The Major Surface Glycoprotein of *Pneumocystis carinii* Induces Release and Gene Expression of Interleukin-8 and Tumor Necrosis Factor Alpha in Monocytes

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Recent studies suggest that interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) may play a central role in host defense and pathogenesis during *Pneumocystis carinii* pneumonia. In order to investigate whether the major surface antigen (MSG) of human *P. carinii* is capable of eliciting the release of IL-8 and TNF-α, human monocytes were cultured in the presence of purified MSG. MSG-stimulated cells released significant amounts of IL-8 within 4 h, and at 20 h, cells stimulated with MSG released 45.5 ± 9.3 ng of IL-8/ml versus 3.7 ± 1.1 ng/ml for control cultures ($P = 0.01$). In a similar fashion, MSG elicited release of TNF-α. Initial increases were also seen at 4 h, and at 20 h, TNF-α levels reached 6.4 ± 1.1 ng/ml, compared to 0.08 ± 0.01 ng/ml for control cultures ($P < 0.01$). A concentration-dependent increase in IL-8 and TNF-α secretion was observed at 20 h with 0.2 to 5 μg of MSG/ml ($P < 0.01$). Secretion of IL-8 and TNF-α from MSG-stimulated monocytes at 20 h was inhibited by 60 and 86%, respectively, after coincubation with soluble yeast mannan ($P = 0.01$). With an RNase protection assay, increases in steady-state mRNA levels for IL-8 and TNF-α were detectable at 4 h. These data show that recognition of MSG by monocytes involves a mannose-mediated mechanism and results in the release of the proinflammatory cytokines IL-8 and TNF-α.

Pneumocystis carinii pneumonia continues to be a leading cause of morbidity and mortality in human immunodeficiency virus (HIV)-infected individuals, as well as among patients receiving immunosuppressive therapy (29, 30).

The inability to culture *P. carinii* in vitro has limited studies of its biology and the host immune response to *P. carinii* and its antigens. However, a major surface antigen can be purified from tissue samples (28). This antigen is an abundant and mannose-rich glycoprotein encoded by a family of genes and known as the major surface glycoprotein (MSG), but also as gp95, gp116, or gpA, depending on host of origin (19, 26, 28). MSG is highly immunogenic, is critical for host recognition and uptake by macrophages, and apparently is involved in organism-host cell binding through interaction with several host proteins (11, 19, 25, 33, 36).

Cytokines, such as interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α), are key components in the initiation of specific inflammatory and immune responses. IL-8 is a major chemotactic factor for neutrophils (34) and T lymphocytes (22) and may be indirectly involved in the modulation of both acute and chronic inflammation because of these properties. TNF-α, on the other hand, is a decisive inflammatory mediator in the host defense against infection through the induction of numerous genes within diverse target cells (41).

We and others have demonstrated the presence of IL-8 in bronchoalveolar lavage (BAL) fluid from patients with *P. carinii* pneumonia and have shown a correlation between IL-8 and BAL neutrophilia (6, 27). Further, we have found that IL-8 has predictive value for progression to respiratory failure and death during *P. carinii* pneumonia (7).

TNF-α activity has been demonstrated in animals with *P. carinii* pneumonia, and alveolar macrophages from patients with *P. carinii* pneumonia release increased amounts of TNF-α (2, 9, 15, 17, 20). Administration of anti-TNF-α immunoglobulin G to reconstituted SCID mice with *P. carinii* pneumonia results in impaired clearance of *P. carinii* in the lung (9).

Little information is available on the cellular cytokine response to MSG (16, 39). Since monocytes and monocyte-derived cells are pivotal cells in normal host defense and both have been shown to phagocytize and degrade *P. carinii* (31, 42), we studied the interaction between monocytes and MSG. Further, we used MSG purified from human *P. carinii* because a recent investigation has demonstrated considerable host-species specificity (13). In the present study, we show that human monocytes produce IL-8 and TNF-α in response to human MSG.

