Neutrophil Deactivation by Influenza A Virus

Role of Hemagglutinin Binding to Specific Sialic Acid-Bearing Cellular Proteins[1]

Kevan L. Hartshorn,[2] Louis S. Liou, Mitchell R. White, Misha M. Kazhdan, Joel L. Tauber, and Alfred I. Tauber

Boston University School of Medicine, Department of Medicine, Boston, MA 02118

Bacterial superinfections are the most common cause of mortality during influenza epidemics. Depression of phagocyte functions by influenza A viruses (IAVs) is a likely contributory cause of such infections. We used an in vitro model of viral depression of neutrophil respiratory burst responses to FMLP and PMA to examine the mechanism of IAV-induced phagocyte deactivation. Respiratory burst responses or intracellular calcium mobilization were triggered by the virus itself, but these were not causally related to deactivation. By treating neutrophils with neuraminidase, and by use of purified IAV hemagglutinin (HA) preparations, cross-linking of sialic acid-bearing neutrophil surface components by the IAV HA was shown to be responsible for deactivation. IAV competed for binding to neutrophils with Abs directed against CD43, sialyl-Le^x, CD45, and gangliosides. Deactivation could be reproduced by treating neutrophils with anti-CD43 or -sialyl-Le^x Abs in the absence of IAV. However, treatment of neutrophils with elastase markedly reduced CD43 expression, without affecting overall IAV binding or the ability of IAV to cause deactivation. Hence, although IAV binding to CD43 can account for deactivation, other IAV-binding proteins exist (e.g., those bearing sialyl-Le^x) that can independently mediate functional depression. *The Journal of Immunology,* 1995, 154: 3952–3960.

The major cause of mortality during IAV[3] epidemics is bacterial superinfection (1). Bacterial pneumonias occur in epidemic fashion during IAV outbreaks and can occur either concurrent with, or shortly after, IAV infection in a given subject (2). Although the bacteria most commonly isolated are Pneumococcus or *Haemophilus influenzae* (as in non-IAV-infected hosts), a particularly increased prevalence of staphyloccocal pneumonia has been documented repeatedly (3–5). A clear temporal association between IAV outbreaks and epidemic outbreaks of meningococcal meningitis or meningococcemia has been established as well (6–8). Of the major respiratory viruses, only IAV and parainfluenza viruses have been associated consistently with substantial increases in hospital admissions for lower respiratory tract infection in adults (9, 10). Although damage to respiratory epithelium is one likely contributor to the development of bacterial superinfection, both IAV and parainfluenza viruses induce another important defect in the host defense barrier by causing phagocyte dysfunction (11–13). Neutrophils and monocytes/macrophages participate in the early inflammatory response to IAV infection in the airway, and both cell types exhibit depressed function in vivo during IAV infection (12). In animal models, this induced functional defect correlates temporally with increased susceptibility to bacterial superinfection (14).

Neutrophil functional depression by IAV or parainfluenza virus can be reproduced in vitro. The functional defect encompasses depressed chemotaxis, degranulation, respiratory burst, and intracellular killing responses (15). In association with this dysfunctional state, depressed intracellular calcium ($[Ca^{2+}]_i$) mobilization (11), arachidonic acid release (11), protein phosphorylation (16), and phagosome-lysosome fusion (17) have been reported. Depressed responses to FMLP, PMA, bacteria, and opsonized

Received for publication September 26, 1994. Accepted for publication December 28, 1994.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants AI92550-03 (K.L.H.) and HL335565-08 (A.I.T.).

[2] Address correspondence and reprint requests to Dr. Kevan L. Hartshorn, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118.

[3] Abbreviations used in this paper: IAV, influenza A virus; GAM, goat anti-mouse; $[Ca^{2+}]_i$, intracellular calcium; HA, hemagglutinin; HAU, hemagglutination units; BHA, bromelain-solubilized HA; CT, cholera toxin; LCA, leukocyte common Ag; GM-CSF, granulocyte-macrophage CSF; NCA, nonspecific cross-reacting Ag.

0022-1767/95/$02.00

particles are all noted. Aggregates of purified IAV hemagglutinin (HA) alone (18) or invertebrate sialic acid-binding lectins (19) depress neutrophil function in a similar manner.

We have also demonstrated that IAV activates neutrophils to generate a respiratory burst response (20–22). This response is not inhibited by pertussis toxin, but is preceded by activation of phospholipase C, phosphatidic acid production, $[Ca^{2+}]_i$ mobilization, and pH changes, and is atypical in that H_2O_2 is generated in the absence of detectable O_2^- elaboration. Respiratory burst activation by IAV is mediated by binding of the viral HA to neutrophil surface sialic acid residues (23). Purified IAV HA preparations are sufficient to trigger H_2O_2 production, but only if the HA molecules are cross-linked by addition of anti-HA Abs (23). In this model, the anti-HA Abs must be added after the HA preparations have first been allowed to bind to the cell surface; if HA preparations are preincubated with anti-HA Abs and then added to the cells, no H_2O_2 is produced (23). Given the fact that IAV both activates and functionally depresses neutrophils, we have termed the latter phenomenon deactivation. The current studies were undertaken to better understand the mechanism of IAV-induced deactivation.

Materials and Methods

Reagents

FMLP, cytochalasin B, PMA, horseradish peroxidase type II, scopoletin, superoxide dismutase, cytochrome *c*, Con A, neuraminidase type X (protease activity <0.002 U/mg protein), trisialoganglioside GT1b, Ficoll, dextran, sodium citrate, citric acid, and staph protein A were purchased from Sigma Chemical Co. (St. Louis, MO), and Hypaque was obtained from Winthrop Pharmaceuticals (Des Plaines, IL). Fura-2/AM and BAPTA/AM were purchased from Molecular Probes (Eugene, OR), organic solvents from Fisher Scientific (Fairlawn, NJ), and Dulbecco's PBS from Flow Laboratories (Costa Mesa, CA). Pertussis toxin and cholera toxin were purchased from List Biochemicals (Campbell, CA). Phosphatidylinositol phospholipase C was purchased from Boehringer Mannheim (Mannheim, Germany). The Leu22 (against CD43), CSLex (directed against sialyl-Le^x Ag), CR1, and the CD35 mAbs (against CR1) were obtained from Becton Dickinson (San Jose, CA). The CD45, CD44, CD11c, CD15, and CD45RO mAbs were obtained from Sigma Chemical Co. CR3 and CDw65 mAbs were obtained from AMAC Inc. (Westbrook, ME). The W6/32-HL (against HLA class I) was obtained from Biodesign Inc. (Kennebunkport, ME). The L2 Ab against CD43 was graciously provided by Dr. E. Remold-O'Donnell (Center for Blood Research, Boston, MA). The Leu22 and L2 mAbs both recognize sialated epitopes on the N-terminus of CD43. mAbs directed against CD16 (i.e., FcRIII) and CD11a were graciously provided by Dr. James Griffin (Dana-Farber Cancer Institute, Boston, MA). Goat anti-mouse (GAM) IgG and IgM Abs (both FITC-labeled and unlabeled) were purchased from The Jackson Laboratory (West Grove, PA). Neutrophil elastase was obtained from Elastin Products Co. (Owensville, MO).

