Inducible Nitric Oxide Synthase in Pulmonary Alveolar Macrophages from Patients with Tuberculosis

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Summary

The high-output pathway of nitric oxide production helps protect mice from infection by several pathogens, including *Mycobacterium tuberculosis*. However, based on studies of cells cultured from blood, it is controversial whether human mononuclear phagocytes can express the corresponding inducible nitric oxide synthase (iNOS; NOS2). The present study examined alveolar macrophages fixed directly after bronchopulmonary lavage. An average of 65% of the macrophages from 11 of 11 patients with untreated, culture-positive pulmonary tuberculosis reacted with an antibody documented herein to be monospecific for human NOS2. In contrast, a mean of 10% of bronchoalveolar lavage cells were positive from each of five clinically normal subjects. Tuberculosis patients' macrophages displayed diaphorase activity in the same proportion that they stained for NOS2, under assay conditions wherein the diaphorase reaction was strictly dependent on NOS2 expression. Bronchoalveolar lavage specimens also contained NOS2 mRNA. Thus, macrophages in the lungs of people with clinically active *Mycobacterium tuberculosis* infection often express catalytically competent NOS2.

A third of the world's human population is thought to be infected with *Mycobacterium tuberculosis* (1). Though the vast majority of individuals hold the organism in check, more than 2.5 million a year fail to do so. As a result, tuberculosis may be the world's leading cause of death from bacterial infection (1). It is not known how human macrophages normally suppress the proliferation of this facultative intracellular pathogen nor how the bacterium sometimes gains the upper hand.

The inducible isoform of nitric oxide synthase (iNOS, NOS2)[1] contributes to the control of viral, bacterial, protozoal, fungal, and helminthic infections in mice (reviewed in references 2 and 3). This is evident from coordinate expression of NOS2 and anti-infectious activity; exacerbation of infection in vitro and in vivo by administration of NOS inhibitors; conferral of anti-infectious activity upon transfection with NOS2 cDNA or exposure to NO-generating organochemicals; and enhanced vulnerability to infection in mice rendered genetically deficient in NOS2 (4, 5). By the first three criteria, control of experimental tuberculosis in mice is NO-dependent (6–9). Moreover, mice rendered genetically deficient in interferon-γ (10), its receptor (11), or interferon regulatory factor-1 (12) are all disadvantaged with respect to expression of NOS2, and all three mouse strains sustain rapid mycobacterial proliferation.

However, the ability of human mononuclear phagocytes to express NOS2 is contested (13–15). Denis obtained the first positive evidence: nitrite in the medium of cytokine-treated, mycobacteria-infected, monocyte-derived macrophages (16). Support followed from at least 16 additional

[1] *Abbreviations used in this paper:* BSA/ggg, 5% BSA and 100 μg/ml goat γ-globulin in Tris-buffered saline; ECL, enhanced chemiluminescence; HUVEC, human umbilical vein endothelial cells; NGS, normal goat serum; NOS, nitric oxide synthase; PAGE, polyacrylamide gel electrophoresis; RT, room temperature; RT-PCR, reverse transcription followed by polymerase chain reaction; TBS, Tris-buffered saline.

Drs. Nicholson, Bonecini-Almeida, and Lapa e Silva contributed equally to this study.

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laboratories experimenting with various forms of induction and detection (17–32). Nonetheless, comparable results have eluded many other investigators (e.g., 33–37), some of whom dismiss the evidence that human mononuclear phagocytes can express NOS2 at all (14) or at a level consonant with host defense (38).

One interpretation of this controversy is that unidentified variables may restrict the consistency with which human monocytes or macrophages can be induced to express NOS2 in vitro. Such suppressive influences may not pertain in vivo. On this premise, we examined pulmonary alveolar macrophages fixed immediately after collection from patients with tuberculosis. Since monocytes can express NOS2 mRNA without detectable NOS2 protein (25), we centered the study on the characterization of an antibody—apparently the first such reagent—with which expression of human NOS2 protein could be demonstrated unequivocally. The results document the frequent expression of catalytically competent NOS2 in human macrophages from a site of infection.

Materials and Methods

Antibody. A peptide (Cys-Arg-Nle-Orn-Ser-Leu-Glu-Met-Ser-Ala-Leu) was synthesized to correspond to the 7 COOH-terminal residues of human NOS2 (underlined) plus 4 additional residues to improve solubility, linkage and quantitation. This peptide was coupled to thyroglobulin as described (39). IgG was purified from serum of a normal rabbit on recombinant staphylococcal protein A columns (Pierce Chemicals, Rockford, IL). The same rabbit was then repeatedly immunized with the peptide conjugate, initially in Freund's complete adjuvant and then in Freund's incomplete adjuvant. IgG was purified from the antisera, collectively termed NO53. $F(ab')_2$ fragments were prepared by digestion with immobilized pepsin (Pierce) and purified as above. The synthetic peptide NO54 (Tyr-Arg-Ala-Ser-Leu-Glu-Met-Ser-Ala-Leu) was used as a competitive blocking agent for immunoblots and immunocytochemistry. Rabbit antibody to pure mouse NOS2 was described (40). Affinity-purified rabbit antibodies to synthetic peptides from rat NOS1 (amino acid residues 1400–1419, containing a single amino acid difference from human NOS1) and human NOS3 (residues 1183–1202) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and were used at 0.1 μg/ml.

