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Acemannan, a β-(1,4)-Acetylated Mannan, Induces Nitric Oxide Production in Macrophage Cell Line RAW 264.7

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SUMMARY

Acemannan is a polydispersed β-(1,4)-linked acetylated mannan with antiviral properties. It is an immunomodulator, and studies in our laboratory have shown that it causes activation of macrophages. Inducible NO synthase is generally expressed after transcriptional induction and is known to mediate some of the cytotoxic action of activated macrophages. Acemannan, in the presence of interferon-γ, greatly increased the synthesis of NO in RAW 264.7 cells. This increase was preceded by increased expression of mRNA for the inducible form of macrophage NO synthase. Preincubation with pyrrolidine dithiocarbamate inhibited the induction, indicating the involvement of nuclear factor-κB. These results suggest that acemannan causes the activation of macrophages by increasing the level of NO synthase at the level of transcription.

Acemannan is a polydispersed β-(1,4)-linked mannan isolated from *Aloe vera* (1). It is believed to be an immunostimulant and is conditionally licensed by the United States Department of Agriculture for the treatment of fibrosarcoma in dogs and cats(2). Acemannan hydrogel (ingredient in Carrasyn Gel Wound Dressings, Carrington Laboratories, Dallas, TX) is approved by the United States Food and Drug Administration for the treatment of aphthous ulcers, wounds, and first- and second-degree burns (3). These approvals, however, are based solely on the ability of acemannan to keep the wounds moist, and little is known about its mechanism of action.

One of the most marked biological activities of mannans in mammals is the activation of macrophages and stimulation of T cells (4–6). Acemannan causes the activation of mouse peritoneal macrophages, and studies in our laboratory have shown that it enhances wound healing in rats (7, 8). Macrophages play a crucial role in wound healing. They are involved in the inflammatory and debridement phase (9), and the growth factors produced by them modulate the angiogenesis phase of wound healing (10). Also, immunologically activated macrophages produce NO, the reactive free radical involved in regulating vascular tone. NO mediates the bactericidal and tumoricidal activities of macrophages (11) and, when produced in excess, is responsible for the endotoxic shock and damage due to inflammation (12). Two major classes of NOSs are known: constitutive and inducible (13). In neurons and endothelial cells, the enzyme is expressed constitutively and is activated by calmodulin and calcium (14). The macrophage enzyme, however, is produced only after activation of the macrophages and requires transcriptional activation of the *iNOS* gene (15, 16). Endogenous calmodulin is a tightly bound subunit of the macrophage iNOS enzyme; therefore, its activity is not regulated by Ca^{2+} levels or exogenous calmodulin (17). The activity of iNOS is, however, dependent on a number of cofactors, such as heme, tetrahydrobiopterin, and flavin nucleotides (18). The synthesis of macrophage NOS is induced by LPS (19) and TNFα (20), and the level of stimulation can be augmented by combining these stimuli with IFNγ (11, 20, 21).

Acemannan is known to cause activation of the macrophages. We report that in the presence of IFNγ, acemannan increases NO production in RAW 264.7 cells. We present evidence that the increased NO production is due to increased transcription of the *iNOS* gene.

Materials and Methods

Reagents and cell culture. We obtained the mouse monocyte/macrophage cell line RAW 264.7 from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Baltimore, MD) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin G and 100

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ABBREVIATIONS: LPS, lipopolysaccharide; G3PD, glyceraldehyde-3-phosphate dehydrogenase; PDTC, pyrrolidine dithiocarbamate; SDS, sodium dodecyl sulfate; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IFN, interferon; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; NFκB, nuclear factor-κB; DEPC, diethylpyrocarbonate.

μg/ml streptomycin). All cell culture reagents were obtained from Life Technologies (Grand Island, NY). Acemannan was obtained from Carrington Laboratories (Dallas, TX). Bacterial LPS (from *Escherichia coli* serotype 0127:B8), cycloheximide, pyrrolidone dithiocarbamate, actinomycin D, and polymyxin B were obtained from Sigma Chemical (St. Louis, MO). Genistein was obtained from GIBCO (Grand Island, NY), and IFNγ was from Genzyme (Cambridge, MA).

RAW 264.7 cells (2×10^6) were seeded into 60-mm tissue culture dishes and incubated overnight at 37° in 5% CO_2 in air to allow for adherence. Cells were incubated with varying concentrations of acemannan, IFNγ, or a combination for 6 hr. At the end of the incubation period, the stimulus was removed, and the cells were rinsed with PBS. The cells were then scraped off the plate, and mRNA was isolated as described below.