Virus preparation

Influenza A viruses were grown in the chorioallantoic fluid of 10-day-old embryonated hens' eggs, and purified on a discontinuous sucrose density gradient, as previously described (11). Virus stocks were dialyzed against PBS, aliquoted, and stored at 70°C until used. Potency of each virus stock was measured by hemagglutination assay, and titers of 1/2,000 through 1/32,000 (as indicated) hemagglutination units (HAU) were measured after samples were thawed from frozen storage at −70°C. Several closely related strains with the H3 hemagglutinin subtype (Bangkok 79, Texas 77, and $Mem71_H3\text{-}Bel_N$) were used. We have previously shown that these strains are similar with respect to causing neutrophil deactivation (24). The Texas 77 strain was used in certain experiments in which use of an anti-hemagglutinin Ab was also required (see below). The PR-8 IAV strain was used as a prototype of the H1 hemagglutinin subtype in certain experiments.

Two viral envelope protein preparations were used: HA/neuraminidase liposomes and bromelain-solubilized HA (BHA). HA/neuraminidase liposomes contained the viral envelope proteins (principally HA and neuraminidase) embedded in viral membrane lipids. These were prepared as previously described (23). In brief, purified Texas 77 IAV was solubilized by using 1.5% octyl-D-glucopyranoside followed by ultracentrifugation (140,000 × *g* for 1 h) to remove nucleocapsid and M protein. The supernatant was then dialyzed against PBS with SM-2 beads for 48 h to remove detergent. The final preparation was concentrated by using Aquacide to 1 mg/ml, and contained 100,000 HAU/ml. SDS-PAGE analysis showed principally bands compatible with HA and neuraminidase. BHA was prepared by digesting Texas 77 IAV with bromelain (Sigma Chemical Co.), as described (23), in the presence of β-mercaptoethanol (final concentration 50 mM). After ultracentrifugation (100,000 × *g* for 60 min) to remove viral cores, the supernatant was concentrated and applied to a preformed 5 to 25% sucrose gradient and ultracentrifuged (140,000 × *g* for 16 h). The BHA-containing fractions were then dialyzed against PBS and stored at −70°C at a concentration of 1.1 mg/ml. The BHA preparations contained only the 58-kDa and 21-kDa bands compatible with HA_1 and BHA_2 (on the basis of Coomassie blue-stained SDS-PAGE gels).

mAbs directed against the HA molecule of the Texas 77 strain of IAV were incubated with the virus in various experiments. The Texas 77 mAb (designation 81/4) was the gift of Dr. R. G. Webster (St. Jude's Hospital, Memphis, TN) and was provided in affinity-purified form in PBS. In some experiments, anti-HA mAbs were preincubated with soluble staph A protein (1:1 protein mixture) to inhibit the ability of the Fc domain of the mAb from interacting with neutrophil Fc receptors (as described (23)).

Neutrophil preparation

Neutrophils from healthy volunteer donors were isolated to >95% purity, as previously described, by using dextran precipitation, followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells, and hypotonic lysis to eliminate contaminating erythrocytes (11). Cell viability was >98%, as determined by trypan blue staining, and cells were used within 5 h of isolation.

Measurement of neutrophil activation

H_2O_2 production was measured by the oxidation of scopoletin, and O_2^- was assessed by the continuous monitoring of the superoxide dismutase inhibitable reduction of cytochrome *c* (20). Changes in $[Ca^{2+}]_i$ were measured by using neutrophils loaded with the acetomethoxy ester of Fura-2/AM, as we have previously detailed (11). Deactivation was assessed by first incubating neutrophils with IAV for various periods of time, followed by measurement of O_2^- production in response to either FMLP or PMA.

Neuraminidase and ganglioside treatments of neutrophils

To desialylate neutrophil surface proteins, 5×10^7 neutrophils were incubated with 0.128 U/ml of neuraminidase at 37°C for 1 h, with constant mixing (23). Cells were subsequently washed three times and resuspended in PBS. To incorporate glycolipids with terminal sialic acids into the neutrophil external membrane, 5×10^7 untreated or previously desialylated neutrophils were incubated with trisialoganglioside GT1b, at a final concentration of 40 μg/ml at 37°C, with constant mixing for 2.5 h (23). Neutrophils were subsequently washed three times and resuspended in PBS at a concentration of 1×10^7 cells/ml.

Measurement of viral binding to neutrophils

Viral binding to neutrophils was measured by preparing FITC-labeled virus and incubating this preparation with neutrophils, followed by evaluation of cell-associated fluorescence by using a flow cytometer. FITC stock was prepared at 1 mg/ml in 1 M sodium carbonate, pH 9.6. The

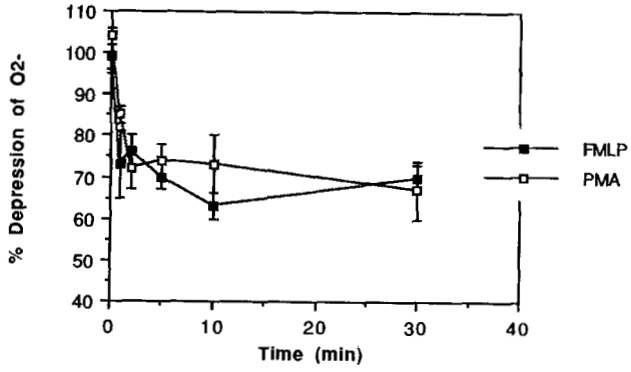

FIGURE 1. Time course of neutrophil deactivation by IAV. Neutrophils were treated with Texas 77 IAV (50 μl/ml of a 1/4000 HAU stock) or control buffer for 0, 1, 2, 5, 10, or 30 min at 37°C before removal of virus by centrifugation, resuspension of cells in control buffer, and measurement of O_2^- generation (by using the ferricytochrome c assay, as detailed in *Materials and Methods*) in response to FMLP (10^{-7} M) or PMA (250 ng/ml). Incubation with virus for 0 min refers to the case in which virus was added, followed immediately by centrifugation to remove virus (actual elapsed time approximately 30 s before initiation of centrifugation). The time of incubation with IAV is shown on the abscissa. The percentage of O_2^- production in IAV-treated cells over that in control cells is indicated on the ordinate. The mean ± SEM percentage of control O_2^- production for 5 experiments is shown. O_2^- production was reduced significantly ($p \leq 0.05$) in IAV-treated cells at all time points (except time 0, e.g., immediately after virus addition), whether PMA or FMLP was used as the stimulus. The degree of depression was significantly greater ($p \leq 0.05$) after 5, 10, and 30 min of virus exposure, as compared with 1 or 2 min. There was, however, no significant difference between the degree of depression at 5, 10, or 30 min.

