Human Mononuclear Phagocyte Inducible Nitric Oxide Synthase (iNOS): Analysis of iNOS mRNA, iNOS Protein, Biopterin, and Nitric Oxide Production by Blood Monocytes and Peritoneal Macrophages

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Nitric oxide (NO) is produced by numerous different cell types, and it is an important regulator and mediator of many processes including smooth muscle relaxation, neurotransmission, and murine macrophage-mediated cytotoxicity for microbes and tumor cells. Although murine macrophages produce NO readily after activation, human monocytes and tissue macrophages have been reported to produce only low levels of NO in vitro. The purpose of this study was to determine if stimulated human mononuclear phagocytes produce inducible nitric oxide synthase (iNOS) mRNA, protein, and enzymatic activity. By reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, we show that human monocytes can be induced to express iNOS mRNA after treatment with lipopolysaccharide (LPS) and/or interferon-γ (IFN-γ). By immunofluorescence and immunoblot analyses, we show monocytes and peritoneal macrophages contain detectable levels of iNOS antigen after stimulations with cytokines in vitro. Control monocytes or those cultured with LPS and/or various cytokines have low levels of NOS functional activity as measured by the ability of cell extracts to convert L-arginine to L-citrulline, and they produce low levels of the NO catabolites nitrite and nitrate. Peritoneal macrophages have significantly enhanced nitrite/nitrate production and NOS activity after treatment with LPS and/or IFN-γ, whereas monocyte nitrite/nitrate production and NOS activity are not altered by the treatments. Monocytes cultured with various live or heat-killed bacteria, fungi, or human immunodeficiency virus (HIV)-1 do not produce high levels of nitrite/nitrate. Antibodies against transforming growth factor-β (TGF-β), a factor known to inhibit iNOS expression and NO production in mouse macrophages, do not enhance NO production in human monocytes or macrophages. Biopterin, an obligate cofactor of iNOS enzymatic activity, is undetectable in freshly isolated or cultured human monocytes and peritoneal macrophages. However, replenishment of intracellular levels of tetrahydrobiopterin by culture with the cell-permeable, nontoxic precursor sepiapterin does not enhance the abilities of the human mononuclear phagocytes to produce NO in vitro. Mixing experiments show no evidence of a functional NOS inhibitor in human mononuclear phagocytes. Thus, we demonstrate that human mononuclear phagocytes can produce iNOS mRNA and protein, and (despite this) their abilities to generate NO are very low.

This is a US government work. There are no restrictions on its use.

Nitric oxide (NO) is produced by numerous different cell types, and it is an important regulator and mediator of numerous processes including smooth muscle relaxation, neurotransmission, and murine macrophage-mediated cytotoxicity for microbes and tumor cells.[1-3] Murine macrophages express high levels of inducible nitric oxide synthase (iNOS) mRNA and protein after stimulation with various agents such as lipopolysaccharide (LPS) and cytokines (including interferon-γ [IFN-γ], interleukin-1 [IL-1], and tumor necrosis factor [TNF]), and they convert L-arginine to NO and L-citrulline. Although normal human hepatocytes, chondrocytes, and cells of the human colon tumor cell line DLD-1 can express iNOS mRNA and produce high levels of NO after treatment with various cytokines and LPS,[4-8] we

and others have reported that human mononuclear phagocytes (monocytes, peritoneal macrophages, and alveolar macrophages) produce no or little NO after various treatments in vitro.[9-15] Some investigators have noted that human mononuclear phagocytes can be induced to produce modest levels of NO in vitro.[15-26] The purpose of the present study was to determine if stimulated human mononuclear phagocytes produce iNOS mRNA, protein, and enzymatic activity. Results demonstrate that human monocytes and macrophages express iNOS mRNA and protein under certain circumstances in vitro, but they produce only low levels of NO in vitro. The basal levels of nitrite/nitrate production and NOS activity of monocytes are not changed by treatments with LPS ± IFN-γ, but those of peritoneal macrophages are significantly elevated by these treatments. Although human mononuclear phagocytes do not contain the cofactor tetrahydrobiopterin, increasing tetrahydrobiopterin levels does not enable them to produce high levels of NO.

MATERIALS AND METHODS

Cells and culture. Human blood monocytes and peritoneal macrophages were obtained from normal donors and from women undergoing laparoscopic investigations for infertility as described earlier.[27-29] Purity of the monocytes and macrophages after centrifugation and adherence with washing[27-29] was 92% to 98% for monocytes and 95% to 100% for macrophages. In peritoneal macrophages, there was no platelet contamination, whereas in monocyte preparations, there were one to five platelets per monocyte. In some instances, blood monocytes were purified by elutriation[30]; these cells had no platelets. All donors gave informed consent according to protocols approved by the Veterans Affairs (VA) and Duke Institutional Review Boards. No individuals were taking medications. The

peritoneal fluids were obtained midcycle (generally on days 12 to 22) of the menstrual cycles. Peritoneal fluids contaminated with blood were not used. Cells of the mouse macrophage cell line J774 are maintained in our laboratory in continuous culture.[31] DLD-1 cells are from the American Tissue Culture Collection (Baltimore, MD).[7] Most cultures were done in Dulbecco's modified Eagle medium (DMEM; without phenol red), and in selected experiments in RPMI-1640 (without phenol red) (Hyclone, Logan, UT). DMEM contained 438 μmol/L L-arginine, and RPMI-1640 contained 870 μmol/L L-arginine. In most experiments, the media contained 100 U/mL penicillin and 100 μg/mL streptomycin, but in selected experiments, the media contained no antibiotics. Fresh human serum was prepared in our laboratory from normal donors, and it was used for all human cells (either unheated or after heating for 30 minutes at 56°C), and 10% heated fetal bovine serum (Hyclone, Logan, UT) was used for the cultures with mouse J774 cells. The media and sera were of very low LPS content as determined by testing the chromogenic assay using Limulus amebocyte lysate (Whittaker Bioproducts, Walkersville, MD).

Measurements and assays. Nitrite and nitrate were measured spectrophotometrically as noted before.[31,32] Nitrate was converted to nitrite for measurement by use of nitrate reductase supplied by *Pseudomonas oleovorans* as noted earlier.[32] The conversion of L-arginine to L-citrulline was done as noted earlier using radiolabeled L-arginine.[31] Briefly, cell extracts from mouse J774 cells and human mononuclear phagocytes were done by three to five freeze-thaw cycles in distilled water containing 100 μmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 1 μg/mL chymostatin, and 5 μg/mL pepstatin A. Lysates were collected after centrifugation, and assayed for protein and NOS activity. The assay buffer contained 50 mmol/L HEPES (pH 7.5), 200 μmol/L nicotinamide adenine dinucleotide hydride (NADPH), 1 mmol/L dithiothreitol, 10 μmol/L flavin adenine dinucleotide (FAD), 100 μmol/L tetrahydrobiopterin, and 10 μmol/L L-arginine. We used L-arginine labeled with ^{14}C in the guanido position (product number NEC-453, New England Nuclear, Wilmington, DE). Thirty microliters of sample was used in a total reaction mixture of 50 microliters. Samples were done in duplicate or triplicate. Measurement of L-citrulline by lack of adherence to Dowex AG 50W-X8 cation exchange resin (from BioRad, Hercules, CA) correlated with determination of L-citrulline using high-performance liquid chromatography (HPLC).[13]

