide production was measured as the total amount of nitrites and nitrates ($\text{NO}_2^- + \text{NO}_3^-$, i.e. NO_x) at different time points after transplantation, and the results are given as nmol/mg tissue (mean $\pm$ SEM). * $P < 0.05$, when compared nontreated allografts (non-parametric Kruskal-Wallis and Dunn tests).

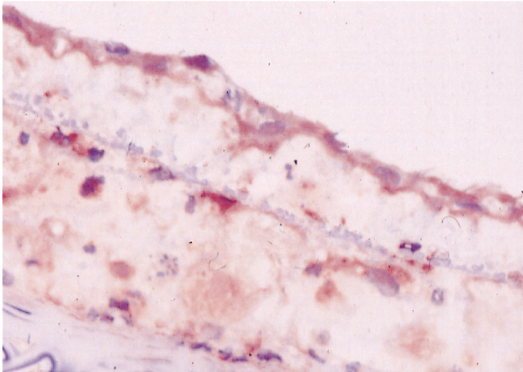
A Normal trachea



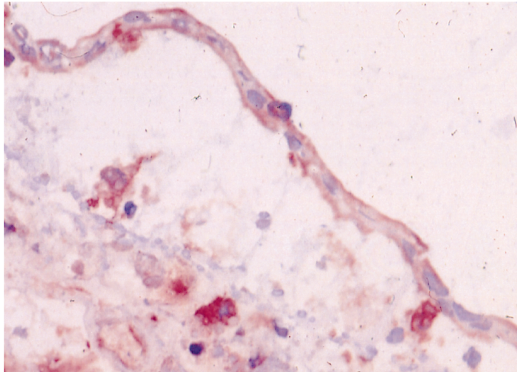
B Syngeneic graft



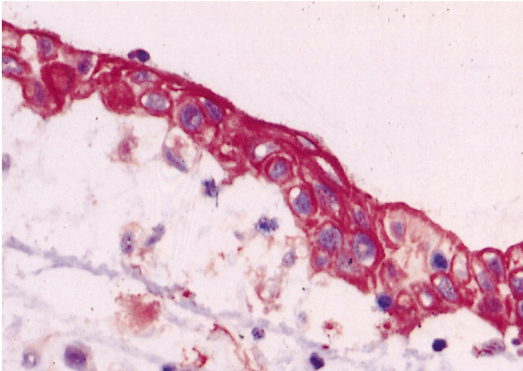
C Allograft



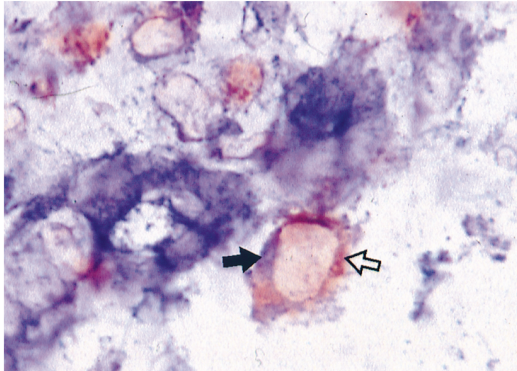
D Allograft+Aminoguanidine



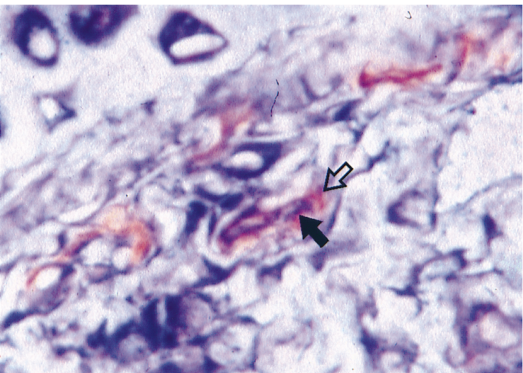
E Allograft+L-arginine



F ED1+iNOS



G α -actin+iNOS



H RECA+iNOS

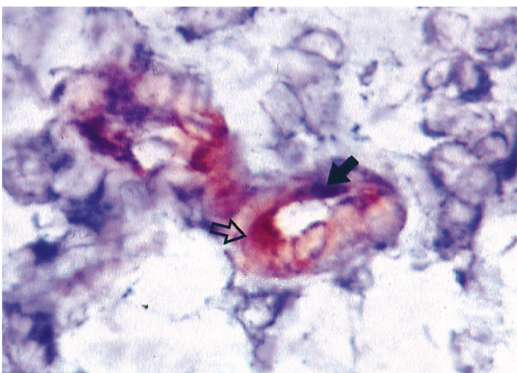


Figure 4. Photomicrographs of iNOS immunoreactivity in the epithelium of (A) normal trachea, (B) syngeneic graft, (C) nontreated, (D) aminoguanidine-treated, and (E) L-arginine-treated allografts 10 d after transplantation (400 $\times$ original). Double stainings for (F) ED1, (G) α -smooth muscle cell actin, and (H) RECA in allografts 10 d after transplantation, revealed iNOS staining in macrophages, smooth muscle cells, and capillary endothelial cells, respectively. Open arrow (red), iNOS; filled arrow (blue), cell subsets (1000 $\times$ original).

Table III. Intra-graft iNOS mRNA Expression

Days after transplantation	n	Normal DA trachea	Syngeneic graft	Allograft	Allograft + aminoguanidine	Allograft + L-arginine
0	6	0.05±0.03				
3	6		0.16±0.03	0.24±0.03	0.27±0.03	0.41±0.06*
10	6		0.09±0.02*	0.34±0.03	0.41±0.02	0.55±0.05*
30	6		0.06±0.01	0.05±0.02	0.24±0.05*	0.23±0.02*

Relative iNOS gene expression levels were measured with semiquantitative RT-PCR after normalization against levels of reference gene GAPDH. Values are given as mean±SEM. **P* < 0.05 when compared with nontreated allografts (Kruskal-Wallis and Dunn tests).

that of nontransplanted tracheas. In nontreated allografts, NO_x production was significantly downregulated at 3 and 10 d when compared with syngeneic controls. Aminoguanidine treatment did not further suppress NO_x production, whereas L-arginine treatment significantly enhanced intra-graft NO_x production early after transplantation.

iNOS mRNA expression. Semiquantitative RT-PCR was used to measure relative iNOS mRNA levels after normalization against levels of the reference gene GAPDH mRNA. In syngeneic grafts, induction of iNOS mRNA synthesis was seen at 3 d returning to the level of nontransplanted tracheas (Table III, Fig. 5). In nontreated allografts compared with syngeneic grafts, a significant induction of iNOS mRNA was observed