Specimens. With informed consent, bronchoalveolar lavage fluids were obtained for diagnostic purposes from 18 consecutive HIV-seronegative patients over 3 mo in 1995 in Hospital Universitario Clementino Fraga Filho (Rio de Janeiro) and 2 such patients at Bellevue Hospital (New York) who presented with clinical findings and roentgenographic evidence suggestive of active pulmonary tuberculosis and whose lavage specimens subsequently proved culture-positive for *M. tuberculosis*. 9 additional patients proved to have other diagnoses. Bronchoscopy specimens were also obtained from 11 healthy volunteers (2 from Rio de Janeiro and 9 from New York). Human study protocols were approved by the institutional review boards. Of the 20 samples collected from patients with tuberculosis, 17 were stored for RNA analysis. In 16 of these, RT-PCR demonstrated β-actin mRNA, which was taken as an indication that the samples were of sufficient quality for further study. 14 of the 20 samples were aliquotted for preparation of Cytospin® slides (Shandon Instruments, Sewicky, PA), and of these, 11 sets were sufficient in number and quality to undergo evaluation by immunocytochemistry and/or diaphorase activity. Samples from 7 of the 11 healthy volunteers were adequate for immunocytochemistry and/or diaphorase studies. Slides were fixed in cold acetone for 10 min, air dried, and stored at −20 to −70°C.

As described (41), human renal epithelial 293 cells (American Type Culture Collection, Rockville, MD) were transiently transfected by the calcium phosphate method with human NOS2 cDNA (42) (a gift of Dr. T. Billiar, University of Pittsburgh, PA) or human NOS1 (a gift of P. Marsden, University of Toronto, Canada) (43) in the pcDNAI vector (Invitrogen, San Diego, CA), with the vector alone, or with a mouse-human NOS2 chimera in which the COOH-terminal 24 amino acid residues of mouse NOS2 were replaced with the cognate 27 residues of the human enzyme (Q.-w. Xie, unpublished observation). Human umbilical vein endothelial cell monolayers were the kind gift of Dr. Siukong Lo (Cornell University Medical College). Lysates of 10^9 *M. tuberculosis* bacilli strain H37Ra (gift of Drs. S. Ehrt and L. Riley, Cornell University Medical College) were immunoblotted in parallel. Peritoneal macrophages were collected from 129/SvEv × C57BL/6 wild-type and homozygous NOS2-deficient mice (4) 24 h after intraperitoneal injection of 10^4 units pure, recombinant mouse interferon-γ (Genentech, Inc., South San Francisco, CA) and 5 μg bacterial lipopolysaccharide (from *Escherichia coli* 0111:B4; Sigma, St. Louis, MO).

Immunoblotting. Human NOS2 is susceptible to primary structural degradation when boiled in Laemmli's buffer (J. Calaycay and Q.-w. Xie, unpublished observations). Accordingly, unheated lysates were electrophoresed under denaturing, reducing conditions on 7.5% polyacrylamide gels, blotted to Immobilon membranes (Millipore Corp., Bedford, MA), and probed with NO53 IgG at 4 ng/ml or its F(ab')2 fragment at 132 ng/ml. Bound rabbit Ig was detected with donkey anti–rabbit IgG conjugated with horseradish peroxidase (Amersham Corp., Arlington Heights, IL) as revealed by enhanced chemiluminescence (Du Pont, Boston, MA).

(Immuno)Cytochemistry. Slides were blocked for 60 min at room temperature (RT) in 10% normal goat serum (NGS) in Tris-buffered saline (TBS), incubated for 60 min in 4 ng/ml preimmune IgG or NO53 IgG or 132 ng/ml of its F(ab')2 in NGS/TBS with or without 10 μg/ml of blocking peptide NO54, washed three times (5 min each) in TBS, and then incubated 60 min in 1:500 dilution of goat IgG anti–rabbit IgG conjugated to biotin (Vector Labs.). After washing, the slides were incubated in avidin-conjugated alkaline phosphatase according to the manufacturer's instructions (ABC Kit: Vector Labs.), washed thrice in TBS, and then incubated in Vector (Texas) Red or Vector Blue substrate for ~20 min. Slides were coverslipped in 10% glycerol and percent positive cells scored among 500 cells per sample under direct illumination (400×). For illustrative purposes, some slides developed with Vector Red were also photographed under epifluorescence with a Nikon B excitation filter (bandpass, 460–485 nm). With this filter, macrophage autofluorescence appeared yellow-green, allowing a clear distinction from positive staining (red). Emission intensity from Texas red under these conditions of excitation was expected to be only ~13% of that for excitation at 596 nm; nonetheless, the signal was brighter than by brightfield.

Some specimens stained with preimmune IgG, and this reactivity was not blocked by peptide NO54. Staining with these characteristics was deemed nonspecific. In an attempt to circumvent nonspecific reactivity, we tried to affinity-purify NO53 on NO54 coupled to solid supports. However, the affinity of NO53 for NO54 was so high that no antibody could be eluted (J. Calaycay

and J.R. Weidner, unpublished observations). We therefore capitalized on the high affinity of specific NO53-antigen interaction by applying an acid wash in situ to disrupt nonspecific interactions, as follows. Slides from samples that showed staining by preimmune serum as developed in Vector Blue were decolorized in xylene for 20 min. Slides developed in Vector Red could not be decolorized; when available, fresh slides from such patients were submitted to the same procedures. The air-dried slides were blocked in 5% BSA, 100 μg/ml goat γ-globulin in TBS (BSA/ggg) for 60 min, then incubated in preimmune IgG, NO53, or NO53 plus NO54 as above, the reagents being diluted in BSA/ggg. The samples were then incubated for 30 min at RT in 50 mM glycine buffer, pH 2.5, washed twice in TBS, and developed with the second antibody and avidin-alkaline phosphatase ABC reagents as above, using either Vector Red or Vector Blue as substrates. As controls, slides that had been processed according to the standard procedure and showed only specific staining were subjected to the alternative procedure; they gave the same percent positive cells as before. Slides that showed nonspecific staining according to the standard procedure showed no staining after the alternate procedure.

