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Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria

(peroxynitrite/host defense/phagocytosis/*in situ* hybridization/immunohistochemistry)

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ABSTRACT **Although the production of NO within rodent phagocytes is well-characterized, its production and function within human phagocytes are less clear. We show here that neutrophils within human buffy coat preparations stimulated with a mixture of interleukin 1, tumor necrosis factor α, and interferon γ contain inducible NO synthase mRNA and protein, one of the enzymes responsible for NO production. The protein colocalizes with myeloperoxidase within neutrophil primary granules. Using an inhibitor of NO synthase, L-*N*-monomethyl arginine, we show that activity of this enzyme is required for the formation of nitrotyrosine around phagocytosed bacteria, most likely through the intermediate production of peroxynitrite, a reaction product of NO and superoxide anions.**

NO is involved in a wide variety of biological processes from vasodilatation (1) to neurotransmission (2). It is produced by the oxidation of L-arginine by an enzyme, NO synthase (NOS), of which there are three isoforms encoded by distinct genes (3, 4). Two of these forms, neuronal and endothelial NOS, are generally constitutively present in the cells where they are expressed, but the third form, inducible NOS (iNOS), is usually only produced following stimulation of cells with agents such as proinflammatory cytokines and lipopolysaccharide (5, 6). In rodents, the induction of this enzyme in activated macrophages is responsible for the generation of NO and NO derived intermediates that contribute toward killing of a variety of microorganisms, such as *Leishmania major*, *Cryptococcus neoformans*, and *Toxoplasma gondii* (7), as well as providing antitumor cell cytotoxicity (8). In addition, rodent neutrophils have been shown to release NO, which is increased following stimulation with a variety of agents such as the cytokines interferon γ, tumor necrosis factor α, and lipopolysaccharide (9, 10); the source of this NO appears to be the iNOS isoform (11).

The production of NO in human neutrophils is more controversial. Some studies have shown low, but detectable, output of NO that can be further increased by activating agents such as fMet-Leu-Phe (12–14). One study (15) provided evidence for NO-dependent bacterial killing in human neutrophil fragments. However, other studies (16, 17) have failed to show NOS activity or the production of the NO metabolites nitrite and nitrate (NO_x) by human neutrophils, even after activation with proinflammatory cytokines. Given the high output of superoxide from activated neutrophils, much, if not all, NO produced may react with superoxide to form the highly reactive anion, peroxynitrite (18). This molecule is microbicidal and cytotoxic, potentially contributing to the endothelial cell injury in conditions such as adult respiratory distress syndrome (ARDS) (19). Since neutrophils are the main cells involved in the initial stages of an inflammatory response, an understanding of the range of mediators that they produce is important in elucidating the pathophysiology of a diverse range of diseases. We demonstrate here that human neutrophils contain iNOS mRNA and protein, and that the iNOS protein is localized to the primary granules. NO produced from this enzyme is responsible for the formation of nitrotyrosine around phagocytosed bacteria, most likely through the intermediate production of peroxynitrite.

METHODS

Cells. Fresh human blood (20 ml) was obtained from volunteers and mixed with 50 μl of preservative-free heparin (1000 units ml^{-1}; CP Pharmaceuticals, Wrexham, U.K.). Sterile dextran was added to a final concentration of 0.6% and the cells were allowed to settle at 37°C until the red cells had sedimented (≈30 min). The buffy coats were then removed, washed once in Hanks' buffered salt solution, and then resuspended in RPMI 1640 medium (ICN) containing 20% fetal calf serum (Tissue Culture Supplies, Botolph Claydon, U.K.), 2 mM glutamine, 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin at a concentration of 5×10^6 cells per ml^{-1}. They were incubated at 37°C in a humidified incubator containing 5% CO_2. Human cytokines (all from Genzyme) were added as follows: 0.5 ng ml^{-1} interleukin 1α, 10 ng ml^{-1} tumor necrosis factor α, 500 units ml^{-1} interferon γ.