FITC-labeled virus was prepared by incubating concentrated virus stocks with FITC (10:1 mixture by volume of virus in PBS with FITC stock) for 1 h, followed by dialysis of the mixture for 18 h against PBS. Neutrophils were preincubated with various agents, followed by washing in PBS and addition of 10-μl aliquots of fluorescent viral samples to neutrophils (10^6 cells in 100 μl PBS). After allowing virus and neutrophils to interact for 15 min at 4°C, the neutrophils were washed, resuspended in virus-free PBS, and fixed with 2% paraformaldehyde. Cell-associated fluorescence was measured on a Becton Dickinson FACScan 2 and analyzed by using the Lysis II program.

Statistics

Statistical comparisons were made by using Student's paired *t*-test.

Results

General features of neutrophil deactivation by IAV

As depicted in Figure 1, whereas O_2^- responses to FMLP or PMA were not inhibited significantly in neutrophils immediately after exposure to IAV, depression of O_2^- production was evident as soon as 1 or 2 min after incubation with IAV at 37°C. Deactivation became more pronounced over the first several minutes of exposure, but the degree of deactivation was no greater at 30 min than after 5 min of incubation. Note that the extent and kinetics of depression are very similar for FMLP- and PMA-induced responses. The time course of deactivation was similar to that of IAV-induced respiratory burst response, as measured by chemiluminescence or H_2O_2 assays (20, 25). H_2O_2 responses to FMLP and PMA were also reduced significantly in IAV-treated cells. The mean H_2O_2 responses to FMLP and PMA were, respectively, 0.7 ± 0.1 and 0.6 ± 0.2 nM/min/4 × 10^6 neutrophils in control cells, as compared with 0.4 ± 0.1 and 0.14 ± 0.1 in IAV-treated cells ($n = 4$; $p \leq 0.05$ for each). In contrast, neutrophils pretreated with concentrations of FMLP (7.5×10^{-9} M) or Con A (50 μg/ml), which stimulated a similar amount of H_2O_2 production as IAV, did not become deactivated (data not shown). The respiratory burst response triggered by IAV per se is, therefore, unlikely to account for deactivation.

Table I. *Effect of intracellular calcium chelation on IAV-induced impairment of neutrophil H_2O_2 response to PMA*

Pre-incubation Condition[a]	*n*	H_2O_2 Response to PMA[b]	
		Lag	Rate
Buffer	4	3.3 ± 0.3	0.4 ± 0.04
IAV	4	8.3 ± 1.5[c]	0.16 ± 0.1[c]
FMLP	6	2.2 ± 0.3[c]	0.65 ± 0.15[c]
Con A	4	2.8 ± 0.2	0.36 ± 0.02

[a] Neutrophils were incubated initially for 10 min with BAPTA/AM (final concentration 20 μM), followed by incubation for an additional 10 min in control buffer or control buffer with either IAV (100 μl of a 1/4000 HAU stock), FMLP (7.5×10^{-9} M), or Con A (50 μg/ml). There was no significant amount of H_2O_2 produced during this preincubation.

[b] Mean ± SEM lag time (in minutes) prior to onset of H_2O_2 production and maximal rate (in nmol/min/4 × 10^6 cells) of H_2O_2 production in response to 250 ng/ml PMA are given.

[c] $p \leq 0.05$, compared with control buffer-treated cells.

Deactivation by IAV is accompanied by a depression of mobilization of $[Ca^{2+}]_i$ in response to FMLP (11). In addition, the respiratory burst response elicited by IAV is preceded by a rise in $[Ca^{2+}]_i$ (20). Pre-incubation of neutrophils for 10 min with the $[Ca^{2+}]_i$ chelator BAPTA/AM (acetomethoxyester; final concentration 20 μmol/liter) caused >90% inhibition of the IAV-induced H_2O_2 response and approximately 80% inhibition of the associated $[Ca^{2+}]_i$ response (data not shown), as previously reported (20). As shown in Table I, however, when neutrophils were pretreated with BAPTA/AM followed by addition of IAV, deactivation of H_2O_2 responses to PMA still occurred to a similar extent as in cells not exposed to BAPTA/AM. PMA was used in this assay instead of FMLP because respiratory burst responses elicited by PMA are not inhibited by BAPTA/AM (20). Note that PMA-induced H_2O_2 responses of neutrophils preincubated with FMLP instead of IAV were actually enhanced, implying that priming had occurred despite the presence of BAPTA (Table I). O_2^- production elicited by PMA was also inhibited by IAV, but not FMLP, in BAPTA/AM-treated cells (data not shown).

Preincubation of neutrophils with cholera toxin (CT) markedly reduces the neutrophils $[Ca^{2+}]_i$ response to IAV, as well as abrogating the IAV-induced respiratory burst response (20). To determine whether CT also inhibits deactivation induced by IAV, neutrophils were pretreated with 64 μg/ml CT, followed by a 25-min incubation with Texas 77 IAV or control buffer. After this procedure, O_2^- responses to PMA were measured. O_2^- responses of neutrophils treated with both IAV and CT were 43 ± 5% of those treated with CT alone (n = 3). This depression of O_2^- responses was not significantly different than that found in cells treated with virus in the absence of CT. Pertussis toxin pretreatment of neutrophils also did not alter the deactivating effect of IAV (data not shown). The BAPTA/AM and CT results indicate that events proximal to IAV-induced $[Ca^{2+}]_i$ or respiratory burst response should be the focus of studies aimed at explaining deactivation.

Deactivation results from binding of hemagglutinin to neutrophil surface sialic acid residues

Effects of neuraminidase treatment of neutrophils on IAV-induced deactivation. Treatment of neutrophils with neuraminidase markedly reduces IAV binding to these cells, as well as abrogating respiratory burst or membrane depolarization responses to the virus (23). Incubation of neuraminidase-treated neutrophils with the trisialoganglioside GT1b for 2 h restores IAV binding to these cells to normal levels. Despite this procedure, neuraminidase-treated, GT1b-loaded neutrophils exhibit no H_2O_2 or membrane depolarization response to IAV (23). In this study, we used the same protocol of neuraminidase and/or ganglioside treatment to determine what effect these manipulations had on deactivation by IAV.

Treatment of neutrophils with IAV (10 μl/ml of Texas 77 strain) significantly depressed O_2^- responses to FMLP to 78 ± 3% of response of non-IAV-treated cells ($p \leq 0.03$; n = 3). Loading neutrophils with GT1b did not significantly alter O_2^- responses of control or IAV-treated neutrophils (data not shown). However, when neutrophils were pre-incubated with neuraminidase, further treatment of the cells with IAV did not cause depression of O_2^- responses whether or not GT1b was added (i.e., FMLP-induced O_2^- responses were 99 ± 4 and 101 ± 4% of control for cells treated with neuraminidase and virus or neuraminidase, GT1b, and virus, respectively; n = 3).

