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Induction of Apoptosis in a Macrophage Cell Line RAW 264.7 By Acemannan, a β-(1,4)-Acetylated Mannan

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ABSTRACT

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. In the presence of IFNγ, acemannan induced apoptosis in RAW 264.7 cells. These cells exhibited chromatin condensation, DNA fragmentation, and laddering characteristic of apoptosis. The induction of apoptosis by acemannan and IFNγ does not seem to be mediated by nitric oxide, since *N*-nitro-L-arginine methyl ester, the nitric oxide inhibitor, had no effect. Acemannan in the presence of IFNγ also inhibited the expression of *bcl-2*. These results suggest that acemannan in the presence of IFNγ induces apoptosis in RAW 264.7 cells through a mechanism involving the inhibition of *bcl-2* expression.

Apoptosis is a form of cell death that can be induced in susceptible cells by a wide variety of normal physiological stimuli as well as by deleterious environmental conditions. Some of the characteristic features of apoptosis include cytoplasmic shrinkage associated with membrane blebbing, followed by chromatin condensation and DNA fragmentation. Although all cells undergoing apoptosis exhibit these changes sequentially, it is believed that these events occur independently and under the control of separate and distinct metabolic pathways. Anticancer drugs are known to induce apoptosis in target cells (Searle *et al.*, 1975; Sen and D'Incalci, 1992; Skladanowski and Konopa, 1993), and although the interaction of these drugs with the cellular targets has been studied extensively, the mechanism by which these chemotherapeutic agents induce apoptosis is unclear (Dive and Wyllie, 1993). One of the mechanisms seems to be by the activation of the sphingomyelin signal transduction pathway (Hannun, 1994; Kolesnick and Golde, 1994).

Acemannan is a polydispersed β-(1,4)-linked mannan isolated from *Aloe vera* (Manna and McAnalley, 1993; Paquet and Pierard, 1996). It is believed to be an immunostimulant and is conditionally licensed by the United States Department of Agriculture (USDA) for the treatment of fibrosarcoma in dogs and cats (Drapier *et al.*, 1988; King *et al.*, 1995). Preliminary trials indicate that acemannan immunostimulant maybe an effective adjunct to surgery and radiation therapy in the treatment of canine and feline sarcomas. However, little is known about the mechanism of action of this compound. Mannans with significant antitumor activity have been isolated from yeasts and it has been shown that they act mainly by the activation of macrophages. Acemannan causes the activation of mouse macrophages and, in the presence of IFNγ, induces nitric oxide synthase in RAW 264.7 cells (Ramamoorthy *et al.*, 1996). In this article, we report that acemannan in the presence of IFNγ also induces apoptosis in RAW 264.7 cells and that this induction seems to be by a nitric-oxide-independent mechanism.

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Materials and Methods

Reagents and cell culture. The mouse monocytic-macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum and antibiotics (100 units/ml of penicillin-G and 100 μg/ml streptomycin). All cell culture reagents were obtained from Life Biotechnologies (Grand Island, NY). Hoechst 33342 dye, propidium iodide and DMSO (dimethyl sulfoxide) were obtained from Sigma Chemical (St. Louis, MO). Acemannan was obtained from Carrington Laboratories (Dallas, TX) and IFNγ was from Genzyme (Cambridge, MA).

Gel analysis of DNA fragmentation. 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 treated with media alone, acemannan (50 μg/ml), IFNγ (1 unit/ml) or a combination of acemannan and IFNγ for 36 hr. The cells were then

ABBREVIATIONS: IFNγ, interferon γ; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; DEPC, diethylpyrocarbonate; G3PD, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC, fluorescein isothiocyanate; L-NAME, *N*-nitro-L-arginine methyl ester.

