Human Mannose-binding Protein Functions as an Opsonin for Influenza A Viruses

Kevan L. Hartshorn,* Kedarnath Sastry,* Mitchell R. White,* E. Margot Anders,§ Michael Super,§ R. Alan Ezekowitz,* and Alfred I. Tauber*

*Departments of Medicine and Pathology, Boston University School of Medicine and the Department of Medicine, Boston City Hospital, Boston, Massachusetts 02118; Divisions of §Hematology/Oncology and Infectious Diseases, Department of Pediatrics, Children’s Hospital and Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115; and 5Department of Microbiology, University of Melbourne, Victoria, Australia

Abstract

Influenza A viruses (IAVs) cause substantial morbidity and mortality in yearly epidemics, which result from the ability of the virus to alter the antigenicity of its envelope proteins. Despite the rapid replication of this virus and its ability to infect a wide variety of cell types, viremia is rare and the infection is generally limited to the upper respiratory tract. The primary host defense response against IAV is generally, therefore, successful. We have previously provided (and summarized) evidence that neutrophils contribute to defense against IAV, although neutrophil dysfunction and local tissue damage may be less salutary byproducts of this response. Here we provide evidence that the serum lectin mannose-binding protein directly inhibits hemagglutinin activity and infectivity of several strains of IAV. In addition mannose-binding protein acts as an opsonin, enhancing neutrophil reactivity against IAV. Opsonization of IAV by mannose-binding protein also protects the neutrophil from IAV-induced dysfunction. These effects are observed with physiologically relevant concentrations of mannose-binding protein. Two different allelic forms of recombinant mannose-binding protein are found to have similar effects. We believe, on the basis of these data, that mannose-binding protein alone and in conjunction with phagocytic cells is an important constituent of natural immunity (i.e., preimmune defense) against IAV. (J. Clin. Invest. 1993. 91:1414–1420.) Key words: neutrophils • hemagglutination • recombinant proteins • mannose-binding protein • influenza A virus

Introduction

The product of influenza A virus (IAV) 1 hemagglutinin (HA) genes is expressed on the surface of infected cells and evokes specific and long-lived humoral and cell-mediated immune re-

1. Abbreviations used in this paper: HA, hemagglutinin; HAU, hemagglutinin units; IAV, influenza A virus; MBP, mannose-binding protein; PFU, plaque-forming units; rMBP, recombinant MBP.

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/93/04/1414/07 $2.00 Volume 91, April 1993, 1414–1420
forms of MBP have been shown to have similar functional properties in vitro. One important difference is that the recombinant MBP containing the Gly to Asp mutation at the fifth collagen repeat was not able to fix complement via the classical pathway (14). In this paper we assess the interaction of both forms of MBP with various influenza viral strains selected for known differences in HA glycosylation.

MBP-ligand complexes would be expected to encounter circulating cells, in particular neutrophils, which have been shown to ingest bacteria opsonized with MBP (11). Neutrophils alone may play a role in the preimmune host defense against influenza (16, 17). We and others have characterized the interaction of human neutrophils with unopsonized IAV and found evidence that these cells are activated by IAV. In the absence of antibody, neutrophils are stimulated by cell-free virus to generate a pertussis toxin–insensitive respiratory burst response, characterized by hydrogen peroxide (H₂O₂), but no superoxide (O₂⁻) production (18, 19). Neutrophils are also reported to specifically adhere to IAV-infected epithelial monolayers without mediation of antibody (20). After exposure to influenza viruses in vitro and in vivo, however, neutrophils and monocytes exhibit depressed responsiveness to various stimuli and depressed microbicidal activity against bacteria (21, 22). In this report we demonstrate that both forms of recombinant MBP inhibit IAV HA activity and act as opsonins, enhancing neutrophil responsiveness to the virus.