Isolation of RNA, reverse transcription, and PCR amplification. RNA was isolated using the MicroFasttrack kit from Invitrogen (San Diego, CA). The cell pellet was suspended in 1 ml of lysis buffer containing 200 mM NaCl, 200 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 2% SDS, and protein/RNase degrader. The cell lysate was passed through an oligo(dT) cellulose column, and poly(A)$^+$ RNA was eluted using a buffer containing 10 mM Tris-Cl, pH 7.5, in DEPC treated water. RNA was precipitated using 0.15 M sodium acetate, 0.1 mg of glycogen carrier, and 2.5 volumes of ethanol. The RNA pellet was washed once with 80% ethanol containing RNasin (0.2 units/μl of DEPC treated water), dried, and resuspended in 11.5 μl of DEPC treated water.

cDNA was synthesized by reverse transcription using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (22). Approximately 1.5 μM of oligo(dT) primer was annealed to 1 μg of poly(A)$^+$ RNA and extended with avian myeloblastosis virus reverse transcriptase (13.3 units/μl) in a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP mix, and RNase inhibitor (1 unit/μl) at 42° for 1 hr. The reaction was terminated by incubation at 80° for 10 min. The cDNA produced was diluted (1:4) with DEPC-treated water and amplified as described below.

PCR amplification was carried out with 10 ng of the cDNA in a buffer containing 0.2 mM)dNTPs, 2.5 mM $MgCl_2$, and 1.5 units of *Taq* polymerase (Promega, Madison, WI). The PCR amplimers for mouse *iNOS* were upstream primer 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′ (nucleotides 2943–2968) and downstream primer 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′ (nucleotides 3440–3415) (15). The PCR amplimers for mouse *G3PD* were upstream primer 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′ (nucleotides 51–76) and downstream primer 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′ (nucleotides 1033–1010) (23). Both of the primer sets were obtained from Clonetech Laboratories (Palo Alto, CA). The amplification was carried out in a PTC 150 thermal cycler (MJ Research, Watertown, MA). The denaturation was at 94° (1 min), the annealing temperature was 60° (2 min), and extension was at 72° (3 min); this was cycled 30 times. The amplified products were analyzed on a 1.5% agarose gel (24). The gels were subjected to densitometric scanning, and the amount of DNA was estimated using the MacBAS 2.0 (Fujix, Fuji Medical Systems USA, Stamford, CT) program. G3PD was used as an internal control, and when appropriate, the iNOS signal was normalized relative to that of G3PD.

Southern and Northern hybridization. The specificity of the products obtained by amplification of the cDNAs were verified by Southern analysis. The Southern transfer of amplified cDNA was from a 1% agarose gel run in Tris/borate/EDTA (1× = 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) buffer to a nylon membrane (Magnagraph; Fisher Scientific, Pittsburgh, PA) according to the manufacturer's recommended protocol. The *Not*I fragment of iNOS and the *Eco*RV/*Bam*HI fragment of G3PD were used as probes. The hybridization probes were radiolabeled with [^{32}P]dATP (3000 Ci/mmol; DuPont-New England Nuclear, Boston, MA) with a random primer labeling kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Southern hybridization was performed at 65° in 5× standard saline/phosphate/EDTA (1× = 150 mM NaCl, 10 mM NaH_2PO_4, 10 mM EDTA, pH 7.4)/2% SDS with 10 μg/ml salmon sperm DNA (24). *mac-NOS* (the 4-kb mouse *iNOS* gene cloned into pBluescript KS vector) was a gift from Dr. William J. Murphy. The PCR fragment obtained using mouse G3PD primers was cloned into pcTR11 vector (TA cloning kit from InVitrogen), and the sequence was verified by restriction analysis and dideoxy sequencing (24).

For Northern analysis, total cellular RNA was isolated from cells solubilized with guanidine isothiocyanate. RNA was purified by centrifugation through a cushion of cesium chloride according to the method of Chirgwin *et al.* (25). Twenty micrograms of RNA was denatured with formaldehyde, separated by electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde, and transferred onto a nylon membrane (Magnagraph; Fisher Scientific) with 10× SSPE. The blots were hybridized with labeled cDNA probes, the *Not*I fragment of NOS, and the *Eco*RV/*Bam*HI fragment of G3PD at 55° in 50% formamide/5× SSPE/1% SDS. The hybridized filters were washed three times at 65° in 0.2× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate)/0.1% SDS for 15 min. Autoradiography was performed by exposure to Kodak X-OMAT film (24).

Immunocytochemistry. RAW 264.7 cells were allowed to adhere in each chamber of two-chamber Lab-tek culture slides (Nunc, Naperville, CT). Adherent macrophages were then cultured for 8 hr with acemannan, IFNγ, acemannan plus IFNγ, or medium containing none of these compounds. The monolayers were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with ice-cold acetone. Nonspecific binding was blocked with 3% bovine serum albumin. Slides were then incubated with anti-iNOS antibody (Transduction Labs, Louisville, KY) (5 μg/ml) for 1 hr at 37°. Specific antibody was detected using FITC-conjugated rabbit anti-mouse IgG (26). Photomicrographs were taken with an Olympus BH2 compound microscope using FITC filters on Kodak Ektachrome 160T color slide film.

