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Inhibition of nitric oxide synthase by antisense techniques: investigations of the roles of NO produced by murine macrophages

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1 An antisense approach to block nitric oxide (NO) synthesis was developed, complementing the widely used chemical inhibitors and overcoming problems associated with their use in studying the roles of NO.

2 Murine macrophage cell lines (J774.2) were generated expressing a 500 bp sequence from inducible NO synthase (iNOS) in either the antisense or sense orientation, driven by the SV40 promoter/enhancer region.

3 Messenger RNA derived from the transfected sequences was detected by a specific cDNA probe. Cells expressing sense and antisense iNOS RNA were characterized further.

4 The antisense lines produced 22–97% less NO than the sense lines on stimulation with lipopolysaccharide (LPS) in the range 1 ng ml^{-1}–10 μg ml^{-1}, as determined by nitrite production. One antisense line in particular, A10, expressed substantially less iNOS protein on LPS stimulation as determined by western blot analysis.

5 Adhesion of the antisense line, A10, to cytokine-stimulated murine endothelial cells (sEnd.1 line) was significantly higher than adhesion of the sense lines. There was a negative correlation between the amount of NO produced, as determined by nitrite accumulation, and the level of adhesion of the transfected lines. This indicates an anti-adhesive role of NO, produced by macrophages during the 15 min of the assay, in adhesion to endothelial cells.

6 This novel approach allowed the roles of NO in adhesion to be investigated with the substantial advantage that the contribution of NO produced rapidly by activated macrophages could be studied separately from that produced in a continuous manner by endothelial cells.

7 These lines, and the extension of this approach, will be of great use in dissecting the contributions of NO produced by different cell types to its many potential functions.

Keywords: Macrophage; nitric oxide; inducible nitric oxide synthase; antisense; adhesion; endothelial cell

Introduction

The physiological messenger nitric oxide (NO), which is synthesized from L-arginine by the enzyme NO synthase (NOS), has been implicated in the biology of most cells of the body. Its functions are wide ranging – from vasodilation to neurotransmission and immune modulation. Several isoforms of NO synthase have been isolated (Morris & Billiar, 1994; Hattori *et al*., 1994); these can be divided into two general categories – constitutive and inducible. Constitutive NO synthase (cNOS) is the predominant isoform found in endothelial cells; its activity is increased by factors such as shear stress and agonists including bradykinin. In contrast, synthesis of inducible NO synthase (iNOS) occurs in response to lipopolysaccharide (LPS) or cytokine stimulation in a wide variety of cells including macrophages, neutrophils, endothelial cells and vascular smooth muscle cells (Bredt & Snyder, 1994). There are a number of ways in which the activity of NOS can be regulated such as phosphorylation, availability of the substrate and cofactors (Wang & Marsden, 1995), feedback inhibition (Assreuy *et al*., 1993) or by inhibition of gene transcription. It appears that a major part of iNOS regulation occurs at a pre-translational level such as transcription (Tonetti *et al*., 1995) or mRNA stability; for example, one of the mechanisms by which transforming growth factor-β suppresses macrophage iNOS expression is via decreased iNOS mRNA stability (Vodovotz *et al*., 1993).

The synthesis of NO can be competitively inhibited by certain guanidino-substituted arginine analogues, including N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA, Palmer *et al*., 1988) and N^G, N^G-dimethylarginine (asymmetric dimethylarginine, ADMA, Vallance *et al*., 1992), and these have been crucial in evaluating the role of NO in many physiological and pathological processes. However, these inhibitors are not suitable for all studies, for example, the widely used inhibitor L-NMMA demonstrates significant effects on arginine transport (Bogle *et al*., 1995). Further difficulties arise in studies designed to examine the interaction between different cell types that both generate NO since the use of inhibitors does not permit the evaluation of NO generated by the individual cell types. The chemical inhibitors of NOS are generally not specific for one isoform of the enzyme and therefore a means of more specific inhibition would be useful. More selective inhibitors of iNOS have been found, such as aminoguanidine (Misko *et al*., 1993); however, a slight inhibitory effect on cNOS is also seen.

An alternative approach to chemical inhibition of the enzyme NOS would be to regulate the amount of protein produced. Antisense techniques are a very specific mechanism for interfering with the production of a single protein. Antisense sequences bind specifically to the complementary portion of the mRNA molecule, producing a short double-stranded sequence which prevents the mRNA from being translated into proteins by a number of mechanisms (Ghosh & Cohen, 1992). Use of antisense techniques to interfere with translation of RNA into proteins would give a specific means of interfering with each enzyme form in a particular cell type; they have been used to inhibit the expression of a number of cellular and viral genes including adhesion molecules (Bennett *et al*., 1994), growth factors (Segal *et al*., 1992) and iNOS in rat smooth muscle cells

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(Thomae *et al*., 1993). The most widely used means of getting antisense molecules into cells is by the addition of synthetic oligonucleotides to cell culture medium. In order to overcome the disadvantages associated with this, the approach taken in this study is different and constructs directing the production of antisense RNA within the cells are used.