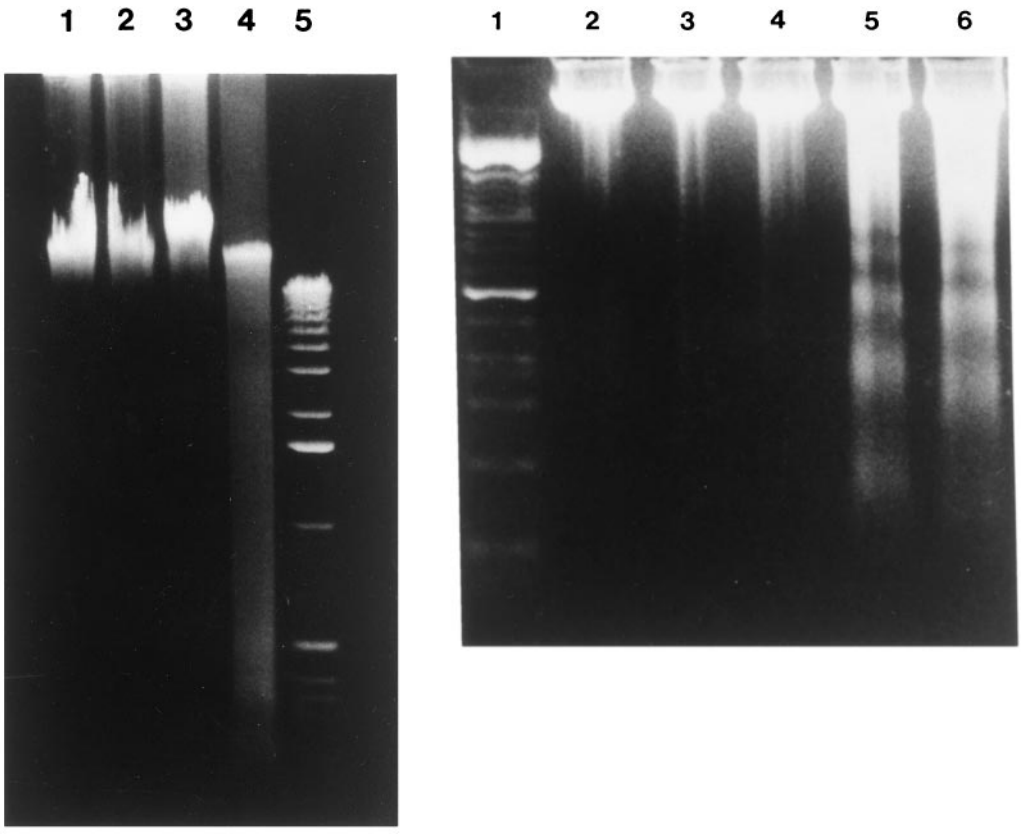

Fig. 1. A, Combination of acemannan and IFNγ causes DNA laddering in macrophages. RAW 264.7 cells were incubated with media alone, acemannan (50 μg/ml), IFNγ (1 unit/ml) or the combination of the two for a period of 36 hr. DNA was isolated and electrophoresed on a 2% agarose gel. The results shown are representative of three independent experiments. *Lane 1*, control; *lane 2* acemannan alone; *lane 3*, IFNγ alone; *lane 4,* acemannan (50 μg/ml) and IFNγ (1 unit/ml); *lane 5,* represents the 123 bp ladder. B, Time course of the induction of apoptosis by acemannan and IFNγ. RAW 264.7 cells were incubated with acemannan (50 μg/ml) in the presence of I FNγ (1 unit/ml) for various time periods at the end of which DNA was isolated and electrophoresed on a 2% agarose gel. The results shown are representative of three independent experiments. *Lane 1*, 123-bp ladder; *lane 2*, medium alone; *lanes 3–6*, treatment with acemannan and IFNγ for 12 hr, 24 hr, 36 hr, and 48 hr.

washed with ice-cold PBS (1 × = 137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), pelleted by centrifugation, lysed in 0.5 ml of lysis buffer (10 mM EDTA, 50 mM Tris-Cl, pH 8, 1% SDS and 250 μg/ml proteinase K) and incubated for 1 hr at 50°. Nucleic acids were extracted from the digested lysates by the phenol/chloroform extraction method (Ausubel *et al.*, 1987; Damle *et al.*, 1993) and then precipitated overnight with cold 100% ethanol at −20°. Nucleic acid precipitates were centrifuged for 15 min at 2000 × *g*, vacuum dried, and resuspended in 20 μl of Tris/EDTA buffer and incubated with 250 μg/ml of RNase at 65° for 5 min to remove RNA. Electrophoresis of the resulting DNA was carried out in 2% agarose gels and DNA was visualized by exposure to UV light and photographed.

Cell staining for apoptotic nuclear morphology. RAW 264.7 cells were plated at a density of 2×10^6 cells/60-mm dish and allowed to adhere for 4 hr. Cells were then treated with media alone or the combination of acemannan (50 μg/ml) and IFNγ (1 unit/ml) for a period of 36 hr and then stained as described (Pelfrey *et al.*, 1995). Briefly, at 36 hr after treatment, the cells were removed and pelleted by centrifugation at 500 × *g*. The supernatant was discarded and the cell pellet was resuspended in 250 μl of Hoechst 33342 dye (stock solution 1 mg/ml in dimethylsulfoxide, diluted 1:200 in PBS). Cells were incubated for 15 min at 37° followed by gentle addition of propidium iodide (20 μg/ml diluted in PBS). The cells were then pelleted, thoroughly resuspended in a very small volume of PBS (15–25 μl) and examined in a fluorescent microscope (Olympus BH2 compound microscope; Olympus, Tokyo, Japan) at 100 × using UV excitation filters. The Hoechst 33342 dye stains morphologically normal nuclei a diffuse pale blue, whereas apoptotic nuclei demonstrate condensed, smaller, and very intensely bright blue nuclei. The cell is stained a diffuse pink with propidium iodide once the membrane of the cell has sustained damage associated with death. Apoptotic cells demonstrate brightly pink condensed nuclei whereas necrotic cells remain diffuse pink.

Thymidine release assay. The thymidine release assay for solubilized low-molecular-weight DNA was performed as a modification of the assay described by Matzinger (1991). Briefly, cells at a concentration of 1×10^5 cells/ml were labeled with 10 μCi/ml [^{3}H]thymidine (86.90 Ci/mmol; DuPont-New England Nuclear, Boston, MA) at 37° for 2 hr. The cells were then washed and plated into 96-well microplates at a cell concentration of 1×10^5/ml in media alone, acemannan (50 μg/ml), IFNγ (1 unit/ml) or a combination of the two for a period of 36 hr. At the end of the incubation period, the cells and their medium were aspirated onto glass fiber filters (Pharmacia Biotech, Piscataway, NJ). In principle, intact chromatin DNA binds to the filter, whereas the fragmented and solubilized DNA does not and thus will be washed away. The filters were washed, dried, and counted in a liquid scintillation counter (Beckman LS3801; Beckman Instruments, Palo Alto, CA). Data are expressed as percentage release of incorporated [^{3}H]thymidine compared with mean control counts ± standard error. Typically, the control wells (no acemannan or IFNγ) had total counts of 20,000–30,000 cpm.

