Neutrophils Do Not Bind To or Phagocytize Human Immune Complexes Formed With Influenza Virus

By Don R. Ratcliffe, Josef Michl, and Eva B. Cramer

Neutrophils appear to form the first line of defense against influenza virus, yet it is unclear how these leukocytes recognize influenza-infected cells. While demonstrating that neutrophils adhere specifically to the sialic acid-binding site on the hemagglutinin molecule (HA) on the surface of influenza-infected [WSN (H1N1)] epithelial cells and not to other viral or epithelial cell antigens, it was observed that human neutrophils do not recognize immune complexes formed with influenza virus. Intact antibodies (mouse monoclonal antibodies [MoAbs] IgG1 and IgG2b, human antibody heat-inactivated serum [predominantly IgG2a], and IgG purified from human immune serum) that block the sialic acid-binding site on HA significantly reduced (> 80%) neutrophil adherence to influenza-infected epithelial cells. Binding and phagocytosis of free influenza virions and neutrophil agglutination by influenza virus were completely prevented by these antibodies. Intact and F(ab'), fragments of mouse MoAbs to other viral epitopes caused increased neutrophil adherence to infected cells. This binding was eliminated by F(ab'), fragments of MoAbs against the sialic acid-binding site on HA, but not by saturating amounts of MoAbs, which block the neutrophil Fc receptors. Thus, it appears that human neutrophils show little ability to bind via their Fc receptors to the immune complexes formed with antibody and either influenza-infected epithelial cells or the free virion. These findings are in contrast to the general dogma, and are the first example of antibody opsonization reducing, rather than enhancing, neutrophil binding and phagocytosis of a pathogen.

© 1993 by The American Society of Hematology.

INFLUENZA INFECTION in humans is an acute, highly contagious respiratory disease that still causes nationwide epidemics in developed countries. The virus replicates in the respiratory epithelia, and peak virus production occurs within 2 days of inoculation.1 Neutrophils are the first leukocytes to accumulate at the site of infection,1 and they have been shown in vitro to adhere to influenza-infected epithelial cells in the absence of antibody and complement,2 to phagocytize influenza virions,3 and to generate toxic oxygen species in response to unopsonized influenza virus.4 These observations suggest that neutrophils form the first line of defense against influenza virus infection, but it is not known how they recognize influenza virus or influenza-infected epithelia.

It is presumed that neutrophils will recognize influenza virus via the same sialic acid-binding site on the viral hemagglutinin molecule (HA) that is used by erythrocytes.4 In the presence of antibodies, neutrophil binding to influenza virus is assumed to be enhanced by opsonization. However, these ideas have not been tested. In this study, it was determined that neutrophils bind to both influenza virus and influenza-infected cells via the sialic acid-binding site on HA. They do not recognize other viral or cellular antigens on the surface of the infected epithelial cells. When antibodies block this site, the majority of neutrophils are unable to detect the virus or the infected cell. Furthermore, in contrast to general assumptions, neutrophils are unable to bind, via their Fc receptors, to immune complexes formed with this or other epitopes on the virus.

MATERIALS AND METHODS

Cell Culture and Virus

The WSN (H1N1) strain of influenza virus was grown in the Madin Darby Canine Kidney (MDCK) cell line as previously reported.2

