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Activation of a mouse macrophage cell line by acemannan: The major carbohydrate fraction from *Aloe vera* gel

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Abstract

Acemannan is the name given to the major carbohydrate fraction obtained from the gel of the *Aloe vera* leaf. It has been claimed to have several important therapeutic properties including acceleration of wound healing, immune stimulation, anti-cancer and anti-viral effects. However, the biological mechanisms of these activities are unclear. Because of this wide diversity of effects, it is believed that they may be exerted through pluripotent effector cells such as macrophages. The effects of acemannan on the mouse macrophage cell line, RAW 264.7 cells were therefore investigated. It was found that acemannan could stimulate macrophage cytokine production, nitric oxide release, surface molecule expression, and cell morphologic changes. The production of the cytokines IL-6 and TNF-α were dependent on the dose of acemannan provided. Nitric oxide production, cell morphologic changes and surface antigen expression were increased in response to stimulation by a mixture of acemannan and IFN-γ. These results suggest that acemannan may function, at least in part, through macrophage activation.

Keywords: Acemannan; Macrophage; Macrophage activation; RAW cells

1. Introduction

Acemannan is a major carbohydrate fraction derived from the central gel of the leaf from *Aloe vera* L. It is predominantly a β-(1,4)-linked galactomannan and the mannose residues are acetylated (Reynolds, 1985).

Acemannan has significant beneficial therapeutic effects. Thus, acemannan immunostimulant is employed for the treatment of fibrosarcomas in dogs and cats (Manna and McAnalley, 1993). Acemannan has been reported to promote healing of aphthous ulcers in humans (Grinalay and Reynolds, 1986) and accelerated wound healing of biopsy-punch wounds in rats (Tizard et al., 1994). Studies in vitro indicate that acemannan has limited anti-viral activity against herpes viruses, measles, and human immunodeficiency virus (Chinnah, 1991). However, the biological mechanisms of these functions are largely unknown. Given this wide diversity of biological activities it has been suggested that a common mechanism may involve macrophage activation. For this reason,

Abbreviations: FcR Fc receptor; IFN-γ Interferon gamma; IL-1β Interleukin 1 beta; IL-2 Interleukin 2; IL-6 Interleukin 6; LPS Lipopolysaccharide; Mac-1 Macrophage antigen 1; NO Nitric oxide; TNF-α Tumor necrosis factor alpha

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we have examined the effects of acemannan on macrophage function.

Macrophage activation is normally mediated by a wide variety of mechanisms. These include exposure to cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2). They are also activated by exposure to bacteria, bacterial products, and particulates (Adams and Hamilton, 1984). Macrophages can destroy tumor cells after treatment with both recombinant IFN-γ and bacterial lipopolysaccharide (LPS), suggesting that at least two stimuli are required for complete activation (Drysdale et al., 1988). It has been shown that each of these molecules interacts with specific high affinity receptors located on the macrophage plasma membrane (Lorsbach et al., 1993).

In the present study, IFN-γ was used as a positive control for macrophage activation. Macrophage activation was expressed as increased cell size, cytoplasmic spreading, increased nitric oxide (NO) production, increased cytokine release, and increased expression of some adhesion molecules and Fc receptors (FcRs). It was found that acemannan could stimulate macrophages to release interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The production of nitric oxide, cell morphological changes and surface molecule expression were not significantly changed in response to acemannan alone. However, these effects could be potentiated by the addition of IFN-γ. Thus these results demonstrate the ability of acemannan to activate RAW cells both directly for cytokine production and indirectly for NO production and some of the surface molecule expression.

2. Materials and methods

2.1. Materials

The mouse macrophage cell line (RAW 264.7 cells) was obtained from the American Type Culture Collection (Rockville, Maryland). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, Grand Island), which was