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 per well. Cells were treated with acemannan (50 μg/ml), IFNγ (1 unit/ml) or the combination of the two, and NO_2^- production was measured after 48 hr (Stuehr and Marletta, 1987). 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% H_3PO_4) in 96-well tissue culture plate for 10 min at room temperature. The absorbance at 550 nm was measured in a ELISA plate reader (Dynatech Labs, Chantilly, VA) along with $NaNO_2$ standards. The concentration of protein was determined using the bicinchoninic acid reagent from Pierce (Rockford, IL) (Bradford, 1976).

SDS-PAGE and immunoblotting. RAW 264.7 cells were treated with media alone, acemannan (50 μg/ml), IFNγ (1 unit/ml) or a combination of the two for a period of 36 hr. At the end of 36 hr, cell monolayers were washed with ice-cold PBS and lysed in a buffer

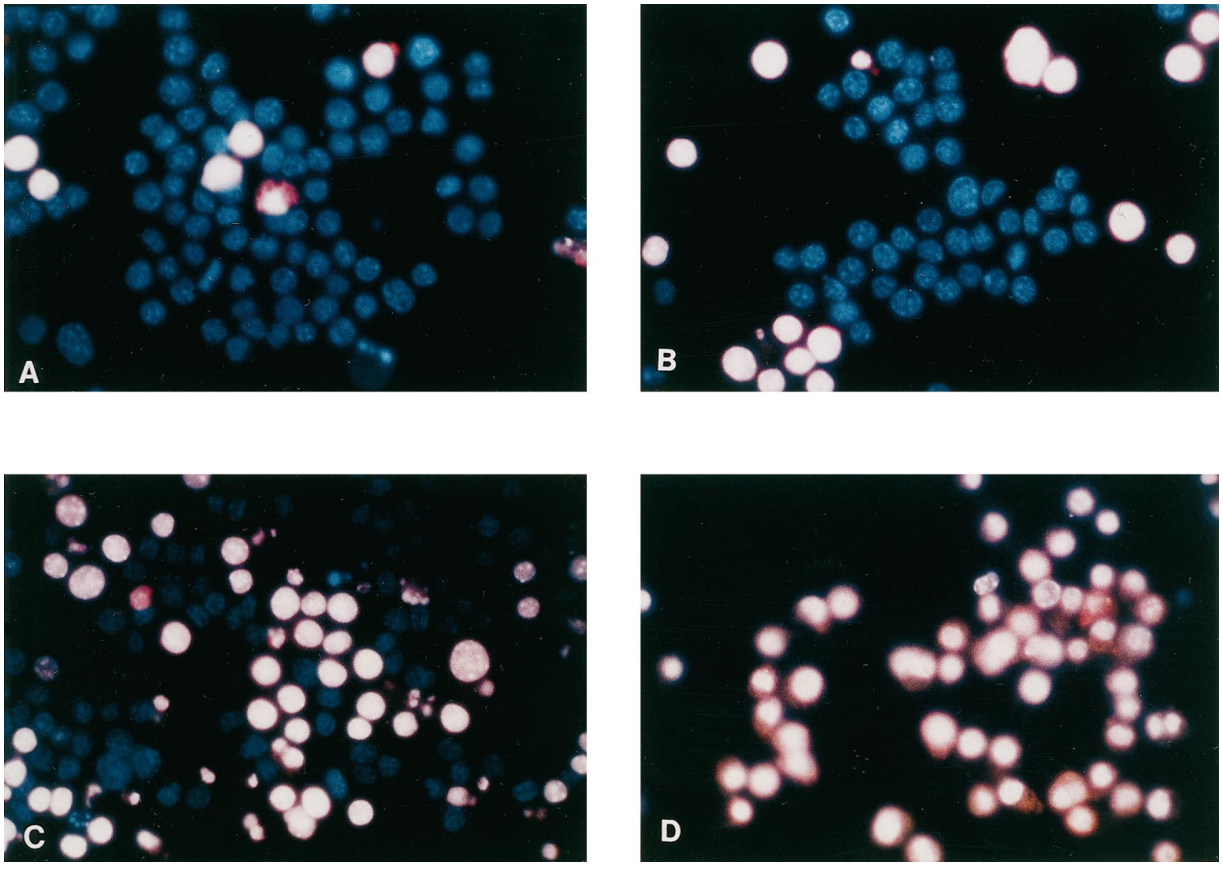

Fig. 2. Acemannan in the presence of IFNγ causes apoptosis in RAW 264.7 cells. RAW 264.7 cells were incubated with either media alone (A), or a combination of acemannan (50 μg/ml) and IFNγ (1 unit/ml) (B-D) for a period of 24 hr, 36 hr, or 48 hr. At the end of the specific time period, cells were harvested, stained with Hoechst 33342 dye and propidium iodide and examined in a fluorescent microscope at 100× using DANS filters. The Hoechst 33342 dye stains morphologically normal nuclei a diffuse pale blue, whereas apoptotic nuclei demonstrate condensed, smaller and very intensely bright blue nuclei. The cell is stained a diffuse pink with propidium iodide once the membrane of the cell has sustained damage associated with death. Apoptotic cells demonstrate brightly pink condensed nuclei, whereas necrotic cells remain diffuse pink.