Preparation of Monoclonal Antibodies (MoAbs) and Antibody Fragments

The MoAb against WSN viral proteins used in this study were produced from hybridomas provided by Dr W. Gerhard (Wistar Institute, Philadelphia, PA). The hybridomas were H17-L19 (mouse IgG1, directed against site CA on HA), H15-C5 (mouse IgG2b, directed against HA), H3-4C5 (mouse IgG1, directed against site CA2 on HA), H18-L17 (mouse IgG1, directed against the neuraminidase molecule [NA]), M2-1C6 (mouse IgG1, directed against the matrix protein [M]), H17-L8 (mouse IgG2a, directed against the nucleoprotein [NP]), and NS1-7A3 (mouse IgM, directed against nonstructural protein [NS]).24 MoAb H17-L19 and MoAb H15-C5 blocked the red blood cell-binding site on the globular head of HA, while MoAb H3-4C5 was more than 1,000-fold less efficient at hemagglutination inhibition. To reduce confusion, MoAb H17-L19, which interferes with the sialic acid-binding site, will be referred to as anti-HA,5 MoAb H3-4C5 as anti-HA,5 and H18-L17 as anti-NA. The hybridoma IV.3, which produces an IgG2b MoAb against the huFcYRII,9 was purchased from American Type Culture Collection (Rockville, MD). MoAb 3G8, an IgG1 against the huFcYRII,9 was provided in ascites form by Dr J. Unkeless (Mount Sinai School of Medicine, New York, NY) and Dr H. Felt (SUNY Health Science Center at Stony Brook, Stony Brook, NY). Hybridomas were grown in tissue culture and as ascites tumors in Balb/c mice (Charles River Laboratory, Boston, MA). MoAbs from supernatant culture medium were purified by affinity chromatography on a Protein A agarose column (Pierce, Rockford, IL) while those from ascites fluids were purified by chromatography on a DEAE Affi-gel blue column (Bio-Rad Laboratories, Rockville Centre, NY) as described by Bruck et al11 or by affinity chromatography on a Protein A Superose HR 10/2 column on a fast-protein liquid chromatography system (Pharmacia, Upsala, Sweden). F(ab')2 and Fab fragments of MoAbs were prepared by affinity chromatography on a Protein A Superose HR 10/2 column on a fast-protein liquid chromatography system (Pharmacia, Upsala, Sweden).
according to the manufacturer’s protocols using immobilized pep-
sin and papain (Pierce), respectively, and were separated from
partially digested and undigested IgG by Protein A affinity chroma-
tography. Purity was assessed by sodium dodecyl sulfate-polyacryl-
amide gel electrophoresis (SDS-PAGE).

To quantitate HA expression on MDCK cells, purified anti-HA\(^+\) MoAb H17-L19 was labeled with \(^{125}\)I to a final specific activity of approximately \(3.7 \times 10^8\) cpm/\(\mu\)g protein with IODO-BEADS (Pierce) as the oxidizing reagent according to the manufacturer’s protocol. Various concentrations of \(^{125}\)I-anti-HA\(^+\) (10 to 120 \(\mu\)g/
\(\mu\)L) were incubated for 1 hour at 4°C with influenza-infected
MDCK monolayers (multiplicity of infection [moi] of 4, 6.5 hours).
Binding saturated at approximately 100 \(\mu\)g/mL and could be inhib-
ited 98% with a 100-fold excess of unlabeled anti-HA\(^+\) MoAb.

Preparation of Human IgG and Serum Minus IgG

Serum from an individual with a high hemagglutination inhibi-
tion titer to influenza virus WSN (1:512) and with low reactivity
to MDCK cells was heat-inactivated at 56°C for 30 minutes (im-
mune HIS) and then stored at \(-20^\circ\)C until used. Human IgG
(hulgG) was purified from this immune HIS by affinity chromato-
graphy on a Protein G Sepharose 4 Fast Flow column (Pharmacia).
The presence of IgG in the bound fraction and its absence in the
flow through material (immune HIS minus IgG) were determined
by immunoblot.

Assay of Neutrophil Adherence to Epithelium

Neutrophils were isolated from the blood of normal volunteers as
previously reported.\(^2\) Confluent MDCK monolayers grown on Ter-
asaki dishes were infected with influenza virus (moi 4). The monol-
ers were washed in Gey’s solution before adding immune HIS,
purified immune hulgG, immune HIS minus IgG, nonimmune HIS,
and intact MoAbs or their F(ab’)_2 fragments (5 to 200 \(\mu\)g/mL)
for 15 minutes at 37°C. Neutrophils (0.005 mL at 10 \times 10^6
cells/mL), suspended in Gey’s solution, were then incubated above
the epithelium for 15 minutes at 37°C either in the presence of anti-
body or after its removal. In certain experiments, the neutrophils
were preincubated for 30 minutes at 4°C with MoAb 3G8 (50 \(\mu\)g/
\(\mu\)L) or its Fab fragments (100 \(\mu\)g/mL) or MoAb IV.3 (50 \(\mu\)g/mL).
The nonadherent neutrophils were removed by inversion the Ter-
asaki dishes in a beaker of Gey’s solution or phosphate-buffered
saline (PBS), and then the epithelial monolayers and any adherent
neutrophils were fixed in ice-cold 4% paraformaldehyde plus 0.1%
paraformaldehyde in PBS (pH 7.4) for 1 hour. The number of neutro-
phils within three 0.06-mm\(^2\) areas per culture was counted by image
analysis (Micro-Comp Data Acquisition System, SMI, Atlanta,
GA) on a Zeiss microscope. The average number of adherent cells
per millimeter squared was determined. Each experimental vari-
able was run in at least five replicate wells and the mean \pm SD
determined. Statistical differences between groups were determined
by Student’s \(t\)-test.