at 10 d, coinciding with the peak inflammatory response. Aminoguanidine treatment did not reduce iNOS mRNA levels but instead was associated with significant upregulation of iNOS mRNA at 30 d, indicating that the inhibition of NO production may inhibit negative feedback of NO to iNOS mRNA synthesis (6). Supplementation of iNOS pathway with L-arginine increased iNOS mRNA expression at all time points compared with nontreated allografts.

Airway wall inflammation and intra-graft cytokine expression of tracheal transplants. In nontreated allografts, a prominent airway wall infiltration of ED1+ and ED3+ macrophages, CD4+, and CD8+ T cells was observed at 3 and 10 d after

transplantation, subsiding thereafter. Many of the infiltrating inflammatory cells showed immune activation with expression of IL-2R and MHC class II. Also, strong epithelial MHC class II expression was recorded. Aminoguanidine or L-arginine treatment did not alter the intensity of airway wall inflammation or the immune activation of inflammatory cells. Allograft expression of proinflammatory cytokines, especially of TNF-α, IFN-γ, IL-1, and IL-2 was upregulated compared to syngeneic grafts (Table IV, Fig. 6). Aminoguanidine treatment did not significantly alter allograft cytokine expression profiles. L-arginine treatment, however, was associated with upregulation of Th2 cytokines, IL-4, and IL-10 in the airway wall during peak inflammation at 10 d (Table IV, Fig. 6).

Discussion

Recent experimental observations in rodents demonstrate that heterotopic tracheal allografts develop myofibroproliferative lesions similar to those seen in small and large airways of human lung allografts with OB (2–4, 25, 26). At the early phase of OB in heterotopic rat tracheal allografts, the respiratory epithelium strongly expresses MHC class II, enabling a direct presentation of foreign antigens to alloreactive T cells (4). In addition, graft-infiltrating recipient-derived professional APC such as dendritic cells and macrophages may process donor alloantigens for indirect T and B cell activation, leading to clonal cell proliferation and Ab production, respectively (4, 27). Recruitment of CD4+ and CD8+ T cells and macrophages into the airway wall induces local cytokine and growth factor production leading to epithelial damage, proliferation of α-smooth muscle actin immunoreactive cells, and gradual occlusion of the airway lumen. In syngeneic grafts and allografts immunosuppressed with sufficient doses of cyclosporin A, obliterative changes do not develop, indicating the key role of alloimmune response in this disease process (2, 4, 28).