Neutrophil deactivation is mediated by purified IAV HA preparations. To test whether IAV envelope proteins alone could cause neutrophil deactivation, we prepared liposomes containing IAV envelope lipids and envelope proteins (HA/neuraminidase liposomes). In addition, we prepared BHA, which is a purified soluble form of the extracellular domain of the HA molecule. The ability of HA/neuraminidase liposomes or BHA to cause neutrophil deactivation was assessed (Fig. 2). HA/neuraminidase liposomes significantly depressed neutrophil O_2^- responses

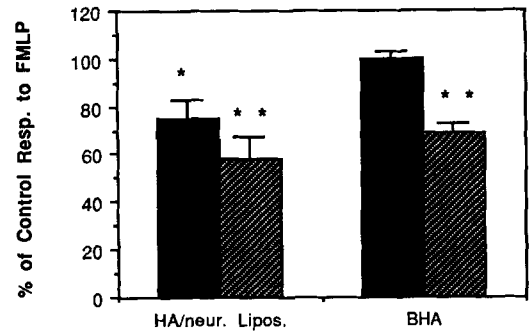

FIGURE 2. Neutrophil deactivation caused by intact IAV or IAV envelope proteins: effect of cross-linking with antiviral Abs. Neutrophils were first treated with 50 μg/ml of either HA/neuraminidase liposomes or bromelain-solubilized hemagglutinin (BHA), as indicated (10 min at 37°C). These neutrophils were then divided into equal aliquots treated either with mAb directed against the Texas 77 IAV hemagglutinin (hatched bars) or an equal concentration of control buffer (solid bars). Afterward, this O_2^- response of the cells to stimulation with FMLP was measured. Mean ± SEM percentage of control O_2^- response from three to five experiments is shown. Pretreatment of the anti-HA mAb with soluble staph A protein did not alter the ability of the mAb to cause deactivation when added to BHA-treated cells (data not shown). Addition of anti-HA mAb in the absence of IAV preparations did not alter O_2^- responses to FMLP (data not shown). *$p \leq 0.05$, compared with neutrophils not treated with IAV preparations. **$p \leq 0.05$, compared with neutrophils treated with IAV preparations but not anti-HA mAb.

to FMLP. BHA alone, in contrast, did not cause deactivation. The concentrations of liposomes and BHA used in these experiments were shown previously to fully inhibit binding of intact IAV to neutrophils (23). When anti-HA mAbs were added to neutrophils after first allowing HA/neuraminidase liposomes or BHA to bind to the cell, a more pronounced deactivation caused by intact HA/neuraminidase liposomes was observed. Most importantly, addition of mAb to BHA-treated cells resulted in deactivation (Fig. 2).

Role of IAV binding to specific neutrophil surface sialoglycoproteins in mediating deactivation

Identification of IAV binding sites. IAV has been reported to bind to CD43 and several other proteins present in solubilized neutrophil membrane preparations (26). Preincubation of neutrophils with saturating concentrations of Leu22, CSLex, CD15, or CD45 mAbs partially, but statistically significantly, reduced IAV binding (Table II). The combination of CSLex, Leu22, and CD45 mAbs inhibited IAV binding to a greater extent than did these mAbs used alone (Table II). Abs directed against other major neutrophil surface proteins did not alter IAV binding (Table II). More striking results were obtained when neutrophils were preincubated with IAV, followed

Table II. *Effect of neutrophil preincubation with Abs directed against various neutrophil surface Ags on subsequent binding of FITC-labeled IAV*[a]

Ab	Ag	*n*	Percentage of Control IAV Binding
Leu22	CD43	5	75 ± 5[b]
CSLex	Sialyl-Le^x	6	77.5 ± 5[b]
CD45	LCA	10	94 ± 2[b]
CSLex + CD45		3	82 ± 2[b]
CSLex + CD45 + Leu22		3	59 ± 3[b]
CD15	Le^x	6	80 ± 5[b]
CDw65	Ganglioside	4	108 ± 12
W6/32-HL	HLA Class I	2	110 ± 2
CD35	CR1	3	98 ± 10
	CR3	3	104 ± 7

[a] Neutrophils were preincubated with the indicated Ab preparations for 15 min at 4°C (or with control buffer), followed by addition of FITC-labeled IAV ($Mem71_H3$-Bel_N strain). Neutrophil-associated fluorescence was measured on a flow cytometer. Results shown are mean ± SEM for percentage of control flourescence for the indicated number of experiments. Percentage of control fluorescence was obtained by dividing fluorescence of neutrophils preincubated with mAbs by that of neutrophils preincubated with control buffer. Concentrations of Abs used were in excess of those determined to maximally saturate the respective neutrophil surface Ags on the basis of indirect immunofluorescence (data not shown). IAV binding was reduced significantly more in neutrophils treated with the combination of CSLex, Leu22, and CD45, compared with those treated with CSLex alone. The combination of CSLex and CD45 caused no greater decrease in IAV binding than CSLex alone.

[b] $p \leq 0.05$, compared with IAV binding to control neutrophils (i.e., not treated with Ab).

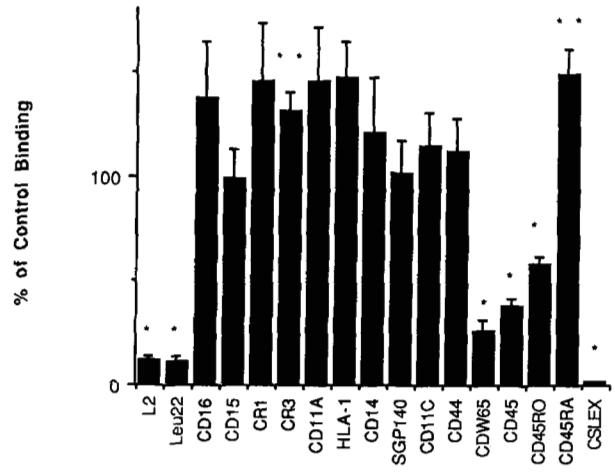

FIGURE 3. Effect of neutrophil preincubation with IAV on ability of the cells to bind Abs directed against neutrophil membrane Ags. Neutrophils were first treated with IAV ($Mem71_H3$-Bel_N strain) or control buffer (15 min at 4°C), followed by resuspension in virus-free buffer. Afterward, this ability of IAV- or buffer-treated neutrophils to bind the indicated Ab preparations was tested by further incubation of the cells with the Abs (15 min at 4°C), followed by FITC-labeled anti-mouse or anti-rabbit $F(ab')_2$ and measurement of cell-associated fluorescence by flow cytometry. *Significantly reduced binding, compared with neutrophils not treated with IAV ($p \leq 0.05$). **Significantly increased binding, compared with neutrophils not treated with IAV ($p \leq 0.05$).

by measurement of the ability of such IAV-treated neutrophils to bind various mAbs (see Fig. 3). Such IAV treatment drastically reduced binding of CSLex, Leu22, and L2 to neutrophils, indicating that IAV competes for binding to sialyl-Le^x and CD43. IAV treatment also inhibited binding of CD45, CD45R0, and CDw65 mAbs to neutrophils, whereas binding of various other mAbs was either unaffected or actually enhanced by the virus. The CD45 mAbs react with members of the leukocyte common Ag (LCA) family of glycoproteins, and CDw65 reacts with a ganglioside Ag. Each of the mAbs whose binding was inhibited by IAV recognizes sialylated epitopes. Of note, binding of the CD45RA and CD15 mAbs was not inhibited by IAV. CD45RA recognizes a nonsialylated epitope on a specific LCA protein. CD15 recognizes the nonsialylated version of the Lewis Ag (Le^x) (27). Note that these experiments were conducted at 4°C, so that the results probably reflect effects of IAV binding and not internalization. For these reasons, we believe the results shown in Figure 3 indicate specific binding competition between IAV and CD43, sialyl-Le^x, CDw65, and certain LCA variants.