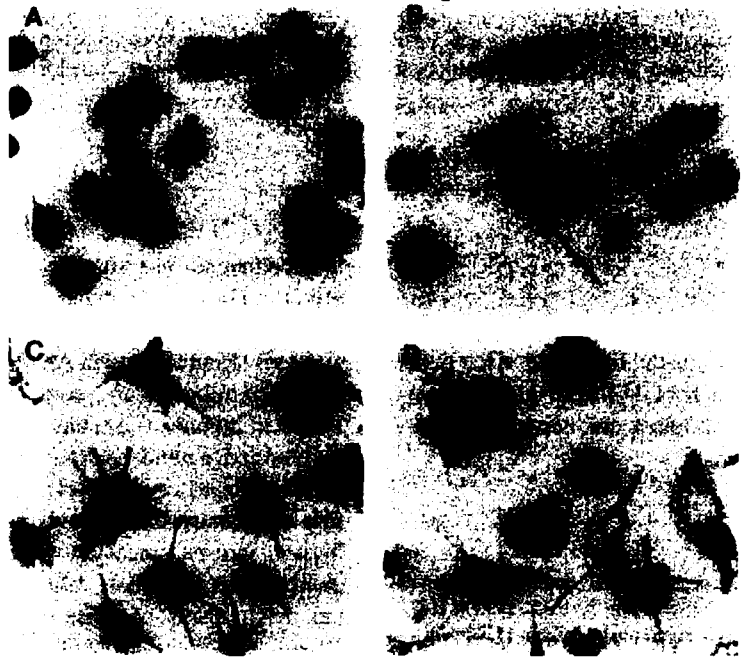

Fig. 1. Macrophage morphological changes in response to acemannan. RAW cells were cultured on cover-slips in the presence of 100 μg/ml of medium (A), acemannan (B), acemannan/IFN-γ (C), or IFN-γ (D) and for 24 h.

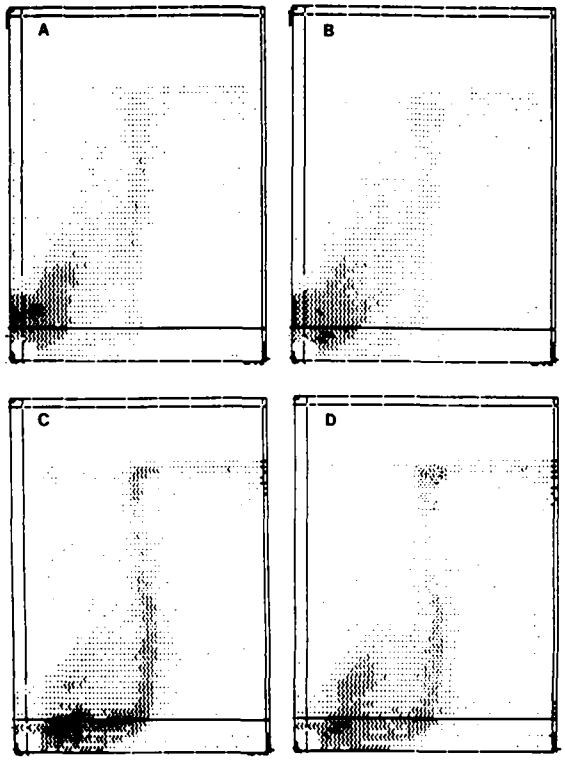

Fig. 2. Flow cytometry analysis of macrophage morphological changes. RAW cells were incubated in the presence of either medium (A), acemannan (100 μg/ml) (B), acemannan/IFN-γ (C), or IFN-γ (D) for 24 h.

supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island), and 1% (v/v) penicillin (10000 U/ml)/streptomycin (10000 U/ml) (P/S) (Gibco BRL). Acemannan (lot # 020401) was obtained as a white freeze dried powder from Carrington Laboratories (Irving, TX). When suspended in saline this formed a cloudy suspension. Recombinant mouse IFN-γ was obtained from Genzyme (Cambridge, MA). Anti-CD11a-FITC (Isotype IgG_{2a}) and anti-CD18-FITC (Isotype IgG_{2a}) were obtained from Sigma (St. Louis, MO). Anti-Mac-1-FITC (Isotype rat IgG_{2b}) (Clone M1/70) was obtained from Boehringer Mannheim (Indianapolis, IN). Anti-CD32/16 (FcRII/III) (Isotype IgG_{2b}) (Clone 2.4 G2) was obtained from Pharmingen (San Diego, CA). Rat-IgG-FITC was obtained from Accurate Chemical and Science (Deerfield, IL).

2.2. Cell staining

To determine the effects of acemannan on macrophage morphology, cells were cultured in sterile glass-slide chambers at a density of 1000 cells/well for three hours. The culture medium was removed. Cells were treated with either acemannan (100 μg/ml), IFN-γ (10 U/ml), acemannan/IFN-γ or medium for 24 h. Following the treatment, the culture supernatant was removed. Cells were fixed and stained in Diff-quick solution (Baxter, Houston, TX). The cells were then examined by a reader unaware of the treatments.