Methods

Reagents. FMLP, cytochalasin B, horseradish peroxidase type II, scopolatin, SOD, cytochrome c, neuraminidase type X (protease activity < 0.002 U/mg protein), ficoll, dextran, sodium citrate, citric acid, and nitroblue tetrazolium were purchased from Sigma Chemical Co. (St. Louis, MO) and Hyapase was obtained from Winthrop Pharmaceuticals (Des Plaines, IL). Fura-2 and bis(1,3-dihydroxyisobutyrlic acid) trimethoxynyl (bisoxynol) were purchased from Molecular Probes, Inc. (Eugene, OR), organic solvents from Fisher Scientific (Fairlawn, NJ), and Dulbecco’s phosphate-buffered saline with Ca²⁺ or Mg²⁺ (PBS) or without Ca²⁺ or Mg²⁺ from Flow Laboratories (McLean, VA). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA).

MBP preparation. Recombinant MBP₈₀ (rMBP₈₀) and rMBP₂₀ were prepared from cDNAs differing only at base pair 230. These cDNAs were subcloned in an eukaryotic expression vector transfected into murine Sp² cells and MBPs were purified from the supernatants by calcium-dependent binding to mannann-Sepharose and EDTA elution or by binding to an affinity matrix of anti-MBP monoclonal antibody coupled to Affigel (Bio-Rad Laboratories, Richmond, CA) as previously described (14). MBPs were dialyzed in PBS and stored at −70°C in aliquots. The specific activities were measured by comparison with a standard preparation using mannann-coated ELISA plates as described (14).

Virus preparation. Virus stocks were grown in the chorioallantoic fluid of 10-day embryonated hen’s eggs and purified on a discontinuous sucrose density gradient as previously described (21). Virus stock was suspended in Dulbecco’s modified PBS, aliquoted, and stored at −70°C until used. Potency of each virus stock was measured by hemagglutination assay, and titers of 1.800–32.000 (as indicated) hemagglutination units (HAU) were measured after samples were thawed from frozen storage at −70°C. The H₃N₂ A Texas 77 (Texas 77) and H₁N₁A/PR/8/34 (PR-8) strains of IAV were graciously gifts of Dr. Jon Abramson (Bowman-Gray School of Medicine, Winston-Salem, NC). The H₁N₁A/Memphis 71p, Bel₄ virus was obtained originally from Dr. W.G. Laver (John Curtin School of Medical Research, Canberra, Australia) and the beta-inhibitor resistant mutant (Mem71p, Bel₄/B5) was developed by Dr. E. Margot Anders (University of Melbourne, Parkville, Victoria, Australia). The H₃N₂A/Bangkok/1/79 (Bankok 79) strain was a gift of Dr. Robert Webster (St. Jude’s Hospital, Memphis, TN). HA titers were determined by titration of virus samples in PBS followed by addition of thoroughly washed human type O red blood cells.

Incubation of MBPs with concentrated IAV stocks was carried out for 0.5 h at 37°C in PBS with 1 mM Ca²⁺ and Mg²⁺, except in certain experiments where 2 mM EGTA or 10 mg/ml mannan (Sigma Chemical Co.) were added, as indicated. For experiments with neutrophils, aliquots of virus or MBP-treated virus were then added to cell suspension at a 1:40 vol/vol ratio. In describing these experiments, the final concentration of MBP in the neutrophil suspension is given.

Virus plaque formation was assessed on Madin-Darby canine kidney cells: duplicate confluent monolayers in six-well plates were exposed to virus preparations with or without MBP in 10-fold dilutions in serum-free CRCM-30 medium (Sigma Chemical Co.) for 1 h at 37°C, followed by washing twice in PBS. The monolayers were then overlaid with CRCM-30 media with 0.1% BSA, 0.4% agarose, 10 µg/ml trypsin, and 10 µg/ml DEAE-Dextran. The agarose solution was warmed until melting and allowed to cool to the touch, and then mixed with the other ingredients and layered over the cells before solidification. The monolayers were then kept at 37°C in a 5% CO₂ incubator for 72 h, followed by removal of the agarose overlay. After staining with crystal violet, the plaques were counted by direct inspection and titers of plaque-forming units (PFU)/ml calculated. Values given are mean PFU/ml for two wells. MDCK cells were obtained from the American Type Culture Collection (Rockville, MD).