Northern analysis of mRNA was done as noted before,[31] using a cDNA probe specific for human iNOS [bases no. 1992 through 2741 with numbering according to the work of Sherman et al].[7] The RNAse protection assay was done as noted before.[33] We used a probe that was a 512 nucleotide antisense transcript of the human iNOS from the human colon adenocarcinoma cell line DLD-1 (bases 610 through 1121).[7] For the reverse transcriptase-polymerase chain reaction (RT-PCR) assays, polyadenylated RNA was extracted from the cells, reverse transcribed into DNA, and then amplified by PCR techniques using iNOS-specific primers designed from sequences of mouse macrophage iNOS[34] or human chondrocyte iNOS.[4] The sense primer was (position no. 1975) CAG GAG GA$\underline{T}$ GCC TTC CGC AGC TGG (no. 1998), and the antisense was the reverse complement of (no. 2701) A$\underline{T}$G ATG AGG TAG TCG AGG AGG G$\underline{T}$C (no. 2724), with numbering according to the work of Lyons et al.[34] The underlined nucleotides signify mouse-human mismatches. RT-PCR products were examined by Southern analysis using a probe specific for human iNOS [bases no. 1992 through 2741 with numbering according to the work of Sherman et al[7]]. cDNA sequencing was done as noted earlier.[35]

Tetrahydrobiopterin content was measured by HPLC.[36,37] Briefly, cell pellets and media samples were collected and stored at −70°C until analysis. One hundred microliters of 0.55 mol/L HClO₄ containing 1% iodine and 2% potassium iodide (KI) was added to cell pellets containing 1 to 2 million cells. Ten microliters 5.5 mol/L HClO₄ containing 10% iodine and 20% KI was added to 100 μL of media samples. After mixing thoroughly, all of the samples were allowed to stand 1 hour at room temperature. Ascorbic acid, 10 μL of a 10% solution, was then added, and the solutions were neutralized with 60 μL of 1 N NaOH. Triethanolamine HCl buffer, 100 μL of 0.1 mol/L, pH 8.0 containing 2 mmol/L EDTA, was added with 2 U of *Escherichia coli* alkaline phosphatase. The solutions were allowed to stand overnight in the dark to dephosphorylate completely. The dephosphorylated solutions (20 to 50 μL) were analyzed directly by high pressure liquid chromatography.[36,37] Dephosphorylation did not change biopterin or media neopterin. The limit of detection in this assay for cellular biopterin was 0.1 pmol/million cells, and 5 nmol/L for medium biopterin. Not all biopterin samples were verified to be tetrahydrobiopterin; however, when representative samples were analyzed for percent reduction via base oxidation, tetrahydrobiopterin was found to be ≥85% of the total biopterin detected.

Indirect immunofluorescence analysis of monocytes adherent to plastic in LabTek chambers (Naperville, IL) was done as noted earlier[38] using a mouse monoclonal antimouse macrophage iNOS from Transduction Laboratories (San Diego, CA). This is an IgG antibody against a 21-kD protein fragment corresponding to amino acids 961 through 1144 of mouse iNOS. It has 78% identity and 85% similarity to human hepatocyte, chondrocyte, and DLD-1 iNOS. Immunoblot analysis of cell extracts was done as described earlier,[39] using the enhanced chemiluminescence (ECL) technique according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Reagents. The following microbes were clinical isolates from the Veterans Affairs and Duke University Medical Centers Clinical Microbiology laboratories: *Listeria monocytogenes, Candida albicans, Staphylococcus epidermidis, Mycobacterium avium complex,* and *Mycobacterium tuberculosis.* In some instances, organisms were killed by autoclaving for 25 minutes. The monocytotropic virus human immunodeficiency virus-1-BaL (HIV-1-BaL) is maintained in our laboratory.[40] Purified, recombinant murine and human IFN-γ and TNF-α were from Genentech (South San Francisco, CA) and Becton Dickinson (Bedford, MA); granulocyte-macrophage colony-stimulating factor (GM-CSF), and stem cell factor were from Amgen (Thousand Oaks, CA); IL-2, IL-4, IL-6, IL-7, G-CSF, and neutralizing chicken antihuman transforming growth factor β1 (TGF-β1) antibody were from R&D Systems (Minneapolis, MN); IL-3 was from Genetics Institute (Cambridge, MA); 1α,25-dihydroxyvitamin D₃ was from Hoffman-LaRoche (Nutley, NJ);[14]C-(guanido)-L-arginine and gamma ^{32}P-CTP were from New England Nuclear (Boston, MA); N^G-monomethyl-L-arginine was from CalBiochem-Behring Corporation (San Diego, CA); tetrahydrobiopterin and sepiapterin were from Dr B. Schircks, Jona, Switzerland. *Taq* polymerase and other reagents used for the RT-PCR were from Perkin-Elmer (Branchburg, NJ); primers and probes for human and mouse glyceraldehyde phosphate dehydrogenase (GAPDH) were from CLONETECH Laboratories (Palo Alto, CA). All other reagents were from Sigma Chemical Co (St Louis, MO).

RESULTS

Cells of the mouse macrophage cell line J774 reproducibly generate large amounts of NO, as detected by measurement of the oxidation products nitrite and nitrate. In general, J774 cells generally simulate well normal mouse peritoneal macrophages in their responses to cytokines and LPS.[3,31] NO is oxidized by oxygen in culture medium to approximate equimolar amounts of nitrite and nitrate, and the combined measurement of nitrite and nitrate fully accounts for formed NO in oxygen containing media; these two chemicals appar-
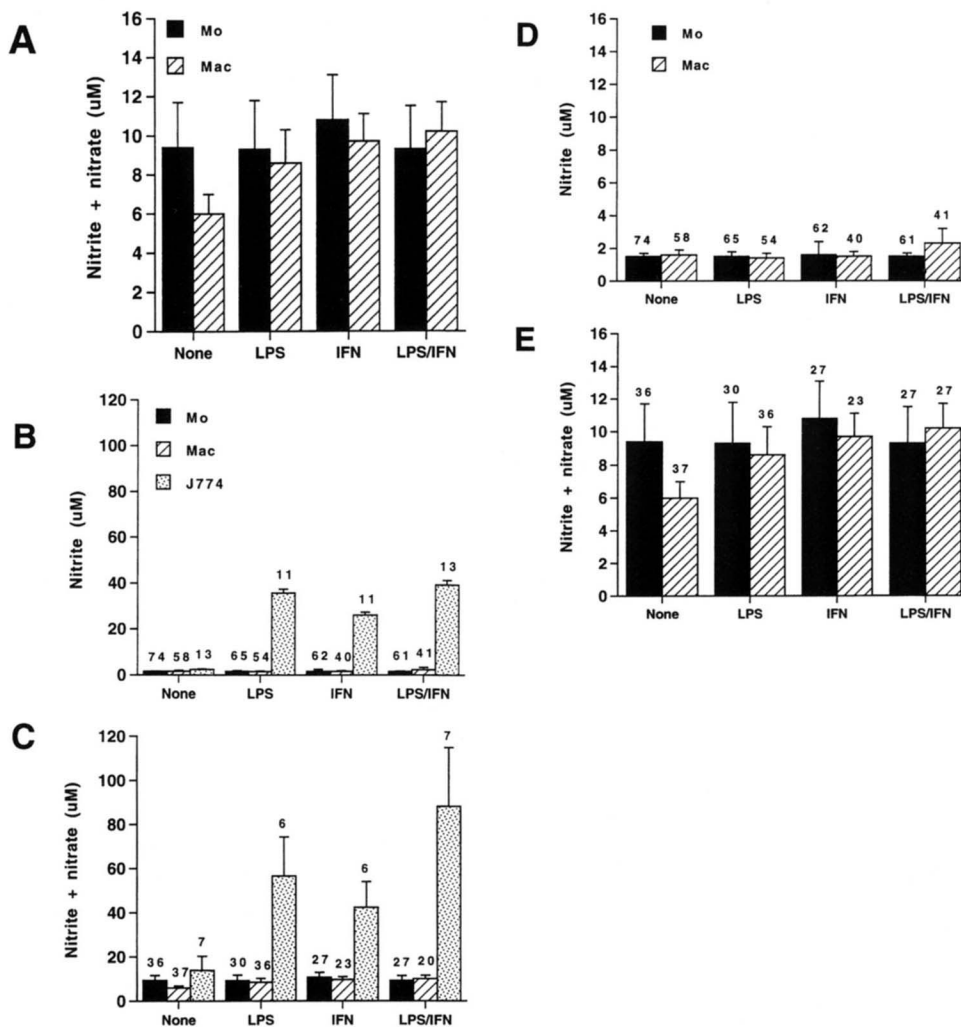
Fig 1. Nitrite and nitrate production by human and mouse mononuclear phagocytes. (A) Nitrite/nitrate production by human monocytes (Mo) and peritoneal macrophages (Mac) from two separate individuals. Results from one experiment using cells from two individuals is shown. Cells were cultured for 4 days with LPS (1 μg/mL) and/or IFN-γ (200 U/mL), and supernatant media were assayed. The error bars show the SEM from triplicate samples. (B and C) Nitrite or nitrite/nitrate production by human monocytes and peritoneal macrophages, and mouse J774 macrophages. The figures show aggregate results from several experiments, with the error bars showing the SEMs. The number of individual experiments from separate donors appear above each bar. For mouse J774 cells, levels of nitrite (B) and nitrite + nitrate (C) produced after treatment with LPS, IFN, or LPS/IFN were all statistically significantly different from that by control cells (at least $P < .01$ Mann-Whitney U test). See (D and E) for analysis of human monocytes and macrophages. (D and E) Nitrite or nitrite/nitrate production by human monocytes and peritoneal macrophages. Using data from (B and C), these figures more clearly show aggregate results from several experiments, with error bars showing the SEMs. The number of individual experiments from separate donors appear above each bar. For monocytes, there were no statistically significant differences in levels of nitrite or nitrite + nitrate after any treatment ($P > .1$ by Mann-Whitney U test). For peritoneal cells, levels of nitrite (D) and nitrite + nitrate (E) produced after treatment with IFN or LPS/IFN were statistically significantly different from that by control cells ($P < .01$ by Mann-Whitney U test).