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% NP-40, and 0.2 mM phenylmethylsulfonyl fluoride. SDS-PAGE (12.5%) was conducted under denaturing, reducing conditions, according to Laemmli (1970). Proteins were transferred onto a PVDF membrane with 0.2-μm pores (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 5% nonfat dry milk in Tris-buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and then incubated with mouse anti-bcl-2 monoclonal antibody (1:5000 dilution; Santa Cruz Biochemicals, Santa Cruz, CA) for 1 hr at room temperature. The membrane was washed with Tris-buffered 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 and 5 mM MgCl2) and developed in a solution of 167.5 μg/ml nitroblue tetrazolium and 167.5 μg/ml 5-bromo-4-chloro-3-indoyl phosphate in alkaline phosphatase buffer.

Isolation of RNA, reverse transcription, and PCR amplification. 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 media alone, acemannan, IFNγ, or the combination of the two, for varying time periods. 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 isolated as described below.

RNA was isolated using the MicroFasttrack kit (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 (Sigma). RNA was precipitated using 0.15 M sodium acetate and 0.1 mg 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 (Searle *et al.*, 1975; Saiki *et al.*, 1985). About 1.5 μM 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 deoxy-NTP 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 deoxy-NTPs (0.2 mM), $MgCl_2$ (2.5 mM), and 1.5 units of *Taq* polymerase (Promega, Madison, WI). The PCR amplimers for mouse *bcl-2* were upstream primer 5′-GAA GTG CCA TTG GTA CCT GC-3′ and downstream primer 5′-GGT CAG ATG GAC ACA TGG TG-3′ (Gonzalez-Garcia *et al.*, 1994; Negrini *et al.*, 1987; Oltvai *et al.*, 1993). The PCR amplimers for mouse *G3PD* were upstream primer 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′ and downstream primer 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′ (Sabath *et al.*, 1990). The primer set for *G3PD* was obtained from Clonetech Laboratories (Palo Alto, CA) while the primers for