Neutrophils from healthy volunteer donors were isolated to > 95% purity as previously described using dextran precipitation, followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes (21). Cell viability was > 98% as determined by trypan blue staining, and cells were used within 5 h of isolation.

Measurement of neutrophil activation. H₂O₂ production was measured by the oxidation of scopoletin, and O₂⁻ was assessed by the continuous monitoring of the SOD-inhibitable reduction of cytochrome c as previously detailed (18). Cell membrane potential was measured using the fluorescent probe bisoxynol as previously detailed (18). Neutrophil deactivation was assessed by first incubating cells with IAV (either unopsonized or opsonized with MBP) for various periods of time followed by measurement of O₂⁻ production in response to FMLP.

In some of the assays of H₂O₂ or O₂⁻ production, mannan alone or MBP treated with mannan were added before stimulation of the cells with IAV or FMLP. It was, however, observed that mannan alone, at quantities sufficient to inhibit the effects of MBP on viral HA activity, inhibited respiratory burst responses to both stimuli. Final concentrations of 250 or 500 µg/ml of mannan reduced both H₂O₂ and O₂⁻ responses by ≥ 30% (n = 3). Concentrations of mannan ≥ 500 µg/ml also inhibited the measured O₂⁻ produced by xanthine oxidase in a dose-related manner. The inhibitory effects of mannan on neutrophil respiratory burst responses was, therefore, at least in part attributable to direct interference with O₂⁻ detection. As detailed below, mannan did not interfere with neutrophil membrane depolarization responses, so this assay was used to assess the ability of mannan to alter the effects of MBP on neutrophil activation responses to IAV.

Results

Effect of MBP on IAV infectivity and hemagglutination titers

The IAV strains used in these experiments were selected to assess the role of high mannose oligosaccharide attachments to the viral HA in mediating MBP–IAV interactions. In Table I
Table I. Inhibition of IAV Hemagglutination Activity and Enhancement of IAV-induced Neutrophil H2O2 Production by Various IAV Strains Opsonized with rMBPα

<table>
<thead>
<tr>
<th>IAV strain</th>
<th>HA Inhibition</th>
<th>rMBPα concentration</th>
<th>H2O2 Enhancement</th>
<th>nmol/min per 4 × 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mem71r-Bel8(H3N1)*</td>
<td>368 ng</td>
<td>0.06±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texas 77 (H3N2)*</td>
<td>224 ng</td>
<td>0.13±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bankok 79 (H3N2)*</td>
<td>112 ng</td>
<td>0.06±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mem71r-Bel8/BS</td>
<td>18 µg</td>
<td>-0.01±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H3N1)†</td>
<td>&gt;35 µg</td>
<td>-0.01±0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HA inhibition was performed by taking 1–2 HAU of the IAV strains and determining the concentration of rMBPα required to fully inhibit red cell hemagglutination by this amount of virus. Final volume of the virus, MBP, and red cell mixture was 100 µl. Results represent mean of two or three determinations. H2O2 enhancement refers to the ability of rMBPα to enhance the H2O2 response of human neutrophils (measured using the scopoletin fluorescence assay) elicited by the IAV strains. The rates of H2O2 production by the unopsonized viruses were, respectively, 0.08, 0.07, 0.05, 0.08, and 0.05 nmol/min per 4 × 10⁶ cells for Mem71r-Bel8, Texas 77, Bankok 79, Mem71r-Bel8/BS, and PR-8. The values given in the table represent mean±SEM difference in response of neutrophils to the various IAV strains opsonized with rMBPα compared with the respective control responses. For these experiments concentrated stocks of the viruses (16,000–32,000 HAU/ml) were preincubated for 30 min at 37°C with rMBPα or control buffer, followed by addition of aliquots of these preparations to neutrophil suspensions for H2O2 assay. The final concentration of rMBPα in the neutrophil suspension was 0.88 µg/ml. Use of fivefold higher concentrations of MBPα did not alter the negative results obtained with Mem71r-Bel8/BS or PR-8.

* IAV strains with high mannose carbohydrate attachment on HA residue 165.
† IAV strains without high mannose carbohydrate attachment on HA residue 165.
‡ P < 0.05 comparing response to unopsonized and rMBPα-opsonized IAV.