Other slides were stained by standard procedures using monoclonal antibodies to CD3, CD4, or CD8 (Coulter Corp., Hialeah, FL).

To detect enzymatic activity, companion slides were incubated in 50 mM Tris, pH 8, with 0.3 mM nitroblue tetrazolium (Sigma), 1 mM NADPH, 0.2% Triton X-100 for 30 min at 37°C, washed, mounted in glycerol, and scored as above under brightfield illumination.

RT-PCR. From 16 culture-positive tuberculosis patients and 2 healthy volunteers each from Rio de Janeiro and New York, bronchoalveolar lavage cells were available that had been stored in RNA Stat-60 (Tel-Test B, Inc., Friendswood, TX) at −70°C. Human 293 cells transfected with NOS2 cDNA (42) or vector alone constituted positive and negative controls. Total RNA (1 μg) was reverse-transcribed using oligo-d$(T)_{16}$ and PCR performed by standard methods (GeneAmp system 9600; Perkin Elmer). First-round PCR was carried out with 20% of the cDNA using oligonucleotide with primers from exon 1 (5′-CAC CTT TGA TGA GGG GAC-3′) and exon 4 (5′-GCA TCC AGC TTG ACC AG-3′) of human NOS2 (44). For nested PCR, 4% of the first-round product was amplified with primers from exon 1 (5′-ATG AGG GGA CTG GGC AGT TC-3′) and exon 4 (5′-GCT TGA CCA GCG ATT CTG GAG-3′). After electrophoresis in 1.5% agarose, reaction products (10 μl) were transferred to nylon membranes (Nytran-Plus; Schleicher & Schuell, Keene, NH) and autoradiographed after probing with ^{32}P-labeled oligonucleotide derived from a sequence of NOS2 internal to the primers (5′-CCT TAC CCC GGG GAG GCA GTG CAG CCA G′3′).

Cloning and Sequencing of NOS2 RT-PCR Products. Nested RT-PCR products from 3 patients and 1 healthy control were excised from the electrophoretic gel and purified (Qiagen, Chatworth, CA). The DNA was ligated into the EcoRV site of the pT7Blue vector containing the lacZ α-peptide (Novagen, Madison, WI) and transformed into *E. coli* NovaBlue competent cells, expressing the LacZ-ω fragment. Transformed cells were grown on LB agar containing ampicillin, X-gal and isopropyl β-D-thiogalactopyranoside. Five clones from each transformant were selected and the presence of the cloned DNA fragment confirmed by restriction with BamHI and SalI. At least one clone from each transformant was sequenced (373 DBA sequencer; Applied Biosystems, Foster City, CA).

Results

Characterization of Anti-NOS2 Antibody. Antibody NO53 was raised against a synthetic peptide that contains the extreme COOH-terminal heptamer of human NOS2, a sequence not represented elsewhere in the currently available protein data base. In particular, this sequence is absent from human NOS1, human NOS3, and mouse NOS2. Antibody NO53 reacted with peptide NO54 in ELISA with a titer of 1:400,000 (not shown). When 293 cells were transfected with human NOS2 cDNA, NO53 immunoblotted a single polypeptide species whose apparent molecular mass (~133 kD) corresponded to that of the NOS2 monomer. Immunoreactivity was abolished by a molar excess of peptide NO54, and was absent using preimmune IgG from the same rabbit (Fig. 1). Immunocytochemistry confirmed the reactivity of NO53 with 293 cells transfected with human NOS2, and not those transfected with vector alone. That the epitope recognized by NO53 was represented in the COOH terminus of human NOS2 was demonstrated by the ability of NO53 to immunoblot 293 cells transfected with a chimeric construct in which the COOH terminus of mouse NOS2 was replaced with the corresponding region from human NOS2 (Fig. 1). In contrast, NO53 could not react with full-length mouse NOS2. Expression of the latter was confirmed by its reactivity with anti-mouse NOS2 antibody (Fig. 1).

When 293 cells were transfected with human NOS1 cDNA, they remained NO53-negative by immunoblot and immunofluorescence. Similarly, lysates of human umbilical vein endothelial cells (HUVEC) were not immunoblotted by NO53. NOS1-transfected 293 cells were immunoblot-positive with anti-NOS1 antibody, and HUVEC were immunoblot-positive with anti-NOS3 antibody, proving the presence of the isoforms with which NO53 did not react (Fig. 1).

Because Freund's complete adjuvant was used to raise NO53, it was necessary to exclude the possibility that the antibody might also react with mycobacterial antigens. NO53 did not immunoblot a lysate prepared from 10^9 bacilli of *M. tuberculosis* H37Ra (the strain used in Freund's complete adjuvant) (Fig. 1). Moreover, when human monocytes were productively infected with *M. tuberculosis* H37Ra, they remained nonreactive with NO53 (Almeida, G., H. Cho, C. Nathan and J. Ho, unpublished observations).

