Role of the Sialophorin (CD43) Receptor in Mediating Influenza A Virus-Induced Polymorphonuclear Leukocyte Dysfunction

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Polymorphonuclear leukocytes (PMNLs) exposed to influenza A virus (IAV) undergo activation of the respiratory burst followed by depression of cell function when subsequently exposed to particulate or soluble stimuli. The effect of IAV on PMNLs is likely to be mediated through the attachment of IAV to one or more specific receptors. Recently, IAV has been shown to bind to the sialophorin protein (CD43) receptor on PMNL plasma membranes. The present study was performed to determine if the sialophorin receptor was responsible for IAV-induced PMNL dysfunction. When PMNLs were incubated with IAV or CD43 monoclonal antibody (MoAb) for 30 minutes and then exposed to a secondary particulate (opsonized zymosan) or soluble (FMLP or phorbol 12-myristate 13-acetate) stimulus, there was significant depression of the PMNL chemiluminescence response compared with the equivalent control (P < .05). When PMNL were incubated with the CD43 MoAb and then cross-linked with a goat antimouse IgG antibody, no depression of PMNL function occurred upon secondary stimulation. Exposure of cells to IAV aggregates also eliminated the PMNL dysfunction that normally occurs due to the virus. Similar to IAV, PMNL dysfunction due to the CD43 MoAb could be overcome by priming the cells with granulocyte-macrophage colony-stimulating factor. These findings indicate that IAV-induced PMNL dysfunction is mediated, at least in part, through the sialophorin receptor.

Influenza Virus-Induced polymorphonuclear leukocyte (PMNL) dysfunction appears to be important in predisposing the host to secondary bacterial infections. Unopsonized influenza A virus (IAV) and its purified hemagglutinin are capable of activating the PMNLs. When virus-treated PMNLs are subsequently exposed to secondary stimuli, depression of cell function occurs. The virus binds to the PMNL plasma membrane and then enters the cell in endocytic vacuoles; however, attachment to the cell membrane appears to be sufficient to activate the respiratory burst and cause the cellular dysfunction.

Exposure of PMNLs to IAV results in a unusual type of cell activation in that hydrogen peroxide is generated in the apparent absence of superoxide production. Furthermore, when PMNLs are incubated with IAV for as little as 2 minutes and then exposed to secondary stimuli, there are alterations in several cellular intermediary activation steps (inhibition of lysosomes-phagosome fusion, altered actin polymerization, and changes in the normal pattern of phosphorylation/dephosphorylation of multiple membrane and cytosolic proteins) and depression of end-stage functions (chemotactic, oxidative, and secretory). This cellular dysfunction is not due to exhaustion of the respiratory burst by IAV. The depressed PMNL function due to IAV occurs when cells are subsequently stimulated with either particulate or soluble compounds. Priming of PMNLs with granulocyte-macrophage colony-stimulating factor (GM-CSF) can completely overcome IAV-induced PMNL dysfunction in cells stimulated with FMLP, but only partially corrects the depressed function in phorbol 12-myristate 13-acetate (PMA)-stimulated cells.

The effect of IAV on PMNLs is likely due to the interaction of IAV with one or more specific receptors on the cell. Recently, Rothwell and Wright have shown that IAV can bind to the predominant sialoprotein on human PMNLs, sialophorin, which serves as a receptor and is recognized by the CD43 monoclonal antibody (MoAb). Additionally, they found that cross-linking the CD43 MoAb with IgG-coated immunobeads can induce hydrogen peroxide production similar to IAV. The present study was performed to examine whether IAV-induced PMNL dysfunction could also be mediated through the sialophorin receptor. The results show that, when PMNL are exposed to the CD43 MoAb without the addition of cross-linking antibody, significant depression of the PMNL chemiluminescence (CL) response occurs. This PMNL dysfunction is similar to that produced by IAV in that it occurs in response to both particulate and soluble stimuli and can be overcome by cross-linking the CD43 MoAb or by priming the cells with GM-CSF.

Materials and Methods

Reagents. Intact goat antimouse IgG was obtained from Pierce Inc (Rockford, IL), intact goat antirabbit IgG from Sigma Co (St Louis, MO), CD43 MoAb from Dako Corp (Carpinteria, CA), LFA-1B anti-CD18 from AMAC Inc (Westbrook, ME), and recombinant human GM-CSF from Immunex Inc (Seattle, WA).