The IAV strains used are grouped according to whether they have a high mannose carbohydrate attachment at residue 165 of the HA. This attachment, when present, is adjacent to the sialic acid–binding pocket of the globular head of the HA. Mem71r-Bel8/BS, which lacks this attachment, is a mutant version of Mem71r-Bel8, selected out by virtue of resistance to bovine serum alpha inhibitors (4). PR-8 is an H1N1 strain of IAV that lacks high mannose oligosaccharide attachments on its HA (23) and specifically has no carbohydrate attachment near the sialic acid–binding site (24).

We carried out detailed studies of the effects of rMBPα on viral HA titers. As shown in Table I, the HA activity of 1–2 HAU of those viral strains with high mannose attachment adjacent to the cell attachment site of the HA (i.e., Texas 77, Mem71r-Bel8, and Bankok 79) was inhibited by preincubation with nanogram concentrations of rMBPα whereas that of the strains without such carbohydrate attachments (i.e., PR-8 and Mem71r-Bel8/BS) were either not inhibited or inhibited at substantially greater rMBPα concentrations. For the Mem71r-Bel8/BS, ~50-fold more MBPα was required to inhibit HA activity than for the parent virus. As depicted in Fig. 1, when preincubated with a highly concentrated stock of Texas 77 IAV, rMBPα inhibited HA titers in a dose-dependent manner, and this effect was abrogated by addition of mannan (or EGTA) to rMBPα before addition of the virus.

To examine whether rMBPα could inhibit viral infectivity we also tested the effects of preincubation with rMBPα on plaque titers of IAV. Aliquots of highly concentrated IAV were preincubated with the lectin exactly as described in fig. 1. For the Texas 77 strain, preincubation of a preparation containing 2 × 10⁹ PFU/ml with 70 µg/ml rMBPα caused a one-log reduction in PFU titer (i.e., to a mean PFU titer of 1.5 × 10⁸; n = 2 experiments). When a starting PFU titer of 2 × 10⁴ was used, 35 µg/ml of rMBPα caused a two-log reduction. A log reduction of PFU titer of concentrated Mem71r-Bel8 resulted from preincubation with 35 µg/ml rMBPα whereas no, or minimal, inhibition of PFU titers of the glycosylation mutant were seen in three experiments (data not shown). These results show that MBPα can inhibit infectivity of H3 IAV strains, probably through binding to high mannose carbohydrate attachments on the viral HA.

Figure 1. Effect of preincubation of Texas 77 IAV with rMBPα on viral HAU titers. Concentrated Texas 77 IAV was preincubated with various concentrations of rMBPα for 0.5 h at 37°C, followed by assessment of viral HAU titers by titration of samples on human type O red blood cells. Results represent mean±SEM of four experiments. HAU titers were significantly reduced (P < 0.05) by all concentrations of MBPα. In three experiments MBPα was preincubated with mannan (10 mg/ml) before addition of IAV, which abrogated the effect of MBPα. Where error bars are not shown identical results were obtained on repeated experiments. Chelation of Ca²⁺ with 5 mM EGTA during incubation of virus with MBPα also substantially inhibited the effect of MBPα (data not shown).
Enhancement of IAV-induced neutrophil activation by preincubation with MBP

Respiratory burst responses. As shown in Table I, when neutrophils were exposed to Texas 77, Bangkok 79, or Mem71r-BelN strains of IAV opsonized with 0.88 μg/ml of rMBP0, significantly enhanced H2O2 production was stimulated compared with that elicited by the unopsonized viruses. H2O2 enhancement was not seen when the PR-8 or Mem71r-BelN/BS strains were opsonized with rMBP0. These results, therefore, parallel the HA inhibition results in that strains lacking high mannose glycosylation on the HA globular head are unaffected. The effects of preincubating MBP with mannans (to block lectin activity) could not be tested in this assay because of direct interference of mannans on respiratory burst assays (see above).