2.3. Flow cytometry

RAW cells (10^6/T25 flask) were cultured in DMEM for three hours. The media were changed and the cells were incubated in the presence of either acemannan (100 μg/ml), IFN-γ alone (10 U/ml), acemannan/IFN-γ (acemannan 100 μg/ml/IFN-γ 10 U/ml) or medium for 24 h. Treated cells were then scraped into PBS–0.1% sodium azide with 1% FBS (PBS-washing buffer) (pH 7.2) and washed twice in PBS-washing solution at 4°C. Before cells were stained with FITC-monoclonal antibody, the cell surface Fc receptors were blocked by incubating cells with 20 μg/10^6 cells of purified anti-mouse CD32/16 for 30 min at 4°C. This antibody was not

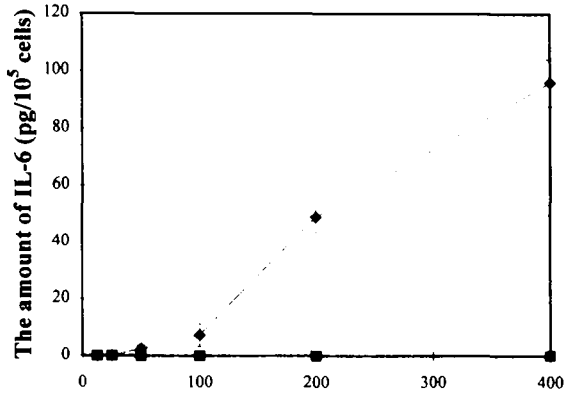

Fig. 3. IL-6 production in response to acemannan. RAW cells were exposed to either acemannan or medium for 24 h. The IL-6 was measured by ELISA. Each dilution was tested in duplicate. – ◆ – Acemannan treatment alone. – ■ – Medium alone.

removed by washing. Anti-Mac-1-FITC, anti-CD11a-FITC, and anti-CD18-FITC were then added and cells were kept at 4°C for 30 min. Cells stained using rat IgG-FITC served as a control for nonspecific binding. Cells were then washed and fixed in cold PBS containing 1% paraformaldehyde (pH 7.2) (Sigma). Flow cytometry analysis was performed on an EPICS V analyzer (Coulter, Hialeah, EL). Fluorescence intensity was determined on 10,000 cells from each sample using logarithmic amplification.

2.4. Cytokine assays

RAW cells were cultured in DMEM in 96-well-flat plates at a density of 10^5 cells/well. Cells were treated in the presence or absence of acemannan at different concentrations. Each dilution was tested in triplicate. The media were removed after 24 h treatment. IL-6 and TNF-α (Genzyme, Cambridge, MA) levels were measured using ELISA kits according to the manufacture's instruction. Data are presented as a mean value from three independent experiments.

2.5. Nitric oxide measurement

NO production was determined by measuring the accumulation of the nitrite using the method described by Stuehr and Nathan (1989). RAW 264.7 cells were cultured in DMEM for three to four hours at a density of 1×10^5 cells/well. After cells adhered to the plate, they were treated with 100 μl of either acemannan, acemannan with 10 U/ml of IFN-γ, or 10 U/ml of IFN-γ, or medium for 48 h (100 μl/well). At the end of the incubation period, 50 μl of supernatant was removed from each well into a new plate. Fifty μl of Griess reagent (Stuehr and Nathan, 1989) was added to each well, mixed and incubated at room temperature for ten minutes. The amount of colored product was determined spectrophotometrically at 570 nm in an automated ELISA reader (MR 600, Dynatech, Torrance, CA). The amount of nitrite was estimated according to the standard curve generated using known concentrations of sodium nitrite. Results are presented as pmol nitrite/10^5 cells.

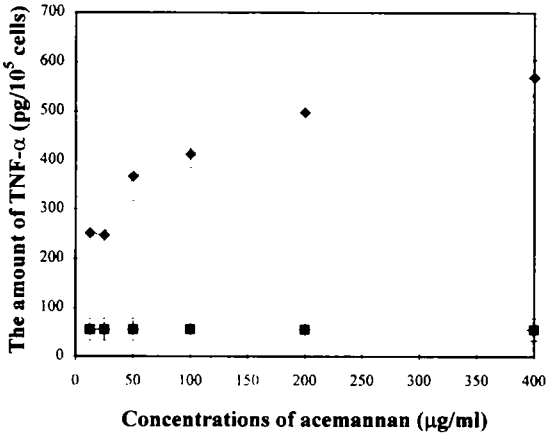

Fig. 4. TNF-α production in response to acemannan. RAW cells were exposed to either acemannan or medium for 24 h. TNF-α was measured by ELISA. Each dilution was tested in duplicate. – ◆ – Acemannan treatment alone. – ■ – Medium alone.