Preparation of IAV. The recombinant IAV (X-42) containing the internal proteins of influenza A Puerto Rico 8/34 (H1N1) and the surface glycoproteins in influenza A Victoria/3/75 (H3N2) was used in this study. This strain of virus causes PMNL dysfunction that is equivalent to other strains of IAV including A/PR/8/34 (H1N1) and A/Texas/77 (H3N2). Virus was harvested from allantoic fluid of embryonated chicken eggs and purified as previously described.

Isolation of PMNLs. Purified populations of PMNLs (≥97%) were obtained from heparinized whole blood by separation of the buffy coat on Isolymph (Gallard-Schlesinger Co, Carle Place, NY) and hypotonic lysis of erythrocytes as previously described.

Respiratory burst activity. The luminol-enhanced CL assay was used to examine the effect of the CD43 MoAb and IAV on PMNL

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It is a measure of the oxidative burst that is dependent on degranulation of myeloperoxidase and correlates well with bactericidal activities.\(^{19}\) We have found this assay to be a sensitive method for detecting both IAV-induced activation and depression of PMNL function.\(^{23}\) Briefly, the CL reaction mixture contained 1 mL of 2.5 \(\times\) 10\(^{-5}\) PMNLs, 275 \(\mu\)g/mL luminol, (5-amino-2,3-dihydro-1,4-phthalalazinedione; Sigma Chemical Co) and Hank's Balanced Saline Solution with Ca\(^{2+}\), Mg\(^{2+}\), and \(1\%\) gelatin. Vials containing cells, luminol, and buffer were placed in a liquid scintillation counter (Beckman LS-100) in the out-of-coincidence mode and, after backgrounds are less than 20,000 cpm, the PMNLs were exposed to CD43 MoAb, IAV, or buffer for 30 minutes. In some experiments, goat antimouse IgG or GM-CSF was then added for an additional 30 minutes. The vials were mixed and counts were analyzed for 12 seconds at 2-minute intervals. These PMNLs were subsequently stimulated with FMLP (10\(^{-5}\) mo/L), opsonized zymosan (16.7 mg/mL), or PMA (100 ng/mL). Time to reach peak CL activity and the cpm at peak activity of duplicate samples were recorded. Superoxide production, as measured by the reduction of ferricytochrome c, was also examined in some experiments.\(^{20}\)

**Statistics.** The CL data were analyzed using the unpaired Student's t-test.

**RESULTS**

**Effect of CD43 MoAb and IAV on PMNL function.** The CL response was measured in PMNLs exposed to the CD43 MoAb or IAV alone or with subsequent stimulation with FMLP, PMA, or opsonized zymosan. Preliminary studies showed that a concentration of 0.9 \(\mu\)g/mL of the CD43 MoAb produced the maximum depression in the CL response (range tested, 0.15 to 9 \(\mu\)g/mL) and this dose was used in all subsequent experiments. Exposure of PMNLs to the CD43 MoAb alone produced no CL response, whereas a small CL response was seen when cells were incubated with IAV (10 to 15 \(\times\) 10\(^5\) cpm above background). Subsequent exposure of these cells to FMLP, PMA, or opsonized zymosan caused significant depression of the CL response compared with equivalently stimulated buffer-treated PMNLs (Table 1). The CL response in cells treated with the CD43 MoAb followed by a particulate or soluble stimulus was more depressed (74% to 88%) compared with IAV (36% to 50%). However, we have previously shown that other harvests of the same strain of IAV (X-47) can cause a similar degree of depression (up to 77%) of the CL response as that seen in CD43 MoAb-treated cells in the present study.\(^2\)

Separate control experiments were performed to confirm that the effect of the CD43 MoAb on PMNL function was not caused by a nonspecific effect of MoAbs. When CD43 MoAb, not bound to the receptor after 30 minutes of incubation with PMNLs, was removed by repeated washing, the PMNL CL response was decreased by 80% ± 2%, 52% ± 2%, and 81% ± 7% compared with buffer-treated cells stimulated with FMLP, PMA, and opsonized zymosan, respectively (P < .05). Incubation of PMNLs with the LFA-18 anti-CD18 antibody, which is not thought to attach to the sialophorin receptor, did not cause inhibition of the PMNL CL response to any stimuli. The effect of the CD43 MoAb on superoxide production was also examined to make certain that this antibody did not cause an artifactual effect on the PMNL CL response. Superoxide production was decreased 71% ± 7% and 63% ± 12% in PMNLs treated with CD43 MoAb compared with control cells exposed to PMA and zymosan, respectively (P < .05); the response to FMLP was too small to measure by this assay.