Antibody. A synthetic peptide corresponding to amino acids 54 to 76 of the human iNOS sequence was prepared with a C-terminal cysteine (Department of Biochemistry, University of Nottingham, U.K.), blocked with citraconic anhydride, and coupled to keyhole limpet hemocyanin using m-maleimidobenzoyl-*n*-hydroxysuccinimide ester as described (20). The resulting conjugate was mixed 1:1 with Freund's complete adjuvant and injected intramuscularly into rabbits at a dose of 300 μg per animal. Further injections using Freund's incomplete adjuvant were repeated at 2-week intervals. The rabbits were killed 1 week after the third injection and the antiserum stored in aliquots at −70°C. Anti-iNOS activity was titrated by an ELISA using the peptide conjugated to BSA as the solid phase antigen. No reactivity to endothelial or neuronal NOS was seen with this antibody.

Western Blotting. Extracted proteins were separated using SDS/PAGE and electroblotted to nitrocellulose in transfer buffer (192 mM glycine/25 mM Tris, pH 8.3/20% methanol). Immunodetection was performed using the Enhanced Chemiluminescence system (Amersham) according to the manufacturer's instructions, with the primary antibody diluted 1:1000 and detected with horse radish peroxidase conjugated goat

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Abbreviations: NOS, NO synthase; iNOS, inducible NOS; NO_x, nitrate plus nitrite; NMMA, *N*-monomethyl arginine; ARDS, adult respiratory distress syndrome; ABC, avidin–biotinylated–peroxidase complex.

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anti-rabbit immunoglobulin (Jackson ImmunoResearch) at 1:2000 dilution.

Immunostaining. After air drying, smears were fixed by immersion for 20 min in a 1% solution of paraformaldehyde in phosphate-buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.3/0.15 M NaCl). Smears were then washed in PBS and stained by either the avidin–biotinylated–peroxidase complex (ABC) method or the indirect immunofluorescence method.

For the ABC method, endogenous peroxidase was blocked by immersing smears in 0.03% hydrogen peroxide in methanol for 20 min followed by washing in PBS. After blocking nonspecific binding by incubating in 3% normal goat serum for 20 min, smears were blotted to remove excess serum and incubated overnight with rabbit antiserum to human hepatocyte iNOS diluted 1:4000 or nitrotyrosine (a gift from Joseph Beckman, University of Alabama, Birmingham) diluted 1:100, in PBS containing 0.05% BSA (PBS-BSA) and 0.1% sodium azide. Smears were washed in PBS and then successively incubated with biotinylated goat antiserum to rabbit IgG (Vector Laboratories) diluted 1:100 in PBS-BSA and freshly prepared ABC reagent (Vectastain, Vector Laboratories) for 30 min and 60 min, respectively. Peroxidase activity was revealed using the hydrogen peroxide-diaminobenzidine method to give brown staining.

For the indirect immunofluorescence method, smears were incubated in PBS containing 0.2% Triton X-100 for 45 min to permeabilize cell membranes. After washing in PBS, nonspecific binding was blocked by incubating in 3% normal goat serum for 20 min. Smears were incubated overnight with antiserum to human hepatocyte iNOS (1:400) or nitrotyrosine (1:20) or a mouse monoclonal antibody to human myeloperoxidase (catalog no. M748, Dakopatts, Glostrup, Denmark) (1:40), all diluted in PBS-BSA, with 0.1% sodium azide. After washing in PBS, smears were incubated with fluorescein- or rhodamine-labeled goat antiserum to rabbit IgG or mouse IgG, respectively (Sigma), to produce green or red fluorescence. For double immunofluorescence staining (iNOS and myeloperoxidase), smears were first incubated with anti-iNOS and stained with fluorescein-labeled goat antiserum to rabbit IgG, followed by anti-myeloperoxidase and detection with rhodamine-labeled goat antiserum to mouse IgG.

Fluorescently labeled smears were visualized using an Olympus BX-60 microscope fitted with a mercury vapor lamp and appropriate filters. For colocalization, photomicrographic film was double exposed with images of fluorescein- and rhodamine-labeled smears.