SDS-PAGE and immunoblotting. Cell monolayers were washed with ice-cold PBS and lysed in a buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 1% Triton X-100, 0.5% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride. SDS-PAGE (10%) was conducted under denaturing, reducing conditions, according to Laemmli (27). Proteins were transferred onto 0.2-μm-pore-sized polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) using 15% methanol, 25 mM Tris, and 192 mM glycine, pH 8.3. The membrane was blocked for 1 hr at room temperature with 1% bovine serum albumin in Tris-buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and then incubated with mouse anti-mac-NOS monoclonal antibody (1/500 dilution; Transduction Labs) for 1 hr at room temperature. The membrane was washed with Tris-buffered

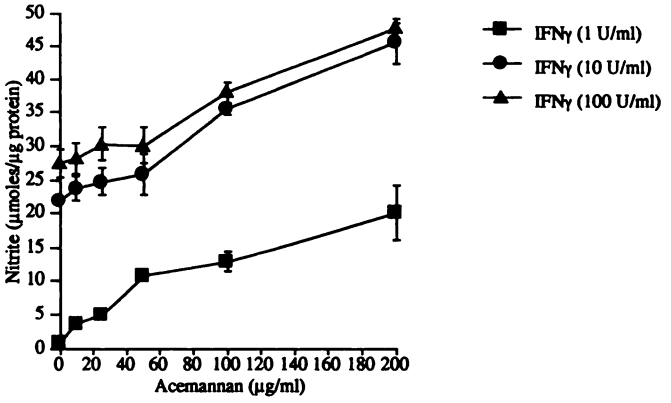

Fig. 1. Combination of acemannan and IFNγ causes increased NO production in macrophages. RAW 264.7 cells were incubated with the indicated concentrations of acemannan either alone or together with 1, 10, and 100 units/ml of IFNγ for 24 hr. NO synthesis was assayed by measuring the NO_2^- present in the culture supernatants.