Murine macrophage cells (J774.2 cell line) have iNOS and produce NO in response to cytokines and LPS. The specific blocking of this enzyme would provide a means of studying the roles of NO produced by macrophages in the immune system (such as killing of bacteria, parasites and tumour cells; Hibbs *et al*., 1988; Bermudez, 1993; Oswald *et al*., 1994), as an anti-adhesive molecule modulating interactions between inflammatory cells and the endothelium (Bath *et al*., 1991; De Caterina *et al*., 1995), in autoimmune disease (Kolb & Kolb-Bachofen, 1992) and in blocking viral replication (Lowenstein *et al*., 1994). J774.2 cells were transfected to generate stable lines which contain plasmids directing the production of antisense RNA to iNOS. This novel approach allowed the roles of NO in adhesion to be investigated with the substantial advantage that the contribution of NO produced rapidly by activated macrophages could be studied separately from that produced in a continuous manner by endothelial cells.

Methods

Cell culture

The mouse macrophage cell line J774.2 (European Collection of Cell Cultures, Centre for Applied Microbiology and Research, Salisbury) was cultured in Dulbecco's modified Eagle's medium containing 5 mM L-glutamine, 0.375% (w/v) sodium bicarbonate, 100 units ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 10% (v/v) foetal calf serum (FCS). Macrophages were removed from the tissue culture flask using a cell scraper, centrifuged at 150 *g* for 10 min and resuspended at the appropriate experimental concentration.

Murine endothelial cells (sEnd.1, Williams *et al*., 1989) were cultured in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 0.375% (w/v) sodium bicarbonate, 100 units ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 10% (v/v) FCS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and were routinely passaged every 4 days.

Preparation of plasmids containing antisense and sense sequences to inducible nitric oxide synthase

A DNA fragment corresponding to bases 2476–2969 of macrophage iNOS mRNA was produced according to methods described in Bogle *et al*. (1994). Briefly, mRNA was isolated from lipopolysaccharide stimulated mouse macrophage cells (J774.2) and this was used for synthesis of first strand cDNA with random primers. The cDNA was then used as a template in a polymerase chain reaction with oligonucleotide primers designed from the published sequence of murine macrophage iNOS (primer 1: base pairs 2476–2498 (sense), primer 2: base pairs 2969–2947 (antisense); Lyons *et al*., 1992). The 493 bp product was purified and subcloned into the plasmid vector pBluescriptII (Stratagene Ltd, Cambridge) at the EcoRV site. The identity of the insert was confirmed by double stranded dideoxy sequencing. The resultant plasmid was digested with HincII, and BamHI linkers were ligated to make the HincII site into a BamHI site. The linearised plasmid was then digested with BamHI to isolate the iNOS DNA plus a 45 bp part of pBluescriptII (total, 538 bp). This fragment was purified and subcloned into the vector pSG5 (Stratagene, Green *et al*., 1988), at its BamHI site, which allows the expression of the inserted DNA under the control of the early SV40 promoter. Restriction enzyme digests were carried out to determine the orientation of the insert (antisense or sense direction with respect to the SV40 promoter/enhancer). CsCl gradients were used to make large scale preparations of plasmids of interest.

Transfection of J774.2 cells

Murine macrophage cells (J774.2) were transfected by use of a modification of the method of Dong *et al*. (1993). Medium was replaced with normal culture medium containing pSG5/iNOS in combination with pSV2neo, in a molar ratio of 10 : 1 respectively, at a final concentration of 10 μg DNA/3ml/9 cm plate and 10 μg ml^{-1} of poly-L-ornithine. Positive transfectants were selected by resistance to geneticin sulphate (G418) encoded by pSV2neo. The pSG5 vectors containing iNOS inserts in either the sense or antisense orientation were used in separate transfections. Mock transfections, without DNA were also carried out. Plates were incubated for 6 h at 37°C with gentle mixing every 1.5 h. The medium was aspirated and replaced with 30% (v/v) DMSO in culture medium for 4 min at room temperature. The plates were then washed rapidly with medium and 10 ml fresh culture medium added. After 5 days, the medium was replaced with culture medium containing 0.5 mg ml^{-1} geneticin sulphate for the isolation of stable transfectants. After a further 3 days, medium was replaced with fresh selection medium and this was repeated every 3 days until G418 resistant colonies appeared. The concentration of geneticin sulphate used had been previously determined as the lowest concentration which caused death of non-transfected J774.2 cells within 7 days. Single colonies were isolated and expanded in culture. Cells were screened by northern analysis using a cDNA probe for macrophage iNOS and positive cell lines were characterized further.