Neutrophil Agglutination and Agglutination Inhibition
Assays

Serial twofold dilutions of WSN virus were made in PBS plus
0.5% bovine serum albumin (BSA), and 0.025 mL of each dilution
was added to triplicate wells of a V-bottomed 96-well tray. Neutro-
phils (4 \times 10^6 cells/mL) were added (0.025 mL/well) and allowed to
settle at room temperature for 1 hour. Hemagglutination assays
were run similarly, except that 0.5% human type 0 erythrocytes in
PBS were substituted for the neutrophils. It required a fourfold
increase in virus concentration to agglutinate neutrophils as com-
pared with the erythrocytes.

To quantitate the amount of antibody required to prevent influ-
enza virus from agglutinating neutrophils, decreasing concentra-
tions of antibody were added to four agglutination units of virus for
1 hour at room temperature. Neutrophils were then added for 1 hour
at room temperature, and the least amount of antibody needed to
prevent agglutination was determined.

Rosette Assay for Fc Receptors

The least amount of rabbit anti-IgG (Diamedix, Miami, FL) needed to agglutinate sheep erythrocytes was diluted twofold and added to 50% erythrocytes in Alsever’s solution.\(^7\) The rabbit anti-IgG (Diamedix) complexes [E(IgG); 0.25% in Gey’s solution] were incubated for 30 minutes at room temperature with
neutrophils bound to various substrates. The nonadherent E(IgG)
were removed by inverting the Terasaki dishes in a beaker of Gey’s solution or PBS. A rosette-positive neutrophil had at least three
bound E(IgG) at the mean. The WSN strain of influenza virus does not aggluti-
nate sheep erythrocytes.

Ultrastructural Analysis

Neutrophils, in suspension with influenza virus or with antibody-
treated influenza virus (15 minutes at 37°C or 4°C), were washed,
pelleted, and embedded in agar. These agar-embedded cells or mi-
cropore filters covered with influenza-infected epithelial mono-
layers and adherent neutrophils were fixed, embedded in Epon 812,
and examined as previously reported.\(^5\)

Indirect Immunofluorescence

Infected monolayers were either initially permeabilized with ace-
tone (5 minutes at 4°C) or incubated directly with an MoAb against
one of the viral proteins of WSN (30 minutes at 4°C). The cultures
were then rinsed in PBS plus 1% fetal calf serum (FCS), fixed in 4%
paraformaldehyde plus 0.1% glutaraldehyde in PBS, and washed in
PBS plus 50 mmol/L NH_4Cl and then PBS plus 1% FCS. The tissue
was then incubated in rhodamine-conjugated, affinity-purified
F(ab’)_2 fragments of goat antimouse (GAM) IgG (heavy and light
chain-specific) or GAM IgM (Accurate Chemical & Scientific,
Westbury, NY) for 1 hour at room temperature, washed in PBS,
and mounted in 90% glycerol in PBS. To determine the subclass
specificity of human Ig in immune HIS that binds to WSN-infected
epithelial cells, infected MDCK cells were fixed, washed in PBS plus
1% normal goat serum, incubated with immune HIS for 1 hour
at room temperature, washed in PBS plus 1% normal goat serum,
and incubated with MoAbs against hulgG\(_1\), hulgG\(_2\), hulgG\(_3\), or hulgG\(_4\)
(Miles Scientific, Elkhart, IN). After 1 hour, the cultures were rinsed
in PBS plus 1% normal goat serum and then incubated with rhoda-
mine-labeled GAM antibodies. All cultures were examined with a
Nikon Diaphot TMD Epifluorescent microscope (Nikon, Tokyo,
Japan) equipped with a Videoscope VS2000N video camera and
intensifier (Videoscope International, Herndon, VA) on a TN-8500
image analysis system (Tracor Northern, Middleton, WI). Fluores-
cence intensity measurements were determined with the camera
gain at 600, the intensifier gain at 400, and the background at less
than 11. The mean \pm SD of the average fluorescence intensity was
calculated from at least three 20\(^\times\) fields of view per culture. Statistical
differences between groups were determined by Student’s \(t\)-test.