**Effect on PMNL function of cross-linking the CD43 MoAb and aggregating IAV.** Studies were performed to examine the effect on the CL response of cross-linking the CD43 MoAb with goat antimouse IgG. When PMNLs were incubated with up to 9.0 \(\mu\)g/mL goat antimouse antibody alone for up to 1 hour, no CL response was detected and, upon subsequent stimulation of the cells with FMLP, the CL response was equivalent to cells exposed to buffer plus FMLP (Fig 1). Exposure of PMNLs to 0.9 \(\mu\)g/mL of the CD43 MoAb for 30 minutes followed by 2.25 \(\mu\)g/mL of the cross-linking antibody for 30 minutes caused no detectable activation of the CL response. These cells were then stimulated with FMLP and the CL response was similar to that seen with buffer-treated cells activated with FMLP (148 cpm ± 20 cpm v 144 cpm ± 21 cpm, respectively; P = .9). Thus, the addition of cross-linking antibody to PMNLs already treated with the CD43 MoAb eliminated the depressing effect of the CD43 MoAb on the cell's CL response noted in Table 1.

To make certain that the loss of PMNL dysfunction was not a nonspecific effect due to Fc receptor binding by antibody, an experiment was performed in which PMNLs were incubated with CD43 MoAb or buffer for 30 minutes followed by a control antibody (goat antirabbit IgG) for 30

<table>
<thead>
<tr>
<th>Initial Agent</th>
<th>Secondary Stimulus</th>
<th>CL Response (cpm × 10(^3))</th>
<th>% Depression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>FMLP</td>
<td>188 ± 36</td>
<td>0</td>
</tr>
<tr>
<td>Buffer</td>
<td>PMA</td>
<td>887 ± 37</td>
<td>0</td>
</tr>
<tr>
<td>Buffer</td>
<td>Zymosan</td>
<td>752 ± 140</td>
<td>0</td>
</tr>
<tr>
<td>CD43 MoAb</td>
<td>FMLP</td>
<td>48 ± 111</td>
<td>74</td>
</tr>
<tr>
<td>CD43 MoAb</td>
<td>PMA</td>
<td>179 ± 34I</td>
<td>79</td>
</tr>
<tr>
<td>CD43 MoAb</td>
<td>Zymosan</td>
<td>84 ± 17I</td>
<td>68</td>
</tr>
<tr>
<td>IAV</td>
<td>FMLP</td>
<td>75 ± 11</td>
<td>50</td>
</tr>
<tr>
<td>IAV</td>
<td>PMA</td>
<td>825 ± 106</td>
<td>47</td>
</tr>
<tr>
<td>IAV</td>
<td>Zymosan</td>
<td>771 ± 60</td>
<td>36</td>
</tr>
</tbody>
</table>

PMNLs (2.5 \(\times\) 10\(^5\)/mL) were incubated with 0.9 \(\mu\)g/mL CD43 MoAb, 20 \(\mu\)g/mL IAV, or buffer and the CL response was measured. After 30 minutes, the cells were stimulated with FMLP, PMA, or opsonized zymosan and the CL response was again recorded. Each experimental condition was performed on ≈3 occasions and the results given are the mean ± SEM.

* The percent depression of the CL response was calculated as follows: 1 – (the peak CL response in PMNLs exposed to the CD43 MoAb or IAV followed by the second stimulus ÷ the peak CL response in buffer-treated cells receiving the equivalent stimulus). The value given is the mean percent depression for all experiments performed with a particular secondary stimulus.

† P < .01 when PMNLs treated with CD43 MoAb followed by a secondary stimulus are compared with the equivalent control.

‡ P < .05 when PMNLs treated with IAV followed by a secondary stimulus are compared with the equivalent control.
INFLUENZA ALTERS PMN FUNCTION VIA CD43 RECEPTORS

Fig 1. The effect on the PMNL CL response of cross-linking CD43 MoAb with goat antimouse IgG. For the representative experiment shown here, PMNLs (2.5 x 10^6/mL) were incubated with CD43 MoAb (0.9 μg/mL) or buffer for 30 minutes with or without the addition of goat antimouse (9.0 μg/mL) for an additional 30 minutes. Cells were then stimulated with FMLP. The CL response was continuously monitored throughout the experiment. Goat antimouse did not elicit any detectable CL response.