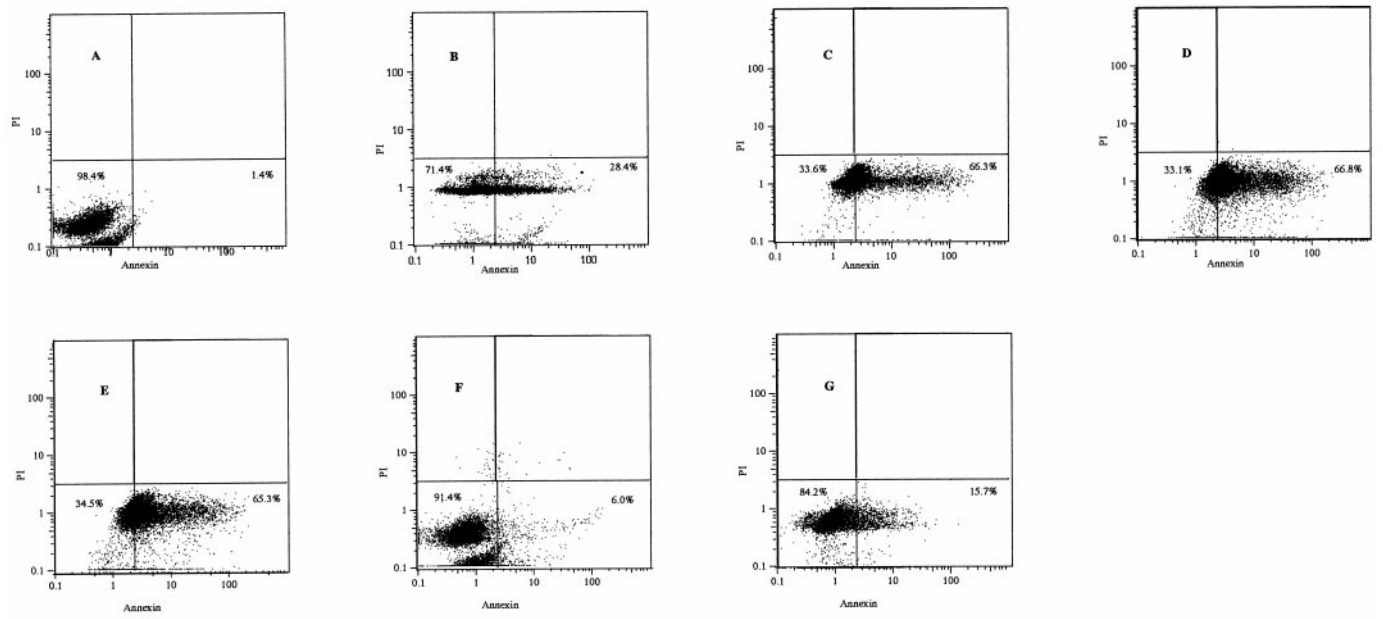

Fig. 3. Annexin V Staining. RAW 264.7 cells were treated with acemannan (50 μg/ml) in the presence of IFNγ (1 U/ml) for various time periods. At the end of the time period cells were stained with FITC conjugated annexin V, and the binding of annexin V was quantified by flow cytometry on a FACS-Calibur. Fig. 4A represents control, Figs. 4B-4E represent treatment with acemannan and IFNγ for 18 hr, 24 hr, 30 hr and 36 hr, Fig. 4F represents treatment with acemannan alone and Fig. 4G represents treatment with IFNγ alone. The percentage of cells staining negative for annexin V (*lower left quadrant*) and the percentage of cells staining positive for annexin V (*lower right quadrant*) is indicated in the figure. The results shown are representative of three independent experiments.

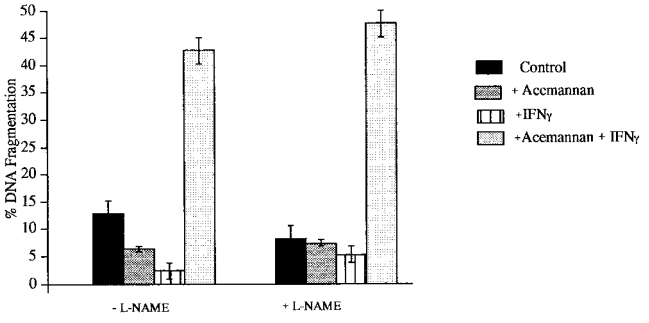

Fig. 4. L-NAME does not inhibit the apoptosis caused by acemannan in the presence of IFNγ RAW 264.7 cells were treated with acemannan and IFNγ in the presence and absence of L-NAME for a period of 48 hr. DNA fragmentation was then analyzed in these samples using the thymidine release assay. Data are expressed as percent DNA fragmentation: 100 $\times$ (1 $-$ cpm in experimental group/cpm of untreated cells) + mean $\pm$ standard error and are representative of three independent experiments.

bcl-2 were synthesized as oligonucleotides by the Gene Technologies Laboratory (Department of Biology, Texas A & M University, College Station, TX). The amplification was carried out in 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) and this was cycled 30 times. The amplified products were analyzed on a 2% agarose gel (Ausubel *et al.*, 1987) .

Southern Hybridization. The specificity of the products obtained by amplification of the cDNAs was verified by Southern analysis. The Southern transfer of amplified cDNA was from a 1.5% agarose gel run in Tris/borate/EDTA (1$\times$ = 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 protocol. The *Eco*RV/*Bam*HI fragment of *G3PD* and the 379-bp fragment of *bcl-2* were used as probes. The hybridization probes were radiolabeled with [^{32}P]deoxy-ATP (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 $\times$ standard saline/phosphate/EDTA (1$\times$ = 150 mM NaCl, 10 mM NaH_2PO_4, and 10 mM EDTA, pH 7.4)/2% SDS with 10 μg/ml salmon sperm DNA (Ausubel *et al.*, 1987). Mouse bcl-2 probe (the 865-bp mouse *bcl-2* gene cloned into pBluescript KS) was also used for southern analysis; this was a gift from Dr. Stanley J. Korsmeyer.

The PCR fragments obtained using mouse G3PD primers and the bcl-2 primers were cloned into pcTR11 vector (TA cloning kit from InVitrogen) and the sequence verified by restriction analysis and dideoxy sequencing (Ausubel *et al.*, 1987).

Annexin V staining. RAW 264.7 cells were plated at a density of 2 $\times$ 10^6 cells/60 mm dish and allowed to adhere for 4 hr. Cells were then treated with media alone or the combination of acemannan (50 μg/ml) and IFNγ (1 unit/ml) for a period of 36 hr and then stained as described (Vermes *et al.*, 1995; Boersma *et al.*, 1997). Briefly, cells were washed with PBS, pelleted by centrifugation at 500 $\times$ *g* and the cell pellet was resuspended in 1$\times$ binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$. To a 100-μl aliquot of the cell suspension, 10 μl of propidium iodide (50 μg/ml) was added, followed by 5 μl of Annexin V [FITC-conjugated Annexin V from PharMingen (San Diego, CA)] and cells were incubated in the dark for 15 min at room temperature. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and the following parameters were measured: forward light scatter, side light scatter, phosphatidylethanolamine fluorescence (575–590 nm) and FITC fluorescence (515–545 nm). Forward light scatter and side light scatter were measured in linear mode, whereas phosphatidylethanolamine and FITC were measured in a logarithmic mode and a total of 10,000 events were analyzed. Analysis of the data was performed using the CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Results

Acemannan in the presence of IFNγ causes apoptosis in macrophages. RAW 264.7 cells were treated with acemannan in the presence and absence of IFNγ for a period of 48 hr and the cells were then analyzed for the occurrence