***In Situ* Hybridization.** A fragment of the human iNOS cDNA corresponding to nucleotides 1919 to 2975 was amplified by reverse transcription-PCR and cloned into pUC18 (21). The plasmid was then digested with *Sph*I and *Sac*I and the 196-bp fragment corresponding to nucleotides 2061 to 2257 of the human iNOS sequence was purified and cloned into the vector pBS (Stratagene). Cleavage of this plasmid with *Hin*dIII or *Eco*RI allowed a linear template to be generated from which either the T3 promoter (*Eco*RI cut) or the T7 promoter (*Hin*dIII cut) can be used to generate antisense and sense mRNA, respectively. Radiolabeled probes were made using [α-^{33}P]CTP and T3 and T7 RNA polymerase for the antisense and sense riboprobes, respectively.

Smears were permeabilized first with 0.2% Triton X-100 in PBS for 15 min and then with a solution of proteinase K at 1 μg/ml in 0.1 M Tris (pH 8) and 50 mM EDTA for 5 min at 37°C. The reaction was stopped by washing smears in 0.1 M glycine in PBS followed by brief postfixation in 4% paraformaldehyde in PBS. Autoradiography background was minimized by immersion in a solution containing 0.255 acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min. Five nanograms of RNA probe ($\approx 5 \times 10^5$ cpm per section; 0.5 ng/μl) was diluted in buffer containing 50% (vol/vol) deionized formamide, 5× standard saline citrate (SSC), 10% (wt/vol) dextran sulfate, 5× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% BSA), 2% SDS, 100 μg denatured salmon sperm DNA per ml, 100 mM DTT. Preparations were covered with dimethyldichlorosilane-coated coverslips to prevent evaporation and incubated at 42°C for 16–24 hr in a humid chamber. After hybridization, the coverslips were removed and smears were subjected to high-stringency washing by immersion four times (10 min each) in 2× SCC, 0.1% SDS, 100 mM DTT at room temperature, twice (15 min each) in 0.1× SSC, 0.1% SDS, 100 mM DTT, and twice (15 min each) in 0.05× SSC, 0.1% SDS, 100 mM DTT at 42°C. Unhybridized, single-stranded RNA probe was removed by treatment with a solution of RNase A at 10 μg/ml in 2× SSC for 15 min at 37°C. The smears were dehydrated through graded concentrations of ethanol containing 0.3 M ammonium acetate, air-dried, dipped in Ilford K-5 emulsion, and stored at 4°C for 4 days. Autoradiographs were developed in Kodak D-19 developer and lightly counterstained with Harris' hematoxylin.

Bacterial Ingestion. After a 16-hr incubation in the presence or absence of cytokines, buffy coat preparations were washed once in Hanks' buffered salt solution and then resuspended in their original volume of growth medium. Treatment with L-*N*-monomethyl arginine (NMMA) or D-NMMA at 0.4 mM was given for 15 min at 37°C following which bacteria (*Staphylococcus aureus* clinical isolate or *Escherichia coli* O11:B4, respectively) were added at a 1:1 ratio with the cells and incubation at 37°C continued. At 15-min intervals thereafter, up to 1 hr after bacterial addition, aliquots of the cell suspension were removed and prepared for immunostaining with the antibody to nitrotyrosine as described above.

RESULTS

iNOS Is Present in Cytokine-Treated Human Neutrophils. An antibody to human iNOS was produced by immunizing rabbits with keyhole limpet hemocyanin conjugated to a synthetic peptide corresponding to amino acids 54 to 76 of the human iNOS sequence. Western blotting of human tissues showed that it recognized predominantly a protein of 130 kDa from atherosclerotic but not normal human aorta (Fig. 1). This is the predicted molecular weight of human iNOS (21), and correlates with the degree of specific immunostaining seen with this antibody in these tissues (22). Human buffy coat preparations were incubated for 16 hr either in medium alone (controls) or with a mixture of interleukin 1, interferon γ, and tumor necrosis factor α (cytokine-treated) and then immunostained with this antibody. Control staining with pre-immune

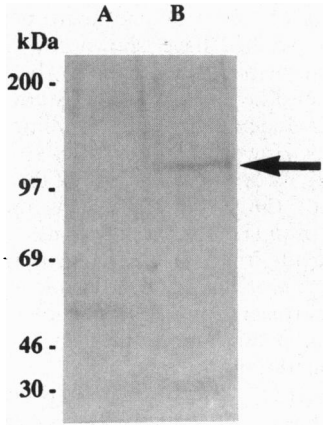

FIG. 1. Western blot of human tissues for iNOS. Proteins from normal (*A*) and atherosclerotic aortae (*B*) were probed with the antibody to iNOS. Molecular mass markers (kDa) are shown to the left of the gel. The arrow indicates the band corresponding to iNOS.