MATERIALS AND METHODS

Isolation of human monocytes and culture. Mononuclear cells in buffy coats obtained from healthy, HIV type 1-seronegative donors were separated by using Ficoll-Paque gradients with a few modifications as described previously (8). The cells were washed, suspended in RPMI 1640 medium (Life Technologies, Roskilde, Denmark), and adjusted to 5×10^6 cells/ml. Cells (5×10^6 or 5×10^8) were seeded in 24-well plastic tissue culture plates (Costar) or 75-cm² flasks (Nunc, Roskilde, Denmark) for 1 h at 37°C and 5% CO_2 to let monocytes adhere. The wells were washed three times with RPMI 1640 to remove nonadherent cells. This resulted in a monocyte population with a purity of >95% (38). Experiments were carried out in RPMI 1640 containing 1 μg of polymyxin B (Sigma Chemical Co., St. Louis, Mo.)/ml in order to avoid stimulation by low levels of endotoxin. Cell cultures were incubated with or without MSG at the indicated concentrations for 0, 1, 4, 8, or 20 h (overnight) at 37°C and 5% CO_2. Supernatants were collected, centrifuged (at $12,000 \times g$ for 1 min) in order to remove cellular debris, and frozen at −20°C until analysis.

To determine the potential role of the macrophage mannose receptor in mediating MSG-induced cytokine production, studies were performed to block this receptor. Cell cultures were preincubated with soluble mannan from *Saccharomyces cerevisiae* (Sigma) for 30 min before MSG was added and throughout the subsequent incubation with or without MSG.

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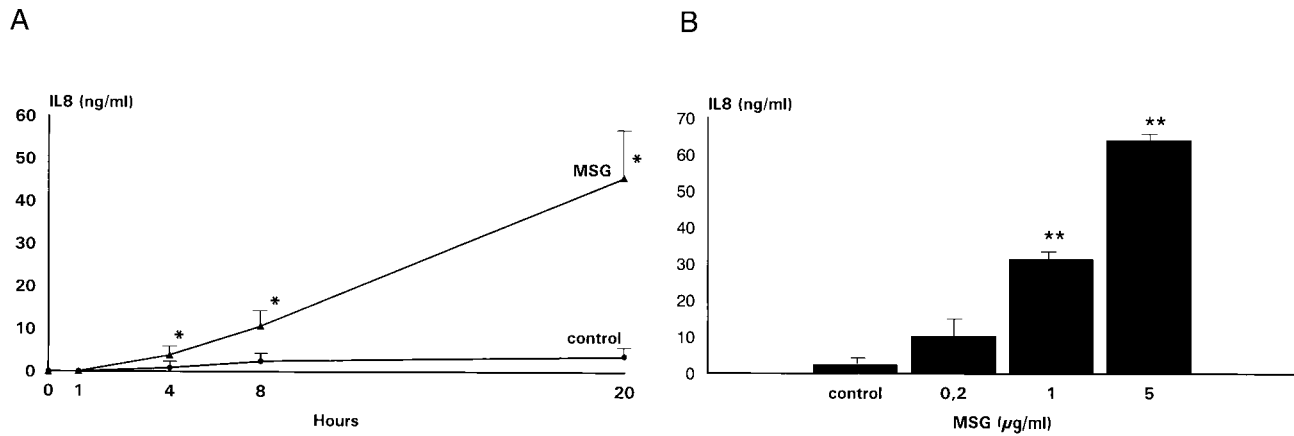
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FIG. 1. Secretion of IL-8 from human monocytes in response to MSG. (A) Time course for IL-8 secretion in response to 5 μg of MSG/ml ($n = 6$; *, $P < 0.01$ by paired t test). (B) MSG in concentrations ranging from 0.2 to 5 μg/ml produced concentration-dependent increases in IL-8 secretion at 20 h ($n = 6$; **, $P < 0.01$ by one-way ANOVA with Bonferroni's adjustment for multiple comparisons).