Thus, NO53 appeared to react with a COOH-terminal epitope of human NOS2, but not with mouse NOS2, human NOS1, human NOS3, any other proteins in a human epithelial cell line, other proteins in human macrophages, or mycobacterial antigens expressed in broth culture or in macrophages.

Immunoreactivity of Macrophages from Patients with Tuberculosis. NO53 stained macrophages from the lungs of 11 of 11 patients presenting with untreated, culture-positive tuberculosis (8 males, 3 females; mean age 39 yr, range 20–74 yr) (Figs. 2, 3). For all 11 tuberculosis patients, NO53 stained 49 ± 20% (mean ± SD) of their bronchoalveolar lavage cells (range 25–80%). Lymphocytes comprised 14 ±

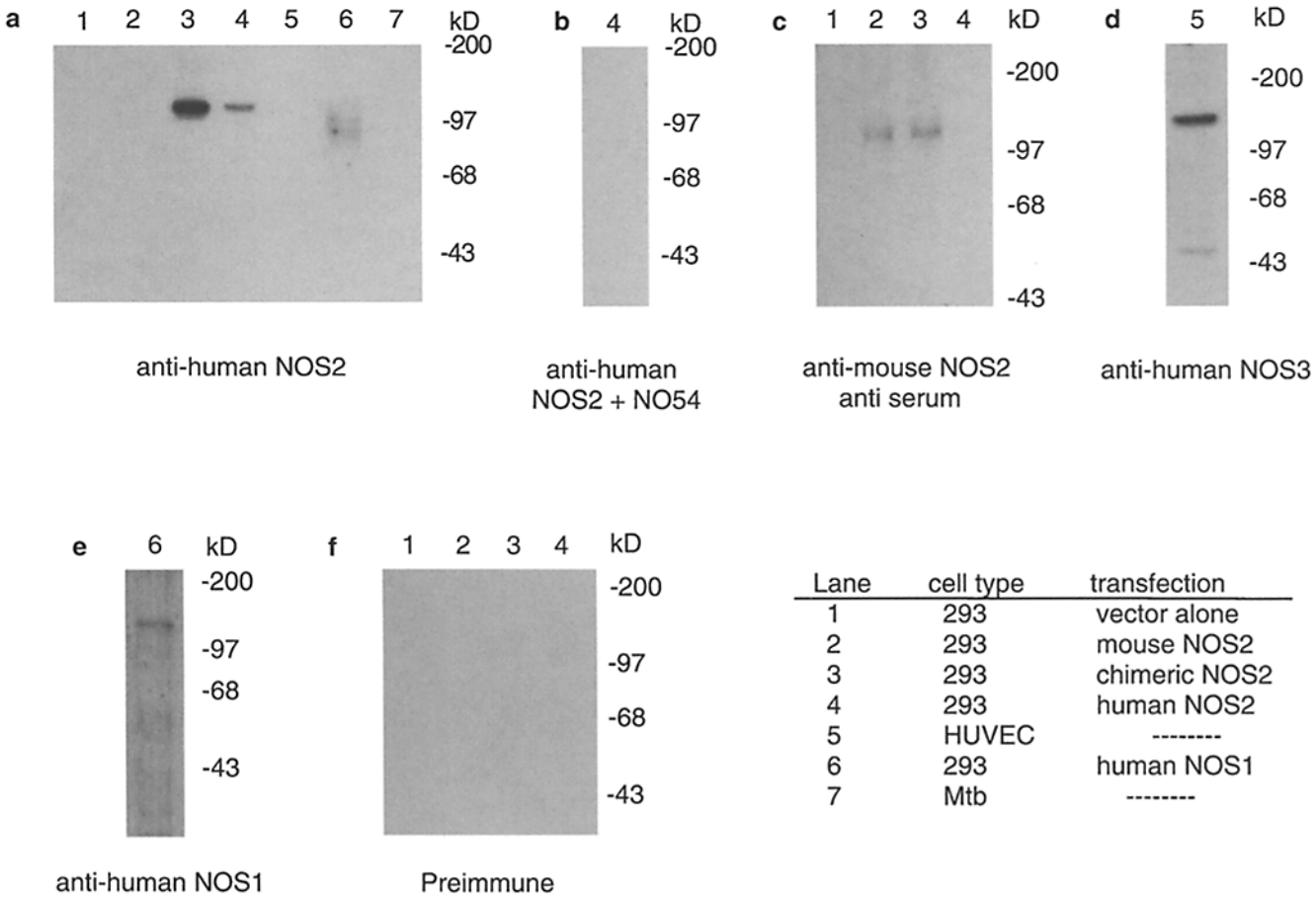

Figure 1. Characterization of antibody NO53 by immunoblot. As summarized in the inset, whole cell lysates of 293 cells (30 μg protein/lane) transfected with vector alone (lanes *1*), with mouse NOS2 (lanes *2*), with chimeric NOS2 consisting of mouse NOS2 bearing the COOH-terminal 24 residues from human NOS2 (lanes *3*), with human NOS2 (lanes *4*); or with human NOS1 (lanes *6*); HUVEC (7×10^5 cells/lane; lanes *5*); or *M. tuberculosis* (10^8 organisms/lane; lane *7*) were electrophoresed in 7.5% SDS-PAGE, then transferred to Immobilon membranes. The membranes were blotted with (*a*) NO53 IgG, (*b*) NO53 + NO54 blocking peptide, (*c*) rabbit anti–mouse holo-NOS2 IgG, (*d*) rabbit IgG against human NOS3-derived peptide; (*e*) rabbit IgG against human NOS1-derived peptide, or (*f*) preimmune IgG from the rabbit in which NO53 was raised. The faint smudge in *a*, lane *6* does not correspond to the molecular mass of human NOS1 monomer (160 kD).