RESULTS

Detection of Influenza Antigens on the Surface
of Infected Epithelial Cells

Indirect immunofluorescence studies were used to detect
the presence of (1) M\(_1\), (2) NS\(_1\), (3) NP, (4) NA, and (5) HA,
were incubated for the virus replication cycle. At 2.5 hours, NP appeared on the infected cells. In contrast, only three of the viral proteins intensity was recorded in arbitrary fluorescent units on a scale of 0 to determined by the intensity of the fluorescence, had in-

Background intensity these proteins on their surface and the amount of protein, as increased significantly.

Site of Neutrophil Binding

Previous experiments in our laboratory have shown that neutrophils adhered only to the influenza-infected MDCK cells and did not adhere to uninfected MDCK cells. Adherence to influenza-infected MDCK monolayers began by 4.5 hours and increased at 6.5 hours. This adherence coincided with the presence and amount of HA and NA on the surface of the epithelial cell. Neutrophil binding to influenza-infected epithelium at 6.5 hours was prevented by F(ab')2, fragments of anti-HA (anti-HA+; Fig 1), but not against the Ca2 site on HA (anti-HA-), or NA (Fig 2).

**Neutrophils Do Not Bind to Immune Complexes Formed on the Surface of Influenza-Infected Epithelia**

*Immune complexes formed with mouse MoAb against HA* and NA. Both intact and F(ab')2 fragments of anti-HA+ and anti-NA caused increased neutrophil adherence (Fig 2). While the intact and F(ab')2 fragments of anti-HA- antibodies appeared by electron microscopy to cause clumping of the virus (Fig 3), they did not increase the total amount of viral protein (as determined by the amount of bound 125I-anti-HA*) associated with the epithelial monolayer. The increased neutrophil adherence was to HA and not to the immune complexes, because F(ab')2 fragments of anti-HA+ completely eliminated neutrophil adherence (Fig 4).

**Immune complexes formed with human antibody. To**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Control</th>
<th>2.5 h</th>
<th>4.5 h</th>
<th>6.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>1% ± 13 ± 1.1</td>
<td>1% ± 13 ± 0.1</td>
<td>1% ± 13 ± 0.8</td>
<td>1% ± 14 ± 0.3</td>
</tr>
<tr>
<td>NS1</td>
<td>1% ± 11 ± 0.1</td>
<td>1% ± 11 ± 0.1</td>
<td>1% ± 11 ± 0.1</td>
<td>3% ± 11 ± 1.0</td>
</tr>
<tr>
<td>NP</td>
<td>1% ± 13 ± 0.2</td>
<td>33% ± 24 ± 4.0</td>
<td>3% ± 17 ± 2.3</td>
<td>3% ± 13 ± 1.2</td>
</tr>
<tr>
<td>NA</td>
<td>1% ± 13 ± 0.4</td>
<td>1% ± 12 ± 0.9</td>
<td>15% ± 21 ± 1.1</td>
<td>82% ± 92 ± 9.9</td>
</tr>
<tr>
<td>HA</td>
<td>1% ± 14 ± 1.3</td>
<td>1% ± 13 ± 1.8</td>
<td>25% ± 54 ± 4.4</td>
<td>85% ± 111 ± 11.2</td>
</tr>
</tbody>
</table>

MDCK cultures were infected with influenza virus at moi of 4 and incubated at 37°C for the times indicated. Uninfected control and infected cultures were incubated for 30 minutes on ice with MoAbs against M1, NS1, NP, NA, and HA, and processed for immunofluorescence. The area of the MDCK monolayer with fluorescence above background and the intensity of the fluorescent positive area were determined (see Materials and Methods). The intensity was recorded in arbitrary fluorescent units on a scale of 0 to 256. The mean intensity ± SD was determined from triplicate samples. The background intensity (13 ± 1.1) was determined from uninfected monolayers.