Purification of human *P. carinii* MSG. *P. carinii* gp95 was purified as described previously (28). Briefly, lung tissue was homogenized, filtered through gauze, and resuspended in 1 mM EDTA (pH 8.5) and phosphate-buffered saline containing 100 U of lyticase (Sigma)/ml for 18 h at 5°C in order to solubilize *P. carinii* antigens. Antigens were purified by high-performance liquid chromatography with a molecular sieve column (Spherogel-TSK G2000SW column; Beckman Instruments, Inc., Fullerton, Calif.) under isocratic conditions at a flow rate of 0.5 ml/min and with 0.1 M KPO₄–0.2 M NaCl (pH 7.0) as the running buffer. Fractions were collected every minute, and aliquots were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining, and Western blot analysis. Fractions containing only MSG and no other bands were pooled, dialyzed, lyophilized, and stored until use. Antigen was reconstituted in pyrogen-free water (BioWhittaker, Walkersville, Md.) and passed three times through Detoxi-Gel columns (Pierce Chemical Co., Rockford, Ill.) to exclude the possibility of contamination with endotoxin. In addition, antigen solutions were analyzed and found to contain less than 0.125 endotoxin units (EU)/ml by the QCL-1000 *Limulus* amoebocyte assay (BioWhittaker). Protein content was quantitated by a colorimetric assay according to the manufacturer's instructions by using bovine serum albumin as the standard (BCA Micro Protein Assay Reagents; Pierce).

ELISA for TNF-α and IL-8. Immunoreactive TNF-α was detected by enzyme-linked immunosorbent assay (ELISA) as previously described (21). Briefly, 96-well microtest plates (Maxisorp; Nunc) were coated overnight with rabbit anti-TNF-α. After washing, samples and human TNF-α standard diluted in buffer containing 10% (vol/vol) normal rabbit serum (Dako) and 0.05% Tween 20 were incubated on a shaker for 1 h at room temperature, followed by the addition of biotinylated rabbit anti-TNF-α, streptavidin-labeled peroxidase, and enzyme substrate. The enzyme reaction was stopped with sulfuric acid, and the absorbance was measured at 492 nm, with a 620-nm reference. The sensitivity of the assay was 30 pg/ml, and undetectable levels were given this value. Immunoreactive IL-8 was measured with a commercially available kit (Quantikine; R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions.

RNase protection assay. Total cellular RNA was extracted by using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio). Experiments were performed with the Ribonuclease Protection Assay Kit II (Ambion, Austin, Tex.). Ten micrograms of total RNA was used for TNF-α, IL-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) experiments. Digoxigenin-11-UTP-labeled (Boehringer GmbH, Mannheim, Germany) riboprobes were generated from plasmids containing cDNA inserts for IL-8, TNF-α, and GAPDH by using the Maxiscript kit (Ambion). RNase protection assay products were subjected to denaturing electrophoresis on 6% Tris-borate-EDTA–urea polyacrylamide gels (Novex, San Diego, Calif.). Labeled RNA molecular mass markers (RNA Century Marker Template Set; Ambion) were used to assess the sizes of the protected fragments. Probe concentrations utilized were in excess of target for TNF-α, IL-8, and GAPDH. Protected fragments and markers were electroblotted onto nylon (Novex) and visualized with the DIG Luminescent Detection Kit (Boehringer Mannheim).

The plasmid containing the TNF-α insert was generated by cloning PCR products into the pCRII vector by using the TA cloning kit (Invitrogen, San Diego, Calif.). A 354-bp TNF-α cDNA fragment was generated via PCR (Geneamp PCR kit; Perkin-Elmer Cetus, Norwalk, Conn.) with the primers 5′-CGG GAC GTG GAG CTG GCC GAG GAG-3′ AND 5′-CAC CAG CTG GTT ATC TCT CTC AGC TC-3′ and with human lung cDNA as a template (Clontech, Palo Alto, Calif.). The plasmid containing the IL-8 cDNA was a generous gift of T. Yoshimura, Laboratory of Immunobiology, National Cancer Institute, Frederick,

Md. (43). This plasmid was further digested with *Hin*dIII and religated, resulting in a 256-bp insert of IL-8 cDNA. The insert sequences were then confirmed by DNA sequencing (Sequenase 2.0; U.S. Biochemicals, Cleveland, Ohio).