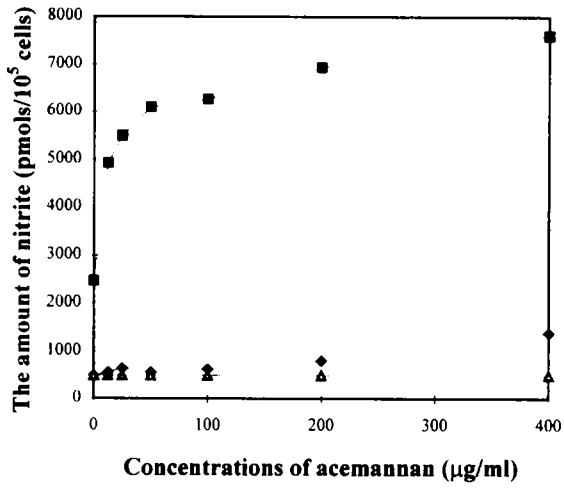

Fig. 5. Nitric oxide production in response to acemannan. The NO produced was measured using Griess reagent. The amount of NO produced was determined using a standard sodium nitrite curve. Interferon-γ was used at a concentration of 10 U/ml. – ◆ – Acemannan treatment alone. – ■ – Acemannan in the presence of 10 U of IFN-γ. – △ – Medium alone.

2.6. Attachment and/or phagocytosis of antibody-coated sheep red blood cells by RAW cells (Rosetting assay)

RAW cells were cultured in sterile culture chambers at a density of 1000 cells/well for three hours. The medium was removed and cells were exposed to

acemannan (50 μg/ml), IFN-γ (10 U/ml) or acemannan/IFN-γ for 24 h. One percent sheep RBC suspension was incubated with heated anti-sheep-RBC antibody at a subagglutinating dilution for one hour. These antibody-coated sheep RBCs (EA) were then added to RAW cell cultures treated by different stimuli and incubated at room temperature for one hour. RAW cells exposed to untreated RBCs were used as a control. After washing to remove the unattached sheep RBCs, cells were fixed and stained. They were examined by a reader unaware of the treatments applied.

2.7. Statistical analysis

Nitrite and cytokine productions were expressed as means ± SD of two to six similar independent experiments. Statistical significance was determined using the Student-*t* test.

3. Results

3.1. Effects of acemannan and IFN-γ on macrophage morphology

Normal RAW cells, when cultured in medium alone, tended to be round. None of them appeared to have spread on the surface (Fig. 1A). Minimal morphological changes (5%) were observed in cells exposed to acemannan alone (Fig. 1B). Approximately 40% of cells had spread cytoplasm in the presence of IFN-γ alone (Fig. 1C). In contrast, approximately 90% of the cells exposed to acemannan and IFN-γ