* Fluorescence intensity significantly greater than control at P < .001.
† Fluorescence intensity significantly greater than control at P < .01.
‡ Fluorescence intensity significantly greater than control at P < .05.
AATCLIFFE, MICHL, AND CRAMER

Q,

S

S

'CI

a

600

500

400

300

200

100

0

0 10 20 50 100 200

E

Anti-NA

IgG

Anti-NA

F(ab')

2

100

0

50

20

10

0

Antibody Concentration (ug/ml)

Fig 2. Neutrophil adherence to anti-HA-- and anti-NA-
treated, influenza-infected epithelia. Confluent monolayers of
MDCK cells infected with influenza virus (moi 4, 7 hours) were in-
cubated (37°C, 15 minutes) with intact or F(ab') fragments of anti-
HA- or anti-NA at the concentrations indicated. Neutrophils (1 0 X
106 cells/mL, 5 pL/well) were added for 15 minutes. The number of
adherent neutrophils (per 0.06 mm2) is expressed as the mean ±
SD for five replicate cultures.

determine if the neutrophils could detect immune com-
exed with human antibody, influenza-infected epi-
thelia were incubated with immune HIS, which had antibod-
ies against the sialic acid-binding site on HA as determined
by hemagglutination inhibition (2256 hemagglutination-
inhibition units/25 pL serum). All immune HIS tested (n =
4) contained predominantly IgG 1 antibodies to the influ-
enza antigens on the surface of the infected MDCK epithe-
"lial cells (Table 2). Low concentrations of immune HIS (di-
luted 10 in Gey’s solution) had no effect on the number of
adherent neutrophils, while 40% immune HIS (hemagglu-
tination inhibition titer equivalent to anti-HA' at 100 pg/
mL) caused an average reduction in neutrophil binding of
83% in three experiments. Purified immune hulG at 5.6
mg/mL (~40% of the IgG concentration in serum) reduced
neutrophil adherence by an average of 93% in three exper-
iments. Immune HIS minus IgG at high concentrations
(23.2 mg/mL, ~40% of the concentration of the non-IgG
proteins in serum) had variable effects on neutrophil adher-
ence. In 2 of 6 experiments, it caused no reduction in bind-
ing and in four experiments it caused some reduction. When immune HIS minus IgG was used at a concentration
equivalent to the purified immune hulG (5.6 mg/mL), it
had no effect on neutrophil binding. Nonimmune HIS
(40%) did not decrease neutrophil binding. These results are
shown in a representative experiment in Fig 5.

Role of the Fc Receptor in Neutrophil Binding to
Influenza-Infected, Antibody-Treated Epithelial Cells

Pretreatment of neutrophils with MoAb 3G8 (50 µg/mL;
anti-huFcγ RIII) had no effect on the binding of neutrophils
to any of the antibody-treated, influenza-infected cells (Ta-
ble 3). In contrast, pretreatment of neutrophils with MoAb
IV.3 (50 µg/mL; anti-huFcγ RI) eliminated the small
(27%) neutrophil adherence to anti-HA'--treated, influ-
enza-infected cells. MoAb IV.3 had no effect on the binding
of neutrophils to anti-HA', immune HIS-, or purified im-
mune hulG--treated monolayers.

ability of neutrophils to bind nonviral immune complexes

Neutrophils were able to bind nonviral immune com-
exes. Ninety-five percent of the neutrophils adherent to
infected epithelial monolayers bound E(IgG). Pretreatment
of neutrophils with MoAb IV.3 (50 µg/mL; anti-huFcγ RI),
MoAb 3G8 (50 µg/mL; anti-huFcγ RIII), or both
MoAb 3G8 (50 µg/mL) and IV.3 (50 µg/mL) reduced
E(IgG) rosetting by 21%, 93%, and 100%, respectively.

Inhibition of Virion-Mediated Agglutination of Neutrophils
by Mouse MoAb and Immune HIS

Agglutination of neutrophils by influenza virions can be
prevented with anti-HA' F(ab')2 fragments (0.6 µg/mL),
but not by anti-HA' and anti-NA (400 µg/mL). Intact anti-
obodies against the sialic acid-binding site on HA (anti-HA'
[IgG1, 0.3 µg/mL], anti-HA' IgG2b [MoAb H15-C5, 0.3
µg/mL], and immune HIS [diluted ≤ 4 X 10-3]) also inhib-
ited agglutination of neutrophils by influenza virus.

Lack of Neutrophil Binding and Phagocytosis of Virions
Coated With Immune Complexes

Electron microscopic examination of neutrophils sus-
pended with influenza virions showed that neutrophils
bound and phagocytized influenza virions at both 4°C and
37°C.