Statistics. All data are expressed as means ± standard errors of the means. Normal distribution was tested with the Kolmogorov-Smirnov test. Differences between experimental and paired control data were analyzed by the paired or unpaired Student t test with Bonferroni's adjustment for multiple comparisons, as appropriate. The concentration-dependent effect of MSG was analyzed by one-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant. Data were analyzed with SPSS software (Statistical Package for Social Sciences, Inc., Chicago, Ill.).

RESULTS

Effects of MSG on secretion of IL-8 and TNF-α by human cultured monocytes. Cumulative secretion of IL-8 and TNF-α following stimulation with MSG (5 μg/ml) was monitored for 20 h. As shown in Fig. 1A, MSG stimulation resulted in significant increases in IL-8 secretion at 4 h ($n = 6$; $P < 0.01$) and 8 h ($n = 6$; $P < 0.01$). At 20 h, a 12-fold increase in IL-8 secretion was seen ($n = 6$; $P < 0.01$). As shown in Fig. 1B, concentration-dependent increases were observed for IL-8 secretion at 20 h with 0.2 to 5 μg of MSG/ml ($n = 6$; $P < 0.01$ by one-way ANOVA). MSG stimulation resulted in an initial increase in TNF-α secretion at 4 h ($n = 6$; $P < 0.05$). At 8 h, MSG-mediated TNF-α secretion had increased further ($n = 6$; $P < 0.05$), and this increase was sustained at 20 h ($n = 6$; $P < 0.05$) (Fig. 2A). As shown in Fig. 2B, concentration-dependent increases in TNF-α secretion were observed at 20 h following stimulation with 0.2 to 5 μg of MSG/ml ($n = 6$; $P < 0.01$ by one-way ANOVA).

We took several measures to ensure that IL-8 and TNF-α secretion was not caused by contaminating lipopolysaccharide (LPS). Antigen stock solutions were passed three times through polymyxin B columns in order to absorb LPS. Following this procedure, stock solutions were assayed by a *Limulus* amoebocyte assay and were found to contain less than 0.125 U of LPS/ml. Finally, we added 1 μg of polymyxin B/ml to all culture media. This concentration was chosen because it was sufficient to inhibit cytokine secretion induced by 100 ng of LPS/ml (ca. 1,000-fold higher than the lower detection limit of the *Limulus* amoebocyte assay) and because this concentration of polymyxin B did not induce cytokine production per se (data not shown).

Soluble yeast mannan inhibits MSG-induced IL-8 and TNF-α secretion from monocytes. Recent studies have dem-
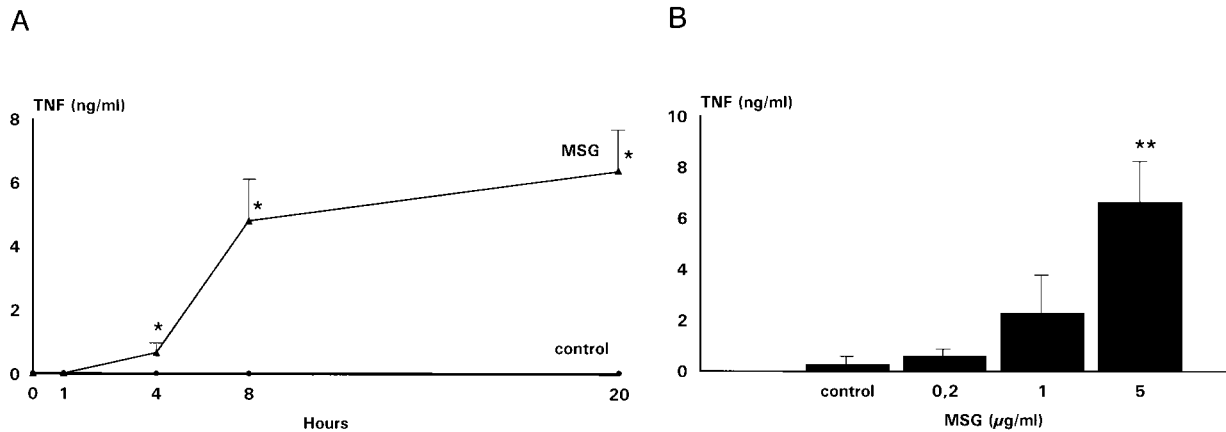
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FIG. 2. Secretion of TNF-α from human monocytes in response to MSG. (A) Time course for TNF-α secretion in response to 5 μg of MSG/ml ($n = 6$; *, $P < 0.05$ by paired t test). (B) MSG in concentrations ranging from 0.2 to 5 μg/ml produced concentration-dependent increases in TNF-α secretion at 20 h ($n = 6$; **, $P < 0.01$ by one-way ANOVA with Bonferroni's adjustment for multiple comparisons).