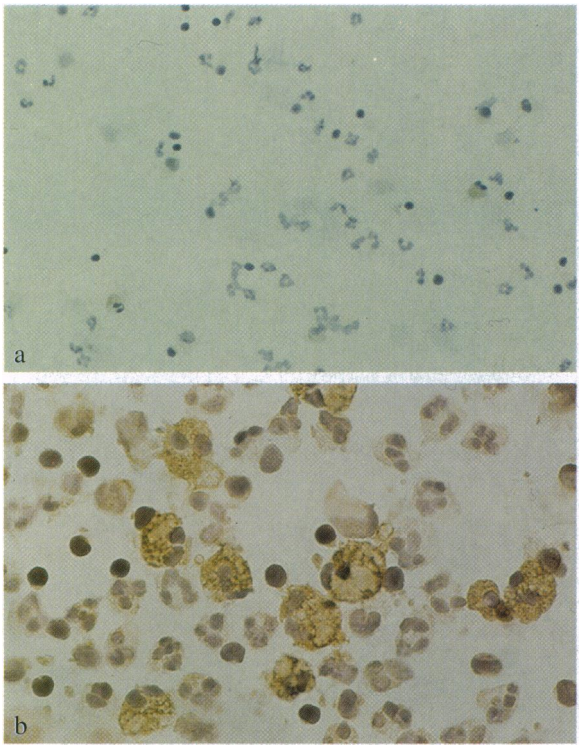

FIG. 2. iNOS staining of human buffy coat preparations. Cells were incubated for 16 hr in the absence (*a*) or presence of cytokines (*b*) before immunodetection with antibody to iNOS. The presence of the iNOS antibody was determined using the ABC method.

rabbit serum showed no immunostaining. With the anti-iNOS antibody, control buffy coats showed very little staining (Fig. 2*a*). However, in the cytokine-treated samples, there was strong immunostaining seen within the cytoplasm of leukocytes, almost exclusively (>90%) within neutrophils (Fig. 2*b*). Not all the neutrophils within the preparation stained; on average, ≈20% of the neutrophil population showed iNOS staining, particularly in those cells that showed homotypic aggregation following cytokine treatment. Control experiments in the presence of a 5-fold molar excess of the peptide that was used to raise the iNOS antibody removed all the iNOS staining (data not shown).

iNOS Colocalizes with Myeloperoxidase in Primary Granules. The pattern of iNOS staining within the neutrophil cytoplasm appeared granular. This was better demonstrated using a fluorescent detection system and confocal microscopy (Fig. 3), which demonstrates that the iNOS is localized to granules within the neutrophil cytoplasm. To determine which

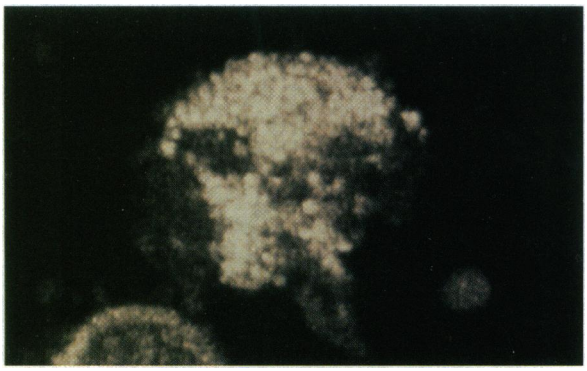

FIG. 3. Immunochemical staining of a cytokine-treated neutrophil with antibody to iNOS. iNOS antibody was detected by fluorescein-labeled anti-rabbit immunoglobulin and visualized using confocal microscopy.