In an attempt to further determine the quantitative contribution of various IAV binding sites to overall IAV binding, we measured concurrently the effect of neutrophil treatment with GM-CSF, PMA, or elastase on IAV and mAb binding. These results again suggested that binding to CD43 alone is unlikely to account for all of IAV binding to the neutrophil. As shown in Table III, GM-CSF modestly reduced Leu22 expression, but it did not alter (or, at higher concentrations, increase) IAV binding. Low concentrations of PMA increased IAV binding but reduced Leu22 expression. Higher concentrations modestly reduced IAV binding and markedly reduced Leu22 expression. More strikingly, treatment of neutrophils with elastase markedly reduced Leu22 expression, but it did not alter binding of IAV nor that of mAbs directed against sialyl-Le^x or CD45 (Fig. 4). Treatment of neutrophils with phospholipase C (0.1 U/ml for 60 min at 37°C) had no significant effect on IAV binding (mean cell-associated fluorescence, 241 ± 26 in control vs 241 ± 36 in phospholipase C-treated cells; $n = 4$). This phospholipase C treatment was effective at removing FcRIII, as assessed by indirect immunofluorescence (mean fluorescence reduced from 686 in control to 144 in phospholipase C-treated cells; $n = 4$; $p < 0.005$). Hence, glycosylphosphatidylinositol-linked proteins are unlikely to be important in IAV binding.

Role of specific IAV binding sites in mediating deactivation. As shown in Table IV, treatment of neutrophils with CSLex mAbs alone significantly depressed O_2^- responses of the cells to FMLP. Leu22 and L2 (anti-CD43) mAbs did so as well, but only when the mAbs were further cross-linked by addition of goat anti-mouse IgG $F(ab')_2$ fragments. Several other mAbs did not depress O_2^- responses, whether or not GAM was added. Of note, when the CSLex

Table III. *Effect of neutrophil preincubation with GM-CSF or PMA on subsequent binding of IAV, or mAbs against Leu22 or CR3*

Preincubation[a] Condition	Percentage of Control Binding of: IAV	Leu22	CR3
GM-CSF (5 ng/ml)		79 ± 4[b]	147 ± 4[c]
GM-CSF (30 ng/ml)	104 ± 3	73 ± 8[b]	154 ± 12[c]
GM-CSF (50 ng/ml)	133 ± 9[c]		
PMA (1.25 ng/ml)	120 ± 3[c]	75 ± 5[b]	
PMA (125 ng/ml)	88 ± 5[b]	18 ± 3[b]	

[a] Neutrophils were preincubated with either the indicated concentrations of GM-CSF (30 min at 37°C) or PMA (15 min at 37°C) prior to assessment of binding of either IAV or mAbs, as indicated. IAV binding was assessed as in Figure 3. mAb binding was assessed by flow cytometry by using FITC-labeled GAM as a secondary Ab. The percentages of IAV or mAb binding to GM-CSF or PMA-treated neutrophils/binding to control neutrophils are shown (mean ± SEM; n = 3–5).

[b] Significantly reduced, compared with binding to control neutrophils ($p \leq 0.05$).

[c] Significantly increased, compared with binding to control neutrophils ($p \leq 0.05$).

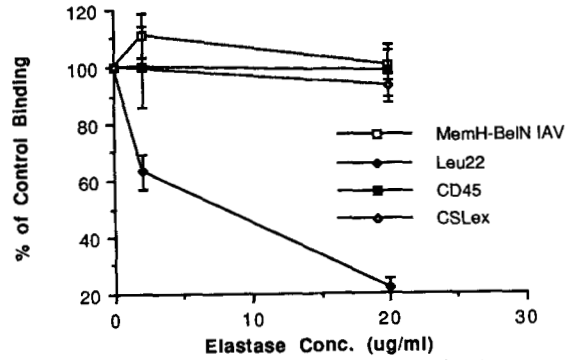

FIGURE 4. Effect of treating neutrophils with elastase on the ability of the cells to bind IAV or mAbs Leu22, CD45, or CSLex. Neutrophils were pretreated (20 min at 37°C) with the indicated concentrations of neutrophil elastase, followed by assessment of IAV or mAb binding by using flow cytometry, as described. Binding of Leu22 mAbs was reduced significantly ($p \leq 0.01$) by either 2 or 20 μg/ml of elastase, whereas binding of IAV or the other mAbs was not altered (n = 3).

mAb (which, along with CD15, is IgM) was cross-linked further by GAM IgM F(ab')$_2$, depression of O_2^- responses was no longer found (in fact, enhancement occurred). Of note, neither O_2^- nor H_2O_2 responses were detected in response to CSLex, L2, or Leu22 mAbs, with or without addition of GAM ($n \geq 3$ for each; data not shown).

Given the ability of elastase to cleave extensively the extracellular domain of CD43 from the neutrophil surface, we determined the effect of elastase treatment on deactivation caused by two prototypical strains of IAV. As shown in Table V, elastase treatment actually significantly enhanced IAV-induced deactivation mediated by both strains. Elastase treatment of neutrophils similarly did not reduce significantly the cells' H_2O_2 response to IAV (mean responses to $Mem71_H3\text{-}Bel_N$ IAV were 0.7 and 0.84 nmol H_2O_2/min/4 × 10^6 cells in control and elastase-treated cells, respectively; n = 4). Finally, phospholipase C treatment of neutrophils did not reduce H_2O_2 responses to IAV, nor did it lessen the degree of deactivation caused by the virus (data not shown).

Discussion

In its own right IAV induces neutrophil activation (e.g., H_2O_2 or $[Ca^{2+}]_i$ responses), but, over a similar time course, it impairs the ability of the cells to respond to other stimuli (termed deactivation). However, IAV-induced deactivation occurred in the absence of activation under certain conditions: inhibition of activation by neutrophil $[Ca^{2+}]_i$ chelation, or by treatment of neutrophils with CT, did not alter deactivation. These results imply that deactivation is triggered by events occurring before, or independent from, the IAV-induced $[Ca^{2+}]_i$ rise or H_2O_2 response. Also, HA/neuraminidase liposomes caused neutrophil deactivation without causing activation. These results suggest that deactivation is not dependent on cell activation per se, and that these functions are distinguishable by postreceptor events.