NO is reported to be an important mediator of inflammatory responses in lungs (11). Recently, induction of NO producing enzyme iNOS and production of NO during rejection were demonstrated. It has also been shown that treatment with aminoguanidine, a preferential inhibitor of iNOS, significantly attenuated the pathogenesis of acute cardiac and lung allograft rejection (15, 16, 18, 19). Supplementation of NO in cardiac allograft ischemia-reperfusion injury prolonged graft survival, which may be due to the protective role of NO to endothelial cells (29, 30).

Although induction of iNOS mRNA and protein expression have been demonstrated in chronic rejection and allograft arteriosclerosis (31, 32), the biological role of NO in this dis-

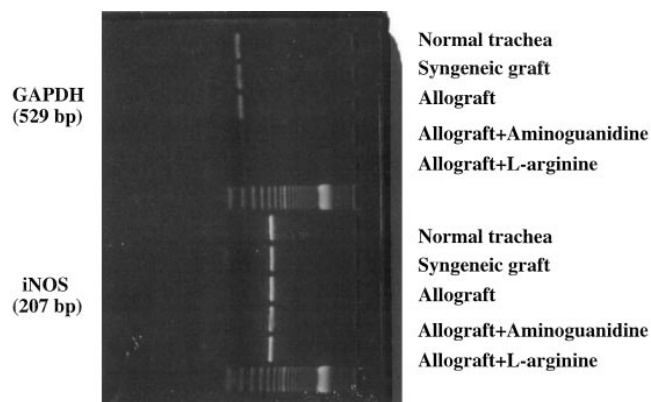


Figure 5. Example of RT-PCR of iNOS mRNA and GAPDH mRNA transcript levels from representative analyses that included one cDNA sample from each group, i.e., normal trachea, syngeneic graft, nontreated, aminoguanidine-treated and L-arginine-treated allografts 10 d after transplantation. The actual determinations were performed simultaneously using the 96-well PCR apparatus, and were completed in triplicate.

Table IV. Inflammatory Cell Subsets, Immune Activation, and Cytokine Expression 10d after Transplantation

Ab	Syngeneic graft		Allograft		Allograft + aminoguanidine		Allograft + L-arginine	
Inflammatory cells								
ED1	0.5±0.1*	MNC	1.1±0.1	MNC	1.1±0.1	MNC	1.2±0.2	MNC
ED3	0.5±0.1		0.7±0.1	Myofibroproliferative lesion, MNC	0.5±0.1	Myofibroproliferative lesion, MNC	0.7±0.2	Myofibroproliferative lesion, MNC
CD4	0.5±0.2	MNC	0.8±0.2	MNC	1.1±0.1	MNC	0.7±0.1	MNC
CD8	0.2±0.1	MNC	0.9±0.2	MNC	1.2±0.3	MNC	0.8±0.3	MNC
Immune activation								
MHC class II	0.6±0.1*	EP	1.3±0.1	EP, MNC	1.4±0.1	EP, MNC	1.5±0.1	EP, MNC
IL-2R	0±0*		1.0±0.1	EP, MNC	1.3±0.2	EP, MNC	0.8±0.1	EP, MNC
Cytokines								
IL-1α	0±0*		0.6±0.1	MNC	0.4±0.2	MNC	0.6±0.1	MNC, SMC
IL-1β	0±0*		0.3±0.1	MNC	0.5±0.1	MNC	0.2±0.0	MNC
TNF-α	0±0		0.3±0.1	MNC, SMC	0.1±0.1	MNC, SMC	0.3±0.1	MNC, SMC
IFN-γ	0±0*		0.9±0.2	MNC	0.3±0.3	MNC	1.3±1.3	MNC
IL-2	0±0*		172±48	MNC	142±45	Airway wall MNC	184±52	MNC
IL-4	0±0		5±2	Myofibroproliferative lesion, MNC, SMC	1±1	Myofibroproliferative lesion, MNC, SMC	16±5	Myofibroproliferative lesion, MNC, SMC
IL-10	0±0		3±1	MNC	3±1	MNC	48±28*	MNC