DISCUSSION

Although neutrophils are the first leukocytes to arrive at
the site of an influenza infection, it is not known how they
recognize influenza virus or influenza-infected cells. Cyto-
toxic T cells recognize and lyse influenza-infected cells in
response to NP and other internal and external influenza
viral proteins. From the present study, it is evident that
neutrophil adherence to influenza-infected epithelia is mediated solely by the sialic acid–binding site on HA, and not by other viral or cellular proteins, since F(ab')₂ fragments against this site completely prevented (>99%) neutrophil binding (Fig 1). The role of sialic acid in this interaction is verified by the finding that preincubation of neutrophils with *Vibrio cholera* neuraminidase (0.75 U/mL, for 30 minutes at 37°C) completely eliminates (100%) neutrophil adherence to influenza-infected epithelia.¹⁴ Although neutrophils readily bind nonviral immune complexes [E(IgG)], they show little adherence to immune complexes formed with influenza virus or influenza viral proteins on the surface of influenza-infected epithelial cells. Our experiments demonstrate that this is not due to (1) a difference in the method used to assess binding, since both experimental situations allow contact to occur by settling and nonadherent cells to be removed by inversion in a beaker of Gey’s solution or PBS; (2) internalization of the immune complexes by the infected epithelial cells, since immunocytochemistry at both the light and electron microscopic levels shows the presence of viral antigen and budding virions on the surface of infected cells in the presence of antibody;² (3) the continued presence of unbound anti-
Neutrophil adherence to influenza-infected epithelia in Gey's solution is significantly less than to infected epithelium in PBS. Neutrophil solution adherence is significantly greater than to infected epithelium in Gey's solution.

Table 3. Role of FcγRII and FcγRIII in Neutrophil Binding to Influenza-Infected Epithelia Incubated With Anti-Influenza Antibodies

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Antibody to Virus</th>
<th>Neutrophil Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Geť's</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>127 ± 39.9</td>
</tr>
<tr>
<td></td>
<td>HIS</td>
<td>16 ± 8.3*</td>
</tr>
<tr>
<td></td>
<td>HulgG</td>
<td>3 ± 0.7*</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>121 ± 24.5</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>33 ± 21.2*</td>
</tr>
<tr>
<td></td>
<td>HA*</td>
<td>59 ± 16.2</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>206 ± 43.2</td>
</tr>
<tr>
<td></td>
<td>HulgG</td>
<td>166 ± 28.4*</td>
</tr>
</tbody>
</table>

MDCK cultures were infected with influenza virus (moc 4, experiments 1 and 2; moc 2, experiment 3) and incubated at 37°C for 6 hours. Anti-HA* (100 μg/mL), anti-HA** (100 μg/mL), immune HIS (40%), or purified immune hulgG (6 mg/mL) were added to the cultures as indicated and incubated at 37°C for 15 minutes. Neutrophils that had been previously treated at 4°C for 30 minutes with 50 μg/mL anti-huFcy RI MoAb IV.3, anti-huFcy RI MoAb 3G8, or both MoAb IV.3 and 3G8 were added to the cultures and incubated at 37°C for an additional 15 minutes. Adherent neutrophils (per 0.06 mm²) are expressed as the mean ± SD for five or six replicate cultures. Neutrophil adherence to uninfected MDCK monolayers was 0 ± 0.2. Abbreviation: ND, not determined.

* Neutrophil adherence is significantly less than to infected epithelium in Geť's solution (P < .001).
† Neutrophil solution adherence is significantly greater than to infected epithelium in Geť's solution (P < .001).
‡ Neutrophil adherence is significantly less than to anti-HA**--treated epithelium (P < .02).
is shown. In some or all of these instances, the enhanced and/or no direct demonstration of Fc receptor involvement made,21-25 experiments are performed in antigen excess though this finding may be unique to influenza virus, the division of virus ability of the immune complexes to cluster on the surface of infected epithelia or on the virions. The density of immune binding."

"Protective effects of neutrophils with Fc receptors. For reasons that are not correct. In many investigations where this assumption is assumption by many that antibody always increases leukocyte binding."

"cytotoxicity reactions require a large effector to target ratio to be effective (typically 100:1).28-30"

"Why the leukocytes are not binding to the influenza immune complexes is not clear. It has been postulated that one of the functions of the carbohydrate on the IgG molecule is to maintain the Fc in a conformation required for binding.31 Influenza virus may be able to alter or distort the Fc portion of the IgG molecule, as viral HA or NA may interact with or modify the carbohydrate on the IgG molecule."