ently accumulate as dead-end products.[41,42] NO formation by mouse macrophages is greatest after stimulation with cytokines (eg, IFN-γ) and LPS. Analysis of monocytes and peritoneal macrophages from an occasional human donor showed NO (nitrite/nitrate) production, which was enhanced by LPS ± IFN-γ (Fig 1A). However, cells from most people did not. When experiments from all individuals were analyzed in aggregate (Fig 1, B-E), it was apparent that human mononuclear phagocytes had very low production in comparison to that of mouse macrophages (Fig 1, B and C).

Figure 1, D and E show details of human monocyte and peritoneal macrophage nitrite/nitrate production. Results show that on averaging results of 23 to 74 separate samples from individual donors, nitrite and nitrite/nitrate production by monocytes was not modified by LPS ± IFN-γ (Fig 1, D and E). However, there was a statistically significant enhancement of nitrite/nitrate production by peritoneal macrophages by LPS ± IFN-γ (Fig 1E). Analysis of the extracts from cells for their ability to convert L-arginine to L-citrulline showed high level activity in mouse J774 cells, while
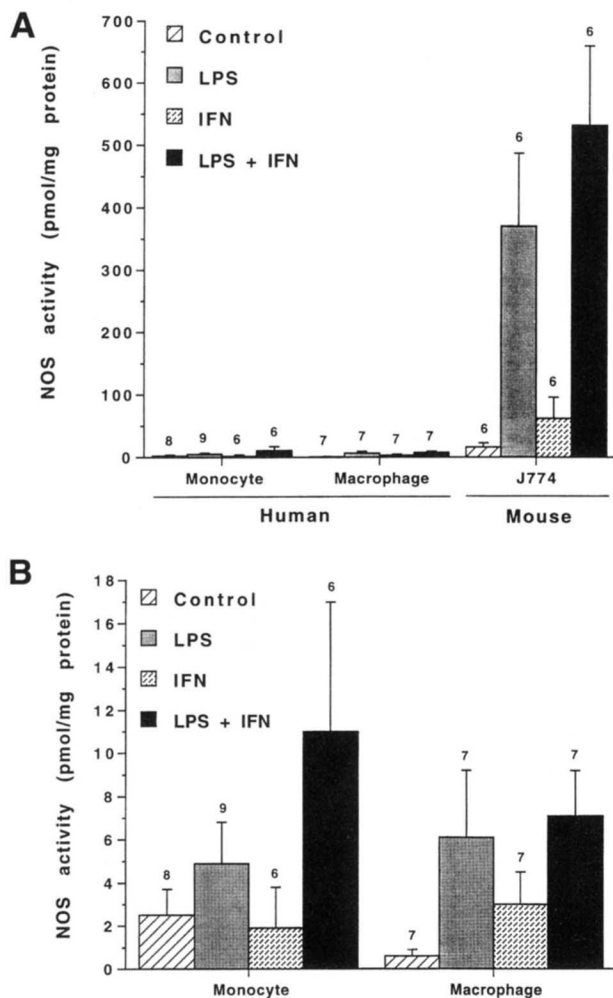
Fig 2. NOS activity in extracts of human monocytes, peritoneal macrophages, and mouse J774 macrophages. (A) NOS activity in human monocytes and peritoneal macrophages, and mouse J774 macrophages. Human monocytes and peritoneal macrophages were cultured for 3 days with 1 μg/mL LPS, 500 U/mL human IFN-γ, or combinations as indicated. Mouse J774 cells were cultured for 3 days with 200 ng/mL LPS, 200 U/mL murine IFN-γ, or LPS/IFN-γ as indicated. They were then assayed for NOS activity. NOS is expressed as picomoles of labeled L-arginine converted to L-citrulline/mg cell protein/h. The figures show aggregate results from several experiments, with the error bars showing the SEMs. The number of individual experiments from separate donors appears above each bar. (B) NOS activity in human monocytes and peritoneal macrophages. Using data from (A), these data more clearly show results from the human cells. For monocytes, none of the treatments caused statistically significant changes. For peritoneal macrophages, differences between control cells and those treated with LPS/IFN-γ are different ($P < .005$ by unpaired t-test). For J774 macrophages, differences between control cells are statistically different from cells treated with LPS or LPS/IFN-γ ($P < .05$ by Mann-Whitney U test).

human monocytes and peritoneal macrophages had much less activity (Fig 2, A and B). LPS and LPS/IFN-γ treatment caused increases in NOS activity; these increases were statistically significant in mouse J774 macrophages and human peritoneal macrophages, but not in human monocytes. In separate experiments (results not shown), we showed that

N^G-monomethyl-L-arginine (2000 μmol/L) inhibited more than 90% of the nitrite/nitrate production by mouse cells and more than 90% of the ability of mouse and human cells to convert L-arginine to L-citrulline.