Total RNA isolation and analysis

Total RNA was isolated from confluent 9 cm plates of J774.2 cells or transfected lines (approximately 2.5×10^6 cells) with RNAzol B (Biogenesis Ltd., Bournemouth) used according to the manufacturer's instructions. Samples of RNA (approximately 10 μg) were subjected to elecrophoresis in 1.5% agarose-formaldehyde gels. The RNA was transferred to a Hybond-N+ membrane (Amersham Life Sciences, Aylesbury, Bucks) according to the manufacturer's instructions. Transfer was by diffusion overnight with 20× SSPE as buffer (3.6 M NaCl, 0.2 M sodium phosphate (pH 7.7) and 0.02 M EDTA). The RNA was fixed to the membrane by placing it on a pad of absorbent filter paper soaked in 0.05 M NaOH. The cDNA probe for iNOS (Bogle *et al*., 1994) was labelled with α-[^{32}P]-dCTP (3000 Ci $mmol^{-1}$) by a multiprime DNA labelling system (Amersham) according to the manufacturer's instructions. Prehybridization solution was prepared as follows: 5× SSPE, 50% (v/v) deionized formamide, 5× Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin), 0.1% (w/v) SDS supplemented by 20 μg ml^{-1} denatured herring sperm DNA and incubated with the membranes for 1 h at 42°C. For hybridization, membranes were incubated for 12 h at 42°C in the above buffer with the addition of denatured ^{32}P-labelled iNOS probe. Hybridization solution was removed and the filters were washed once with 50 ml of 2× SSPE 0.1% (w/v) SDS for 15 min at room temperature, twice with 50 ml of 2× SSPE 0.1% (w/v) SDS for 30 min at 65°C and once with 50 ml of 1× SSPE 0.1% (w/v) SDS for 30 min at 65°C. The membranes were air dried, wrapped in Saran-wrap and autoradiographed with an intensifying screen at −70°C.

Stimulation of nitric oxide production by J774.2 cells

Transfected J774.2 cell lines were seeded to 96-well tissue culture plates at a density of 4×10^6 cells ml^{-1}, 100 μl/well and incubated at 37°C overnight. Lipopolysaccharide (LPS) was added at the stated concentrations in a volume of 100 μl, to give a final volume of 200 μl, and incubated for 24 h. NO production was assessed by measuring the amount of nitrite accumulated over the 24 h using the Greiss reaction. The remainder of the tissue culture medium was removed, the cells washed with 200 μl PBS and 200 μl 1 M sodium hydroxide

added. The plates were left for 30 min at room temperature and then stored frozen until assayed for protein (Bradford, 1976; Bio-Rad, Hemel Hempstead, Herts).

Measurement of nitric oxide produced by J774.2 cells (Greiss reaction)

Nitrite and nitrate are stable products formed from NO upon release from cells (Hibbs *et al*., 1988). The production of NO by transfected J774.2 cell lines was determined by measuring the accumulation of nitrite in the tissue culture medium (Greiss reaction) according to Di Rosa *et al*. (1990). Standards of sodium nitrite in the range 0–100 μM were prepared in J774.2 culture medium. Aliquots of 75 μl of either standards or supernatants to be assayed were added to 96-well microtitre plates followed by 25 μl of Greiss reagent (1% (w/v) sulphanilamide, 0.1% (w/v) napthyl-ethylenediamine, 2.5% (v/v) phosphoric acid). The plates were left for 10 min at room temperature and then the absorbance at 540 nm was determined by use of a Titertek Multiskan microtitre plate reader.

Western blot analysis

Macrophage cells (J774.2) or transfected lines were either unstimulated or stimulated with LPS (0.1 μg ml^{-1} or 1 μg ml^{-1}) for 24 h. Stimulation with 1 μg ml^{-1} LPS had been previously determined to give the maximum detectable iNOS by western blot analysis. To detect iNOS, confluent 9 cm plates of J774.2 cells or transfected lines (approximately 2.5×10^6 cells) were washed and scraped in phosphate-buffered saline. The pellets were lysed in 50 mM Tris (pH 7.4), 10 mM EDTA containing 0.2 mM leupeptin, 1 mM PMSF, 0.05 mM pepstatin A and 1% (v/v) Triton X-100 by passing repeatedly through a needle and two cycles of freeze-thawing. The lysate was spun at 12,000 *g* for 5 min and the amount of solubilized proteins determined (Bradford, 1976; Bio-Rad). The proteins were separated by SDS-polyacrylamide gel electrophoresis on a 7% polyacrylamide gel and transferred to a nitrocellulose membrane as described previously (Towbin *et al*., 1979). Following incubation in blocking buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 5% (w/v) milk powder) for 1 h, the membrane was incubated with monoclonal anti-mouse iNOS (Transduction Laboratories, Kentucky, U.S.A.) at 1/2500 for 1 h. After the membrane had been washed it was incubated with anti-mouse IgG peroxidase (A4416, Sigma Chemical Co., Poole, Dorset) at a 1/500 dilution for 1 h. Detection of membrane bound antibodies was carried out according to the manufacturer's instructions by use of a Chemiluminescence Western Blotting Kit (Boehringer Mannheim, Germany). Densitometry was carried out with a Bio-image Omnimedia scanner and Whole Band Analyser.

Cytokines

Recombinant murine cytokines, interferon-γ (IFN-γ), tumour necrosis factor α (TNFα) and interleukin-1β (IL-1β), were reconstituted from lyophilized preparations in sterile distilled water and stored at -70°C. Stock solutions were diluted in culture medium just before use.