"The inability of most neutrophils to bind and/or phagocytize influenza virions is not due to an inability of the immune complexes to cluster on the surface of infected epithelia or on the virions. The density of immune complexes on each virion could be approximately 48,000 IgG molecules/μm² (1,500 HA binding sites/0.1 μm²). This clustering of ligand should further increase neutrophil binding."

"Intact anti-HA− and anti-NA cause a significant increase in neutrophil adherence to influenza-infected cells. This increased adherence is not due to the presence of immune complexes as (1) F(ab)² of anti-HA− and anti-NA cause the same reaction, (2) treatment of neutrophils with MoAb 3G8 and MoAb IV.3 does not significantly affect neutrophil adherence to anti-HA− -treated infected epithelia (Table 3), and (3) anti-HA+ F(ab)² fragments can completely eliminate neutrophil adherence to anti-HA−-treated infected epithelia (Fig 4). Electron microscopy of influenza-infected epithelia treated with anti-HA− shows aggregation of virions on the epithelial surface. It is possible that the aggregation of virus particles increases neutrophil adherence, since clustering of ligand on the surface of a particle increases the efficiency of neutrophil binding."

"The inability of most neutrophils to bind and/or phagocytize influenza-antibody complexes was not expected. Although this finding may be unique to influenza virus, the assumption by many that antibody always increases leukocyte binding to virus by way of the Fc receptor may not be correct. In my investigations where this assumption is made,21-25 experiments are performed in antigen excess and/or no direct demonstration of Fc receptor involvement is shown. In some or all of these instances, the enhanced response may have been due to antibody-induced aggregation of virus (as seen with anti-HA− MoAb), rather than to Fc receptor interaction with immune complexes."

"In several reports, antibodies appear to increase viral infectivity of cells with Fc receptors. For reasons that are not clear, this only occurs when low (antigen excess), rather than high, concentrations of antibody are used.26,27 Antibody has also been implicated in increasing the ability of neutrophils to kill virus-infected cells.28 In our study, a small population of neutrophils did recognize the immune complexes with mouse anti-HA+ (Table 3). It may be this small subpopulation of cells that is involved, as neutrophil cytotoxicity reactions require a large effector to target ratio to be effective (typically 100:1).28-30"

"The authors thank Edmund Folkes, Alex Fulop, and Louis Scala for their expert technical assistance.

ACKNOWLEDGMENT

The authors thank Edmund Folkes, Alex Fulop, and Louis Scala for their expert technical assistance.

REFERENCES


Table 4. Effect of Antibody on the Number of Virions Bound to and Inside Neutrophils

<table>
<thead>
<tr>
<th>Treatment of Virions</th>
<th>Virions Associated With Neutrophils</th>
<th>37°C</th>
<th>Inside</th>
<th>4°C</th>
<th>Inside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus alone</td>
<td>27.3 ± 28.0*</td>
<td>8.9 ± 11.8</td>
<td>30.9 ± 19.1</td>
<td>1.9 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Virus + anti-HA+</td>
<td>0.1 ± 0.3†</td>
<td>0.2 ± 0.8†</td>
<td>0 ± 0†</td>
<td>0 ± 0†</td>
<td></td>
</tr>
<tr>
<td>Virus + anti-HA+ F(ab)²</td>
<td>0.0 ± 0.0†</td>
<td>0.1 ± 0.8†</td>
<td>0 ± 0†</td>
<td>0 ± 0†</td>
<td></td>
</tr>
<tr>
<td>Virus + immune HIS</td>
<td>0.1 ± 0.6†</td>
<td>0.7 ± 1.6†</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Equal volumes of antibodies (100 μg/mL anti-HA+ or its F(ab)² fragments or 50% immune HIS in PBS) or PBS were added to influenza virus (320 HA units) and incubated for 1 hour at 4°C. Neutrophils (1 × 10⁶) were added at either 37°C or 4°C to the virus-antibody mixtures and incubated in suspension for 15 minutes. The cells were pelleted and processed for electron microscopy. Results are expressed as the mean ± SD of virions attached to the surface or within vacuoles of the leukocytes per ~70-nm thick section.

Abbreviation: ND, not determined.

* Mean ± SD.
† Number of cells examined.
‡ P < .001 when compared with virus alone.


Neutrophils do not bind to or phagocytize human immune complexes formed with influenza virus

DR Ratcliffe, J Michl and EB Cramer