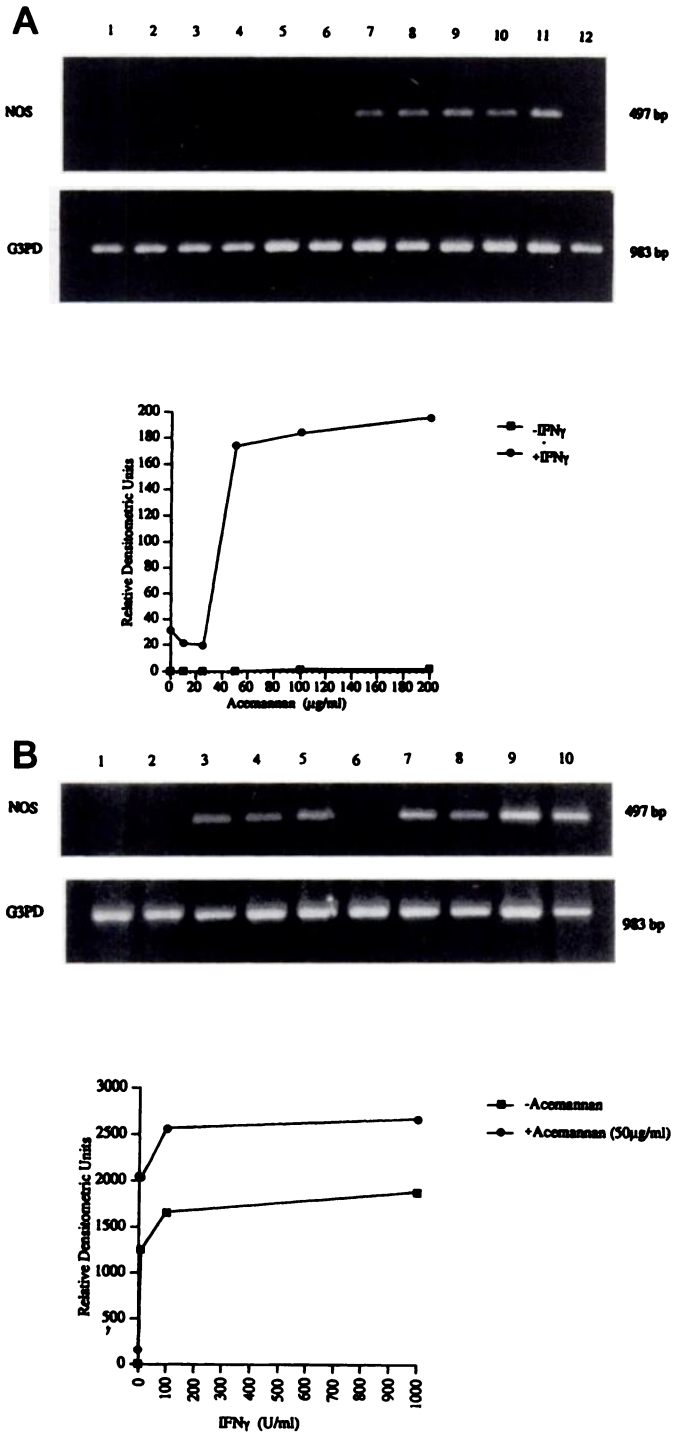

Fig. 2. Dose-response analysis of induction of iNOS mRNA by acemannan and IFNγ. RAW 264.7 cells were incubated for a period of 6 hr with indicated concentrations of acemannan in the presence or absence of IFNγ (1 unit/ml) (A) or indicated concentrations of IFNγ in the presence or absence of acemannan (50 μg/ml) (B). mRNA was isolated and reverse-transcribed using oligo(dT) primers. Identical amounts of the cDNA was amplified by PCR in two separate reactions using primers for either iNOS or G3PD and electrophoresed on 1.5% agarose gels. The levels of iNOS and G3PD were quantified by densitometric scanning. *Graphs*, relative level of NOS mRNA after normalization to the respective G3PD signal to account for variability in the RNA preparation, cDNA synthesis, and PCR amplification. The results shown are representative of three independent experiments. *Graphs* correspond to *lanes* in G3PD and NOS. A: *Lane 1*, media alone; *lanes 2–6*, media with 10–200 μg/ml acemannan (10, 25, 50, 100, and 200 μg/ml); *lanes 7–11*, media with 1 unit/ml IFNγ and 10–200 μg/ml acemannan (10, 25, 50, 100, and 200 μg/ml); *lane 12*, medium with 1 unit/ml IFNγ. B: *Lanes 1–5*, media with 0.1–1000 units/ml IFNγ (0.1, 1, 10, 100, and 1000 units/ml); *lanes 6–10*, media with 50 μg/ml acemannan and 0.1–1000 units/ml IFNγ (0.1, 1, 10, 100, and 1000 units/ml).

saline and then subsequently incubated for 1 hr with anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical). After washing, the membrane was equilibrated in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and developed in a solution of 167.5 μg/ml nitro blue tetrazolium and 167.5 μg/ml 5-bromo-4-chloro-3-indoyl phosphate in alkaline phosphatase buffer.

Assay for NO synthesis. Synthesis of NO was determined by assay of culture supernatants for NO_2^-, a stable reaction product of NO with molecular oxygen. RAW 264.7 cells were seeded into 12-well tissue culture plates at a density of 10^6 cells/well. Cells were treated with acemannan, IFNγ, or a combination, and NO_2^- production was measured after 48 hr (28). Briefly, 50 μl of culture supernatant was incubated with an equal volume of Greiss reagent [0.5% sulfanilamide, 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% $H3PO_4$] in a 96-well tissue culture plate for 10 min at room temperature. The absorbance at 550 nm was measured in an enzyme-linked immunosorbent assay plate reader (Dynatech Labs, Chantilly, VA) along with $NaNO_2$ standards. The concentration of protein was determined using the bicinchoninic acid reagent from Pierce Chemical (Rockford, IL) (29).

Results

Combination of acemannan and IFNγ increased NO production in macrophages. In the presence of IFNγ, acemannan caused an increase in the production of NO by macrophages. RAW 264.7 cells were stimulated with acemannan, IFNγ, or various combinations for 24 hr, after which accumulation of nitrite in the media was measured using the Greiss reagent. Treatment of the cells with a low concentration of IFNγ (1.0 unit/ml) had no effect, whereas higher concentrations (10 and 100 units/ml) induced NO synthesis (Fig. 1). Macrophage NO production was enhanced in the presence of IFNγ and various concentrations of acemannan. The combinatorial effect of acemannan and IFNγ on NO production was evident when cells were stimulated with a lower concentration of IFNγ (1 unit/ml) together with acemannan (10–50 μg/ml) (Fig. 1). However, treatment of macrophages with acemannan alone had no effect (data not shown).

Acemannan and IFNγ together increase iNOS mRNA production. Acemannan in the presence of IFNγ caused an increase in the level of iNOS mRNA (Fig. 2). The macrophages were incubated with acemannan, IFNγ, or a combination, and the mRNA was isolated, reverse transcribed, and amplified. The amplified cDNAs were electrophoresed onto agarose gels, and the amount of cDNA present was indicative of the mRNA levels in the various treatments. Low concentrations of acemannan (10–50 μg/ml) did not cause any detectable induction of iNOS mRNA. However, in the presence of IFNγ (1 unit/ml), abundant iNOS mRNA was observed (Fig. 2A). This increase was very evident with 50 μg/ml acemannan. A high concentration of acemannan (200 μg/ml) by itself caused a very low induction of iNOS mRNA (<5 relative densitometric units), but in the presence of IFNγ, there was a significant increase (190 relative densitometric units) in iNOS mRNA. This observed increase in mRNA parallels the increase seen in NOS assay, in which 50 μg/ml

acemannan in combination with IFNγ showed more increased activity than occurred in response to acemannan at 10–25 μg/ml.

Low concentrations of IFNγ (0.1–1 unit/ml) by itself did not cause a marked increase in the level of iNOS mRNA (Fig. 2B). However, when cells were treated with IFNγ (1 unit/ml) in the presence of acemannan (50 μg/ml), there was a 25-fold increase in mRNA levels. Higher concentrations of IFNγ (10–100 units/ml) increased the level of iNOS, and this increase was further augmented by acemannan (Fig. 2B).