This general low level NO production by human monocytes and human peritoneal macrophages was found under numerous different culture conditions, and after stimulation with numerous different agents. The following culture conditions did not render the monocytes or macrophages capable of producing high levels of nitrite-nitrate: culture in different amounts or different types of sera [3% to 50% (vol/vol) human, fetal bovine, and dog serum—heated or unheated]; culture for 3 to 30 days to allow maturation of the cells with subsequent addition of cytokines and LPS; culture in low oxygen atmosphere (3% oxygen-92% nitrogen with 5% CO_2, instead of 95% air with 5% CO_2), culture in medium with excess L-arginine (4,000 μmol/L L-arginine), or culture with 2 mmol/L NADPH. Comparable results with reference to nitrite/nitrate formation were obtained when using either DMEM or RPMI-1640, and when analyzing blood monocytes isolated by sequential ficoll-Hypaque-Percoll-adherence steps or by elutriation. Table 1 summarizes results of experiments using different known activators for macrophage functions. These agents have been noted to activate mouse or human mononuclear phagocytes for a variety of functions, including enhancement of NO production. None of the treatments and culture conditions induced the human mononuclear phagocytes to produce nitrite/nitrate levels above baseline (control) levels.

Denis[19] has noted that after incubation of monocytes with TNF and GM-CSF, *Mycobacterium avium intracellulare* (complex) causes these monocytes to produce NO. We tested *Listeria monocytogenes, Candida albicans, Staphylococcus epidermidis, Mycobacterium avium complex,* and *Mycobacterium tuberculosis* (living or heat-killed) for their abilities to enhance NO production by monocytes. The microbes (1 $\times 10^5$ to 1×10^6 per well) were incubated in antibiotic-free media alone or with monocytes for 3 days, and nitrite/nitrate was determined. The microbes alone did not produce nitrate/nitrate. Likewise, the microorganisms did not induce NO production by human monocytes or macrophages, even by those cultured previously (or at the same time) with different cytokines and LPS, including TNF and GM-CSF (data not shown). To determine if HIV-1 infection of human monocytes caused elaboration of nitrite/nitrate, we inoculated cells with HIV-1-BaL (multiplicity of infection of ≈ 0.01); this caused a productive infection with resultant high levels of viral reverse transcriptase in the supernatant medium and induction of monocyte polykaryons.[40] When analyzed at day 14 and 21 after inoculation (times of high level infection and polykaryon infection), supernatant media from the infected cells showed no nitrite/nitrate.

Tetrahydrobiopterin is a necessary cofactor for NOS activity.[43] Others have noted that while mouse macrophages can synthesize tetrahydrobiopterin, human monocytes synthesize none or very little.[44,45] This lack of tetrahydrobiopterin production is because of low levels of the second enzyme in the tetrahydrobiopterin biosynthetic pathway (pyruvoyl-tetrahydrobiopterin synthase)[46,47] in human cells. We found that

Table 1. List of Agents Tested for Their Abilities to Stimulate Human Monocyte Production of Nitrite/Nitrate

LPS & IFN-γ	IL-2	IL-6	D₃

LPS & IFN-γ
  LPS; IFN-γ; LPS + IFN-γ;
TNF
  TNF; LPS + TNF; IFN-γ +
  TNF; LPS + IFN-γ + TNF
GM-CSF
  GM-CSF; LPS + GM-CSF;
  IFN-γ + GM-CSF; LPS + IFN-γ
  + GM-CSF; TNF + GM-CSF;
  IFN-γ + TNF + GM-CSF; LPS
  + IFN-γ + TNF + GM-CSF
IL-1
  IL-1; LPS + IL-1; IFN-γ + IL-1;
  LPS + IFN-γ + IL-1; TNF + IL-
  1; LPS + TNF + IL-1; IFN-γ +
  TNF + IL-1; LPS + IFN-γ +
  TNF + IL-1

IL-2
  IL-2; LPS + IL-2; IFN-γ +
  IL-2; LPS + IFN-γ + IL-2;
  TNF + IL-2; LPS + TNF +
  IL-2; IFN-γ + TNF + IL-2;
  LPS + IFN-γ + TNF + IL-2
IL-4
  IL-4; LPS + IL-4; IFN-γ +
  IL-4; LPS + IFN-γ + IL-4
IL-7
  IL-7; LPS + IL-7; IFN-γ +
  IL-7; LSP + IFN-γ + IL-7;
  IL-4 + IL-7; LPS + IL-4 +
  IL-7; IFN-γ + IL-4 + IL-7;
  LPS + IFN-γ + IL-4 + IL-7

IL-6
  IL-6; LPS + IL-6; IFN-γ +
  IL-6; LPS + IFN-γ + IL-6;
  IL-1 + IL-6; LPS + IL-1 +
  IL-6; IFN-γ + IL-1 + IL-6;
  LPS + IFN-γ + IL-1 + IL-6;
  IL-1 + IL-4 + IL-6; LPS +
  IL-1 + IL-4 + IL-6; TNF +
  IL-6; LPS + TNF + IL-6;
  IFN-γ + TNF + IL-4 + IL-6;
  LPS + IFN-γ + TNF + IL-4
  + IL-6; IFN-γ + TNF + IL-6;
  LPS + IFN-γ + TNF + IL-6;
  TNF + IL-1 + IL-6; LPS +
  TNF + IL-1 + IL-6; TNF +
  IL-1 + IL-4 + IL-6

D₃
  D₃; LPS + D₃; IFN-γ + D₃; LPS
  + IFN-γ + D₃
PMA
  PMA; LPS + PMA; IFN-γ +
  PMA; LPS + IFN-γ + PMA;
A23187
  A23187; LPS + A23187; IFN-γ
  + A23187; LPS + IFN-γ +
  A23187
ConA
  ConA; LPS + ConA; IFN-γ +
  ConA; LPS + IFN-γ + ConA
PHA
  PHA; LPS + PHA; IFN-γ +
  PHA; LPS + IFN-γ + PHA

None of the individuals agents or combinations of agents induced an increase in supernatant media nitrite/nitrate levels above control levels of 5 to 10 μmol/L. In all experiments except those with PMA, A23187, ConA, and PHA, cultures were done with and without 20 to 200 μmol/L sepiapterin (to assure adequate levels of tetrahydrobiopterin). All experiments were done at least two times; several were done >15 times with separate donors. The cells were cultured for 3 to 4 days, and supernatant media were analyzed for nitrite/nitrate content. All reagents were present throughout the culture period. The following doses were analyzed: LPS, 0.01 to 10 μg/mL; IFN-γ, 200 or 500 U/mL; GM-CSF, 500 U/mL; IL-1α, 200 U/mL; IL-2, 20 or 100 U/mL; IL-4, 200 or 500 U/mL; IL-6, 200 U/mL; IL-7, 10 or 100 ng/mL; 1,25 dihydroxyvitamin D₃ (D₃), 5, 25, or 125 nmol/L; PMA, 1, 100, or 200 nmol/L; A23187, 5 μmol/L; conA, 2 or 10 μg/mL; and PHA, 1 or 5 μg/mL.