Fig. 2 A depicts the dose–response relationship for H2O2 enhancement by rMBP0 for the Texas 77 and PR-8 strains. Preincubation of Texas 77 IAV with rMBP0 caused a concentration-related enhancement of neutrophil H2O2 production in response to the virus. This enhancement was most consistent with 0.88 and 4.4 μg/ml of rMBP0. Note again that rMBP0 did not alter H2O2 responses to the PR-8 strain of IAV (Fig. 2). A representative tracing of scopoletin fluorescence decrease (indicative of H2O2 production) in response to MBP-opsonized IAV is given in Fig. 2 B. Note that preincubation of the virus with the lectin was necessary to achieve enhancement. When MBP alone was added to neutrophils, followed by addition of IAV, no significant enhancement was seen (Fig. 2 B). Preincubation of neutrophils with MBP alone similarly did not enhance H2O2 responses to FMLP (data not shown), indicating that the lectin did not have a general priming effect on neutrophil responses. Also note that neither H2O2 nor O2− production was induced by MBP alone (Fig. 2 B and data not shown).

Neutrophil membrane depolarization. IAV induces neutrophil membrane depolarization in a time course similar to the induction of H2O2 production (18, 19). Preincubation of IAV with MBP significantly enhanced this response. Texas 77 IAV opsonized with 2.2 μg/ml rMBP0 increased the rate of neutrophil membrane depolarization (as measured by changes in bis-oxyxynol fluorescence) from 7±1 for virus alone to 15±3 for MBP-opsonized virus. These results are mean±SEM change in arbitrary fluorescence units (n = 4; P < 0.03 for IAV alone vs. MBP-opsonized IAV). When MBP was preincubated with mannans (10 mg/ml) before addition of virus, the enhancing effect of MBP was lost: mean±SEM depolarization rate of 7±2 for IAV incubated with MBP and mannans. In contrast to its effects on respiratory burst assays, mannans alone (at the same concentration) did not significantly alter the membrane depolarization response to IAV (mean±SEM depolarization rate was 7±2 for mannans plus IAV) nor did it alter the depolarization response to FMLP (data not shown).

MBP protects neutrophils from the deactivating effects of IAV

In addition to causing neutrophil activation, influenza A viruses also suppress respiratory burst and other responses of the infected neutrophil to subsequent stimulation by a wide range of agonists (21, 22). This dysfunction (termed deactivation) can be shown by first incubating neutrophils with IAV and then measuring O2− responses to FMLP. As shown in Table II, a 10-min incubation with either Texas 77, Mem71r-BelN, Texas 77, or PR-8 IAV with rMBP0 on human neutrophil H2O2 production induced by these viruses. Aliquots of Texas 77 or PR-8 IAV (final concentrations of 384 and 320 HAU/ml, respectively) were added to a stirred suspension of human neutrophils (4×10⁶ cells in 2 ml), and H2O2 production was measured by decreases in scopoletin fluorescence. The viruses were either unopsonized or opsonized with rMBP0 (final concentrations of MGP 0.088, 0.88, and 4.4 μg/ml). Results represent mean±SEM of four experiments. For Texas 77 IAV, H2O2 production rates (in nmol/min per 4×10⁶ cells) were significantly elevated by samples opsonized with either 0.88 or 4.4 μg/ml MBP compared with those stimulated by IAV alone (P < 0.025). Responses to PR-8 were not significantly affected by MBP. (B) Representative tracings of neutrophil H2O2 production (as indicated by a decline in fluorescence of scopoletin) in response to Texas 77 IAV that had been preincubated for 30 min with rMBP0 (MBP-IAV) as compared with the response to the same amounts of rMBP0 alone followed by IAV alone. The final concentrations of rMBP0 and IAV in the cell suspension were, respectively, 2.2 μg/ml and 384 HAU/ml. Two or more additional experiments showed similar results.
Various virus strains were preincubated with control buffer or various concentrations of rMBP$_{D}$ for 30 min at 37°C. Neutrophils (10$^6$ in 1 ml) were then incubated for 10 min at 37°C with aliquots of these viral samples (final concentrations of Texas 77, Mem71$_{r}$-Bel$_{n}$, Mem71$_{r}$-Bel$_{n}$/BS, and PR-8 were, respectively, 384, 192, 192, and 320 HAU/ml), followed by addition of cytochalasin-B (5 $\mu$g/ml) and FMLP (10$^{-7}$ M/liter) and measurement of O$_2$ production. The final concentrations of MBP in the neutrophil suspension are those indicated. Results represent mean±SEM ($n = 6$ experiments, except for the 6.6 $\mu$g/ml concentration of MBP where $n = 3$) of FMLP-stimulated O$_2$ production in IAV-treated divided by control cells (i.e., percent of control). No O$_2$ production was seen in response to opsonized or unopsonized IAV alone.