onstrated that *P. carinii* uptake by macrophages is mediated through the macrophage mannose receptor and that uptake is inhibited by the presence of yeast mannan (11). To determine the potential role of this receptor for recognition of MSG by monocytes, experiments were performed with MSG in the presence of mannan. As shown in Fig. 3A, the presence of mannan significantly inhibited the release of IL-8 from monocytes by 60%, compared to MSG alone ($n = 6$; $P < 0.01$).

Mannan inhibited MSG-induced TNF-α release by 86%, as shown in Fig. 3B ($n = 6$; $P < 0.01$).

Induction of steady-state IL-8 and TNF-α mRNA in monocytes by MSG. Experiments were performed to assess whether MSG up-regulates IL-8 and TNF-α mRNA in human monocytes. Monocytes were incubated with either medium or MSG (5 μg/ml) for 1 and 4 h, total RNA was extracted, and RNase protection assays were performed. As shown in Fig. 4, MSG induced time-dependent increases in both IL-8 and TNF-α mRNAs at 4 h, whereas changes were minimal at 1 h. GAPDH mRNA levels are shown in the bottom panel to control for the amount of mRNA utilized in each reaction. No signal was detected when yeast tRNA was used as a negative control.

DISCUSSION

Our results show that human MSG elicits the release of IL-8 and TNF-α protein and up-regulates mRNA expression by human monocytes in a dose- and time-dependent manner. To our knowledge this is the first report of IL-8 release by monocytes in response to MSG, whereas our study confirms findings reported by others on animal-derived MSG and TNF-α release by monocytes (16). The mechanism apparently involves the mannose receptor, because MSG-induced IL-8 and TNF-α production is significantly inhibited by the addition of soluble mannan.