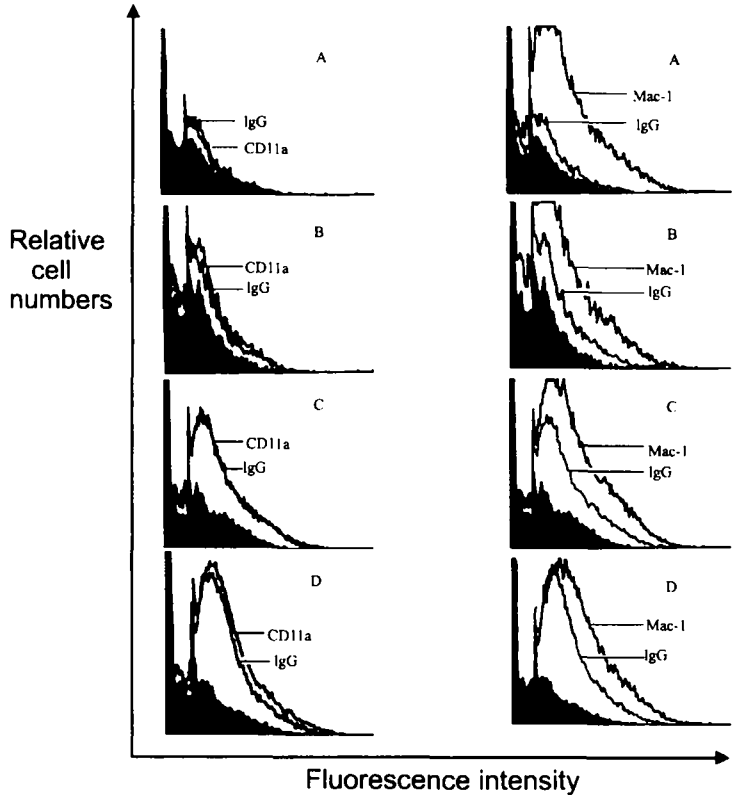

Fig. 6. Cell surface CD11a and Mac-1 expression. RAW cells were cultured in the presence of medium (A), acemannan (B), IFN-γ (C), or acemannan/IFN-γ (D) for 24 h. Cell surface CD11a and Mac-1 molecules were labeled with either anti-CD11a-FITC or anti-Mac-1-FITC. The *X*-axis shows the fluorescence density. The *Y*-axis shows relative cell numbers. The shaded curve denotes background fluorescence.

were greatly enlarged with extensive cytoplasmic spreading (Fig. 1D).

In addition, the cell sizes and surface characteristics were examined by measuring forward angle light scatter (FALS) and orthogonal light scatter (OLS) with flow cytometry. As shown in Fig. 2A, normal RAW cells consisted of one major group of cells with low FALS and low OLS. Cells exposed to acemannan alone were comparable in FALS and OLS to the control cells although occasional high FALS cells were observed (Fig. 2B). Cells exposed to IFN-γ alone consisted of two major populations. One had low to medium FALS with high OLS. The other population showed high FALS with high OLS (Fig. 2C). In contrast to the above results, most cells showed medium to high FALS with high OLS when exposed to acemannan/IFN-γ (Fig. 2D). Since the forward angle light scatter gives an estimate of cell size while orthogonal light scatter gives an estimate of membrane ruffling and granularity, these results suggested that cells treated with acemannan/IFN-γ were larger and rougher than those exposed to either acemannan or IFN-γ alone. Together, results were consistent with the light microscopy observations and suggested that more activated macrophages were obtained by exposure to the combination of acemannan and IFN-γ. Acemannan alone induced minimal macrophage morphologic changes, IFN-γ stimulated more macrophage activation, and much greater macrophage morphological changes were generated by the combined action of acemannan and IFN-γ.

3.2. Direct cytokine production in response to acemannan

To determine whether acemannan had a direct effect on cytokine production, the amounts of IL-6 and TNF-α released were measured. IL-6 and TNF-α were released in a dose dependent manner in response to acemannan stimulation (Fig. 3 and Fig. 4). Results suggested that the cytokine production did not require the presence of IFN-γ. The presence of IFN-γ (10 U/ml) in medium did however increase IL-6 and TNF-α release by approximately 40% (data not shown).

3.3. The effects of acemannan on nitric oxide production

As shown in Fig. 5, macrophages did not release nitric oxide in response to medium. A minimum amount of NO was released when the cells were