Macrophage-endothelial cell binding assay

Endothelial cells (sEnd.1) were grown in 96-well flat-bottomed tissue culture plates (10^5 cells/100 μl/well) in the endothelial cell culture medium described above until confluent. Diluted murine cytokines (50 μl, IL-1β, 10 ng ml^{-1} and TNFα, 25 ng ml^{-1}) or medium control were added to the wells 24 h before the assay. Transfected J774.2 cell lines were stimulated with LPS (10 μg ml^{-1}, 10 ml/9 cm plate) for 24 h and were labelled with ^{51}Cr by incubating 10^7 cells with 200 μCi of $Na^{51}CrO_4$ at 37°C for 60 min. The cells were washed a total of three times with culture medium before resuspension in the same medium to a concentration of 1×10^6 cells ml^{-1}. Labelled transfected J774.2 cell lines (50 μl, 0.5×10^5 cells) were then added to the endothelial cell monolayers and incubated at 37°C for 15 min. The wells were washed four times with 100 μl of culture medium before the addition of 0.1 M NaOH (100 μl/well). After 30 min at 37°C, the contents of each well were transferred to a fresh tube and the radioactivity determined by an LKB Wallac 1275 Minigamma counter. The total radioactivity in the 50 μl of labelled cells added was also determined. The binding of macrophages to endothelial cells was calculated as a percentage of the total radioactivity in the cells added:

$$\%\ \text{cell binding} = (\text{c.p.m. remaining in well/total c.p.m. added}) \times\ 100\%$$

Statistical analysis

Results were analysed by the non-parametric Mann–Whitney U test and parametric one-way ANOVA with Tukey–Kramer post test. Results were considered to be significant if the P value was less than 0.05. In all cases the same degree of significance was obtained with both tests. Linear regression analysis (Prism, Graphpad, San Diego, U.S.A.) was used to determine correlation.

Results

Screening of cell lines by northern blot analysis

Cell lines were screened by northern blot analysis of RNA by use of a ^{32}P-labelled double-stranded DNA probe corresponding to the 493 bp murine macrophage iNOS insert. An analysis of seven of the J774.2 cell lines derived with the antisense construct is shown in Figure 1a and b. Untransfected J774.2 cells stimulated with LPS (lanes 8 and 11) showed a band at approximately 3.6 Kb corresponding to the endogenous iNOS. No bands were detectable in lanes 7 and 10 which contain RNA from untransfected unstimulated J774.2 cells. A band corresponding to RNA of a smaller size can be seen for antisense transfected unstimulated cells A7, A11, A18, A6 and A10 (lanes 2, 3, 5, 6 and 9, respectively). The size of this band corresponds to the antisense sequence plus β-globin from pSG5 (approximately 1.2 Kb). Lines A7, A18 and A10 (lanes 2, 5 and 9 respectively) also have a band of even smaller RNA, for which there is no obvious explanation. The antisense lines A10 and A18, which had bands corresponding to the antisense RNA, were characterized further.

An analysis of six of the J774.2 cell lines derived with the sense construct is shown in Figure 1c. LPS-stimulated untransfected J774.2 cells have a band of RNA corresponding to endogenous iNOS (lane 19) and unstimulated untransfected J774.2 cells have no detectable bands (lane 18). The sense lines S1, S17, S16, S15, S14, and S13 (lanes 12–17 respectively) had very strong 1.2 Kb bands corresponding to the size of the sense insert plus β-globin. The sense line S9 gave similar results (not shown). The sense lines S9 and S17 were characterized further.

Nitric oxide production by transfected J774.2 cells

The production of NO by the transfected cell lines on stimulation with LPS was measured by detecting nitrite in the tissue culture medium (Greiss reaction). The sense transfected cells, S9 and S17, showed a high level of nitrite accumulation in response to LPS in the range 1 ng ml^{-1} to 10 μg ml^{-1}. The antisense transfected cells, A10 and A18, produced between 22–97% less NO than the sense lines in response to LPS (Figure 2).

Amount of inducible NO synthase in transfected J774.2 cells

Western blot analysis of the iNOS content of J774.2 cells and tranfected cell lines is presented in Figure 3. Unstimulated J774.2 cells and unstimulated sense and antisense lines had no