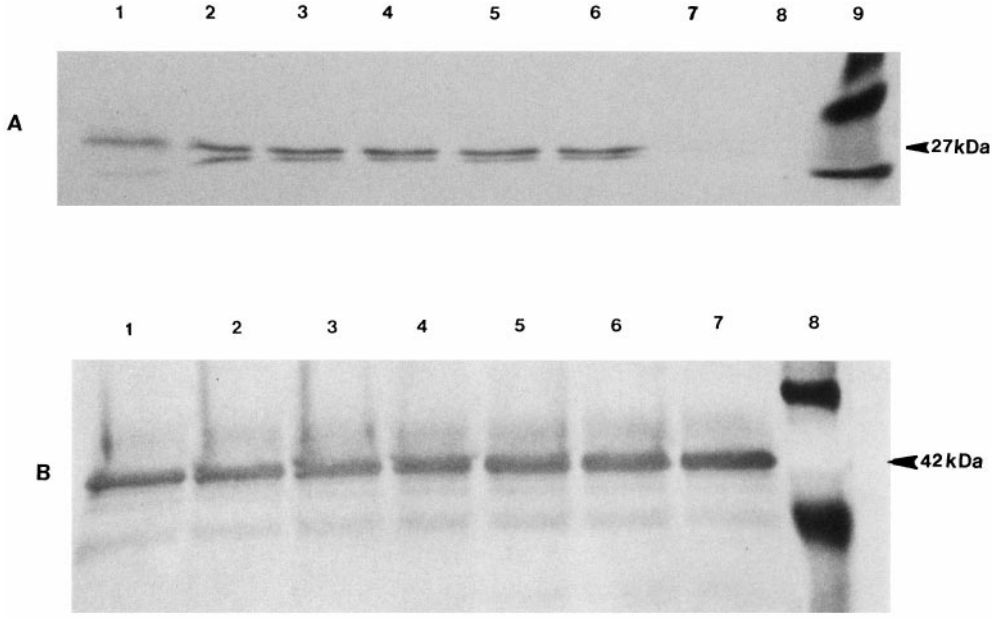

Fig. 5. Time course of bcl-2 expression. RAW 264.7 cells were treated with acemannan (50 μg/ml) and IFNγ (1 unit/ml) for various time periods. Total cell lysates were prepared and subjected to SDS-PAGE (12% gel for bcl-2 and 10% gel for actin) blotted on to PVDF membranes and probed (A) anti-bcl-2 antibody and (B) anti-actin antibody. The results shown are representative of three independent experiments. A, *lane 1*, the positive control (M1 cell lysate from PharMingen); *lane 2* represents treatment with media alone and lanes 3–8 represent treatment with acemannan and IFNγ for 6 hr, 12 hr, 18 hr, 24 hr, 30 hr, and 36 hr, and lane 9 represents the migration of the molecular weight markers. B, *lane 1*, represents media alone; *lanes 2–7*, treatment with acemannan and IFNγ for 6 hr, 12 hr, 18 hr, 24 hr, 30 hr and 36 hr; *lane 8;* the migration of the molecular weight markers.

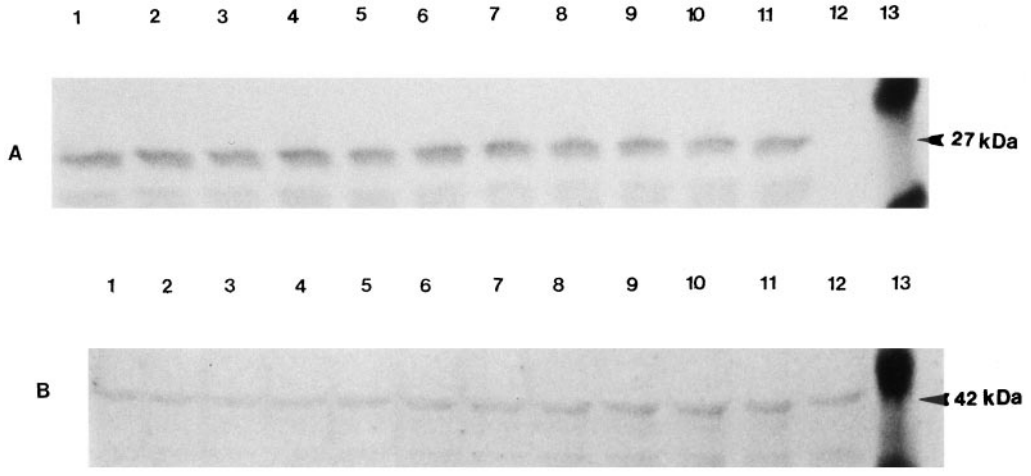

Fig. 6. Acemannan in the presence of IFNγ causes a decrease in the expression of bcl-2. RAW 264.7 cells were treated with media alone, acemannan (50 μg/ml), IFNγ (1 unit/ml) or the combination of the two for varying time periods. Total cell lysates were prepared and subjected to SDS-PAGE (12%) blotted on to a PVDF membrane and (A) probed with anti-bcl-2 antibody or (B) probed with anti-actin antibody. Lane 1, represents media alone, *lane 2,* acemannan treatment for 12 hr, *lane 3,* IFNγ treatment for 12 hr; *lane 4,* treatment with acemannan and IFNγ for 12 hr; *lane 5,* media alone for 24 hr; *lane 6*, acemannan alone for 24 hr; *lane 7,* IFNγ for 24 hr, *lane 8,* acemannan and IFNγ for 24 hr; *lane 9*, media alone for 48 hr; *lane 10,* acemannan alone for 48 hr; *lane 11,* IFNγ alone for 48 hr; *lane 12* acemannan in the presence of IFNγ for 48 hr; *lane 13,* the migration of molecular weight markers. The bcl-2 band is 27 kDa.