Abbreviations: PMA, phorbol myristate acetate; conA, concanavalin; PHA, phytohemagglutinin.

freshly isolated human monocytes and peritoneal macrophages contained no measurable tetrahydrobiopterin (Fig 3). However, both cell types contained neopterin. Although the more differentiated peritoneal macrophages appeared to have higher levels than blood monocytes, the difference was not statistically significant. When monocytes were cultured in vitro with LPS or IFN-γ for 3 to 4 days, they still had no measurable tetrahydrobiopterin, but (as expected[48]), their
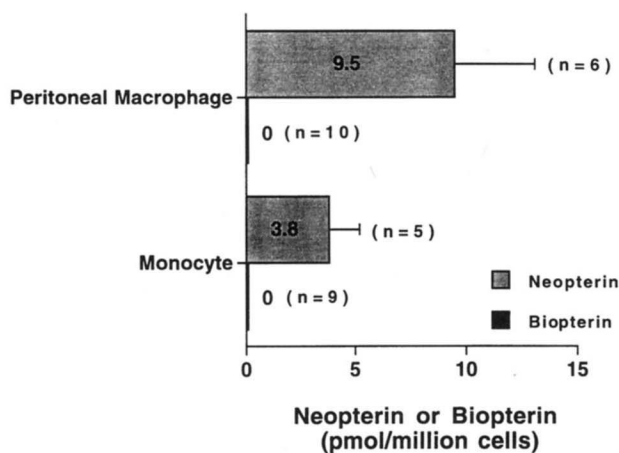

Fig 3. Neopterin and biopterin content in freshly-isolated human blood monocytes and peritoneal macrophages. Cells were isolated and then cell-associated neopterin and biopterin were measured. Biopterin was undetectable in all samples. The displayed values are the means, and the error bars show one SEM. The difference between the levels of neopterin in peritoneal macrophages and blood monocytes is not statistically significant ($P > .05$ by Mann-Whitney U test).

levels of neopterin increased after incubation with IFN-γ (Fig 4A). Addition of the nontoxic, cell-permeable tetrahydrobiopterin precursor sepiapterin to cultures increased cellular tetrahydrobiopterin levels, and decreased neopterin levels (Fig 4B). Comparable changes in tetrahydrobiopterin and neopterin levels have been observed by others.[49] The decreased neopterin production likely resulted from a combination of the inhibition of GTP cyclohydrolase I enzyme by pterins (both sepiapterin and tetrahydrobiopterin[46]), and by protein synthesis inhibition of guanosine triphosphate (GTP) cyclohydrolase I by this treatment.[49] Mouse J774 macrophages contained tetrahydrobiopterin (Fig 5A), while human monocytes did not (Fig 5B). Adding sepiapterin to the cultures increased tetrahydrobiopterin levels in both mouse cells and human mononuclear phagocytes (Fig 5, A and B). Despite this normalization of tetrahydrobiopterin levels, the human cells still did not produce high levels of nitrite/nitrate (Fig 5, C and D).

TGF-β reduces NO production by mouse macrophages (1) by enhancing iNOS mRNA degradation, (2) by reducing protein translation from iNOS mRNA, and (3) by enhancing iNOS protein degradation.[50] To determine if the human mononuclear phagocyte-produced TGF-β was inhibiting iNOS expression and NO production, we cultured monocytes with amounts of chicken IgG against human TGF-β1 that would neutralize 100% of the TGF-β1 activity, or with normal chicken IgG. Supernatant media from control monocytes, and those treated with 1 μg/mL LPS, 500 U/mL IFN-γ, or LPS with IFN-γ had less than 5 μmol/L nitrite/nitrate, and this was not altered by the presence of 1 or 10 μg/mL anti–TGF-β1 Ig or 10 μg/mL of control chicken IgG.
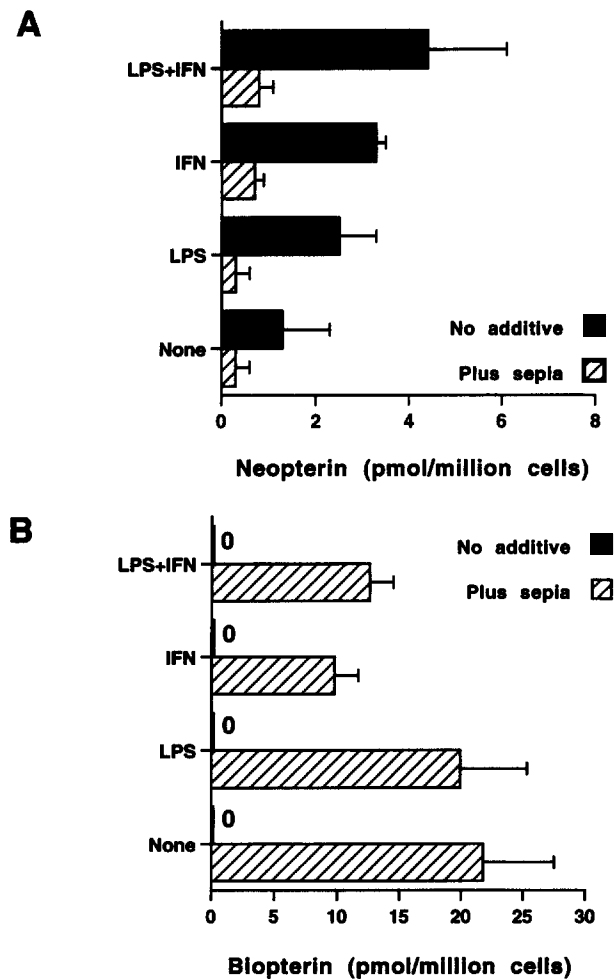
It has been noted that treatment of mouse macrophages

Fig 4. The influence of added sepiapterin, LPS, and IFN-γ on human monocyte neopterin and biopterin levels. Monocytes were isolated and then cultured with or without 100 μmol/L sepiapterin, 1 μg/mL LPS, or 500 U/mL IFN-γ for 3 days. Then cell-associated neopterin (A) and biopterin (B) were measured. This is one experiment that is representative of two performed. The bars display the mean and one SEM. In some, the small error makes it not possible to see the error bar.

with cytokines and LPS increases the steady state content of mRNA for iNOS to levels consistent with the induced high levels of NO production.[51] Genes for the three classes of nitric oxide synthases (constitutive neuronal NOS, constitutive endothelial cell NOS, and inducible NOS) have been cloned, sequenced, and analyzed. For humans, iNOS has been characterized for chondrocytes,[4] hepatocytes,[8] and a colon cancer cell line.[7] When mRNA from stimulated mouse J774 cells and human DLD-1 colon cancer cells were examined by Northern analysis, we noted high levels of induced mRNA; however, by use of northern analysis, we could never detect iNOS mRNA in human control monocytes and those treated with LPS, IFN-γ, or LPS with IFN-γ (data not shown). When we used the more sensitive RNAse protection assay, we could detect mRNA for iNOS in stimulated human DLD-1 cells, but none could be found in monocytes treated

with a variety of different stimuli (Fig 6A). In our experiments, this protection assay can detect levels of iNOS mRNA in DLD-1 human colon cancer cells in as little as 0.1 μg of total cellular RNA per reaction.

We next used RT-PCR techniques to analyze expression of iNOS mRNA in human monocytes and peritoneal macrophages. By use of this more sensitive technique, we were able to detect iNOS mRNA in human mononuclear phagocytes. After isolation (before adherence to plastic), there was no (or very little) expression of the iNOS mRNA (data not shown). Culture of the cells with LPS and/or IFN-γ for 8 hours caused increased expression of iNOS mRNA (Fig 6B). On southern analysis of the RT-PCR DNA using an iNOS-specific cDNA probe, there was a band at the predicted size of 749 bp. Time course analysis of mRNA expression showed maximum expression after 6 to 8 hours stimulation, with a waning in culture noted at 24 to 36 hours. We found comparable results when we studied monocytes isolated by gradient separation or elutriation, and peritoneal macrophages. This indicates that platelet iNOS[52,53] probably did not contribute to our findings, because the elutriated monocytes and the peritoneal macrophages were platelet-free. Although the RT-PCR assays are qualitative or semiquantitative, it appeared that the human phagocytes produced much lower levels of iNOS mRNA than did the mouse J774 cells and the human DLD-1 colon cancer cells. Based on the sensitivity of the RNAse protection assay, the level of mRNA noted in these experiments was at least 100-fold less than that capable of being found in the RNAse protection assay (see above). We sequenced cDNA derived from the RT-PCR and demonstrated that bases 568 through 3459 were identical to sequences for human chondrocyte, hepatocyte, and colon cancer line DLD-1 iNOS cDNA.[4,7,8] We were unable to amplify cDNA from 1-567 in experiments using a variety of primers based upon 5' sequence of human DLD-1 iNOS. Thus, it is possible that there are sequences in this region of the human monocyte iNOS mRNA that are uniquely different from that of other iNOSs.

