Human neutrophil respiratory burst response to influenza A virus occurs at an intracellular location

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Abstract: We have studied in detail the in vitro interactions of influenza A viruses (IAVs) with human neutrophils to clarify why these cells become dysfunctional during IAV infection. Unosponized IAV elicited a respiratory burst response in neutrophils which, like that triggered by formylmethionyl-leucyl-phenylalanine (fMLP), involved mediation of signal-transducing GTP-binding proteins and tyrosine kinase activation. The IAV-induced response differed from that provoked by fMLP in that H_2O_2 was produced without concomitant O_2^- release. IAV also did not cause extracellular release of granule enzymes in cytochalasin B-treated neutrophils. Using chemiluminescence assays, the respiratory burst response to IAV was found to occur at an intracellular location. These findings may, in part, explain the anomalous nature of the respiratory burst response elicited by IAV and suggest strategies for determining the mechanism of IAV-induced neutrophil deactivation. *J. Leukoc. Biol.* 56: 59-64; 1994.

Key Words: *influenza virus • neutrophils • chemiluminescence • respiratory burst • degranulation*

INTRODUCTION

Our interest in studying the interactions of influenza A viruses (IAVs) with human neutrophils derives from the long-standing observation that IAV-infected subjects are prone to suffer bacterial superinfection. These superinfections represent the major cause of morbidity and mortality during IAV epidemics [1]. There is abundant evidence that an acquired phagocyte (both neutrophil and monocyte) functional defect resulting from IAV infection is an important contributory factor to these superinfections [1-3]. Depression of neutrophil functional responses can be reproduced in vitro by addition of IAV [4] or purified IAV hemagglutinin preparations [5]. In the process of studying the mechanisms of this IAV-induced depression, we observed that the virus acts as a neutrophil respiratory burst stimulant in its own right [6]. Neutrophil activation by IAV is unusual in that hydrogen peroxide (H_2O_2) but no superoxide (O_2^-) is formed [6-8]. The cellular signals that precede activation by IAV bear many similarities to those triggered by chemoattractants: inositol trisphosphate and phosphatidic acid are released, and an intracellular calcium (Ca_i^{2+}) rise, intracellular pH changes, and membrane depolarization occur [9]. The H_2O_2, pH, and membrane potential responses are inhibited by protein kinase antagonists, suggesting that protein kinase activation plays a role in neutrophil activation by IAV. In contrast to chemoattractant activation, pertussis toxin has no inhibitory effect on IAV-induced activation events [6]. Activation by IAV is triggered by binding of the IAV hemagglutinin to the neutrophil surface possibly through a crosslinking mechanism [10], although the specific surface receptors involved are unknown. How these various events relate to neutrophil deactivation by IAV also remains to be established.

The ability of neutrophils to be activated by unoposonized IAV, and to adhere specifically to IAV-infected epithelia [11], suggests that these cells may play a role in the early phase of IAV containment in vivo. In fact, neutrophils predominate in the early inflammatory infiltrate in humans [12] and animals [13] infected with IAV. Indirect evidence suggests that these neutrophils do play a protective role in nonimmune hosts infected with IAV [3, 8].

In this study we address some of the major anomalous features of neutrophil activation by IAV. We provide further evidence of similarities between the signal transduction events triggered by IAV and fMLP and data which indicates that neutrophil activation events in response to IAV occur largely at an intracellular site.

MATERIALS AND METHODS

Reagents

Formylmethionyl-leucyl-phenylalanine (fMLP), cytochalasin B, phorbol 12-myristate, 13-acetate (PMA), horseradish peroxidase type II, scopoletin, luminol, catalase, 4-aminoantipyrine, superoxide dismutase, cytochrome *c*, Ficoll, dextran, sodium citrate, citric acid, GTPγS, and GDPβS were purchased from Sigma Chemical Co. (St. Louis, MO). Hypaque was obtained from Winthrop Pharmaceuticals (Des Plaines, IL). Organic solvents were purchased from Fisher Scientific (Fair Lawn, NJ) and Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} or Mg^{2+} or without Ca^{2+} or Mg^{2+} from Flow Laboratories (Costa Mesa, CA). Genistein was obtained from LY Services Corp. (Woburn, MA). Tumor necrosis factor α and interferon γ were gifts of Genentech (San Francisco, CA), and granulocyte-macrophage colony-stimulating factor (GM-CSF) was a gift of ICN Immunobiologicals (Costa Mesa, CA).