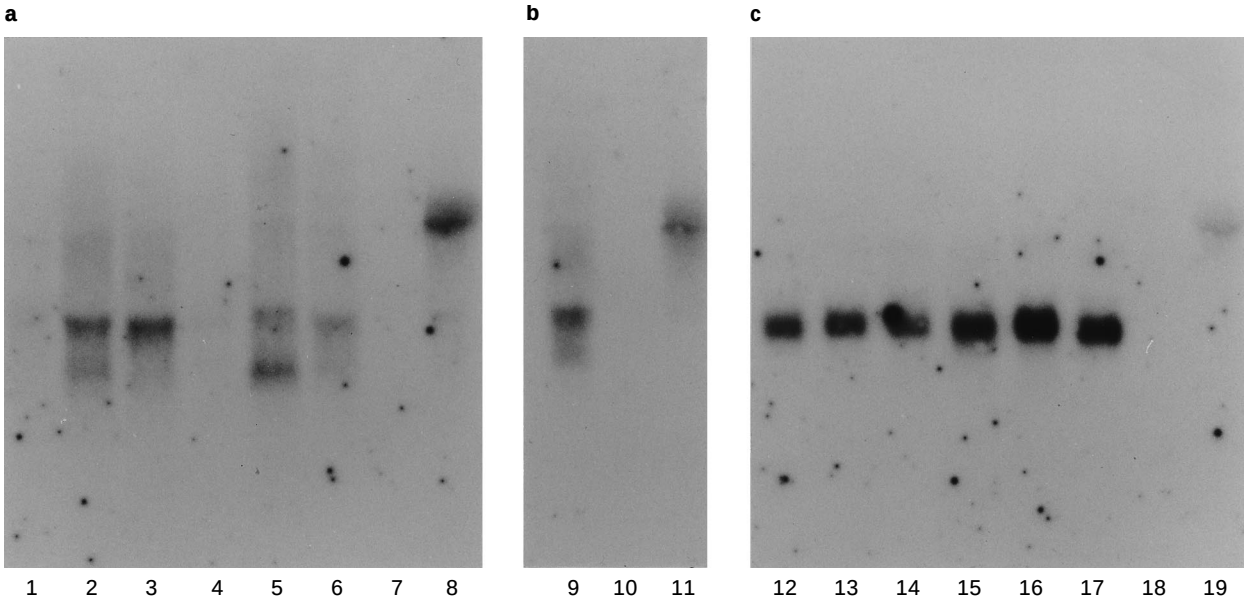

Figure 1 Northern blot analysis of cell lines transfected with pSG5/iNOS. RNA was isolated from transfected lines and from J774.2 cells and electrophoresed through a 1.5% agarose gel. Autoradiography was performed after transfer of the RNA to Hybond N+ membranes and hybridization with ^{32}P-labelled probe to murine macrophage iNOS. Lanes 8, 11 and 19 are RNA from untransfected J774.2 cells stimulated for 4 h with LPS ($10\,\mu g\,ml^{-1}$, 10 ml/9 cm plate/2.5×10^6 cells) and have a band at approximately 3.6 Kb. Lanes 7, 10 and 18 are unstimulated untransfected J774.2 cell RNA. Lanes 1–6 are unstimulated antisense transfected cells A12, A7, A11, A4, A18 and A6, respectively. Lane 9 is unstimulated antisense line A10. Lanes 12–17 are unstimulated sense transfected cells S1, S17, S16, S15, S14 and S13, respectively. The bands of antisense or sense sequence plus β-globin from pSG5 are approximately 1.2 Kb.

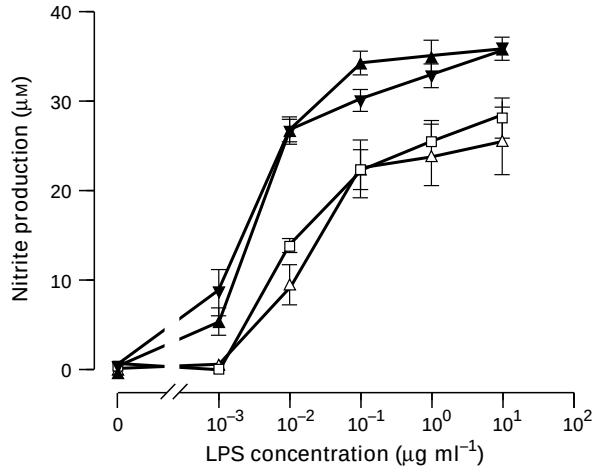

Figure 2 Nitrite accumulation from LPS-stimulated cell lines transfected with pSG5/iNOS. Transfected lines were seeded at 4×10^6 cells ml^{-1} (96-well plate, 100 μl/well) and incubated for 24 h. LPS was added at the stated concentrations in a volume of 100 μl and incubated for 24 h. Nitrite accumulation over the 24 h was measured by the Greiss reaction on 75 μl medium. The remainder of the medium was removed, the cells washed with PBS and lysed with 1 M NaOH. Protein concentration in each well was measured and the Greiss reaction results adjusted to a standard protein concentration. Results presented are mean (vertical lines show s.e.mean) of pooled results from 4 separate experiments, each in triplicate. S17 (▲), S9 (▼), A10 (△) and A18 (□).

detectable iNOS present. Untransfected J774.2 cells had detectable levels of iNOS protein on stimulation with 0.1 μg ml^{-1} and 1 μg ml^{-1} LPS for 24 h (lanes 7 and 8, a and b). The sense lines S17 and S9 produced similar amounts of iNOS after LPS stimulation (lanes 1 and 2, S17 b, S9 a). In contrast, the antisense line A18 contained less iNOS than either the untransfected J774.2 cells or the sense transfected line S17 after LPS stimulation (Figure 3b) and the iNOS content of A10 cells was even lower. On stimulation of A10 cells, no iNOS was detected with 0.1 μg ml^{-1} LPS and only a very small amount was observed with 1 μg ml^{-1} LPS (Figure 3a).