The acemannan-induced increase in iNOS expression was further characterized by a time course experiment. The macrophages were incubated with acemannan (50 μg/ml) and IFNγ (1 unit/ml) for various time periods, and the mRNA was isolated, reverse transcribed, and amplified. Acemannan and IFNγ together caused an increase in iNOS mRNA levels, and this increase was seen within 3 hr of treatment (Fig. 3). Quantification of the signal by densitometric scanning and normalization with the G3PD showed that the mRNA levels continued to increase until 12 hr after treatment; then, it leveled off (Fig. 3).

Acemannan and IFNγ together increase iNOS protein production. The increase in iNOS mRNA levels induced by the combination of acemannan and IFNγ was reflected in the increase in iNOS protein. When RAW 264.7 cells were treated with acemannan in the presence of IFNγ, the inducible NOS appeared at ~6-8 hr (Fig. 4). The size of the protein seems to be ~130 kDa. The increase in iNOS protein was confirmed by immunohistochemistry (Fig. 5). RAW 264.7 cells were incubated with acemannan in the presence of IFNγ, and iNOS in these macrophages was identified *in situ*. The staining for NOS appeared in the perinucleararplasm (Fig. 5D). Acemannan alone (Fig. 5B) or IFNγ alone (Fig. 5C) showed background level of staining.

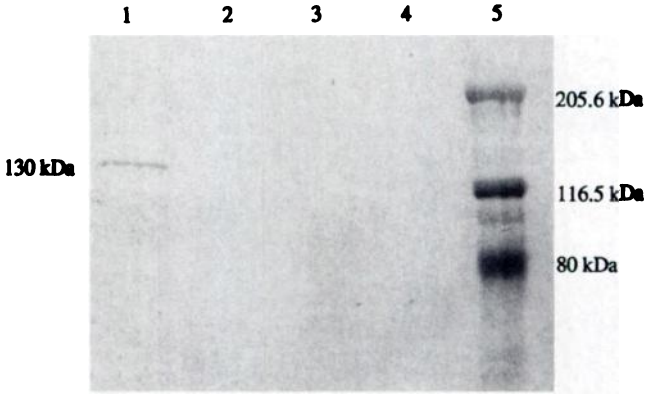

Fig. 4. Western blot analysis of iNOS in macrophage cell lysates. RAW 264.7 cells were treated with media alone (*lane 4*), acemannan (50 μg/ml) (*lane 3*), IFNγ (1 unit/ml) (*lane 2*), or a combination (*lane 1*) for 8 hr. Total cell lysates were subjected to SDS-PAGE (10%), blotted onto a polyvinylidene difluoride membrane, and probed with anti-mac-NOS antibody. *Right*, migration of molecular mass markers (in kDa). The 130-kDa iNOS band is marked.

Effect of cycloheximide and pyrrolidine dithiocarbamate on iNOS induction by acemannan. Cycloheximide, a reversible inhibitor of protein synthesis, greatly suppressed the level of NOS mRNA in cells treated with acemannan and IFNγ (Fig. 6A). Macrophages were preincu-