We and others have previously implicated IL-8 in the pathogenesis of *P. carinii* pneumonia. BAL IL-8 levels were demonstrated to correlate positively with BAL neutrophilia and inversely with PO_2 (6, 27). Further, increased IL-8 levels were found to predict subsequent progression to death and need of mechanical ventilation (7). IL-8 is a mediator of several inflammatory events central in host defense, but these also potentially provide the cellular basis for sustained inflammation. Among the mechanisms that may contribute to inflammation
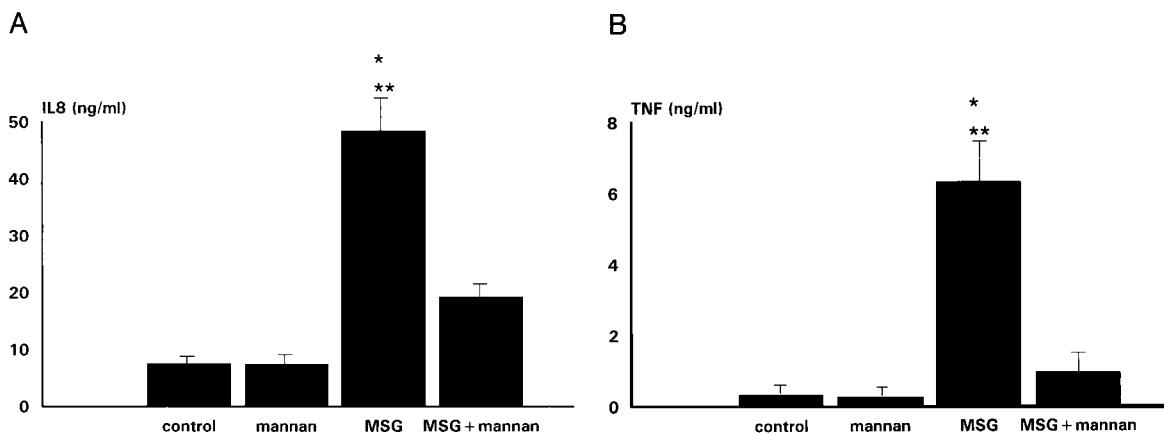
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FIG. 3. Effect of yeast mannan on MSG-induced IL-8 and TNF-α secretion from human monocytes. (A) MSG alone resulted in significant stimulation of IL-8 secretion compared to controls (*, $P < 0.01$). Yeast mannan resulted in a significant inhibition of IL-8 secretion (60%) from monocytes stimulated with MSG (**, $P < 0.001$) compared to monocytes stimulated with MSG in the absence of mannan ($n = 6$). (B) Similarly, MSG resulted in significant stimulation of TNF-α secretion compared to controls (*, $P < 0.01$). Yeast mannan resulted in an 86% inhibition of TNF-α secretion from monocytes stimulated with MSG (**, $P < 0.01$) compared to monocytes stimulated with MSG in the absence of mannan ($n = 6$). Cultures were incubated for 24 h with 5 μg of MSG/ml and/or 100 μg of mannan/ml.
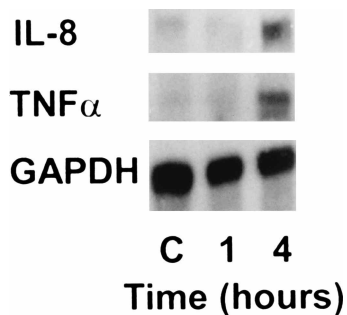
FIG. 4. MSG induces time-dependent increases in IL-8 and TNF-α mRNAs in human monocytes. Steady-state levels of mRNA were assessed by RNase protection assay after stimulation with MSG (5 μg/ml) for 1 and 4 h. Ten micrograms of total RNA was used for each assay. (Top and center) MSG induced increases in IL-8 and TNF-α mRNA, respectively, at 4 h. (Bottom) GAPDH mRNA levels are shown in order to control for the amount of mRNA in each reaction. This figure is representative of three separate experiments demonstrating the same result. C, control.

are recruitment of neutrophils and lymphocytes (22, 34), up-regulation of integrin/selectin expression on endothelial and epithelial cells (1), and degranulation of neutrophils (10). Although these aspects were not studied, we speculate that IL-8-mediated superimposed inflammation may explain the connection between elevated IL-8 levels and disease severity.

Several lines of evidence imply that TNF-α is an important cytokine in host defense during *P. carinii* pneumonia. In reconstituted SCID mice TNF-α is essential for clearance of *P. carinii*, since administration of antibodies against TNF-α significantly impairs resolution of the infection (9). This is supported by a recent study by Kolls et al. in which clearance of murine *P. carinii* infection was delayed in animals treated with an adenovirus encoding a TNF inhibitor gene (18). Krishnan et al. found a correlation between elevated TNF-α concentrations and low cyst counts during *P. carinii* pneumonia, indicating a protective role of TNF-α in human *P. carinii* infection (20). Interestingly, a recent report found a correlation between decreased alveolar macrophage TNF-α mRNA levels and decreased arterial PO$_2$ (a marker of disease severity) in HIV-infected patients (37). In T helper cell-deficient mice, *P. carinii* infection persisted despite initial up-regulation of TNF-α production, indicating that other mediators are necessary for clearance of the infection (17). Several of these studies (17, 20, 37) identified the alveolar macrophage as a source of TNF-α during *P. carinii* pneumonia. Our findings support this and suggest that monocyte hypersecretion of TNF-α is mediated partly through recognition of MSG.