Graft-infiltrating inflammatory cell subsets, immune activation, and expression of inflammatory cytokines in the grafts. EC, capillary endothelial cells; EP, epithelial cells; MNC, mononuclear inflammatory cells; SMC, smooth muscle cells. For IL-2, IL-4 and IL-10 the number of positive cells were scored per cross-section. For others, the intensity of staining was scored from 0 to 3. Values are mean±SEM. **P* < 0.05, when compared with non-treated allografts (Kruskal-Wallis and Dunn tests).

ease process remains unknown. In models of ordinary atherosclerosis, inhibition of NOS has been shown to accelerate atherogenesis (33) and to increase cardiac fibrosis (34), whereas supplementation of NOS pathway with L-arginine reduced hypercholesterolemia and endothelial denudation-induced atherosclerosis (20, 35). The same beneficial effect with L-arginine, however, was not achieved in a chronic rejection model of femoral artery allografts in the rat (36).

In this communication we demonstrate a protective regulatory role of NO in OB development, i.e., chronic rejection. Supplementation of NO pathway by L-arginine resulted in significant reduction in the proliferation of α-smooth muscle actin immunoreactive cells, and thereby in downregulation of tracheal allograft occlusion. L-arginine treatment also reduced epithelial damage and necrosis. In contrast, inhibition of iNOS by aminoguanidine treatment significantly accelerated development of OB, particularly early after transplantation.

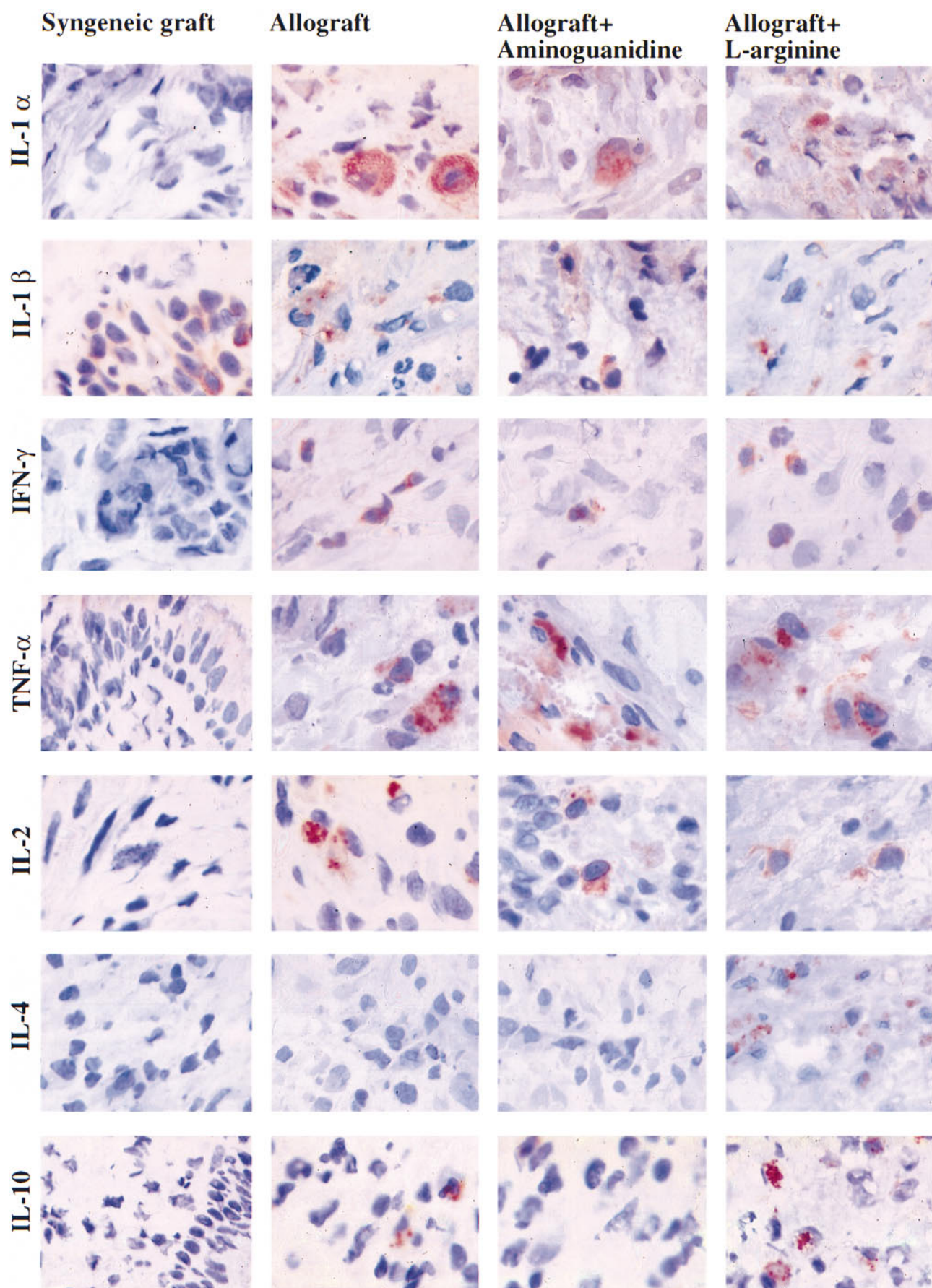
In normal tracheas and syngeneic grafts, iNOS was localized in the epithelium, suggesting that it may be the main source of NO. This result is consistent with an earlier report demonstrating continuous NO production by epithelial iNOS (10, 11). In nontreated allografts, an increase in the relative intragraft iNOS mRNA and mononuclear inflammatory cell iNOS immunoreactivity was demonstrated during progressive loss of respiratory epithelium and airway occlusion. The intragraft NO production was, however, decreased, possibly due to alloimmune damage to the epithelium, and thus decreased epi-