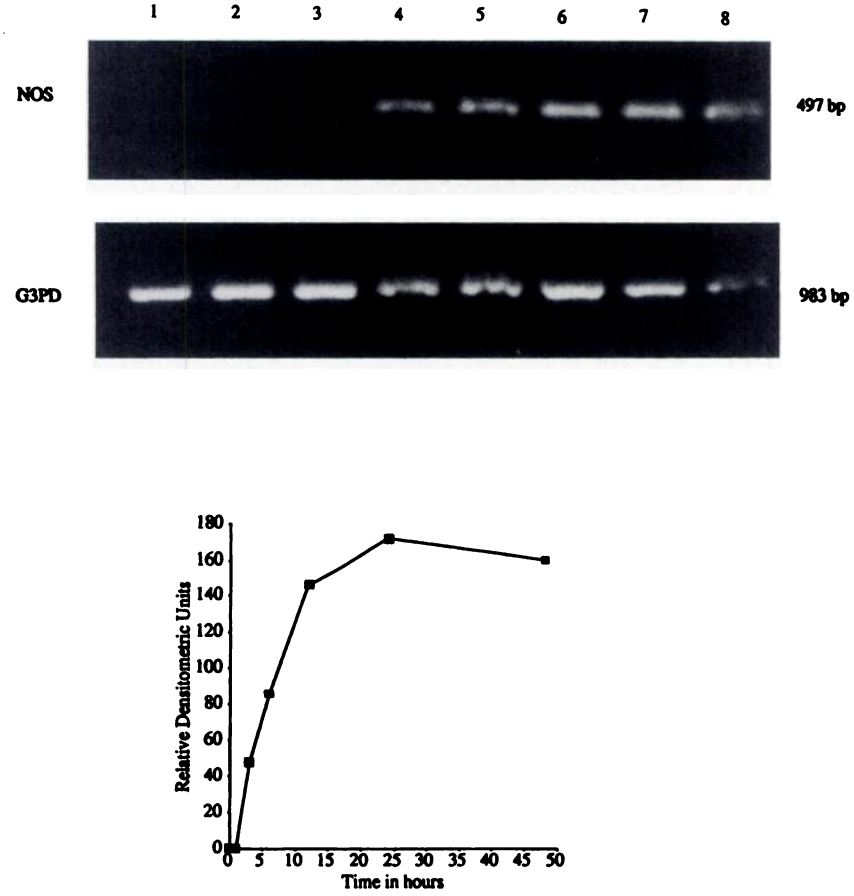

Fig. 3. Kinetics of induction of iNOS mRNA by acemannan and IFNγ. RAW 264.7 cells were incubated with acemannan (50 μg/ml) and IFNγ (1 unit/ml) for the indicated time periods. mRNA was isolated and reverse-transcribed using oligo(dT) primers. Identical amounts of the cDNA was amplified by PCR in two separate reactions using primers for either iNOS or G3PD and electrophoresed on 1.5% agarose gels. The levels of iNOS and G3PD were quantified by densitometric scanning. *Graph*, relative level of NOS mRNA after normalization to the respective G3PD signal to account for variability in the RNA preparation, cDNA synthesis, and PCR amplification. Results are representative of three independent experiments. *Graph* corresponds to the eight lanes in G3PD and NOS; *lane 1*, medium alone. *Lanes 2–8*, different incubation time periods (0.5, 1, 3, 6, 12, 24, and 48 hr).

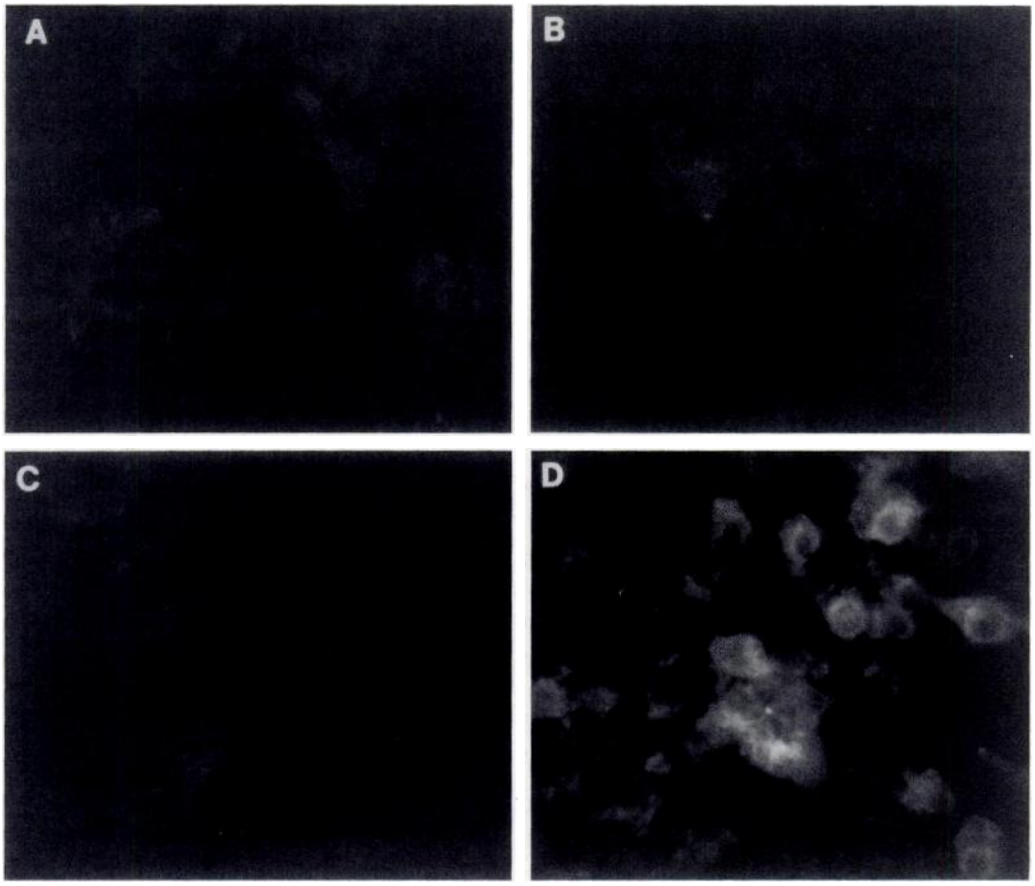

Fig. 5. *In situ* localization of iNOS in macrophages. RAW 264.7 cells were treated with media alone (A), 50 μg/ml acemannan (B), 1 unit/ml IFNγ (C), or a combination of 50 μg/ml acemannan and 1 unit/ml IFNγ (D) for 12 hr. Cells were then fixed, stained with a monoclonal antibody mac-NOS, and detected using FITC-conjugated anti-mouse IgG.

bated with cycloheximide for 30 min, after which they were treated with acemannan, IFNγ, or a combination for 6 hr. Preincubation with cycloheximide inhibited the induction of *iNOS* by acemannan and IFNγ but had no effect on the transcription of a constitutive gene *G3PD* (Fig. 6A). This indicates that the increased expression of iNOS caused by acemannan and IFNγ is dependent on *de novo* protein synthesis.