Deactivation was, however, mediated by IAV binding to neutrophil surface sialic acid-bearing sites. Neuraminidase treatment of neutrophils prevented deactivation. Incubation of neuraminidase-treated neutrophils with gangliosides (in a manner we have previously shown to restore IAV binding) did not restore the ability of IAV to cause deactivation of neuraminidase-treated cells. This result implies that HA binding to endogenous sialoglycoproteins mediates deactivation. Purified BHA alone could mediate deactivation, but only after the BHA was cross-linked with anti-HA Ab. We have reported similar results with respect to neutrophil activation (23). These findings support, and extend upon, those of Cassidy et al. (18). Because BHA lacks the transmembrane component of the HA, this portion of the molecule is unlikely to be involved in causing deactivation. This stands in contrast to the case of phagocyte dysfunction caused by the feline leukemia virus envelope protein (28). In addition, our results indicate that a certain degree of cross-linking of HA binding sites is required to obtain deactivation.

The critical issue, therefore, in accounting for IAV-induced neutrophil deactivation, is identifying which endogenous, sialated neutrophil membrane components are bound by the HA. By testing for binding competition between IAV and mAbs directed against various neutrophil surface membrane components, we have identified several highly sialated neutrophil membrane components as IAV binding sites, including CD43, sialyl-Le^x, CD45, and CDw65. By testing IAV binding to neutrophil membrane proteins present in Western blots, Rothwell and Wright (26) identified 160- and 125-kDa bands, one or both of which were identified as CD43. By using IAV particles to

Table IV. *Neutrophil deactivation caused by mAbs directed against neutrophil membrane Ags*[a]

Ab	Ag	Percentage of Control O_2^- Response to FMLP	
		−GAM	+GAM
Leu22	CD43	120 ± 2[c]	81 ± 2[b]
L2	CD43	99 ± 6	75 ± 5[b]
CSLex	Sialyl-Le^x	76 ± 5[b]	112 ± 2[c]
CD15	Le^x	99 ± 8	102 ± 10
CD45	LCA	100 ± 4	94 ± 2
CD11a	Integrin	109 ± 7	99 ± 7
CD35	CR1	104 ± 11	91 ± 14
W6/32-HL	HLA-1	92 ± 4	88 ± 5

[a] Neutrophils were preincubated with control buffer or the indicated mAbs directed against known neutrophil surface Ags (as indicated) for 15 min at 4°C. The concentrations of mAbs used were those found to cause maximal neutrophil fluorescence (assessed by flow cytometry; data not shown). After washing cells to remove unbound mAb, the cells were divided into aliquots, one of which was treated with F(ab')$_2$ fragments of goat Abs directed against mouse IgG or IgM, depending on whether the primary mAb was IgG or IgM (+GAM), and the other with an equal quantity of control buffer (−GAM) for 15 more min at 15°C. Neutrophils were then washed and resuspended in fresh buffer, and O_2^- responses to FMLP were measured, as in Figure 1. The percentage of control O_2^- response was calculated by comparing responses in mAb-treated ± GAM with those in control cells (i.e., cells not treated with mAb). Mean ± SEM of three to six experiments is given. No O_2^- production was observed prior to addition of FMLP. Addition of goat anti-mouse IgG or IgM alone did not significantly alter O_2^- responses (mean 107% and 103% of control, respectively; $n = 5$).

[b] Significantly reduced, compared with O_2^- response control neutrophils ($p \leq 0.05$).

[c] Significantly increased, compared with O_2^- response control neutrophils ($p \leq 0.05$).

Table V. *IAV-induced neutrophil deactivation: effect of neutrophil pretreatment with elastase*[a]

Stimulus	IAV Strain	Percentage of Control O_2^- Response to Stimulus	
		Control neutrophils	Elastase-treated neutrophils
FMLP	H3N2	60 ± 14[b]	54 ± 1[c]
	H1N1	73 ± 15	66 ± 8[c]
PMA	H3N2	33 ± 5	40 ± 3
	H1N1	78 ± 2[b]	68 ± 2[c]

[a] Neutrophils were pretreated either with neutrophil elastase (20 μg/ml) or control buffer alone for 20 min at 37°C. These cells were then subdivided further into aliquots treated with either control buffer or IAV for a further 20 min at 37°C. Either an H3N2 (Bangkok 79) or H1N1 (PR-8) IAV, respectively, was used, as indicated. After washing off unbound IAV, O_2^- responses of the cells upon stimulation with either FMLP or PMA were measured, as described in Figure 1. Mean ± SEM of three experiments is shown (except in the case of neutrophils pretreated with Bangkok 79 IAV and stimulated with PMA, where $n = 2$).

[b] $p \leq 0.05$, compared with neutrophils not treated with IAV.

[c] $p \leq 0.05$, compared with neutrophils treated with IAV, but not with elastase.

precipitate solubilized neutrophil membrane proteins, several additional proteins were found associated with the virus. Our results suggest that IAV does indeed bind to CD43, but to additional sites as well, including proteins bearing the sialyl-Le^x Ag, CD45, and neutrophil surface gangliosides. Our findings with GT1b-loaded neutrophils (see above and Ref. 23) suggest that IAV binding to gangliosides is unlikely to be involved in IAV-induced activation or deactivation.

Of the various binding proteins, CD43 and proteins bearing the sialyl-Le^x contributed most extensively to IAV binding, because mAbs directed against these proteins could inhibit IAV binding to a modest but reproducible extent. Results obtained by using the virus to block subsequent binding of mAbs were qualitatively similar, but quantitatively more striking, suggesting that the virus is a more effective blocking agent than were the mAbs. These results were nonetheless specific, in that the virus only inhibited binding of mAbs directed against sialylated epitopes. Discordant findings were obtained with the anti-CD15 mAb: this mAb significantly inhibited IAV binding (Table IV), but IAV did not in turn inhibit binding of the CD15 mAb (Fig. 3). This result might possibly be explained by the fact that CD15 is an IgM mAb that recognizes nonsialylated carbohydrates on some of the same glycoproteins recognized by CSLex (e.g., the nonspecific cross-reacting Ag (NCA) 160; see Ref. 27).