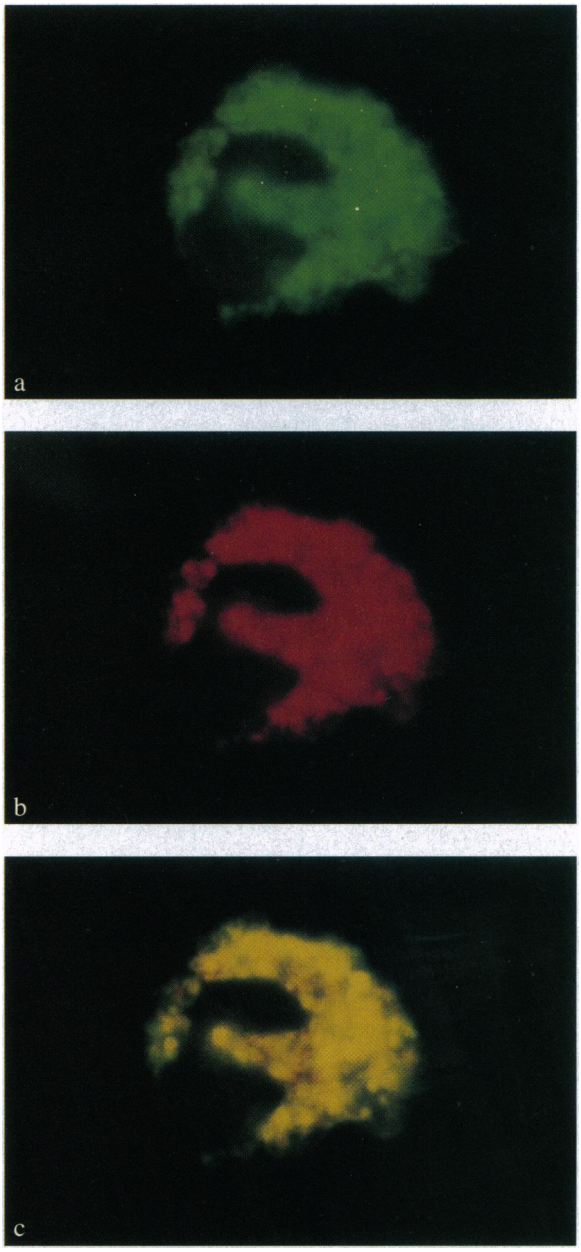

FIG. 4. Colocalization of iNOS with myeloperoxidase in primary granules. Cytokine-treated neutrophils were stained with antibody to iNOS and myeloperoxidase. Staining in one neutrophil is shown with iNOS showing as green staining (*a*) and myeloperoxidase as red (*b*). (*c*) Colocalization was determined by producing a double exposure of this preparation to detect both the myeloperoxidase and iNOS. Granules labeled with both antibodies stain yellow.

population of neutrophil granules contained iNOS, we performed dual antibody staining with the iNOS antibody, and antibodies to marker proteins contained within each of the three granule classes. Such double staining with anti-iNOS and antibodies against myeloperoxidase, a component of primary granules (23), showed colocalization of most of the iNOS protein with myeloperoxidase within the same granule population (Fig. 4). Thus, the majority of the iNOS protein is contained within neutrophil primary granules. No such colocalization was seen using anti-iNOS and antibodies directed against the secondary and tertiary granule populations (data not shown).

iNOS mRNA Is Present in Cytokine-Treated Neutrophils. To substantiate further the presence of iNOS within cytokine-treated neutrophils, we sought to demonstrate the existence of

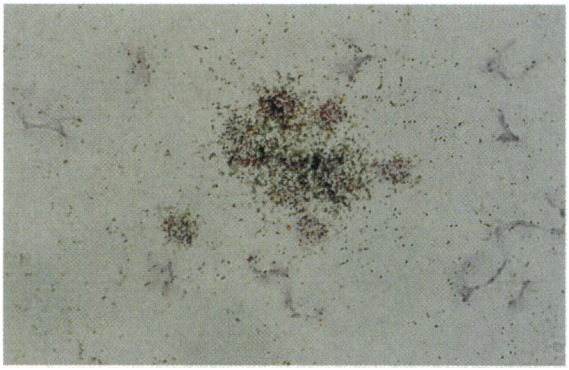

FIG. 5. *In situ* hybridization of a riboprobe to iNOS with a cytokine-treated buffy coat preparation.