PDTC, an antioxidant that acts as a relatively specific inhibitor of NFκB activation, inhibited the induction of *iNOS* by acemannan in the presence of IFNγ. Activation of NFκB, a transcription factor, has been shown to be an essential process in the induction of NOS in macrophages. To verify whether the induction of *iNOS* by acemannan involved the activation of NFκB, RAW 264.7 cells were preincubated with PDTC for a period of 30 min, after which they were treated with acemannan, IFNγ, or a combination for 6 hr. Preincubation of cells with PDTC inhibited the induction of *iNOS* but had no effect on the transcription of *G3PD* (Fig. 6B). This inhibition of iNOS mRNA by PDTC was also reflected in the enzymatic assay of iNOS protein. The NO produced by the macrophages was assayed by measuring nitrite in the media using the Greiss reagent. At a concentration of 50 μM, PDTC completely inhibited NO production (data not shown). This suggests that the induction of *iNOS* by acemannan in the presence of IFNγ involves the activation of NFκB.

The induction of iNOS by acemannan and IFNγ does not involve LPS. Treatment of RAW 264.7 cells with acemannan and IFNγ caused an induction of *iNOS* gene and an increase in the level of iNOS mRNA. This effect was very similar to that caused by the combination of LPS and IFNγ. To rule out the possibility that the induction observed was an endotoxin-mediated response, acemannan was incubated with polymyxin B before its addition to the cells. Polymyxin B greatly inhibited the induction caused by LPS and IFNγ but had no effect on the increase in mRNA levels caused by acemannan in the presence of IFNγ (Fig. 7). The endotoxin levels were monitored using the Limulus assay (30), and the results indicate that there were no endotoxins present in the acemannan samples used. Therefore, the effect of acemannan on NO production is not due to endotoxins.

Discussion

Acemannan, a β-(1,4)-linked acetylated mannan, has several important therapeutic properties, including acceleration of wound healing, inhibition of inflammation, and antiviral effects (7, 8, 31). However, it is unclear how acemannan exerts this wide variety of effects. We believe that acemannan mediates some of its response through the macrophages, which are known to play a pivotal role in antimicrobial immune response and are important components of the host inflammatory response. The results presented here suggest that acemannan in the presence of IFNγ causes transcriptional activation of the *iNOS* gene in a macrophage cell line. It is known that effective induction of iNOS requires activation by both IFNγ and LPS (11); however, IFNγ also synergizes with TNFα in the induction of NO production (32), suggesting that multiple mediators can act on the *iNOS* gene to achieve inducible NO synthesis. Unlike endothelial cells, neurons, and other cell types that express the NOS constitutively (14), NO production in macrophages is observed only