*M P < 0.05 comparing FMLP-stimulated O$_2$ production in virus-treated to control cells.

*† P < 0.05 comparing FMLP-stimulated O$_2$ production rates in neutrophils treated with virus alone vs. virus opsonized with MBP.

Mem71$_{r}$-Bel$_{n}$/BS, or PR-8 viruses caused significant deactivation of FMLP-stimulated O$_2$ responses. Treatment of neutrophils with the same amount of Texas 77 IAV opsonized with 4.4 or 6.6 $\mu$g/ml of rMBP$_{D}$ caused significantly less deactivation than did unopsonized Texas 77. Opsonization with 1.76 or 4.4 $\mu$g/ml of rMBP$_{G}$ reduced deactivation caused by Mem71$_{r}$-Bel$_{n}$ significantly. Of note, no such protective effect was observed with respect to the Mem71$_{r}$-Bel$_{n}$/BS mutant or PR-8 strains, indicating that binding to carbohydrate attachments near the globular end of the HA is necessary for MBP to inhibit deactivation.

The concentrations of rMBP$_{D}$ necessary to protect from deactivation were those that caused substantial inhibition of HA activity of the virus. The HA titers of Texas 77 and Mem71$_{r}$-Bel$_{n}$ samples opsonized with 4.4 $\mu$g/ml of rMBP$_{G}$ were reduced, respectively, by 98 and 93% compared with unopsonized controls. Enhancement of neutrophil H$_2$O$_2$ production in response to IAV could be demonstrated at lower concentrations than those that were necessary to protect from deactivation (Fig. 2). Note also that neither IAV nor MBP-opsonized IAV caused superoxide production in their own right. This is in accord with our previous findings using IAV (18, 19). It is unlikely, therefore, that the ability of MBP to protect neutrophils from the deactivating effects of IAV is a direct result of its ability to enhance respiratory burst responses to the virus itself.

**Effects of rMBP$_{D}$ on IAV and on the IAV–neutrophil interaction**

Human serum MBP exists in two allelic forms that differ as a result of a single base pair change at base pair 230 that results in a Gly to Asp substitution. The latter form is less prevalent and is referred to as rMBP$_{D}$. It has been suggested (15) that this allelic form may be associated with susceptibility to infection. We therefore performed experiments to determine whether rMBP$_{D}$ differed from rMBP$_{G}$ in its interaction with IAV or neutrophils.

rMBP$_{D}$ had similar effects to rMBP$_{G}$ on IAV HA titers, inhibiting HA activity of the Texas 77 and Mem71$_{r}$-Bel$_{n}$ strains of IAV while having no significant effect against the Mem71$_{r}$-Bel$_{n}$/BS mutant strain. Fig. 3 depicts the dose–response relationship for HA inhibition of the Texas 77 strain of IAV resulting from preincubation with rMBP$_{D}$. Note that inclusion of 5 mM EGTA during the incubation of the virus with rMBP$_{D}$ substantially inhibited the effects of the lectin on viral HA activity. Preincubation of concentrated Mem71$_{r}$-Bel$_{n}$ IAV (2,000 HAU/ml) with 250, 66, and 33 $\mu$g/ml of rMBP$_{D}$ caused, respectively, 90±10, 70±10, and 50±0% (mean of two determinations) reductions in the HA titers whereas 100 $\mu$g/ml of rMBP$_{D}$ had no effect on HA titers of 2,000 HAU of the Mem71$_{r}$-Bel$_{n}$/BS strain. Inhibition of infectivity of Texas 77 was also found with rMBP$_{D}$ (PFU reduced from 7 × 10$^9$ to 6 × 10$^8$ PFU/ml by preincubation with 88 $\mu$g/ml rMBP$_{D}$).