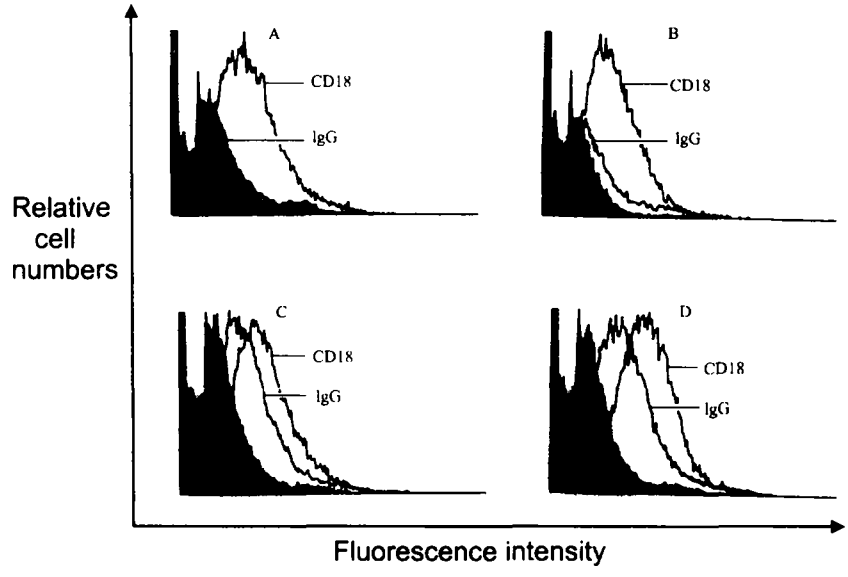

Fig. 7. Cell surface CD18 expression. RAW cells were cultured in the presence of medium (A), acemannan (B), IFN-γ (C), or acemannan/IFN-γ (D) for 24 h. Cell surface CD18 was labeled with ant-CD18-FITC. The *X*-axis shows the fluorescence density. The *Y*-axis shows relative cell numbers. The shaded curve denotes background fluorescence.

exposed to acemannan alone. A dose dependent increase in nitric oxide production was observed in IFN-γ treated cells. A plateau in nitric oxide production was observed when cells were exposed to high concentration of IFN-γ (data not shown). Significantly more NO was produced when cells were