The figure shows a graph with the CL response (cpm) on the y-axis and time (min) on the x-axis. Three conditions are compared: Control, CD43, and CD43 + IgG. The CL response is plotted at different time points, indicating the inhibition of the CL response in the presence of CD43 and IgG compared to the control.

In the representative experiment shown here, PMNLs (2.5 x 10^6/mL) were incubated with CD43 MoAb (0.9 μg/mL) or buffer for 30 minutes with or without the addition of goat antimouse (9.0 μg/mL) for an additional 30 minutes. Cells were then stimulated with FMLP. The CL response was continuously monitored throughout the experiment. Goat antimouse did not elicit any detectable CL response.

We have previously reported that some harvests of IAV did not depress PMNL function when cells were stimulated with particulate or soluble stimuli. Experiments were performed to determine if the inability of some of the viral harvest to depress PMNL function could be due to aggregation of the virus. IAV obtained from the same harvest of eggs underwent an equivalent purification procedure except that the virus was pelleted by high-speed centrifugation with or without putting a 60% sucrose cushion at the bottom of the tube. The sucrose cushion allows the virus to be pelleted without individual virions aggregating together. Incubation of PMNLs with either preparation of IAV caused a small CL response (10 to 20 x 10^3 cpm). When cells were subsequently stimulated with FMLP, only virus from the preparation spun on the sucrose cushion caused PMNL dysfunction (34% and 3% depression in cells treated with virus prepared by pelletting with or without a sucrose gradient, respectively, when compared with buffer-treated cells stimulated with FMLP). Similar results were obtained in two additional experiments. Electron microscopy experiments showed that nondepressing virus was often found in aggregates of various sizes, whereas the depressing virus was usually seen as single virions.

Capacity of GM-CSF to overcome PMNL dysfunction due to the CD43 MoAb. We previously reported that incubating PMNLs with the cytokine GM-CSF at a dose of 50 ng/mL for 30 minutes completely overcame the depressing effect of IAV on the PMNL CL response to FMLP. Studies were therefore undertaken to determine if GM-CSF was also capable of overcoming PMNL dysfunction due to the CD43 MoAb. Preliminary studies were performed to determine the concentration of GM-CSF that was best able to reverse the PMNL dysfunction due to the CD43 MoAb. The concentration range of GM-CSF tested was 0.1 pg/mL to 500 ng/mL and some reversal of cell dysfunction was seen at concentrations as low as 100 pg. Similar to IAV, the maximum effect was seen at a concentration of 50 ng/mL of GM-CSF. At this dose, GM-CSF was able to completely overcome the PMNL depression induced by IAV (Table 2). PMNL treated with the CD43 MoAb and then incubated with GM-CSF before FMLP stimulation exhibited a CL response that was greater than that occurring in FMLP-stimulated control cells that were not primed with GM-CSF. Although the CL response to FMLP in GM-CSF-primed, CD43 MoAb-treated cells was not as high as primed control cells, the magnitude of the priming effect of GM-CSF on the cells was, if anything, greater; ie, GM-CSF-primed control cells had a 2.5-fold increase in the CL response compared with nonprimed, control cells, whereas there was a 5.7-fold increase in GM-CSF-primed, CD43 MoAb-treated cells compared with nonprimed, CD43 MoAb-treated cells.