Macrophage-endothelial cell binding studies

The adhesion of antisense and sense transfected J774.2 macrophage cells to murine endothelial cells (sEnd.1) is shown in Figure 4. Less than 5% of A10, A18, S9 and S17 cells stimulated with LPS bound to unstimulated sEnd.1 cells. On cytokine stimulation of sEnd.1 cells (IL-1β/TNFα) the adhesion of LPS-stimulated macrophage lines increased by between 6 and 8 fold compared with adhesion to unstimulated sEnd.1 cells. The antisense lines A10 and A18 showed higher levels of adhesion to sEnd.1 cells than either of the sense lines, S9 or S17. In particular, A10 showed 2.9 fold higher binding compared with S9 ($P<0.001$, $n=15$) and 2.6 fold compared with S17 ($P<0.001$, $n=15$). Antisense line A18 had significantly higher adhesion to sEnd.1 cells than S9 cells (1.5 fold higher, $P<0.05$, $n=15$). The adhesion of A18 to sEnd.1 cells was higher than that of S17 (1.4 fold higher, $P>0.05$, $n=15$), but not statistically significant. Greiss reactions performed on supernatants of J774.2 cells stimulated with LPS for 24 h (from the same cultures in the same experiments), before labelling with ^{51}Cr showed that A10 produced the lowest amount of NO followed by A18, S17 and S9 (Figure 5). The sense line S9 produced 2 fold more ($P<0.01$, $n=9$) and S17 produced 1.7 fold more ($P<0.05$, $n=9$) NO than A10 cells. A negative correlation was found for the cell lines between the amount of NO produced and the level of adhesion observed (Figure 6, coefficient of determination: $r^2=0.77$).

Discussion

The use of antisense techniques to inhibit iNOS has a number of advantages over conventional methods in which competitive

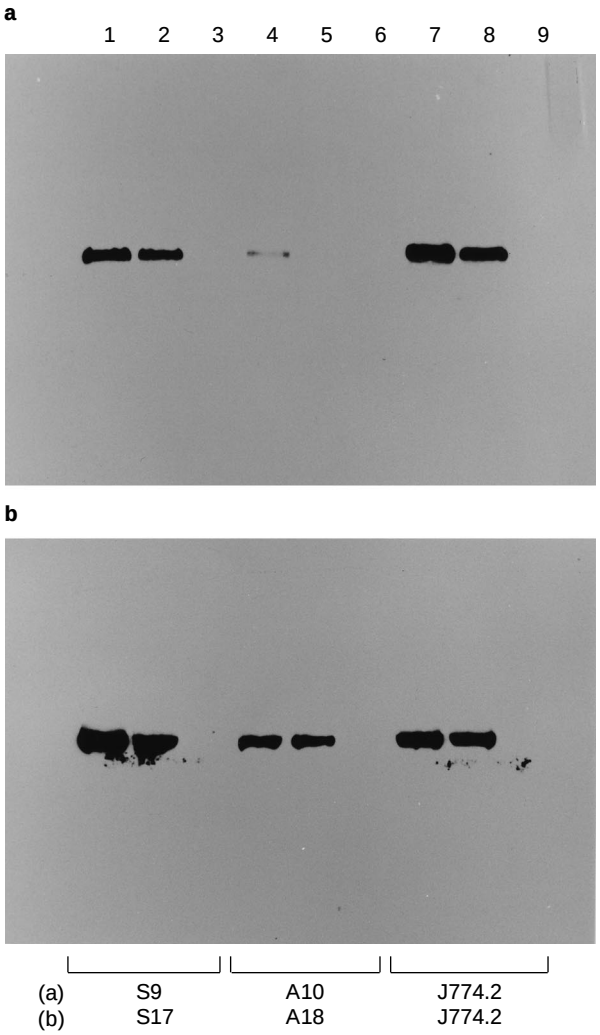

Figure 3 Western blot analysis of untransfected J774.2 cells and cells transfected with pSG5/iNOS. Transfected lines and J774.2 cells (2.5×10^6 cells/9 cm plate) were stimulated with LPS at the stated concentrations for 18 h, harvested and lysed. Proteins (40 μg/track) were separated by SDS-polyacrylamide electrophoresis and transferred to a nitrocellulose membrane. Following incubation with monoclonal anti-mouse iNOS and anti-mouse IgG peroxidase, membrane bound antibodies were detected by chemiluminescence. (a) S9 (lane 1, 1 μg ml^{-1} LPS; lane 2, 0.1 μg ml^{-1} LPS; lane 3, unstimulated), A10 (lane 4, 1 μg ml^{-1} LPS; lane 5, 0.1 μg ml^{-1} LPS; lane 6, unstimulated) and untransfected J774.2 cells (lane 7, 1 μg ml^{-1} LPS; lane 8, 0.1 μg ml^{-1} LPS; lane 9, unstimulated); (b) S17 (lane 1, 1 μg ml^{-1} LPS; lane 2, 0.1 μg ml^{-1} LPS; lane 3, unstimulated), A18 (lane 4, 1 μg ml^{-1} LPS; lane 5, 0.1 μg ml^{-1} LPS; lane 6, unstimulated) and untransfected J774.2 cells (lane 7, 1 μg ml^{-1} LPS; lane 8, 0.1 μg ml^{-1} LPS; lane 9, unstimulated). Data in (a) and (b) are from two separate experiments. Densitometry of these autoradiographs gave the following density values for the bands (ND, denotes not detectable); (a) – lane 1, 18.48; lane 2, 13.46; lane 3, ND; lane 4, 2.06; lane 5, ND; lane 6, ND; lane 7, 28.02; lane 8, 19.97 and lane 9, ND; (b) – lane 1, 41.48; lane 2, 32.56; lane 3, ND; lane 4, 16.46; lane 5, 14.59; lane 6, ND; lane 7, 23.92; lane 8, 19.97 and lane 9, ND.