When analyzed by indirect immunofluorescence using a mouse monoclonal anti-iNOS antibody, we noted that mouse J774 macrophages treated for 3 days with IFN-γ or LPS with IFN-γ had increased numbers of cells reacting with the antibody (Fig 7). Also, human monocytes treated for 3 days with IFN-γ contained antigen that reacted with the antibodies, whereas control cells and those treated with LPS or LPS with IFN-γ did not (Fig 8). Immunoblot analysis of human monocytes and peritoneal macrophages showed iNOS antigen detected by a mouse monoclonal anti-iNOS antibody (Fig 9). In control monocytes and peritoneal macrophages cultured for three days, there was very little reactivity. Treatment of the cells for 3 days with LPS ± IFN-γ caused appearance of a band at approximately 130 to 133 kD molecular mass; this band corresponded well with the major immunoreactive material seen in extracts of activated mouse J774 and RAW macrophages, as well as in activated human DLD-1 colon cancer cells. The presence of an appreciable amount of iNOS protein (antigen) that has little ability to produce NO suggests either a nonfunctional enzyme protein, lack of a cofactors, or existence of an enzyme inhibitor. In an at-
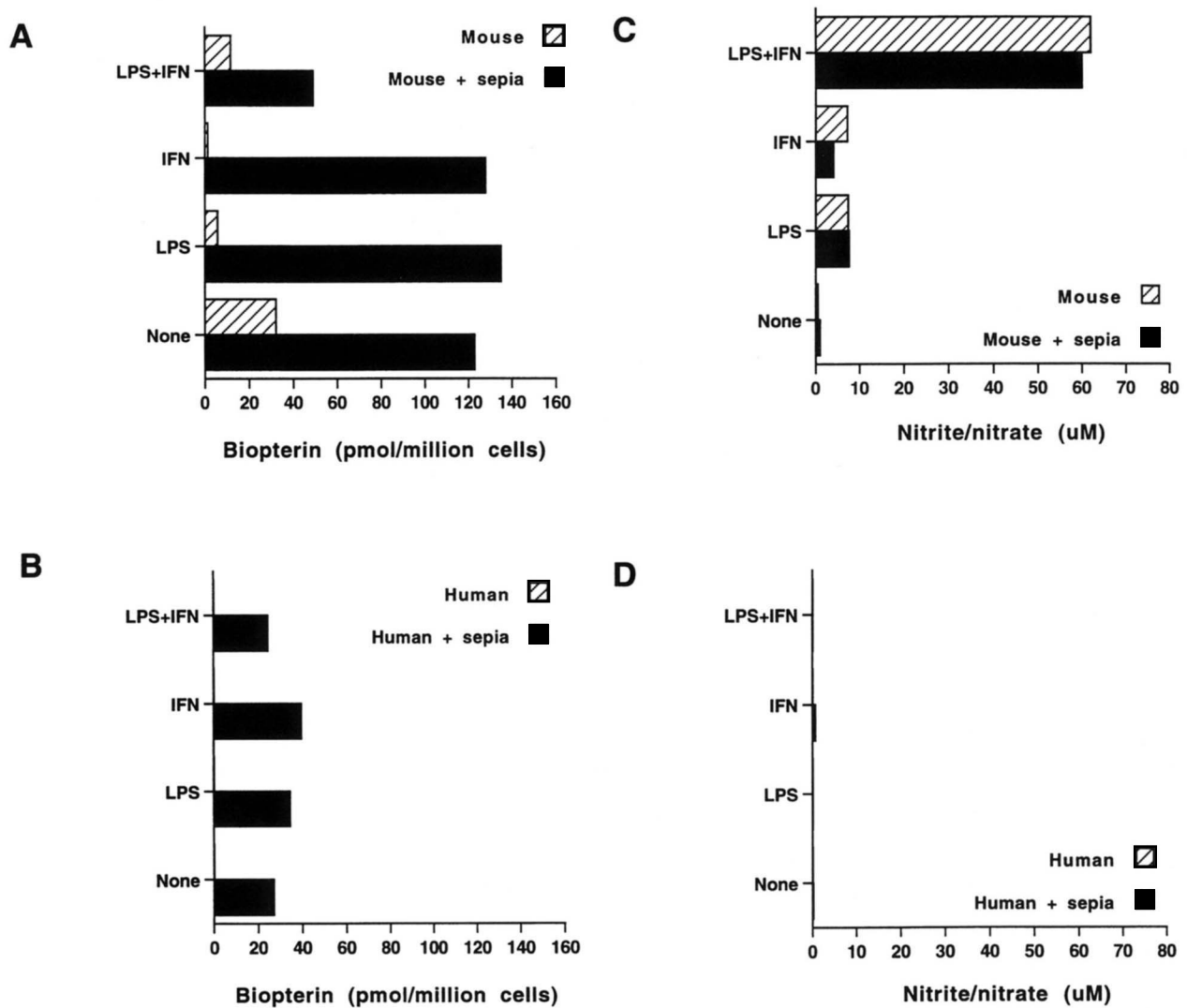
Fig 5. Nitrite/nitrate production and biopterin content in mouse J774 macrophages and human monocytes after culture with sepiapterin, LPS, and IFN-γ. Cells were cultured as noted in Fig 3 for 3 days, and their production of biopterin content (A and B) and nitrite/nitrate (C and D) were measured. This is one experiment that is representative of three performed. The bars display the mean.

tempt to find a functional iNOS inhibitor in human mononuclear phagocytes, we mixed extracts from J774 cells treated with LPS and IFN-γ with equal amounts of extracts from human monocytes (control cells, and those treated with 1 μg/mL LPS, 500 units/mL IFN-γ, or LPS with IFN-γ). We found no inhibition of the iNOS enzyme activity of J774 cells (conversion of ^{14}C-L-arginine to ^{14}C-citrulline) by human monocyte extracts.

DISCUSSION

Although some investigators have been unable to show NO production by human monocytes or macrophages,[9-15] others have noted induction of only low to modest levels of NO production.[15-26] In our work reported here, we too are unable to find high level NO production despite finding iNOS mRNA and protein in the human mononuclear phagocytes.