3% of the total cell population as assessed by expression of CD3 or the combination of CD4 + CD8 (whichever was greater), whereas 10 ± 4% of the cells were polymorphonuclear leukocytes and 0.9 ± 0.3% were scored morphologically as epithelial cells. The rest of the lavage cells (75 ± 5%) and 100 ± 0% of the NO53-positive cells had morphologic characteristics of macrophages (Figs. 2, 3). Thus, on average, 65% of the macrophages were NOS2-positive. Results were no higher for the subset of subjects who smoked tobacco (39 ± 15% of lavage cells; $n = 6$) than those who did not (60 ± 15% of lavage cells; $n = 4$); the smoking status of one subject was unknown.

In lavage specimens from 5 nonsmoking normal donors, 10 ± 5% of the cells (almost all of which were macrophages) stained with NO53 (range 2–16%) (Fig. 2). In two clinically normal donors who smoked, 24 and 62% of the cells were NO53-positive.

Diaphorase Cytochemistry. Only small numbers of cells could be diverted from the diagnostic studies for which the bronchoscopies were carried out, and these were promptly fixed for transport. These constraints precluded any direct measurement of NO production by explanted cells. However, mouse NOS2 in fixed sections can reduce tetrazolium salts (45, 46). Consistent with this, 293 cells became diaphorase-positive after transfection with NOS2 cDNA (Fig. 4). With live cells, diaphorase activity can also reflect superoxide produced by the respiratory burst oxidase (47), an enzyme system present in human pulmonary alveolar macrophages (48). However, peritoneal macrophages that were activated in vivo and then collected and fixed were diaphorase-negative when they derived from NOS2-deficient mice, whereas those from NOS2-replete mice were diaphorase-positive (Fig. 4), even though the respiratory burst is normal in macrophages from the NOS2-deficient mice (4). Thus, as applied herein to fixed cells, the diaphorase assay strictly depended on expression of NOS2.

The proportion of tuberculosis patients' bronchoalveolar lavage cells that were positive by diaphorase cytochemistry (45 ± 27%, 9 subjects) (Fig. 3) closely matched the proportion positive for NO53 reactivity (49 ± 20%).

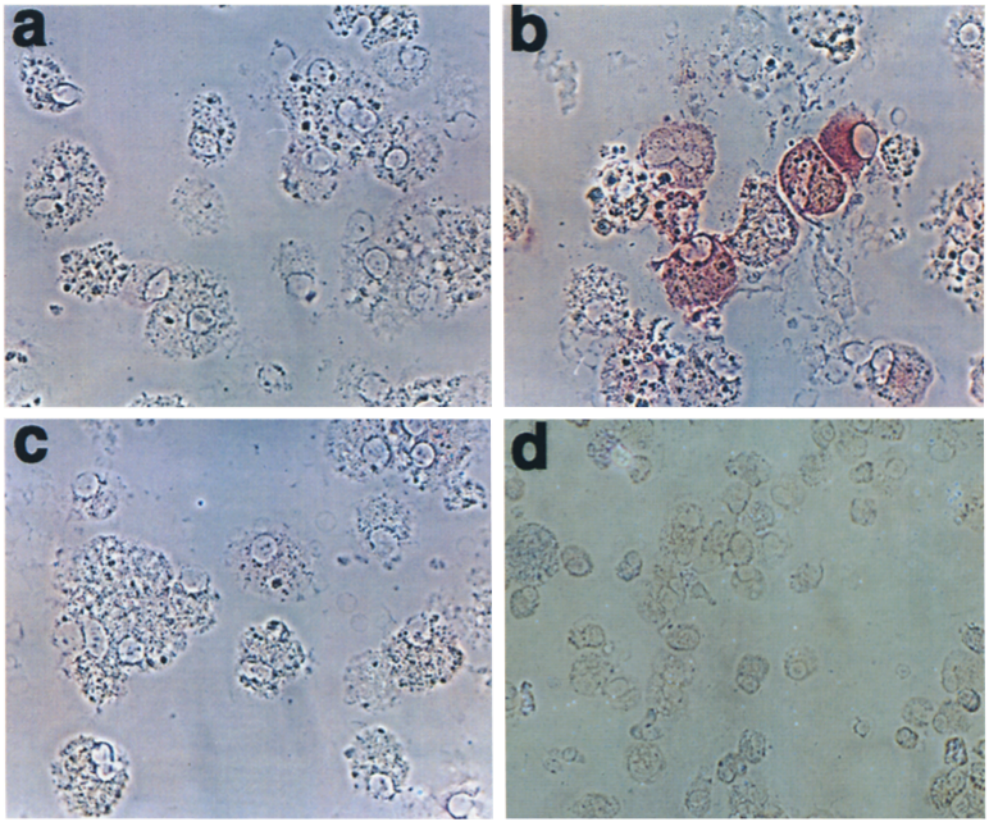

Figure 2. Immunocytochemical detection of NOS2 in pulmonary alveolar macrophages from a patient with tuberculosis but not in cells from a normal donor: brightfield studies. Cells were freshly isolated by bronchoalveolar lavage from (*a–c*) a patient with tuberculosis or (*d*) a non-smoking normal donor, and stained with (*a*) preimmune IgG or (*b–d*) NO53 IgG, with (*c*) or without (*b* and *d*) blocking peptide NO54. (*a–c*) Stained with Vector Red; original ×400. (*d*) Stained with Vector Blue; original ×200.