Several other findings indicated that IAV binding to CD43 cannot fully account for binding of the virus to neutrophils. Treatment of neutrophils with low doses of PMA or with GM-CSF or TNF enhances IAV binding (Table III and Ref. 29). These results suggest that a granular pool of receptors contributes to some extent to IAV binding (30). Among the various likely IAV-binding proteins, CD45 (31) and NCA 160 (27) are known to be up-regulated in this manner. CD43, in contrast, was down-regulated under these circumstances. Conditions that cause extensive proteolytic cleavage of CD43 from the neutrophil membrane, including treatment with higher dose PMA or elastase (32), did not substantially affect IAV binding. These findings suggest that other nonelastase sensitive binding sites must contribute importantly to IAV binding under certain conditions. During IAV infection, neutrophils predominate in the initial inflammatory infiltrate into the infected airway (33). It is presumably in interacting with these cells that IAV has its most important impact on antibacterial defenses. It would be important, therefore, to establish whether CD43 expression is reduced in neutrophils recruited to these sites.

The key question remains in determining which IAV binding sites are of most functional importance. A key candidate, CD43, is a large molecule that protrudes from the neutrophil surface and that appears to function to impede neutrophil spreading and respiratory burst activation of adherent neutrophils (34). It has been shown to mediate activation of monocytes through a $[Ca^{2+}]_i$-dependent, staurosporine-inhibitable mechanism (35). However, cross-linking of neutrophil CD43 epitopes by adding anti-CD43 mAbs followed by GAM was not found to trigger a respiratory burst response (see above and Ref. 36). In this study, we show that cross-linking of CD43 causes neutrophil deactivation. The ability of CD43 to mediate monocyte and lymphocyte activation has been localized to a specific domain of the

molecule (37). Rothwell and Wright (26) showed that anti-CD43 mAbs did elicit neutrophil H_2O_2 reponses, suggesting that the mode of presentation of the mAb (e.g., on a particle) may be important.

In any case, our finding that elastase treatment actually enhanced IAV-induced deactivation, and did not inhibit IAV-induced H_2O_2 production, suggests that binding proteins other than CD43 may contribute importantly to these effects. Sialyl-Le^x is the ligand for P- and E-selectins, and is required for neutrophil attachment to, and rolling on, endothelium (38, 39). Interference with selectin binding to this Ag has markedly inhibitory effects on neutrophil-mediated inflammatory responses. The sialyl-Le^x Ag is a carbohydrate expressed on several neutrophil surface proteins including NCA 160 (as well as lower m.w. glycosyl phosphatidylinositol-linked NCAs), L-selectin (40), and the recently identified P-selectin ligand PGSL (41, 42). Our finding that elastase treatment did not significantly reduce CSLex reactivity suggests that L-selectin and lower m.w. NCAs (likely to be cleaved under these conditions) may not contribute greatly to CSLex reactivity (nor to IAV binding or deactivation). It is intriguing to speculate that the 160-kDa IAV-binding protein band identified by Rothwell and Wright (26) may include NCA 160. How IAV interacts with sialyl-Le^x-bearing proteins is an important subject for further research.

Ligation of either CD43 or sialyl-Le^x with mAbs caused depression of neutrophil respiratory burst responses (Table IV). Anti-CD43 mAbs caused depression only after further cross-linking with GAM IgG. Anti-sialyl-Le^x mAbs caused depression without further cross-linking, possibly because the mAb used was IgM and capable of causing cross-linking of neutrophil surface receptors without additional GAM IgM. In fact, further cross-linking of CSLex with GAM IgM reversed the depressing effect of the mAb. These findings suggest that explaining IAV-induced neutrophil deactivation involves not only identifying the specific neutrophil Ags bound by IAV, but also establishing the manner in which these Ags are complexed further by the virus.

Binding to CD45 may also contribute in a more limited way to functional effects of IAV, as this tyrosine phosphatase has a critical role in signal transduction and activation of lymphoid and NK cells. Anti-CD45 mAbs do not inhibit neutrophil respiratory burst responses, but have been shown to inhibit chemotaxis (43). It is important to note that IAV-induced deactivation involves depression not only of respiratory burst responses, but of chemotaxis, degranulation, and intracellular killing responses as well (12). It may be that these diverse functional effects of IAV result from binding of IAV to more than one functionally important membrane glycoprotein.

References

1. Kilbourne, E. D. 1987. *Influenza.* Plenum, New York.
2. Louria, D. B., H. L. Blumenfeld, J. T. Ellis, E. D. Kilbourne, and D. E. Rogers. 1959. Studies on influenza in the pandemic of 1957–1958. II. Pulmonary complications of influenza. *J. Clin. Invest. 38: 213.*
3. Chickering, H. T., and J. H. Parker, Jr. 1919. *Staphylococcus aureus* pneumonia. *JAMA 72:617.*
4. Finland, M., O. L. Peterson, and E. Strauss. 1942. Staphylococcic pneumonia occurring during an epidemic of influenza. *Arch. Int. Med. 70:183.*
5. Schwartzmann, S. W., J. L. Adler, R. J. Sullivan, and W. M. Marine. 1971. Bacterial pneumonia during the Hong Kong influenza epidemic of 1968–1969. *Arch. Int. Med. 127:1037.*
6. Hubert, B., L. Watier, P. Garnerin, and S. Richardson. 1992. Meningococcal disease and influenza-like syndrome: a new approach to an old question. *J. Infect. Dis. 166:542.*
7. Cartwright, K. A. V., D. M. Jones, A. J. Smith, J. M. Stuart, E. B. Kaczmarski, and S. R. Palmer. 1991. Influenza A virus and meningococcal disease. *Lancet 338:554.*
8. Young, L. S., F. M. LaForce, J. J. Head, J. C. Feeley, and J. V. Bennett. 1972. A simultaneous outbreak of meningococcal and influenza infections. *N. Engl. J. Med. 287:5.*
9. Glezen, W. P. 1982. Serious morbidity and mortality associated with influenza epidemics. *Epidemiol. Rev. 4:25.*
10. Mufson, M. A., V. Chang, V. Gill, S. C. Wood, M. J. Romansky, and R. M. Chanock. 1967. The role of viruses, mycoplasmas and bacteria in acute pneumonia in civilian adults. *Am. J. Epidemiol. 86:526.*
11. Hartshorn, K. L., M. Collamer, M. Auerbach, J. B. Myers, N. Pavlotsky, and A. I. Tauber. 1988. Effects of influenza A virus on human neutrophil calcium metabolism. *J. Immunol. 141:1295.*
12. Hartshorn, K. L., D. E. Daigneault, and A. I. Tauber. 1992. Phagocyte responses to viral infection. In *Inflammation: Basic Principles and Clinical Correlates.* Gallin, J. I., I. M. Goldstein, and R. Snyderman, eds. Raven Press, New York, p. 1017.
13. Jakab, G. J., and G. M. Green. 1976. Defect in intracellular killing of *Staphylococcus aureus* within alveolar macrophages in Sendai virus-infected murine lungs. *J. Clin. Invest. 57:1533.*
14. Abramson, J. S., G. S. Giebink, and P. G. Quie. 1982. Influenza A virus-induced polymorphonuclear leukocyte dysfunction in the pathogenesis of experimental pneumococcal otitis media. *Infect. Immun. 36:289.*
15. Abramson, J. S., and E. L. Mills. 1988. Depression of neutrophil function induced by viruses and its role in secondary microbial infections. *Rev. Infect. Dis. 10:326.*
16. Caldwell, S. E., L. F. Cassidy, and J. S. Abramson. 1988. Alterations in cell protein phosphorylation in human neutrophils exposed to influenza A virus. *J. Immunol. 140:3560.*
17. Abramson, J. S., J. C. Lewis, D. S. Lyles, K. A. Heller, E. L. Mills, and D. A. Bass. 1982. Inhibition of neutrophil lysosome-phagosome fusion associated with influenza virus infection in vitro: role in depressed bactericidal activity. *J. Clin. Invest. 69:1393.*
18. Cassidy, L. F., D. S. Lyles, and J. S. Abramson. 1989. Depression of polymorphonuclear leukocyte functions by purified influenza virus hemagglutinin and sialic acid-binding lectins. *J. Immunol. 142:4401.*
19. Hartshorn, K. L., D. E. Daigneault, M. R. White, and A. I. Tauber. 1992. Anomalous features of human neutrophil activation by influenza A virus are shared by related viruses and sialic acid-binding lectins. *J. Leukocyte Biol. 51:230.*
20. Hartshorn, K. L., M. Collamer, M. R. White, J. H. Schwartz, and A. I. Tauber. 1990. Characterization of influenza A virus activation of the human neutrophil. *Blood 75:218.*
21. Hartshorn, K. L., D. E. Daigneault, M. R. White, M. Tuvin, J. L. Tauber, and A. I. Tauber. 1992. Comparison of influenza A virus and formyl-methionyl-leucyl-phenylalanine activation of the human neutrophil. *Blood 79:1049.*
22. Hartshorn, K. L., J. Wright, M. Collamer, M. R. White, and A. I. Tauber. 1990. Human neutrophil stimulation by influenza virus: relationship of cytoplasmic pH changes to cell activation. *Am. J. Physiol. 258:C1070.*
23. Daigneault, D. E., K. L. Hartshorn, L. S. Liou, G. M. Abruzzi, M. R. White, S. K. Oh, and A. I. Tauber. 1992. Influenza A virus binding to human neutrophils and cross-linking requirements for activation. *Blood 80:3227.*