We show that human blood monocytes and tissue (peritoneal) macrophages cultured under a variety of conditions with several different combinations of cytokines, growth factors, and microbial stimulants produce only low levels of NO. Peritoneal macrophages have a statistically significant small increase in NOS activity and NO production after treatment with LPS ± IFN-γ, whereas monocytes do not. Although the level of in vitro NO production by human mononuclear phagocytes we show here is low compared with that produced by mouse macrophages in vitro, this low level production could cause functional changes in cells, especially if NO were generated in areas where volumes are limited (eg, within the vacuolar system or in areas of phagocyte-target cell contact).[54]

Vodovotz et al[55] have noted that mouse peritoneal macrophages incubated with IFN-γ and LPS, though initially producing iNOS protein and NO, eventually with time in culture
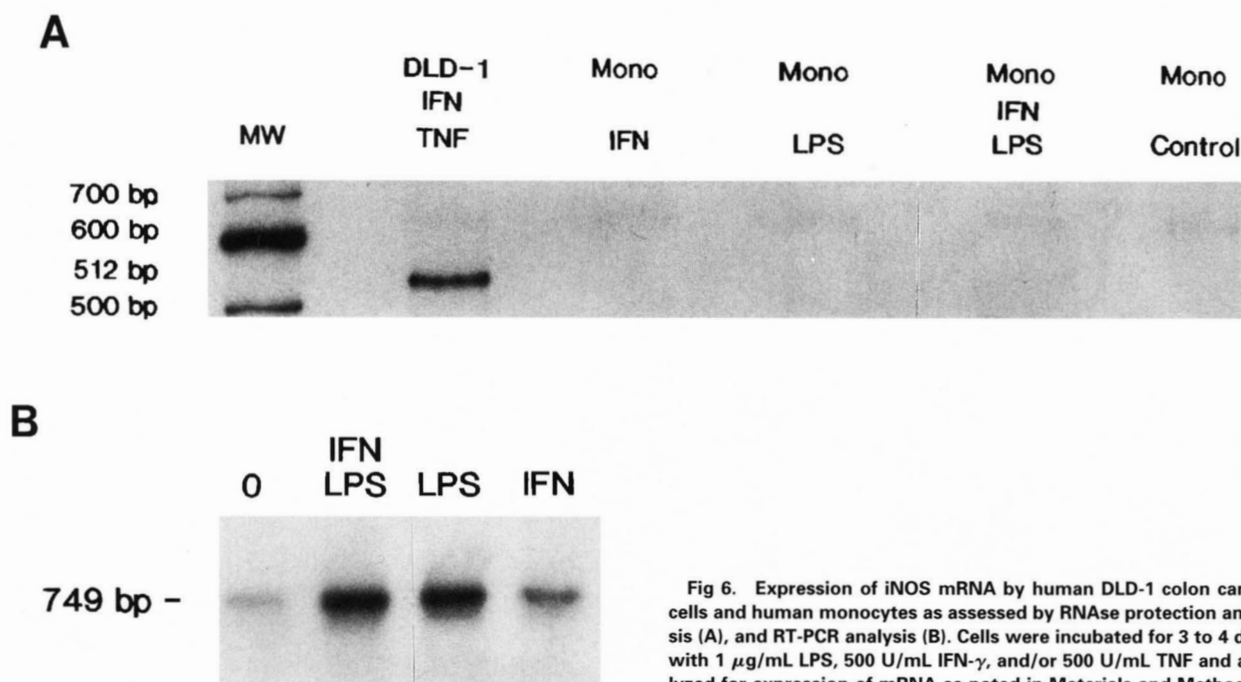
A

B

Fig 6. Expression of iNOS mRNA by human DLD-1 colon cancer cells and human monocytes as assessed by RNAse protection analysis (A), and RT-PCR analysis (B). Cells were incubated for 3 to 4 days with 1 μg/mL LPS, 500 U/mL IFN-γ, and/or 500 U/mL TNF and analyzed for expression of mRNA as noted in Materials and Methods.

have a posttranslational and nondegradative inactivation of the enzyme. These cultured cells with inactivated enzyme are somewhat comparable with what we see with human mononuclear phagocytes—cells expressing iNOS mRNA and containing iNOS antigen with appropriate levels of iNOS cofactors, but lacking ability to produce NO. They noted that the inactivation of mouse macrophage iNOS required the presence of LPS.[55] Also, exposure of mouse macrophages to low amounts of LPS before treatment with IFN-γ has been noted to block expression of iNOS.[56] In our system, we think that LPS is not causing inactivation of iNOS or preventing expression of iNOS because IFN-γ alone induced iNOS, and because LPS contamination was minimal (not detectable by limulus testing).

We cannot fully explain the differences in NO production by human and mouse mononuclear phagocytes. Because the same detection systems for NO (measurement of nitrite and nitrate, and conversion of radioactive L-arginine to L-citrulline) were used for both mouse and human cells, it is unlikely that the differences are related to the assays. One unlikely possibility is that human (unlike mouse) mononuclear phagocytes have the capability to metabolize nitrite and nitrate to products undetectable in our assay. Lack of iNOS cofactor activity in human mononuclear phagocytes is a possibility, with deficiency of tetrahydrobiopterin the most likely candidate, because we and others[10,14,49,57] find no detectable biopterin in human monocytes or macrophages. However, raising biopterin levels by addition of sepiapterin does not correct this problem. Other investigators have found comparable results.[14] If human mononuclear phagocytes express functional iNOS activity, they must derive tetrahydrobiopterin from other cells capable of synthesizing it.

Addition of NADPH or the calcium ionophore A23187 to

the cultures of human mononuclear phagocytes also did not enhance NO-producing abilities. Likewise, enzymatic assays of lysates of the human cells in the presence of added NADPH, tetrahydrobiopterin, dithiothreitol, FAD, and L-arginine showed no or relatively little activity. Because Vallance et al[58] had noted the presence of an arginine derivative in urine and plasma of patients with renal failure that inhibited NOS action, we postulated that human monocytes might contain a comparable inhibitor. However, in our experiments adding excess L-arginine (which should overcome such an inhibitor) did not enhance NO production, and extracts from the human monocytes did not inhibit NOS activity of mouse macrophage extracts.

Although we detected monocyte iNOS mRNA production, the levels appeared to be lower than those seen in mouse macrophages. Thus, the reduced levels of NO production could simply be related to an overall lower level mRNA transcription. We were not able to sequence the 5' end of the cDNA from human monocytes, but of that sequenced, there was identity to bases 568 through 3459 of human DLD-1 iNOS cDNA. We noted that the apparent molecular mass of human monocyte iNOS was comparable with that of mouse iNOS and human DLD-1 cell iNOS. It is possible that there are cell lineage–specific differences in the 5' region of the mRNA between human monocyte iNOS and human hepatocyte, chondrocyte, and DLD-1 iNOS. Other researchers have noted tissue-dependent transcriptional heterogeneity generated by alternative splicing in cytochrome P-450–related genes,[59] including that of neural NOS mRNA.[60,61] Likewise, generation of tissue-specific or cell lineage–specific molecular isoforms by alternative splicing of exons in response to environmental stimuli in different cell types (eg, during embryogenesis, or with differentiation of certain
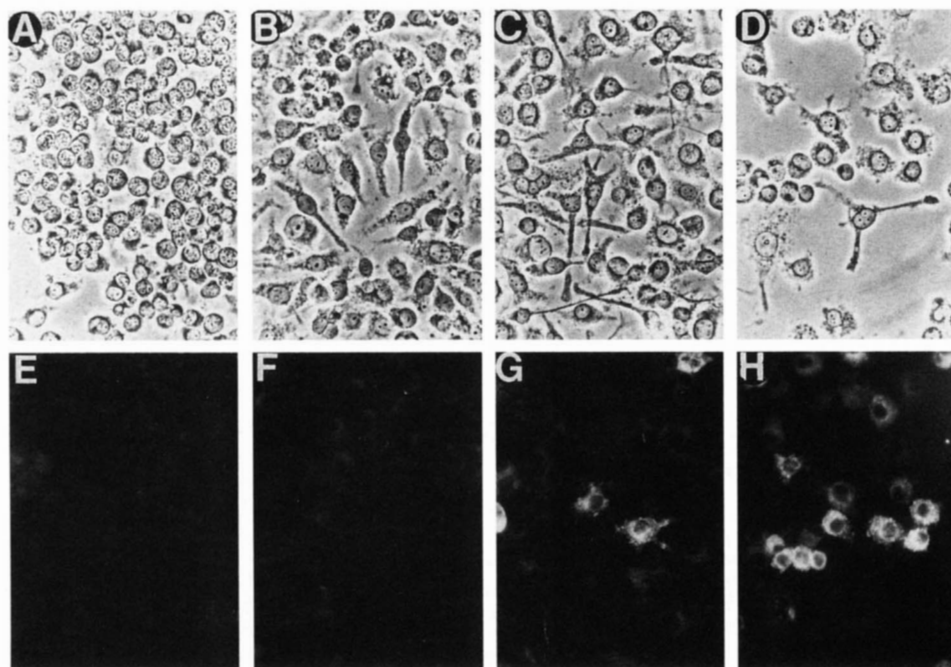
Fig 7. Anti-iNOS indirect immunofluorescence and immunoblot analyses. Immunofluorescence analyses of mouse J774 are shown. Cells were cultured for 3 days with no additives in A and E, with LPS 200 ng/mL for J774 cells in B and F, with murine IFN-γ (200 U/mL for J774) in C and G, and with LPS and IFN-γ in D and H.