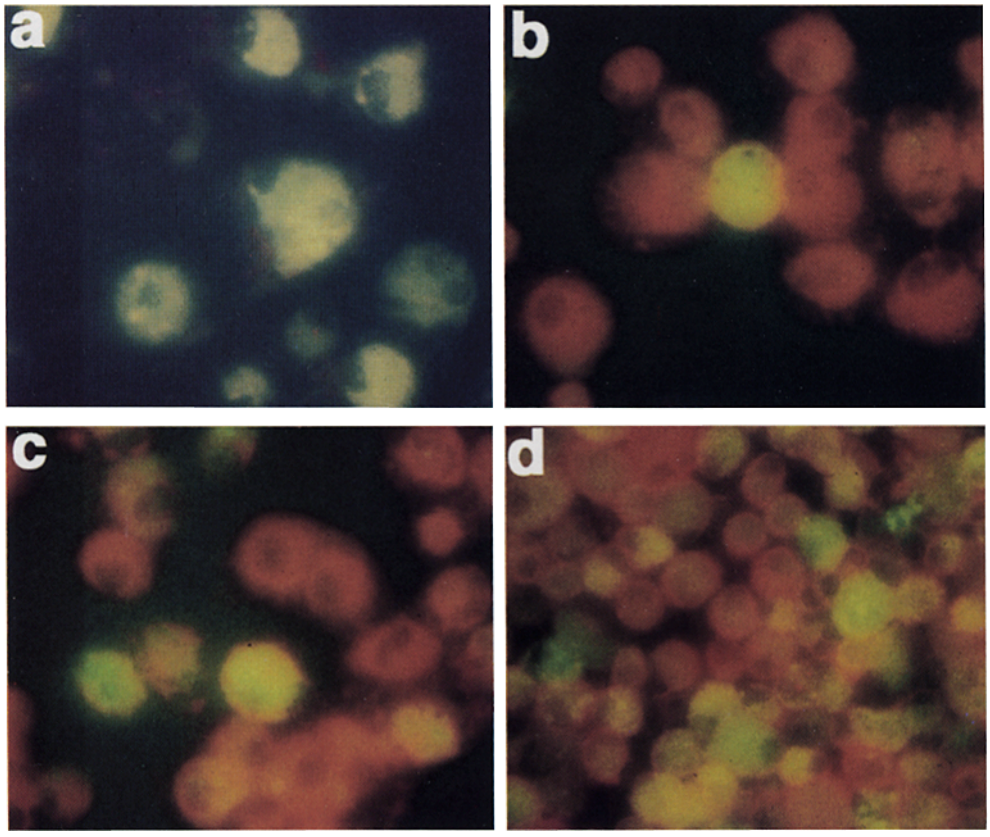

Figure 3. Immunocytochemical detection of NOS2 in pulmonary alveolar macrophages from three additional patients with tuberculosis: immunofluorescence. Cells were freshly isolated by bronchoalveolar lavage and stained with (*a*) preimmune IgG or (*b–d*) NO53 IgG. Results with NO53 IgG plus blocking peptide resembled those in (*a*) and are not shown. (*a–c*) Originals ×400; (*d*) original ×200. (*a* and *b*) Cells are from one patient; (*c* and *d*) two additional patients. Under a 460-485-nm bandpass excitation filter, positive cells fluoresce red and negative cells autofluoresce yellow-green.