DISCUSSION

Rothwell and Wright have recently shown that IAV can bind to the sialoglycoprotein receptor on PMNLs. When the cells are incubated with the CD43 MoAb plus antimouse IgG immunobeads, the respiratory burst is activated. The investigators note that a previous study had found that the CD43 antibody, when used alone, was not capable of inducing the respiratory burst in PMNLs and speculated that the IgG beads served the purpose of presenting the antibody to the cell's CD43 receptors in an orientation that resembled IAV. We also found that, when PMNLs were incubated with the CD43 MoAb alone, there was no activation of the respiratory burst, whereas unopsonized IAV elicited a small CL response, as previously reported. When either CD43 MoAb or unopsonized IAV-treated PMNLs were subsequently stimulated with particulate or soluble stimuli, there was marked inhibition of the cell's respiratory burst. When CD43 MoAb-treated PMNLs were cross-linked with goat antimouse IgG and then stimulated with FMLP, no inhibition of cell function was observed. Similarly, aggregation of IAV resulted in loss of the ability of the virus to depress the respiratory burst of the PMNLs. We have previously shown that both GM-CSF and G-CSF can overcome IAV-induced PMNL dysfunction in vitro and in vivo. The results of this study show that an equivalent priming dose of GM-CSF can reverse PMNL dysfunction induced by the CD43 MoAb. These data indicate that the PMNL dysfunction induced by IAV is very similar to that caused by the CD43 MoAb.
Neither this study nor that performed by Rothwell and Wright have excluded the possibility that IAV is binding to sites other than the sialophorin receptor. Therefore, although it appears likely that IAV-induced PMNL dysfunction is mediated, at least in part, through the attachment of the virus to the sialophorin receptor, a definitive conclusion awaits further study.

The exact site within the sialophorin receptor to which IAV or the CD43 MoAb binds may be critical to whether the signal transduction pathway is activated and PMNL dysfunction occurs. IAV binds to the sialyoligosaccharide N-acetylmuramic acid galactose. The linkage between the N-acetylmuramic acid and galactose is crucial to the specificity of binding by different viral strains. The exact site of binding of the CD43 MoAb in the sialophorin receptor is uncertain. Cross-linking the CD43 MoAb or aggregating IAV is likely to change the way these ligands attach to the receptor and thereby alter the nature of the signal transmitted to the interior of the cell. This may explain why cross-linking of CD43 MoAb and aggregating IAV eliminates PMNL dysfunction. It also offers a potential reason for the apparent difference between the study of Rothwell and Wright and our study in which cross-linking the CD43 MoAb with immunobeads, but not goat antimouse IgG antibody, caused activation of the respiratory burst (ie, these two cross-linking agents may cause the CD43 MoAb to bind to the sialophorin receptor in a different manner).

The activation pathway and subsequent PMNL functions elicited by binding of ligand to the sialophorin receptor are currently under investigation. The sialophorin receptor is a long, rigid molecule with a strong negative charge and when cells are activated (eg, by adherence to a surface) the receptor is shed from the membrane. Nathan et al have shown that blocking shedding of this receptor with human serum albumin inhibited the respiratory burst of cells adhered to protein-coated tissue culture plates and stimulated with tumor necrosis factor, CSa, or FMLP, but not with PMA. In contrast to these findings, in our study exposure of PMNLs to CD43 MoAb or IAV caused inhibition of the respiratory burst when cells were subsequently stimulated with FMLP or PMA. The different response of PMNLs to PMA in these two studies could be due to our use of PMNLs in suspension, rather than cells already activated through adherence to a surface with blocking of sialophorin shedding with albumin. Although presently unknown, it is possible that the amount of sialophorin on the PMNL membrane required to inhibit the PMNL response to PMA versus FMLP is different.

The exact mechanism by which IAV inhibits PMNL function and GM-CSF reverses this process has not been determined. Based on several lines of evidence, we have previously postulated that IAV mediates its effect by altering the function of one or more trimeric G-proteins. The virus alters phosphorylation of multiple proteins in the membrane and cytosol fraction, suggesting that the signal transduction step at which IAV exerts its effect occurs before activation of protein kinases. Similar to pertussis toxin, which inhibits the function of several trimeric G-proteins, IAV depresses oxidative, chemotactic, secretory, but not phagocytic activity. These data raise the possibility that IAV is altering one or more G-proteins in the PMNLs. GM-CSF could be directly reversing the effect of IAV on the PMNLs by upregulating the G2a subunit. Alternatively, GM-CSF could be indirectly reversing PMNL dysfunction by augmenting alternative pathways.

The inhibition of PMNL function induced by IAV and the CD43 MoAb and the reversal of this cell dysfunction by GM-CSF are consistent with the possibility that ligand binding of the sialophorin receptor alters G-protein function. The antibodies and cytokines used in this study will provide useful tools for further exploring the mechanism of IAV-induced activation and inhibition of PMNL function and the role that this has in clinical disease.

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REFERENCES

Role of the sialophorin (CD43) receptor in mediating influenza A virus-induced polymorphonuclear leukocyte dysfunction

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