of apoptosis by DNA fragmentation assay, gel electrophoresis, and propidium staining. Analysis of the DNA by agarose gel electrophoresis indicated the occurrence of apoptosis. The DNA laddering characteristic of apoptosis was observed in cells treated with acemannan and IFNγ (Fig. 1A). Acemannan by itself or IFNγ (1 unit/ml) did not cause any DNA laddering. In cells treated with acemannan (50 μg/ml) and IFNγ (1 unit/ml), DNA fragmentation occurred around 36 hr after treatment, and by 48 hr, most of the cells had undergone apoptosis (Fig. 1B). Staining of the cells with Hoechst 33342 followed by propidium iodide also indicates that apoptosis is occurring in RAW 264.7 cells treated with acemannan and IFNγ (Fig. 2).

Cells undergoing apoptosis lose membrane phospholipid asymmetry and expose phosphatidylserine on the outer leaflet of the plasma membrane. The detection of phosphatidylserine exposure by annexin V during the redistribution of the plasma membrane has been shown to be a general and early marker of apoptosis. Annexin V staining of RAW 264.7 cells treated with acemannan and IFNγ showed that apoptosis occurred in these cells at 18 hr after treatment and by 36 hr about 65% of the cells had undergone apoptosis (Fig. 3). All these results suggest that acemannan in the presence of IFNγ causes apoptosis in RAW 264.7 cells.

Apoptosis caused by acemannan in the presence of IFNγ is not NO- dependent. Acemannan in the presence of IFNγ causes the induction of the inducible nitric oxide synthase in RAW 264.7 cells (Ramamoorthy *et al.*, 1996). Nitric oxide is known to cause apoptosis in some cell types and this could account for the apoptosis observed in these cells. To verify this, an inhibitor of nitric oxide production L-NAME (Sigma) was used. RAW 264.7 cells were treated with ace-

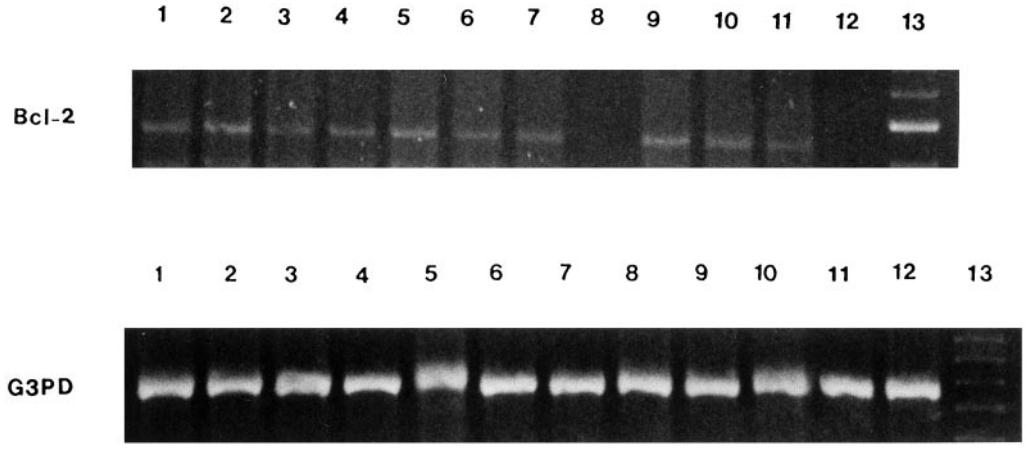

Fig. 7. Acemannan in the presence of IFNγ causes a decrease in the level of bcl-2 mRNA. RAW 264.7 cells were incubated media alone, acemannan (50 μg/ml), IFNγ (1 unit/ml), or the combination of the two for the indicated time periods. mRNA was isolated and reverse transcribed using oligo dT primers. Identical amounts of the cDNA were amplified by PCR in two separate reactions using primers for either bcl-2 or G3PD. The bcl-2 product was electrophoresed on a 2% agarose gel while the G3PD product was analyzed on a 1.2% agarose gel. The amount of bcl-2 PCR product used for electrophoresis was 3 times that of G3PD. *Lane 1*, media alone; *lane 2*, acemannan treatment for 12 hr; *lane 3,* IFNγ treatment for 12 hr; *lane 4,* treatment with acemannan and IFNγ for 12 hr; *lane 5,* media alone for 24 hr; *lane 6* acemannan alone for 24 hr; *lane 7*, IFNγ for 24 hr; *lane 8*, acemannan and IFNγ for 24 hr; *lane 9,* media alone for 48 hr; *lane 10*, acemannan alone for 48 hr; *lane 11*, IFNγ alone for 48 hr; *lane 12,* acemannan in the presence of IFNγ for 48 hr; *lane 14*, the migration of the 100-bp ladder. The amplified bcl-2 fragment is 379 bp, whereas the amplified G3PD fragment is 983 bp. The results shown are representative of three independent experiments.

mannan and IFNγ in the presence and absence of L-NAME and occurrence of apoptosis was studied at the end of 48 hr using the thymidine release assay. Acemannan by itself did not cause any significant DNA fragmentation, but the combination of the two caused a 3–4-fold increase in the fragmentation of DNA. L-NAME inhibited the production of nitric oxide by cells treated with acemannan and IFNγ (data not shown) but did not inhibit DNA fragmentation in these cells, which suggests that nitric oxide may not be involved in this induction of apoptosis caused by the combination of acemannan and IFNγ (Fig. 4).