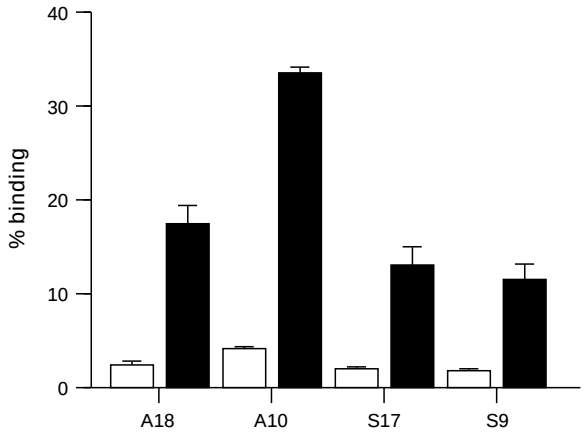

Figure 4 Adhesion of J774.2 cells transfected with pSG5/iNOS to sEnd.1 cells. Endothelial cells (sEnd.1) were cultured in 96-well plates (10^5/100 μl/well) and 24 h before the assay, 50 μl of diluted murine cytokines (IL-1β, 10 ng ml^{-1} and TNFα, 25 ng ml^{-1}) or medium control were added to the wells. Transfected J774.2 cell lines were stimulated with LPS (10 μg ml^{-1}, 10 ml/9 cm plate) for 24 h. ^{51}Cr-labelled LPS-stimulated transfected lines derived from J774.2 cells (0.5×10^5 cells/well) were added and incubated at 37°C for 15 min. Nonadherent cells were removed by washing and the remaining radioactivity was measured and used to determine the percentage of cells which had bound to the sEnd.1 monolayer. The mean + s.e.mean presented are derived from three independent experiments each consisting of replicates of five. Open columns, control unstimulated sEnd.1 cells; solid columns, cytokine-stimulated sEnd.1 cells.

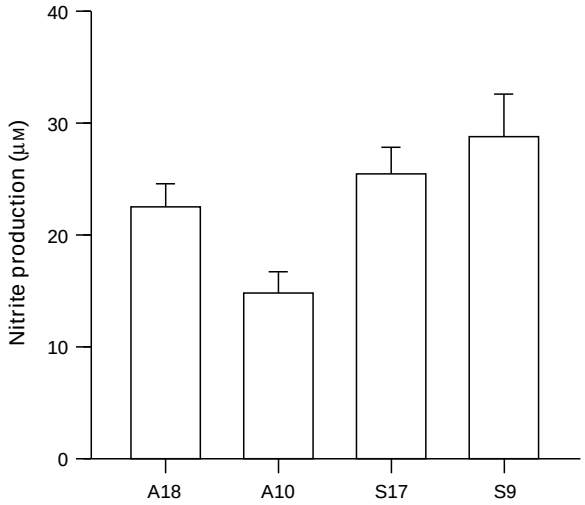

Figure 5 Nitrite accumulation from LPS-stimulated cells transfected with pSG5/iNOS before being used in macrophage-endothelial binding assay. Transfected lines were stimulated with LPS (10 μg ml^{-1}) for 24 h. Before the cells were labelled with ^{51}Cr, NO production was measured by the Greiss reaction on 75 μl medium. Results presented are mean + s.e.mean of pooled results from three separate experiments (as presented in Figure 4), each in triplicate.

inhibitors of NOS are used. The arginine basis of inhibitors such as L-NMMA makes effects on other cell systems possible (eg. arginine has significant effects on L-NMMA transport; Bogle *et al*., 1995) which may be exacerbated by metabolism of the chemical during longer assays (MacAllister *et al*., 1994). When studying systems involving the interaction between more than one cell type, the effect of inhibiting the NOS activity separately in one of the cells cannot be assessed using chemical inhibition. Inhibitors such as L-NMMA will, to varying extents, inhibit all isoforms of NOS and so the separate functions of the different types cannot be investigated. By selection of the region targeted, the antisense approach can be made specific for a particular enzyme form and will work on one defined cell type within a mixed population.