types, as has been noted with CD44[62]) might also occur with human iNOS. We continue work to fully explore these possibilities in human monocyte iNOS.

The promoter regions of mouse macrophage iNOS genomic DNA have been partially analyzed and consensus sequences for several known DNA-binding protein promoters have been noted.[63,64] The promoter region for the human hepatocyte iNOS genomic DNA has been partially analyzed,[65] and although the mouse and human iNOS promoter regions were generally similar, some differences were noted. Thus, it is possible that the promoter region in the human mononuclear phagocyte iNOS gene is different from that in the mouse gene, and that human mononuclear phagocytes require different stimuli for activation for high level transcription and subsequent NOS and NO production. Further analysis of the promoter region of the human mononuclear phagocyte iNOS genomic DNA should give important information regarding this possibility.
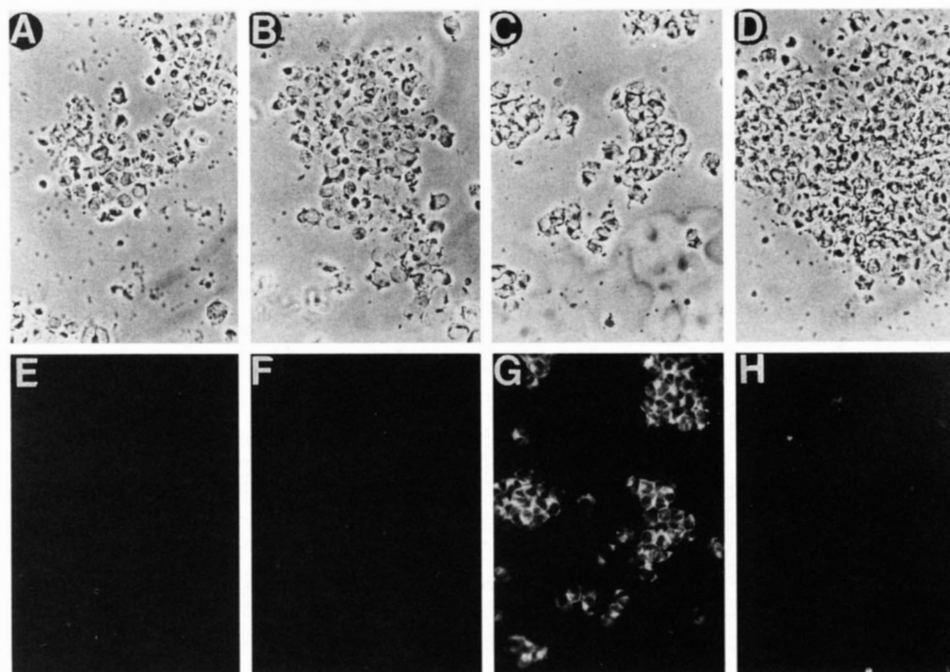

Fig 8. Anti-iNOS indirect immunofluorescence and immunoblot analyses of human monocytes. Cells were cultured for 3 days with no additives in A and E, with LPS (1 μg/mL) in B and F, with human IFN-γ (500 U/mL) in C and G, and with LPS and IFN-γ in D and H.
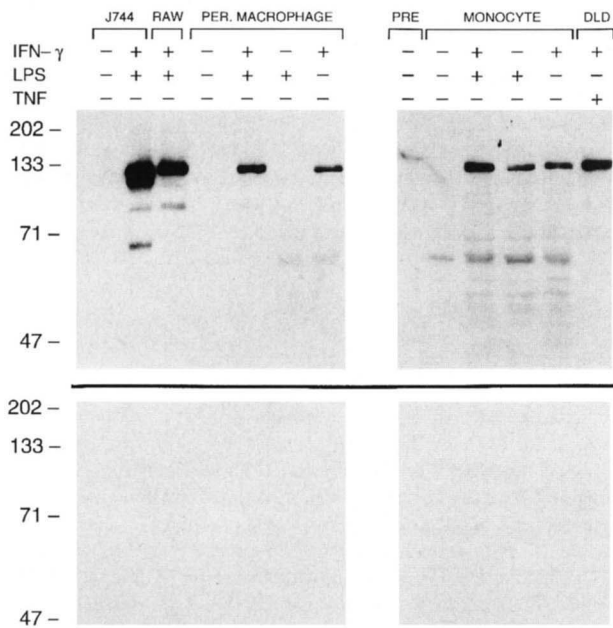
Fig 9. Immunoblot analyses were done for mouse J774 cells, mouse RAW cells, human peritoneal macrophages, human monocytes, and human DLD-1 colon cancer cells. The cells were treated 3 days with LPS (1 µg/mL for monocytes and 200 ng/mL for J774 cells and RAW cells), IFN-γ (500 U/mL human for monocytes, macrophages, and DLD-1 cells, and 200 U/mL murine for J774 and RAW cells), and TNF (500 U/mL) as indicated. Approximately 40 to 50 µg of cellular protein per lane was analyzed. "PRE" refers to extracts from monocytes before any adherence or culture. The anti-iNOS antibody (upper panel) or control P3 antibody (lower panel) was used at a dilution of 1:500.

It is clear that humans can be induced to produce increased amounts of NO in vivo with infection and shock,[66] and when receiving IL-2 treatment for cancer.[67,68] The cells producing the excess NO in vivo in these conditions are not known. Mononuclear phagocytes, hepatocytes, smooth muscle cells, endothelial cells, and/or other cells could be overproducing NO. Based on our findings of only low NO production by stimulated human mononuclear phagocytes in vitro, and undetectable levels of the NOS cofactor tetrahydrobiopterin in freshly isolated and cultured/stimulated human mononuclear phagocytes, one could also speculate that humans have evolved to a state in which mononuclear phagocytes do not "need" to produce large amounts of NO in physiologic or pathologic conditions, and that alternate antimicrobial and antitumor effector mechanisms exist in these cells. A full understanding of human mononuclear phagocyte iNOS and the NO production pathway will be valuable in understanding physiology and pathology. Understanding the reason for the relative low level NO production and correcting this relative "deficiency" might provide an important means of increasing the effector function of human mononuclear phagocytes and enhancing host defense against microbes and malignant cells.

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