Effect of acemannan and IFNγ on the expression of *bcl-2*. Acemannan in the presence of IFNγ caused a decrease in the expression of *bcl-2*. RAW 264.7 cells were treated with acemannan in the presence of IFNγ for various time periods and the expression of *bcl-2* was studied by Western analysis. The expression of *bcl-2* could be detected in all cells at 12-hr, 18-hr, and 24-hr post-treatment. However, bcl-2 expression could not be detected in cells treated with acemannan and IFNγ for 30 hr and the effect continued even at 36 hr after treatment (Fig. 5). This was observed only when the cells were treated with acemannan in the presence of IFNγ. Acemannan and IFNγ by themselves did not cause any decrease in the expression of *bcl-2* (Fig. 6) To verify if the inhibition of bcl-2 occurred at the level of transcription, we examined the steady state mRNA levels of bcl-2.

RAW 264.7 cells were treated with acemannan in the presence and absence of IFNγ for varying time periods, at the end of which mRNA was isolated and the level of *bcl-2* was studied using RT-PCR. The *bcl-2* gene was expressed in all cells at 12 hr after treatment, but by 24 hr, the gene seems to be turned off in cells treated with acemannan and IFNγ and continues to be so even at 48 hr after treatment (Fig. 7). Thus acemannan in the presence of IFNγ causes the induction of apoptosis in RAW 264.7 cells by a mechanism involving *bcl-2*.

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. It has also been shown to have antitumor activity; injection of acemannan has been shown to offer increased immune protection against implanted malignant tumor cells (Merriam *et al.*, 1995). Animals that recovered from the Norman murine sarcoma transplant rejected subsequent tumor transplants. However it is unclear how acemannan exerts this wide variety of effects and we believe that some of these effects are mediated through the macrophages.

Apoptosis, or programmed cell death, is believed to be an intrinsic death program that cells activate; thus, they actively contribute to their own deaths. The nucleus undergoes a relatively characteristic metamorphosis during apoptosis, first chromatin condensation, then nuclear condensation (Pelfrey *et al.*, 1995). This characteristic feature of DNA fragmentation and laddering was observed in RAW 264.7 cells treated with acemannan and IFNγ. Murine macrophages are extremely sensitive to the induction of apoptosis by inhibitors of macromolecular synthesis. Many cancer chemotherapeutic agents, such as dactinomycin, doxorubicin, and cycloheximide, have been shown to cause apoptosis in mouse peritoneal macrophages (Lewis *et al.*, 1995). RAW 264.7 cells treated with acemannan (50 μg/ml) and IFNγ (1 unit/ml) undergo the classical morphological changes associated with apoptosis. DNA fragmentation and the characteristic DNA laddering was observed in these cells. However, the mechanism by which this apoptosis is induced is not very clear. The transcriptional activator interferon regulatory factor 1 has been shown to play a critical role in the induction of apoptosis (Tanaka *et al.*, 1994; Tamura *et al.*, 1995). Because apoptosis could be induced by acemannan only in the presence of IFNγ, it is quite likely that it involves the activation of interferon regulatory factor 1. However more stud-

ies need to be performed before its role can be clearly demonstrated.

Acemannan in the presence of IFNγ causes the induction of nitric oxide synthase in RAW 264.7 cells. Nitric oxide has been shown to induce apoptosis in these cells (Messmer *et al.*, 1995). However the induction of apoptosis by acemannan does not seem to be mediated by nitric oxide, because L-NAME had no effect.

Expression of *bcl-2* was inhibited in RAW 264.7 cells treated with acemannan and IFNγ for a period of 36 hr. Members of the *bcl-2* family (*bcl-2, bcl-x, bax,* etc.) play a prominent role in apoptosis or preventing it (Meikrantz *et al.*, 1994; Kroemer *et al.*, 1995; Merino *et al.*, 1995; Wang *et al.*, 1995). They are important molecular switches. Overexpression of bcl-2 can delay or block growth factor withdrawal-induced apoptosis indicating that bcl-2 plays an important role in preventing cell death. However, bcl-2 does not prevent apoptosis of ciliary neurons after ciliary neurotrophic factor withdrawal suggesting that the effect of bcl-2 is context dependent. RAW 264.7 cells treated with acemannan (50 μg/ml) and IFNγ (1 unit/ml) do not express *bcl-2* and this is when apoptosis is observed in these cells. These results suggest that acemannan in the presence of IFNγ induces apoptosis in RAW 264.7 cells through a mechanism involving the inhibition of bcl-2 expression.

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