iNOS mRNA within these cells. An antisense riboprobe containing a segment of the human iNOS cDNA sequence was hybridized to control and cytokine-treated buffy coat preparations. Autoradiography of these specimens showed little hybridization to control untreated cells. Following cytokine treatment, however, there was strong hybridization of the iNOS riboprobe to neutrophils within the cell population (Fig. 5). As with the antibody staining, about 20% of the neutrophils hybridized to the probe, particularly in those cells showing homotypic aggregation after cytokine stimulation. Control incubations with sense iNOS riboprobes and other unrelated sequences showed no hybridization (data not shown); the iNOS riboprobe does not cross-hybridize under the conditions used here to the mRNAs for the neuronal or endothelial isoforms of NOS.

Nitrotyrosine Formation Within Phagolysosomes. Coproduction of NO and superoxide within phagolysosomes may give rise to the formation of peroxynitrite. Peroxynitrite, but not NO or superoxide, can nitrate tyrosine residues of proteins to 3-nitrotyrosine, either directly or by a metal-ion catalyzed reaction (24, 25). Detection of nitrotyrosine thus serves as a marker of the generation of peroxynitrite. To detect whether peroxynitrite is generated within phagolysosomes, we exposed cytokine-treated buffy coat suspensions to bacteria. At various times thereafter, samples of the suspension were removed and nitrotyrosine content was assayed by immunohistochemistry, using a specific antibody directed against 3-nitrotyrosine. Under these conditions, neutrophils within the suspension rapidly phagocytose the added bacteria into phagolysosomes before they are completely lysed, usually together with the cell (Fig. 6*a*; the Giemsa stains the nucleus dark purple and the ingested bacteria dark blue). Nitrotyrosine staining of these suspensions showed the presence of nitrotyrosine on the surface of ingested bacteria that increased with time following addition of bacteria, reaching a maximum after 60 min incubation (Fig. 6 *c* and *d*). The staining was seen with both *S. aureus* (as shown in Fig. 6) and *Escherichia coli* (data not shown). No staining was seen in cytokine-treated cells that had not ingested bacteria, nor in unstimulated cells that were allowed to ingest bacteria (data not shown). To demonstrate that this nitrotyrosine production was dependent on the formation of NO, we repeated the experiment, but added the specific NO synthase inhibitor L-NMMA (0.4 mM) for 15 min before the addition of the bacteria and throughout the remainder of the incubation period. This treatment completely abolished the nitrotyrosine staining (Fig. 6*b*); no inhibition of this staining was seen using the same concentration of the inactive stereoisomer, D-NMMA (Fig. 6*c*).

DISCUSSION

The generation of superoxide by human neutrophils is thought to be an essential component of their microbicidal and cyto-