24. Hartshorn, K. L., K. Sastry, D. Brown, M. R. White, T. B. Okarma, Y. M. Lee, and A. I. Tauber. 1993. Conglutinin acts as an opsonin for influenza A viruses. *J. Immunol. 151:6265.*

25. Kazhdan, M., M. R. White, A. I. Tauber, and K. L. Hartshorn. 1994. Human neutrophil respiratory burst response to influenza A virus occurs at an intracellular location. *J. Leukocyte Biol. 56:59.*

26. Rothwell, S. W., and D. G. Wright. 1994. Characterization of influenza A virus binding sites on human neutrophils. *J. Immunol. 152: 2358.*

27. Stocks, S. C., M. Albrechtsen, and M. A. Kerr. 1990. Expression of the CD15 differentiation antigen (3-fucosyl-*N*-acetyl-lactosamine, Le^x) on putative neutrophil adhesion molecules CR3 and NCA-160. *Biochem. J. 268:275.*

28. Harrell, R. A., G. J. Cianciolo, T. D. Copeland, S. Oroszlan, and R. Snyderman. 1986. Suppression of the respiratory burst of human monocytes by a synthetic peptide homologous to envelope proteins of human and animal retroviruses. *J. Immunol. 136:3517.*

29. Little, R., M. R. White, and K. L. Hartshorn. 1994. Interferon α enhances neutrophil respiratory burst responses to stimulation with influenza A virus and FMLP. *J. Infect. Dis. 170:802.*

30. Richter, J., T. Andersson, and I. Olsson. 1989. Effect of tumor necrosis factor and granulocyte colony-stimulating factor on neutrophil degranulation. *J. Immunol. 142:3199.*

31. Kuijpers, T. W., A. T. Tool, C. E. Schoot, L. A. Ginsel, J. J. Onderwater, D. Roos, and A. J. Verhoeven. 1991. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood 78:1105.*

32. Remold-O'Donnell, E., and D. Parent. 1994. Two proteolytic pathways for down-regulation of the barrier molecule CD43 of human neutrophils. *J. Immunol. 152:3595.*

33. Sweet, C., and H. Smith. 1980. Pathogenicity of influenza virus. *Microbiol. Rev. 44:303.*

34. Nathan, C., Q. Xie, L. Halbwach-Mecarelli, and W. W. Jin. 1993. Albumin inhibits neutrophil spreading and hydrogen peroxide release by blocking the shedding of CD43 (sialophorin, leudosialin). *J. Cell Biol. 122:243.*

35. Wong, R. K., E. Remold-O'Donnell, D. Vercelli, J. Sancho, C. Terhorst, F. Rosen, R. Geha, and T. Chatila. 1990. Signal transduction via leukocyte antigen CD43 (sialophorin). *J. Immunol. 144: 1455.*

36. Lund-Johansen, F., J. Olweus, V. Horejsi, K. Skubitz, J. Thompson, R. Vilella, and F. Symington. 1992. Activation of human phagocytes through carbohydrate antigens (CD15, sialyl-CD15, CDw17, and CDw65). *J. Immunol. 148:3221.*

37. Remold-O'Donnell, E., and F. S. Rosen. 1990. Proteolytic fragmentation of sialophorin (CD43). *J. Immunol. 145:3372.*

38. Lowe, J. B., L. M. Stoolman, R. P. Nair, R. D. Larsen, T. L. Berhend, and R. M. Marks. 1990. ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. *Cell 63:475.*

39. Mulligan, M. S., J. C. Paulson, S. D. Frees, Z. Zheng, J. B. Lowe, and P. A. Ward. 1993. Protective effects of oligosaccharides in P-selectin-dependent lung injury. *Nature 364:149.*

40. Kuijpers, T. W., M. Hoogerwer, L. J. W. Laan, G. Nagel, C. E. Schoot, F. Grunert, and D. Roos. 1992. CD66 nonspecific cross-reacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. *J. Cell Biol. 118:457.*

41. Sako, D., X. Chang, K. M. Barone, G. Vachino, H. White, G. Shaw, G. Veldman, K. Bean, T. Ahern, B. Furie, D. A. Cumming, and G. R. Larsen. 1993. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell 75:1179.*

42. Norgard, K. E., K. L. Moore, S. Diaz, N. Stults, S. Ushiyama, R. P. McEver, R. D. Cummings, and A. Varki. 1993. Characterization of a specific ligand for P-selectin on myeloid cells. *J. Biol. Chem. 268: 12764.*

43. Harvath, L., J. A. Balke, N. P. Christiansen, A. A. Russell, and K. M. Skubitz. 1991. Selected antibodies to leukocyte common antigen (CD45) inhibit human neutrophil chemotaxis. *J. Immunol. 146:949.*