As was the case with rMBP$_{G}$, significant enhancement of IAV-induced neutrophil H$_2$O$_2$ production was also observed when Texas 77 or Mem71$_{r}$-Bel$_{n}$ IAV were opsonized with rMBP$_{D}$. As shown in Fig. 4, significantly enhanced H$_2$O$_2$ responses were obtained with Texas 77 IAV opsonized with 1.1, 2.75, or 5.5 $\mu$g/ml of this MBP preparation. For Mem71$_{r}$-Bel$_{n}$, opsonization with 3.3 $\mu$g/ml of rMBP$_{D}$ increased H$_2$O$_2$ production from 0.98 ± 0.18 to 2.7 ± 0.11 nmol/min per 4

![Figure 3](https://doi.org/10.1172/JCI116345)
× 10⁶ cells (n = 3; P < 0.02). In parallel experiments no effect of rMBP₃ was found on H₂O₂ responses to the glycosylation mutant Mem7₁₁₁⁻Bel₉/BS (control response 1.04±0.19 vs. 0.42±0.04 for MBP-treated samples). (Responses were higher in the experiments involving the effects of rMBP₀ on H₂O₂ production elicited by the Mem7₁₁ strains than in earlier experiments due to use of cytochalasin-B before virus).

As described above for rMBP₀, opsonization with rMBP₀ enhanced neutrophil membrane depolarization responses to Texas 77 IAV, an effect again inhibited by preincubation of the MBP with mannan (see Fig. 5).

Preincubation of Texas 77 IAV with rMBP₀ also significantly inhibited neutrophil deactivation caused by this virus strain. The O₂⁻ response to FMLP was 77±3% of control when neutrophils were preincubated with 100 HAU/ml of Texas 77 IAV and 97±3% of control when neutrophils were pretreated with this amount of virus opsonized with a 1.1 µg/ml concentration of rMBP₀ (n = 4; P < 0.03). It thus appears that both forms of MBP have comparable functional activity as determined by these assay systems.

Discussion

In this report we demonstrate that two allelic variants of recombinant MBP inhibit HA activity of several H3 strains of IAV. Binding to a specific high mannose attachment present near the sialic acid–binding pocket of the HA of H3 IAV strains is crucial to these effects, as demonstrated using the glycosylation mutant of Mem7₁₁₁⁻Bel₉/IAV. Opsonization of IAV with both forms of MBP also enhances neutrophil respiratory burst and membrane depolarization responses to cell-free IAV. Of further interest, the MBPs appear to protect neutrophils from the deactivating effects of IAV. This protective effect occurs at somewhat higher concentrations of MBP than are necessary to provide significant enhancement of the respiratory burst. Protection from deactivation requires substantial inhibition of viral HA activity. This finding is similar to results we have obtained using antibodies that neutralize IAV HA activity (unpublished data) and supports the concept that viral HA binding to neutrophil surface sialic acid residues is critical for causing deactivation. This corroborates the finding of Cassidy et al. (25) that purified IAV HA preparations can mediate deactivation. It is of further interest that, in the setting where deactivation and HA activity are strongly inhibited by MBP, the opsonized virus still activates more vigorous neutrophil respiratory burst responses than unopsonized controls. Whether MBP-opsonized IAV activates neutrophils in a qualitatively different manner than the unopsonized virus requires further study. Activation by MBP-opsonized IAV is similar to that triggered by the unopsonized virus in that only H₂O₂, without accompanying superoxide, is produced. We have studied this phenomenon extensively with respect to unopsonized IAV (18, 19, 26).

Our findings indicate that the lack of superoxide production may be accounted for by postulating that the virus-induced respiratory burst occurs largely at an intracellular site from which H₂O₂, but not O₂⁻, can diffuse out to the extra-cellular space and be detected (19). This may also be the case for the burst elicited by MBP-opsonized IAV, although this has yet to be proven.