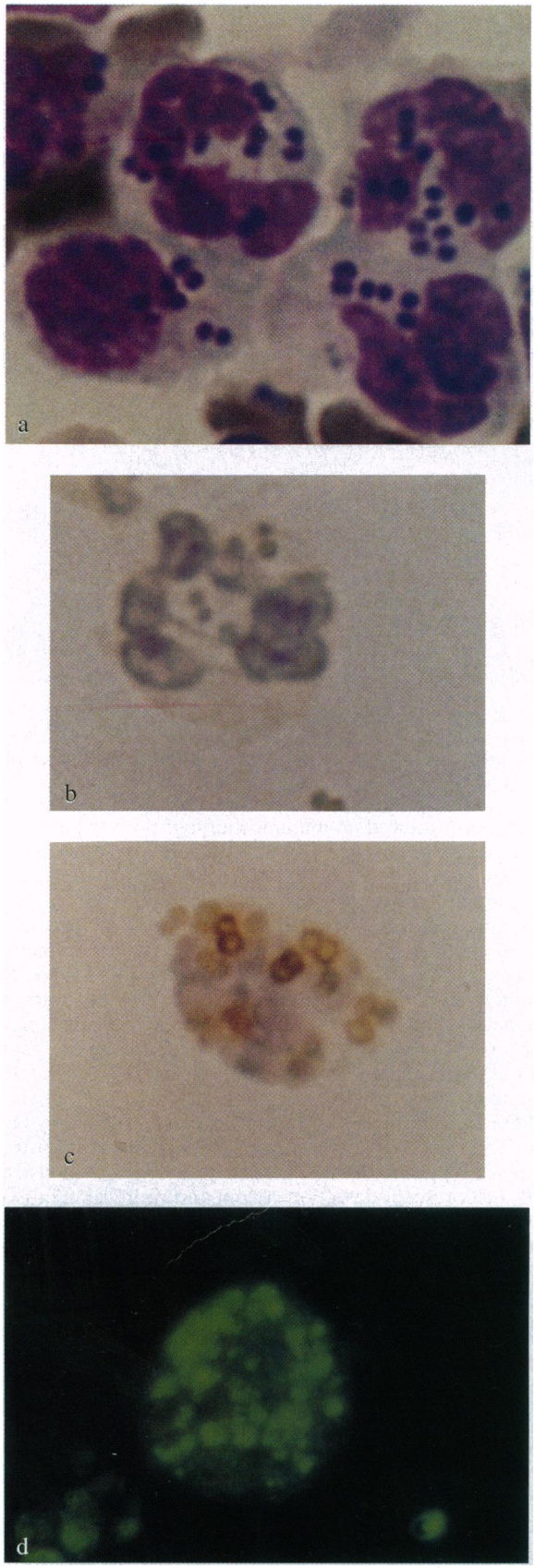

FIG. 6. Bacterial ingestion by buffy coat preparations. Cytokine-treated buffy coats were allowed to ingest *Staphylococcus aureus* for 60 min. Cells were stained with May–Grünewald Giemsa (*a*) or with antibody to nitrotyrosine (*b–d*). (*b* and *c*) The antibody to nitrotyrosine revealed by the ABC method, in the presence of 0.4 mM L-NMMA (*b*)
(Fig. 6 *legend continues on the opposite page.*)

toxic action (23). However, although superoxide can be directly toxic, some studies have shown a limited reactivity with biological molecules, suggesting that the secondary production of more reactive intermediates may be important (18, 26). We demonstrate here that human neutrophils contain inducible NO synthase mRNA and protein. This enzyme produces NO that on reacting with superoxide can form peroxynitrite, the most important mediator of protein tyrosine nitration. Earlier reports have described the production of NO and peroxynitrite by activated human neutrophils (27, 28), but this is the first report showing vacuolar localization of NOS and NO dependent production of nitrotyrosine around phagocytosed bacteria within human neutrophils.

Previous studies using human neutrophils have provided conflicting data on NO production, with some reports of basal NO output increased by agents such as fMet-Leu-Phe (12, 14), while other reports found no NOS activity or NO_x production even after cytokine treatment (16). While some of these reports may have missed significant NO production through a failure to measure nitrate levels in cell supernatants (17), direct measurement of cytoplasmic enzyme activity by the conversion of L-arginine to citrulline has also failed to show the presence of NOS activity in cytokine-treated neutrophils (16). We too were unable to demonstrate such enzymatic activity (data not shown). However, we were clearly able to show the presence of the mRNA for human iNOS within cytokine-activated neutrophils (Fig. 5). In addition, using an anti-peptide serum that recognizes a protein of the expected molecular weight in human tissues (Fig. 1), we were able to detect immunoreactive iNOS within primary granules of human neutrophils (Figs. 2–4). Evidence that this protein is functionally active is provided by the experiments using the antibody to nitrotyrosine. This showed that nitrotyrosine is produced by cytokine-activated neutrophils on ingestion of bacteria, and that this is prevented by the NOS specific inhibitor L-NMMA but not by its inactive stereoisomer, D-NMMA (Fig. 6). Importantly, only cells that had ingested bacteria showed evidence for nitrotyrosine production. Thus, one explanation for the inability to detect iNOS enzymatic activity in cell homogenates may be that the enzyme is only active following fusion of primary neutrophil granules with an ingested foreign particle. This brief production of NO also accounts for the difficulty in detecting nitrite or nitrate, stable metabolites of NO, in neutrophil supernatants. The mechanism for such activation of iNOS on phagocytosis is not known, but might involve posttranslational modification such as phosphorylation.