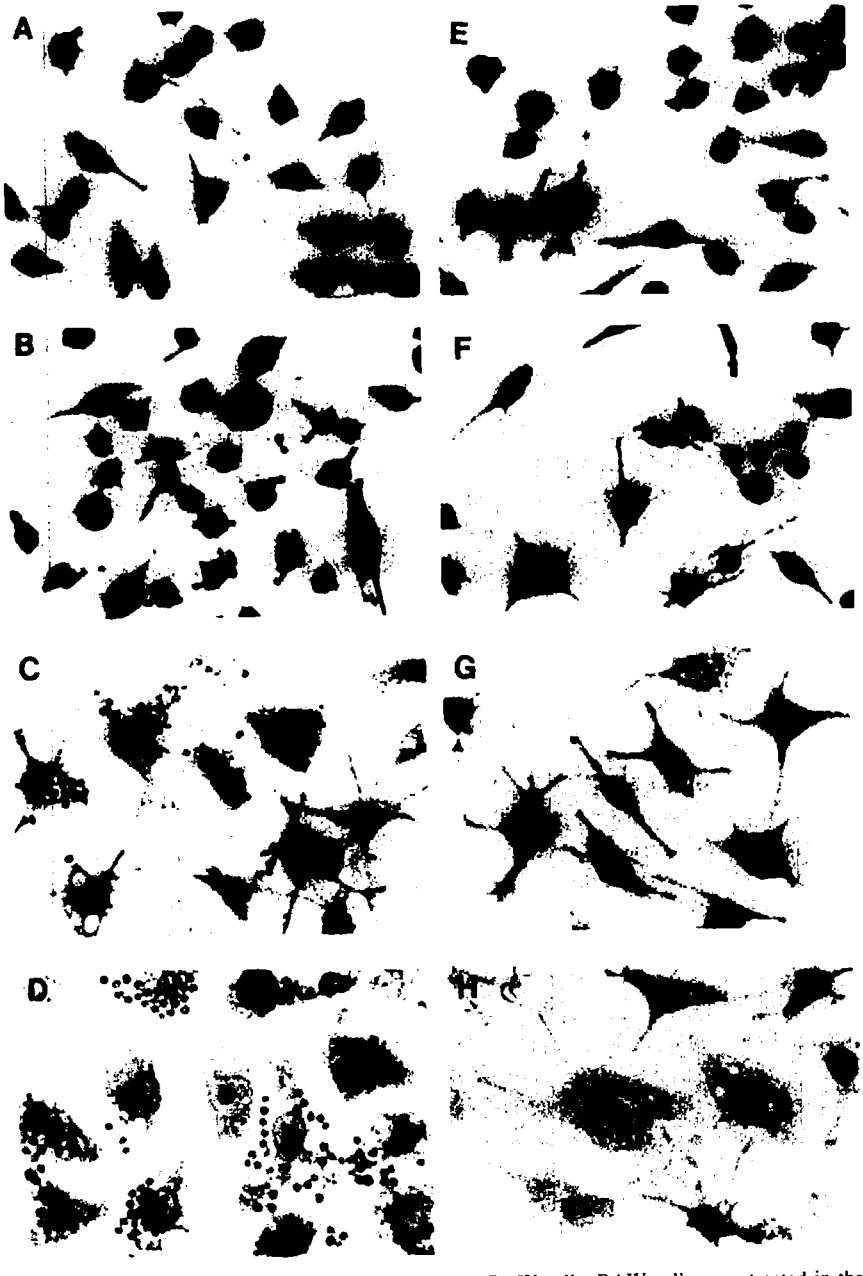

Fig. 8. The attachment and/or phagocytosis of antibody coated RBCs by RAW cells. RAW cells were treated in the presence of medium alone (A and E), acemannan alone (B and F), IFN-γ (C and G), or acemannan/IFN-γ (D and H) for 24 h. Cells were incubated in the presence of either 1% antibody coated sheep red blood cells in saline (A–D) or 1% sheep red blood cells in saline (E–H) at room temperature for one hour.

Table 1
The effects of nitric oxide on short term exposure to acemannan. RAW cells were treated with the first reagents for four hours. Cells were washed three times in DMEM and exposed to the second signal for another 44 h. The nitrite level was determined at 48 h post-treatment

First signal (4 h)	Second signal (44 h)	Nitrite production (nmol/10^5 cells)
Medium	medium	421 ± 278
Acemannan (100 μg/ml)	medium	430 ± 291
Acemannan (100 μg/ml)	IFN-γ	4430 ± 378
Acemannan (100 μg/ml)	acemannan	600 ± 282
IFN-γ (10 U/ml)	medium	1860 ± 413
IFN-γ (10 U/ml)	acemannan	4300 ± 516
IFN-γ (10 U/ml)	IFN-γ	2430 ± 393
Acemannan/IFN-γ	medium	5026 ± 198
Acemannan/IFN-γ	acemannan/ IFN-γ	5964 + 246

exposed to combination of acemannan and IFN-γ. This suggested that more activated macrophages or higher level of macrophage activation were obtained from cells treated with acemannan and IFN-γ together.

3.4. The effects of acemannan on surface molecule expression

Since the adhesion molecules play an important role in macrophage activation process, RAW cell surface expression of CD11a, Mac-1 and CD18 (Fig. 6 and Fig. 7) were examined by flow cytometry. Surface CD11a molecules were not expressed on resting cells. Their expressions were not increased by exposure to acemannan. Increased CD11a expression occurred when cells were treated with IFN-γ. Greatly increased CD11a expression occurred when cells were exposed to both acemannan and IFN-γ. In contrast, the CD18 and Mac-1 molecules were constitutively expressed on resting cells. Their expressions were not increased when treated with acemannan. Increased expressions of CD18 and Mac-1 were obtained on cells treated with IFN-γ. Significantly increased CD18 expression was observed on cells treated with acemannan/IFN-γ. However, the expression of Mac-1 on cells treated with acemannan/IFN-γ was not changed.

In addition, minimum EA rosetting reflecting FcR expression (Fig. 8) was observed on resting macrophages. A similar result was obtained from cells exposed to acemannan. Greater EA rosette formation was obtained on IFN-γ treated cells. Greatly increased EA rosette formation was observed on cells treated with ACM/IFN-γ.

3.5. Effects of short term exposure to acemannan

To determine the mechanism of acemannan induced macrophage activation, the effects of short term exposure to acemannan were examined. Nitrite production by macrophages pre-exposed to acemannan, IFN-γ or acemannan/IFN-γ and medium for 4 h followed by extensive washing and treated with the second agent for another 44 h was measured (Table 1). Results showed that macrophages exposed to either acemannan or IFN-γ alone in the first four hours followed by treatment with either IFN-γ or acemannan respectively generated a large amount of NO. When macrophages were exposed to a combination of acemannan and IFN-γ for four hours and then washed, it also resulted in maximum NO production at 48 h. A reduced effect was observed when macrophages were exposed to these agents for less than four hours.