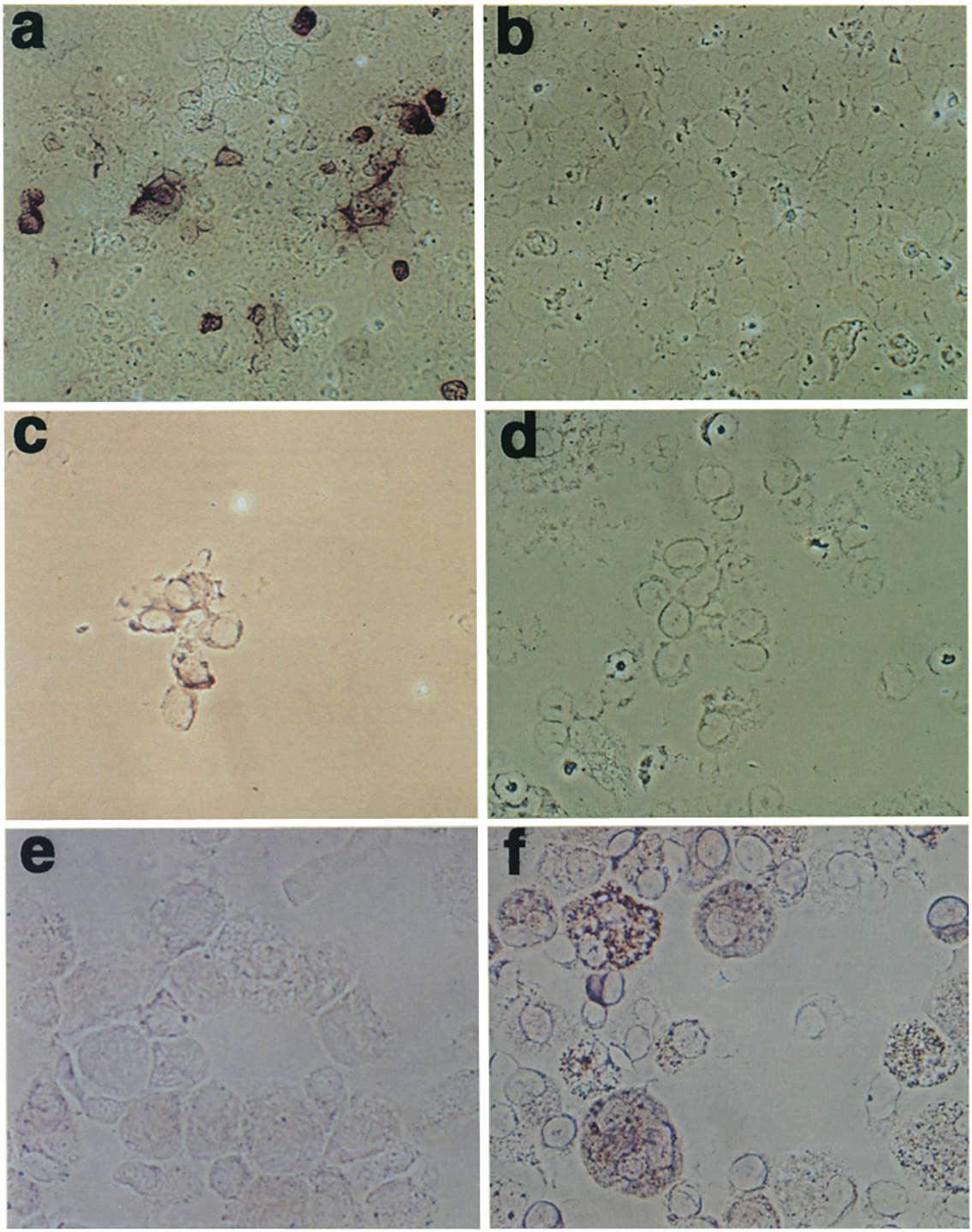

Figure 4. Diaphorase histochemistry. (*a* and *b*) 293 cells were transfected (*a*) with human NOS2 cDNA or (*b*) vector alone. Activated mouse peritoneal macrophages were collected from (*c*) wild-type or (*d*) NOS2-deficient mice. Bronchoalveolar lavage cells were collected from (*e*) a non-smoking, healthy volunteer and (*f*) a patient with tuberculosis. (*a–d*) Originals ×200; (*e* and *f*) originals ×400.

Detection of NOS2 mRNA. RT-PCR provided evidence for the presence of NOS2 independent of antibody. Of the 16 tuberculosis patients' samples tested, 12 supported the amplification from NOS2-specific primers of a product that hybridized with a NOS2-specific probe encoded between but not within the regions of the NOS2 gene used for designing the primers. Visualization of this product was evident after first-round PCR in 7 cases and required nested PCR in the remaining 5. The NOS2-hybridizing product was of the expected size (413 bp) in 9 of the 12 cases (Fig. 5, *A* and *B*). In the other 3 patient samples, the amplified product was larger (~500 bp; not shown). In none of the 4

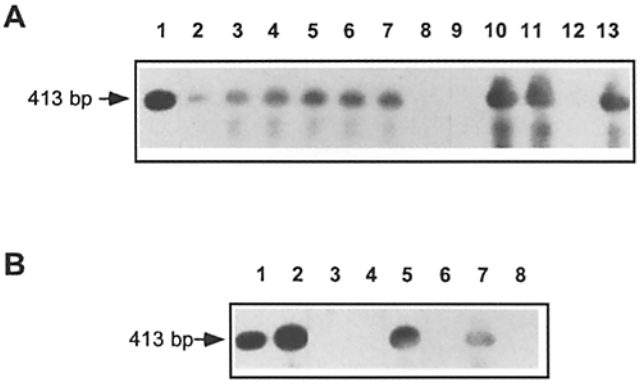

Figure 5. Detection of NOS2 mRNA. All PCR reactions illustrated were nested, and detection was by means of Southern hybridization to an internal NOS2-specfic probe. Positive control reactions illustrated were direct PCR amplification of NOS2 cDNA itself (*A*, lane *1*), and RT-PCR of 293 cells transfected with NOS2 cDNA (*A*, lane *2*; *B*, lane *1*). Negative control reactions illustrated were RT-PCR of human peripheral blood monocyte-derived macrophages (*B*, lane *8*), and RT-PCR in the absence of a template (*A*, lane *8*). Experimental results were from RT-PCR of bronchoalveolar lavage samples from 13 tuberculosis patients (*A*, lanes *3–7*, *9–13*; *B*, lanes *2–4*) and from 3 healthy volunteers (*B*, lanes *5–7*).

healthy volunteers' samples tested did first-round PCR yield a NOS2-specific product. However, nested PCR yielded a 413-bp, NOS2-hybridizing product in 2 samples from normal donors (Fig. 5 *B*) and a ~500 bp product in a third; the fourth remained negative by nested PCR. As a control, 293 cells were transfected with NOS2 cDNA; application of nested RT-PCR demonstrated the expected 413-bp product (Fig. 5 *A*, lane *2*; *B*, lane *1*) comigrating with that produced by direct PCR on NOS2 cDNA (Fig. 5 *A*, lane *1*).