That nitrotyrosine is a marker of the production of peroxynitrite and that its detection is specific are supported by the following observations. Firstly, neither NO nor superoxide alone is capable of nitrating tyrosine residues (25, 29); one report showing apparent, although slow, direct NO production of nitrotyrosine used high concentrations of NO (100 μM), which also contained unquantified higher nitrogen oxides (30). Nitrogen dioxide can also nitrate tyrosine, but it is itself principally formed by the decomposition of peroxynitrite at low pH (31). Tyrosine nitration can also be produced by hydrogen peroxide and nitrite, but this proceeds through the production of peroxynitrite as an intermediate (32). The antibody to nitrotyrosine used in these experiments has been well-characterized. Its specificity is demonstrated by inhibition of binding by excess nitrotyrosine (19), but not by aminotyrosine or phosphotyrosine (33). In addition, it does not react with BSA that has been treated with activated oxygen or halogen compounds (31). Thus, we believe that our data support the conclusion that cytokine-treated human neutrophils are able to synthesize NO, which can react with superoxide to give the reactive compound peroxynitrite.

The production of peroxynitrite by human neutrophils may be important in a number of pathological processes. Peroxynitrite is known to be bactericidal, and thus may contribute to neutrophil killing mechanisms (34). Its generation within phagolysosomes is ideally placed to deliver a high concentration of the molecule directly to ingested microorganisms. In addition, NO generation within primary granules is particularly efficient at producing protein nitration, since myeloperoxidase (a major component of the primary granule) catalyses rapid peroxynitrite dependent tyrosine nitration (35, 36). Since the expression of iNOS within neutrophils requires a 16-hr incubation with cytokines and only about 20% of treated cells express the protein, it would be difficult to demonstrate NO-dependent killing in these cells, unless one could select out only those neutrophils that express iNOS; indeed, our preliminary experiments have not been able to show an NO-dependent component to bacterial killing by cytokine-treated neutrophils (data not shown). However, it is certainly a potential additional neutrophil killing mechanism. Its microbicidal action may be accounted for by a number of processes, including lipid peroxidation (37), oxidation of critical sulfhydryl groups, or nitration of important tyrosine residues (34). Why only about 20% of the stimulated neutrophils express iNOS is not clear. The cells showing iNOS expression were those that had undergone homotypic or self aggregation, a process that requires the participation of L-selectin, β-2 integrins, and other glycoproteins (38). It may be that only a subset of neutrophils are able to respond to the cytokine stimulus, such as those cells at a relatively earlier stage of their differentiation.

Neutrophils are involved in a large number of pathological processes. In many cases, they are agents of tissue damage, such as in the lung in ARDS. A number of studies have demonstrated the presence of nitrotyrosine, and hence peroxynitrite, in damaged lung from both animals and humans (19, 33). The current study thus provides a cellular source, neutrophils, for the peroxynitrite involved in this tissue damage—a cell that is known to be of central importance in the development of acute lung injury in ARDS (39). The importance of peroxynitrite in lung injury has considerable therapeutic implications. Inhaled NO has been used as a therapeutic agent in the treatment of ARDS, where it can reduce pulmonary hypertension and diminish intrapulmonary shunting (40). However, clearly it may also exacerbate the lung injury of ARDS through its reaction with superoxide to form peroxynitrite. The outcome of these beneficial and detrimental effects remains to be determined.

In conclusion, the current study demonstrates that cytokine-treated human neutrophils contain iNOS mRNA and protein that produces nitrotyrosine around ingested bacteria, most likely from the production of peroxynitrite, a highly reactive anion that may contribute to microbial killing and tissue damage. Further work will be required to elucidate the mechanisms responsible for the activation of the iNOS enzyme after phagocytosis and the molecular targets of the resulting peroxynitrite.

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or 0.4 mM D-NMMA (*c*), 15 minutes before the addition of bacteria and throughout the incubation period. (*d*) The nitrotyrosine staining revealed by indirect immunofluorescence.

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