In most studies synthetic antisense oligonucleotides designed to hybridize to specific RNA sequences are used (Chiang *et al*., 1991; Thomae *et al*., 1993). However, there are several disadvantages associated with this approach. Nucleases in serum degrade oligonucleotides and so the experiments must be performed in serum-free medium and the oligonucleotides have to be added at high concentration with repeated addition. This may be toxic, increases the expense and makes the study

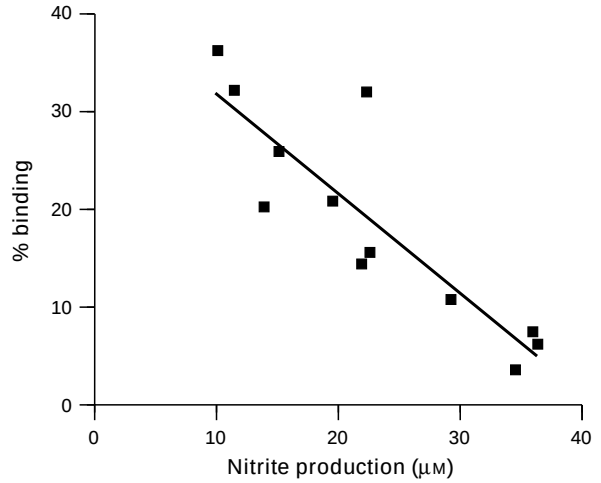

Figure 6 Linear regression analysis showing correlation between nitrite accumulation and % binding. The mean nitrite concentration (μM, $n=3$) was plotted against the mean % binding ($n=5$) for each of the four cell lines A10, A18, S9 and S17. These values were from the three independent experiments pooled together in Figures 4 and 5.

of longer term phenomena difficult. Chemical modification of the oligonucleotides increases their half-life (Stein & Cheung, 1992) but may reduce the uptake into cells. The oligonucleotides are relatively short (because of expense and to allow entry into cells) and so may not demonstrate a significant effect on the target mRNA.

The approach taken in this study differs from most antisense studies in that stable cell lines, containing plasmids directing the production of antisense RNA to iNOS, were generated. This allows the inhibition of protein production to be permanent with constitutive transcription of this antisense gene giving rise to antisense RNA sequences. Antisense overexpression vectors have been used to regulate the production of a number of proteins (Jaattela, 1993; Xu & Ware, 1995).

Murine macrophage J774.2 cells transfected with a region of iNOS in the antisense orientation produced less iNOS protein (Figure 3) and less nitrite (Figure 2) than sense transfected cells on induction with LPS. These macrophage antisense lines will be useful in investigating some of the proposed functions of NO with the sense transfected cell lines acting as true controls rather than untransfected cells, a deficiency of some antisense studies.

In the present study, the role of NO in adhesion of macrophages to the endothelium has been investigated. Due to its strategic position, the endothelium is important in the mediation and modulation of both inflammatory and immunological responses. The endothelium normally maintains blood flow by inhibiting coagulation and resisting the adhesion of blood leukocytes. However, in response to infection and tissue injury endothelial cells become activated and regulate the movement of fluid and leukocytes which is characteristic of inflammation. This is achieved through the generation of vasoactive compounds (for example prostacyclin and NO), lipid-based leukocyte activators (for example platelet activating factor), chemotactic cytokines and specific cell surface adhesion molecules. The continuous release of NO from the endothelium has an important role in blood flow modulation. It is apparent from recent findings that NO may also be an important modulator in interactions between inflammatory cells and the endothelium.

By use of NO donors and chemical inhibitors of NOS, NO has been shown to reduce platelet aggregation and neutrophil and platelet adhesion to endothelial cells (Radomski *et al*., 1987; 1993; Kubes *et al*., 1991; 1993). Monocyte adhesion is also decreased by NO (Bath *et al*., 1991; De Caterina *et al*., 1995) but lymphocyte adhesion is not (Cartwright *et al*., unpublished observations). The adhesion studies presented here (Figure 4) support these results and provide definitive evidence of the importance of NO derived from macrophages (as opposed to the endothelial cells in the culture) in modulating the adhesion during the short period of the assay (15 min). The antisense lines, A10 in particular, had a higher level of adhesion to cytokine stimulated endothelial cells than the sense lines. Determination of the NO produced by the macrophages in the same experiments (Figure 5) showed that the more NO produced, the lower the adhesion to sEnd.1 cells (Figure 6), consistent with the proposed anti-adhesive role of NO.

Previous studies on the effect of NO on monocyte/macrophage adhesion have used exogenously applied NO (Bath *et al*., 1991) or NO donors (De Caterina *et al*., 1995). NO donors do not have predictable kinetics and the release of NO requires redox or metabolic activation making the concentration of NO in the study difficult to predict. These are not as physiologically relevant as studies inducing the production of NO from either macrophages or endothelial cells themselves. By stimulating the macrophages with LPS to produce endogenous NO, other pathways involved in the regulation of adhesion may also be activated giving a more realistic picture of the events *in vivo*. The sEnd.1 cells have both constitutive NOS and iNOS (Walter *et al*., 1994) and produce NO on stimulation. Investigating the contribution of NO produced by endothelial cells and macrophages in adhesion would be difficult using inhibitors of NOS as they could act on both cell types. The advantage of the antisense lines is that less NO is produced by a defined cell population in the assay, compared with controls and the contribution of NO produced by this population can be studied.

These macrophage antisense lines will also be useful in investigating some of the other proposed functions of NO in the immune system (such as killing of bacteria, parasites and tumour cells; Hibbs *et al*., 1988; Bermudez, 1993; Oswald *et al*., 1994), in autoimmune disease (Kolb & Kolb-Bachofen, 1992) and in blocking viral replication (Lowenstein *et al*., 1994). The application of these antisense techniques to other forms of NOS and other cell types has great potential for increasing our understanding of the functions and interactions of NO.

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