thelial iNOS. The aminoguanidine treatment-enhanced OB was not associated with reduction of relative iNOS mRNA synthesis, but was linked to upregulation of relative iNOS mRNA at the end-point, indicating that inhibition of NO production may inhibit negative feedback of NO to iNOS mRNA synthesis (6). Aminoguanidine did not affect total intragraft NO production, but decreased iNOS immunoreactivity in smooth muscle cells. In contrast, the antiproliferative effect of L-arginine supplementation was linked with enhanced relative intragraft iNOS mRNA synthesis, iNOS immunoreactivity in capillary endothelial and smooth muscle cells, and intragraft NO production. L-arginine treatment, however, did not affect the number of macrophages, known to be an important source of iNOS (37).

Our findings suggest that one of the possible mechanisms of NO in OB prevention may be mediated by smooth muscle cell proliferation inhibition or enhanced revascularization of grafts by better-preserved capillary endothelium (30), indicated by increased iNOS expression in smooth muscle cells and capillary endothelium during L-arginine treatment. On the growth factor level of smooth muscle cell proliferation, NO inhibits platelet-derived growth factor (PDGF) expression (38), which is a major mitogen for smooth muscle cells, and may have an essential role in OB development (3, 39, 40). NO also appears to inhibit smooth muscle proliferation directly through elevation of cyclic-GMP levels (41, 42).

Several mechanisms exist whereby NO may modulate immune responses in the graft. Supplementation of L-arginine has

Figure 6. Photomicrographs of cytokine stainings of syngeneic grafts, nontreated, aminoguanidine-treated, and L-arginine-treated allografts. Nontreated allografts expressed pro-inflammatory cytokines IL-1α, IL-1β, TNF-α, IFN-γ, and IL-2 when compared with syngeneic grafts. Aminoguanidine treatment did not significantly alter allograft cytokine expression profiles, while L-arginine treatment was associated with significant upregulation of Th2 cytokines, IL-4, and IL-10 in the airway wall during peak inflammation 10 d after transplantation.



been shown to reduce platelet aggregation (43, 44) and leukocyte adhesion and emigration (45). In this study, supplementation of NO pathway or inhibition of iNOS pathway had no effect on the number of graft-infiltrating CD4⁺ or CD8⁺ T cells or macrophages, or on the level of immune activation with expression of IL-2R or MHC class II. Supplementation of NO pathway by L-arginine, however, modulated the immune response towards Th2 cytokine IL-4 and IL-10 production, which has been linked with prolonged graft survival and tolerance induction (46). NO has been shown to inhibit the expansion of cloned Th1, but not Th2 cells (47). Furthermore, observations in iNOS-deficient mice demonstrate that a low concentration of endogenously generated NO by cNOS may be required for Th1 cell proliferation, but a high concentration of NO produced by iNOS is necessary for prevention of potential overexpansion of Th1 cells, which is implicated in a variety of autoimmune diseases (48).

In conclusion, we demonstrate that NO has a protective role in the OB development: while NO pathway supplementation by L-arginine attenuated obliterative changes, preferential inhibitor of iNOS pathway enhances these changes. Our results indicate furthermore that potential mechanisms of NO in the prevention of OB include either inhibition of smooth muscle cell proliferation or altered immune response towards Th2 cytokines.

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