Alveolar macrophages bind and phagocytize *P. carinii* (31, 42). We show here in monocytes that this may be mediated in part by recognition of mannose-rich MSG, as release of IL-8 and TNF-α was clearly suppressed by coincubation with yeast-derived mannan. This is in accordance with the findings of Ezekowitz et al. (11), who showed that the macrophage mannose receptor is critical for both binding and uptake of organisms through mannose-rich components of the *P. carinii* cell wall. Further, they were able to block uptake by coincubation with mannan. However, Hoffman et al. found that binding of *P. carinii* to macrophages involves both mannose and β-glucan components (14). They further showed that TNF-α release was mainly mediated through interaction with cell wall β-glucan, as TNF-α release was suppressed by blocking of macrophage β-glucan receptors or by digestion of the β-glucan cell wall with lyticase. However, digestion with lyticase also removes MSG from the cell wall (28), and this may in part account for

the reduced release of TNF-α. Another explanation for the apparent discrepancy between the latter study and ours may be that free MSG interacts differently with host cell receptors than surface-bound MSG does, through either steric hindrance, engagement of additional receptors, or mechanisms induced by other *P. carinii* antigens. Species variation of MSG may also explain the discrepancy, since we used human-derived MSG with human monocytes whereas other studies have used rat-derived *P. carinii* organisms with rat alveolar macrophages. Finally, monocytes and macrophages are phenotypically different and may show differences in the amount of mannose and β-glucan expressed on the surface. Future studies should investigate the reactivity of macrophages to human MSG.

Several molecules, including fibronectin, vitronectin, and immunoglobulin, bind to the surface of *P. carinii* and enhance macrophage and epithelial recognition of the organism (25, 31, 35, 36). Data indicate that this may be mediated by binding to MSG. Therefore, since MSG binding to monocytes enhances proinflammatory cytokine release, further studies should investigate whether MSG recognition is enhanced by these mechanisms.

Clinically, moderate to severe *P. carinii* pneumonia is characterized by a further impairment of lung function 3 to 5 days after initiation of antimicrobial therapy, unless adjuvant corticosteroids are administered (32). Pulmonary dysfunction is also accompanied by an initial increase in several inflammatory markers after the start of therapy; these are completely suppressed by corticosteroids (3, 5). In this respect, it is of interest that a surface antigen that presumably is released in large quantities upon killing of the microorganism is capable of inducing considerable production of two important inflammatory cytokines. Clearly, overproduction of IL-8 and TNF-α may account for the cellular events leading to the observed lung injury (4, 24, 40).

LPS is a potent inducer of both IL-8 and TNF-α release by monocytes, as well as many other cell types (23, 41). Since preparations of MSG are obtainable only through purification of tissue infected with *P. carinii*, the possibility of contamination exists. Our preparations were pure as assessed by protein staining with Coomassie blue, silver staining, and Western blot analysis using monoclonal antibodies (28). To further eliminate the presence of LPS, we took several measures: (i) stock solutions were passed through columns containing polymyxin B; (ii) these solutions were assessed by a highly sensitive *Limulus* amoebocyte assay and found to contain less than 0.125 EU of LPS/ml; and (iii) polymyxin B was added to the media in order to neutralize trace amounts of LPS. Also, our cytokine responses with MSG concentrations of 5 μg/ml are comparable to those reported by others using concentrations of 1 to 20 μg/ml (16, 39). We therefore find it unlikely that LPS influenced the secretion of IL-8 and TNF-α in our experiments.

In conclusion, we have shown that MSG is a potent inducer of IL-8 and TNF-α production in monocytes. We speculate that these proinflammatory cytokines, in addition to having beneficial host defense effects, may contribute to the lung inflammation observed during *P. carinii* pneumonia and attenuated by corticosteroid treatment (32). In this respect, recent novel therapeutic modalities, such as specific antibodies, soluble receptors, or receptor antagonists, directed against cytokine hyperproduction may be of benefit (12).

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