We have conducted experiments with MBP isolated from human serum (gift of Dr. Jens Jensenius, University of Aarhus, Aarhus, Denmark) and found HA inhibition, enhancement of neutrophil respiratory burst responses, and inhibition of IAV-induced neutrophil deactivation with this preparation as well (data not shown). We have also observed similar effects using bovine conglutinin (Hartshorn, K. L., K. Sastry, M. R. White, D. Brown, T. Okarma, Y. M. Lee, and A. I. Tauber, manuscript submitted for publication). There are extensive similarities between MBP and conglutinin in terms of lectin activity and macromolecular structure (6, 7). Both molecules also have the ability to bind to the cellular C1q receptor via their highly conserved collagen domains (27). Recent studies indicate that conglutinin may be the major constituent of the bovine serum beta inhibitor of IAV infectivity whereas another lectin, possibly MBP, plays this role in murine serum (5). The
finding that these lectins also favorably alter neutrophil interactions with IAV adds an important new dimension to our understanding of their potential role in antiv-antibody immunity to this virus. It may be that the related lectins found in pulmonary surfactant (6) have similar effects vis-a-vis IAV as well. These surfactant lectins may be particularly well positioned to contribute to the initial defense against IAV in the lung.

The various effects of rMBP\(_6\) in our experiments were observed at concentrations of the lectin normally present in human serum (14). All of the observed effects would be expected to influence favorably the outcome of IAV infection in vivo, either through direct antiviral effects of the lectin or through enhancing neutrophil antimicrobial mechanisms and protecting the neutrophil from virus-induced dysfunction. The effective concentrations of rMBP\(_6\) and MBP\(_8\) appear to be very similar from our data. In experiments using the two preparations of MBP to opsonize bacteria (14), major differences in ability to bind to or opsonize bacteria were not found. It appears likely, therefore, that the ability to opsonize organisms is not dramatically different between the two allelic variants of MBP. However, MBP\(_6\) was able to fix complement via the classical pathway, whereas rMBP\(_8\) was not, despite the fact that both recombinant preparations were capable of forming hexamers (i.e., Cl\(_4\)-like configurations). The precise role of man-nose-binding protein in IAV infection in vivo remains to be determined. One intriguing possibility that one can conclude from our in vitro studies is that absent or dysfunctional MBP may contribute to bacterial superinfection as a result of neutrophil deactivation. In this regard it would be interesting to identify patients who had bacterial superinfection after IAV infec-tion and to determine the concentrations of MBP in their serum and respiratory secretions.

Further studies are still required on how mannose-binding proteins alter IAV binding, uptake into and survival in phago-cytes. The relative contribution of putative IAV and MBP receptors on the neutrophil to activation needs to be established. Addition of complement to the MBP-opsonized virus model is likely to result in more potent neutrophil activation and viral killing. Studies of the interaction of MBP (or conglutinin) with other respiratory viral strains may reveal expanded clinical relevance of the lectins in preimmune antiviral responses. Specifically, H1 IAV isolates other than PR-8 may interact with MBP. The WSN strain has more mannose residues on its HA than PR-8 (28), and more recently isolated H1 strains (since the reintroduction of this subtype into active circulation in the human population in 1976) have acquired changes in oligosaccharide attachments as compared with PR-8 (29) and are sensitive to beta-inhibitors (5). The effects of MBP on influenza B and parainfluenza viruses are also of interest. We believe that the results of these various studies will confirm an important role for mammalian C-type lectins, in conjunction with phago-cytes, in the natural immune response to influenza and perhaps other related virus infections.

Acknowledgments

We thank Ann Marie Happnie for her expert secretarial assistance in the preparation of this manuscript.

This study was supported by National Institutes of Health grants: AI-29550-03 (K. L. Hartshorn), HL-33556-08 (A. I. Tauber), and a Grant-in-Aid from the Bristol Myers Medical Foundation (R. A. B. Ezekowitz). R. A. B. Ezekowitz is an established investigator of the American Heart Association.

References