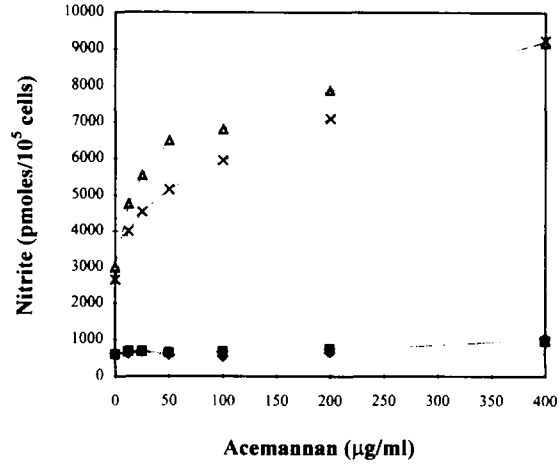

Fig. 9. The effect of polymyxin B on RAW cell nitric oxide production. RAW cells were treated with acemannan alone, IFN-γ, acemannan/IFN-γ or bacterial endotoxin in the presence or absence of polymyxin B for 48 h. The culture supernatants were analyzed for nitrite as described in Materials and Methods in three independent experiments. – △ – Acemannan/IFN-γ w/o polymyxin B. - × – Acemannan/IFN-γ with polymyxin B. – ◆ – Endotoxin alone. - ■ – Endotoxin with polymyxin B.

3.6. Endotoxin contamination

To ensure that the effects on macrophages were not due to endotoxin contamination of acemannan, all solutions were pre-treated with polymyxin B for three hours at 37°C and then tested for their effectiveness on macrophage activation as judged by nitric oxide production. Acemannan, IFN-γ, and LPS incubated under similar condition in the absence of polymyxin B were used as controls. As shown in Fig. 9, nitrite production in LPS treated cells was substantially blocked by polymyxin B treatment. In contrast, the amount of nitrite released from acemannan, IFN-γ, or acemannan/IFN-γ treated cells was decreased by less than five percent. These results suggested that the macrophage activation effects were not due to endotoxin contamination in any of the solutions.

4. Discussion

Maximal activation of macrophage in vitro requires a minimum of two signals such as IFN-γ and LPS (Adams and Hamilton, 1987). In the system described here, we have shown that acemannan can stimulate macrophage IL-6 and TNF-α production by itself. Acemannan also synergistically enhanced macrophage sensitivity to IFN-γ as reflected by either nitric oxide production or morphological changes. The present studies demonstrate that acemannan alone can activate macrophage for cytokine and nitric oxide production and for expression of some cell surface markers. In addition, the inability of polymyxin B to block the effect of acemannan suggested that stimulation was not caused by endotoxin contamination in the acemannan.

However, an interesting aspect of acemannan activation of macrophages for nitric oxide production is noted in Table 1. NO production following four hours incubation with acemannan followed by washing and then continued incubation in the presence of IFN-γ is about 80% of that of the full response upon acemannan/IFN-γ challenge. Similar results were observed in the IFN-γ pre-treated cells. Therefore, short term incubation with acemannan is able to prime macrophages but is unable to generate sufficient transmembrane signalling for nitric oxide production. The long-term incubation with acemannan (treated with acemannan/IFN-γ for 48 h), is capable of activating macrophages for nitric oxide production. Evidence suggests that maximum NO production requires a two-signal pathway (Stuehr and Nathan, 1989). Thus, the results of NO production following transient exposure of macrophages to either acemannan or IFN-γ suggested that acemannan could increase macrophage sensitivity to the second signal for the generation of NO. Evidence from LPS and IFN-γ induced macrophage activation suggest that LPS and IFN-γ have bi-directional synergetic effects leading to markedly higher macrophage activation at limiting concentrations of either LPS or IFN-γ (Hibbs et al., 1988). This evidence, along with the absence of detectable amounts of endotoxin, suggest that the macrophage response to acemannan may be similar to that induced by LPS although acemannan's effect is not caused by endotoxin contamination.

Attachment of acemannan to RAW cells is probably mediated by specific receptors. Studies of acemannan binding proteins indicate that acemannan binds to four macrophage membrane proteins (data not shown). Thus, it has been suggested that acemannan induces macrophage activation not only through phagocytosis but also through a specific receptor-mediated pathway.

Our observations suggest that acemannan/IFN-γ can act synergistically to increase the Fc receptor expression in association with increased cell activation. The increased expression of Fc receptors in response to acemannan and IFN-γ suggested that these cells may have an enhanced phagocytic ability in response to combined treatment.

In summary, the present study demonstrates the ability of acemannan to activate macrophages directly or indirectly and affecting both NO production and cytokine production, as well as the expression of some surface molecules. The acemannan effect can be potentiated by IFN-γ but does not require its presence for macrophage cytokine production. With this exception, acemannan does not activate macrophages directly and its role in the unstimulated host may therefore be minimal. The ability of acemannan to induce macrophage activation directly has important implications in the physiologic mechanisms of host defense against invading bacteria, virus

and may explain the accelerated wound healing effects.

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