Cloning and sequencing of amplification products of the 413-bp products from one patient and one normal donor confirmed their identity as NOS2. The larger amplificand was also cloned and sequenced from two patients. From the 5′ end, the first ~110 bp were identical to NOS2 exon 1 and its junction with intron 1. The 3′ end closely matched the exon 4 primer used in PCR, while the intervening portion was of indeterminate origin. These larger amplificands were considered artefacts, and samples yielding them were scored as negative.

Correlation of Results from Three Assays for NOS2. Samples from 12 tuberculosis patients and 4 healthy volunteers were sufficient for testing by 2 or more of the assays for NOS2 (immunocytochemistry, diaphorase cytochemistry, and RT-PCR). For the patients, 2 of 2 samples were positive by all 3 assays, 8 of 10 by each of 2 assays, and the remaining 2 by 1 of 2 assays. In contrast, samples from none of the 4 healthy volunteers tested by 2 assays were positive by both.

Discussion

Many studies of NOS2 expression in human cells have been clouded by lack of evidence for the specificity of the antibodies used or by their subsequently revealed lack of specificity. For example, a mAb reactive with mouse NOS2 and widely used as an anti-human NOS2 reagent was recently shown to bind human NOS1 (49). To our knowledge, the present report provides the first documentation of the monospecificity of an antibody against human NOS2, and the first evidence for expression of NOS2 protein in human mononuclear phagocytes using such an antibody.

Inducible NOS was detected in a mean of 65% of the pulmonary alveolar macrophages of 100% of 11 patients with newly diagnosed, untreated pulmonary tuberculosis. In contrast, in 5 normal subjects, NOS2-positive alveolar cells averaged 10% of the bronchoalveolar lavage population. In two clinically normal smokers, NOS2-positive cells were much more frequent (mean 43%). Likewise, extensive NOS2 reactivity was evident in alveolar macrophages from patients with a variety of other pulmonary inflammatory disorders, such as nontuberculous pneumonia (52 ± 9% of alveolar lavage cells; $n = 5$), cancer (24 ± 24%; $n = 2$), sarcoidosis (20%; $n = 1$) (Bonecini-Almeida, M.G., S. Nicholson, J.R. Lapa e Silva, C. Nathan, and J.L. Ho, unpublished observations), and nocardiosis (90%; $n = 1$; Cho, H., W. Rom, and C. Nathan, unpublished observations). Smoking cannot account for all of the results in patients with tuberculosis, since at least 4 of the 11 did not smoke, and the NO53-positivity of macrophages in this subset was if anything more extensive than among the smokers. The substantially greater degree of NOS2 expression in alveolar macrophages of tubercular compared to normal subjects may signify that NOS2 was induced in the patients' alveolar macrophages by cytokines and/or mycobacterial products (50).

First-round RT-PCR was positive for NOS2 with 7 samples from 16 tuberculosis patients but not with samples from the 4 normals. The latter results are consistent with a recent study in which first-round RT-PCR was negative for NOS2 when applied to pulmonary alveolar macrophages from 4 normal subjects (51). In the present work, however, nested PCR with Southern blot detection was positive in 2 of 4 normal subjects' samples, consistent with the presence of a few NOS2-positive cells by immunocytochemistry. The difference between the present work and the study of Guo et al. (51) with respect to an occasional minor representation versus a complete lack of NOS2-positive macrophages in normal subjects might be attributable to the presence of subclinical pulmonary inflammation in some of our volunteers and/or to differences in assay sensitivity.

Circumstances have been described with human (21) and mouse (52) macrophages in which NOS2 protein was detectable but little or no NO was generated. To test for enzymatic activity in fixed preparations of NOS2-positive cells, we used diaphorase cytochemistry. Under the conditions tested, expression of NOS2 was strictly required for a positive reaction. The proportion of diaphorase-positive pulmonary lavage cells from tuberculous patients matched that of NOS2-positive cells. There is no precedent for expression of a NOS that preserves diaphorase activity but has lost the ability to produce NO. Thus, the expression of di-

aphorase activity is strong evidence for the presence of the high-output NO pathway before fixation.

When present in human macrophages in vitro, NOS2 appears to contribute to their antimicrobial activity against *Mycobacterium avium* (16), *Trypanosoma cruzi* (18), and *Leishmania major* (32). While there are apparently no such reports for *M. tuberculosis*, human macrophages do not appear to have been infected in vitro with the latter organism under conditions in which they abundantly expressed NOS2, and thus the experimental conditions seem not to have adequately reproduced the milieu of the infected lung. Given that NOS activity appears to be essential for the control of tuberculous infection in mice (6–8), the present findings suggest that NOS2 may constitute an element of the antimycobacterial armamentarium of human macrophages in vivo.

These findings bring new questions to the fore. What host and mycobacterial products augment or suppress the expression of NOS2 in tuberculosis? Are there any NOS2-positive cells in the lungs of patients with tuberculosis cells besides the macrophages recovered by lavage, such as epithelial cells (51, 53), and if so, do they contribute to control of the infection, considering that NOS2 can exert *trans*-cellular anti-viral effects (2, 54)? Are the consequences of NOS2 expression predominantly beneficial, such as tuberculostasis (55), or potentially harmful, such as lymphostasis (56), epithelial destruction (53, 57) or cavitation? What is the impact of endogenous NO on pulmonary ventilation and perfusion in tuberculosis (58)? Might persistent expression of NOS2 within the granuloma help maintain tubercular dormancy? Is reactivation of disease associated with a lapse in NOS2 expression by the host, or a loss of susceptibility to NO on the part of the pathogen?

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