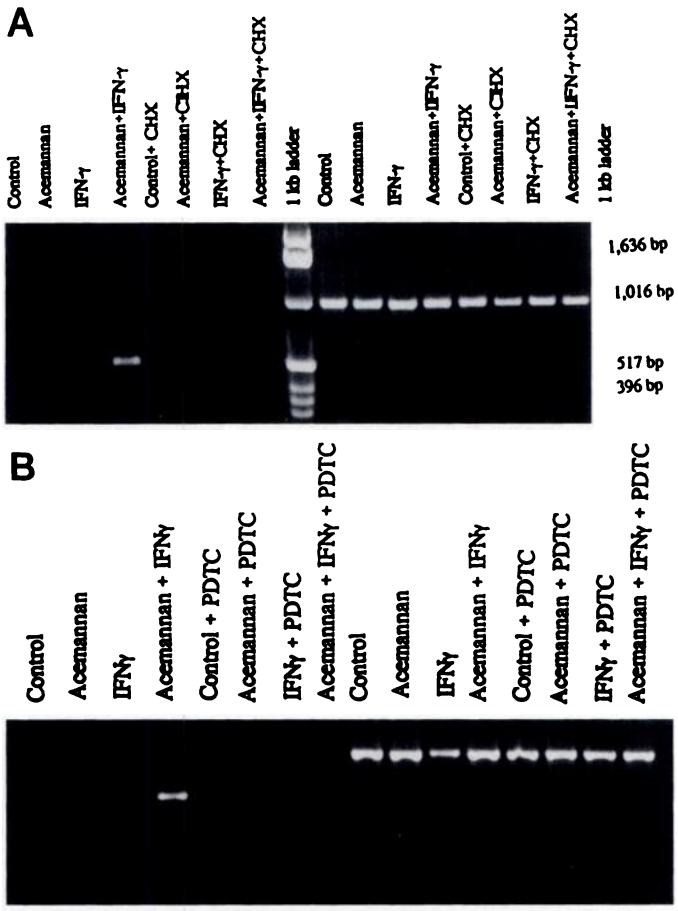

Fig. 6. Cycloheximide (*CHX*) and PDTC inhibit acemannan-induced increase in iNOS mRNA. RAW 264.7 cells were preincubated for 30 min with 100 μg/ml cycloheximide (A) or 50 μM PDTC (B) and then treated with acemannan (50 μg/ml), IFNγ (1 unit/ml), or a combination for 6 hr. mRNA was isolated and reverse-transcribed using oligo(dT) primers. Identical amounts of the cDNA were amplified by PCR in two separate reactions using primers for either iNOS or G3PD and electrophoresed on 1.5% agarose gels. The individual treatment for each lane is indicated *above the lane*. A: *Lanes 1–8*, iNOS; *lanes 10–17*, G3PD. *Lane 10*, 1-kb ladder. B: *Lanes 1–8*, iNOS; *lanes 9–16*, G3PD.

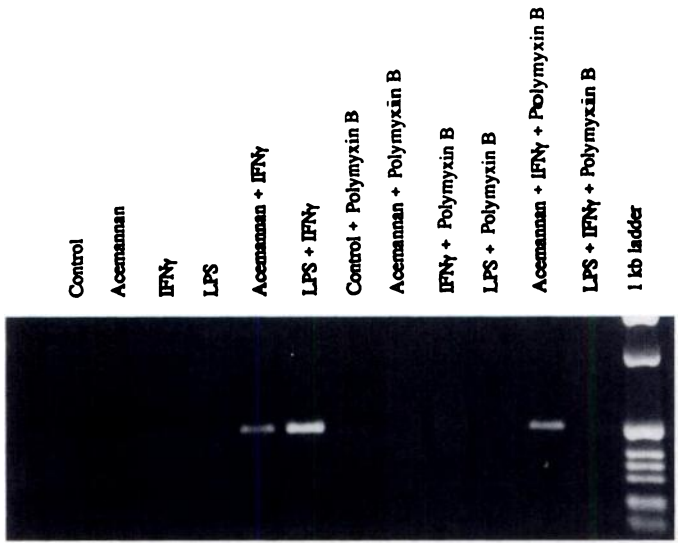

Fig. 7. Polymyxin B does not affect the acemannan-induced increase in iNOS mRNA. RAW 264.7 cells were treated with acemannan (50 μg/ml), IFNγ (1 unit/ml), or LPS (100 pg/ml) alone or a combination of acemannan and IFNγ in the presence or absence of polymyxin B (100 units/ml) for 6 hr. mRNA was isolated and reverse-transcribed using oligo(dT) primers. The cDNA was amplified by PCR using primers for iNOS and electrophoresed on a 1.5% agarose gel. The individual treatment for each lane is indicated *above the lane*. *Last lane*, 1-kb ladder.

after exposure of the cells to cytokines or bacterial products, and induction of *iNOS* represents a response associated with the activation process (15, 16).

Acemannan causes the activation of macrophages and at high concentrations (200 μg/ml) was able to cause only a small increase in the level of iNOS mRNA. However, in the presence of IFNγ, low concentrations of acemannan (50 μg/ml) caused a significant increase in the level of iNOS mRNA, expression of the iNOS protein, and NO production. In this respect, it is very similar to the induction caused by LPS (11). However, this induction is not inhibited by polymyxin B, and this together with the results of the Limulus assay for endotoxins suggests that the effect of acemannan is not due to endotoxins that may be present in the preparation.

Cycloheximide suppressed the level of NOS mRNA in cells treated with acemannan and IFNγ. This indicates that the increased expression of iNOS caused by acemannan and IFNγ is dependent on *de novo* protein synthesis. Various transcription factors have been implicated in the regulation of the mouse iNOS promoter (20). The promoter for the macrophage *iNOS* gene contains consensus sequence motifs for the binding of several known transcription factors involved in the induction of other cytokine-responsive genes, such as IFNγ-responsive element, γ-activated site, TNF-responsive element and NB-κB site (13). The induction of *iNOS* gene by LPS is dependent on the presence of NF-κB heterodimers p50-c-rel and p50-rel A (33), whereas the synergistic inductive contribution of IFNγ requires IRF-1 (34). The inhibition of iNOS induction by PDTC suggests that activation of NFκB is involved. PDTC is an antioxidant that has been shown to inhibit the activation of NFκB by preventing the binding of NFκB/Rel to the NFκBd (the binding site on the iNOS promoter) (33, 35). Preincubation of RAW 264.7 cells with PDTC completely inhibited the induction of *iNOS* by acemannan in the presence of IFNγ. These results suggest that activation of NFκB is involved in the induction of *iNOS* by acemannan and IFNγ.

Taken together, the results presented here suggest that acemannan in the presence of IFNγ causes transcriptional activation of the *iNOS* gene. Inducible NOS plays an important role in angiogenesis, acting as a vasorelaxant, and this induction of iNOS could be one of the ways by which acemannan enhances wound healing.

Acknowledgments

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