Virus preparation

Influenza A virus strains H3N2 A/Texas/77 (Texas 77) and H3N3 A/Bangkok/79 (Bangkok 79) were grown in the chorioallantoic fluid of 10-day-old embryonated hens' eggs and purified on a discontinuous sucrose density gradient as

Abbreviations: CL, chemiluminescence; IAV, influenza A virus; fMLP, formylmethionyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage colony-stimulating factor; HAU, hemagglutination unit; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PT, pertussis toxin.

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previously described [4]. Virus stock was measured by hemagglutination assay, and titers of 1:2000 through 1:32,000 (as indicated) hemagglutination units (HAU) were measured after samples were thawed from frozen storage at −70°C.

Neutrophil preparation

Neutrophils from healthy volunteer donors were isolated to >95% purity as previously described using dextran precipitation, followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes [4]. Cell viability was >98% as determined by trypan blue staining, and cells were used within five hours of isolation.

Measurement of neutrophil activation

H_2O_2 production was measured by the oxidation of scopoletin, and O_2^- was assessed by continuous monitoring of the superoxide dismutase-inhibitable reduction of cytochrome *c* [6]. For the H_2O_2 assay sodium azide (final concentration 50 μM) was added except where otherwise indicated (e.g., Table 3).

Neutrophil chemiluminescence (CL) responses were measured by two methods. In method I (used in experiments presented in Table 3 only), a 2-ml preparation of PBS containing 400 HAU/ml of Bangkok 79 IAV or 2.5×10^{-7} M fMLP and 1.1×10^{-5} M luminol was incubated at 37°C, followed by addition of 400 μl of PBS containing 4×10^6 neutrophils. This method was devised to mimic as closely as possible the conditions used in the H_2O_2 assay. Glass scintillation vials containing 900 μl of PBS at 37°C were prepared in advance, and at 2-min intervals 100-μl aliquots were removed from the mixture of neutrophils and IAV or fMLP, added to the glass vials, and counted in a TM Analytic Delta 300 beta counter for 30 s. In method II, samples containing 9 ml of PBS, 1.1×10^{-5} M luminol, and 100 HAU/ml of Texas 77 or Bangkok 79 IAV to which 10^7 neutrophils were added, followed by incubation at 37°C. One-milliliter aliquots of these samples were taken immediately after addition of cells and at 2-min intervals thereafter, placed in glass vials, and counted for 30 s in the beta counter. In certain experiments, 200 μg/ml catalase or 50 μM sodium azide was incorporated (where indicated) into the reaction mixture of neutrophils with fMLP or IAV. This amount of catalase was employed after determining that it was sufficient to reduce by >90% the H_2O_2 detectable by the scopoletin assay after addition of fMLP.

Neutrophil degranulation was assessed by measuring lysozyme or myeloperoxidase (MPO) release into the cell supernatant. The cells were first incubated with cytochalasin B (5 μg/ml) for 5 min followed by addition of IAV, 5×10^{-7} M fMLP, or control buffer and incubation for various periods of time (as indicated) at 37°C. Lysozyme and MPO contents of cell supernatants and lysed cell pellets were determined using the *Micrococcus lysodeikticus* and 4-amino antipyrine assays as described [14]. The virus preparations did not interfere with detection of a standard lysozyme preparation. Results are expressed as the lysozyme or MPO activity detected in the supernatant divided by the total activity in the lysed cell pellet and supernatant × 100.

Electropermeabilization of neutrophils

Electropermeabilization was performed in a manner similar to that reported by Therrien and Naccache [15]. Neutrophils were incubated in permeabilization buffer (PBS without added Ca^{2+} or Mg^{2+} but with 5 μM glucose and 100 μM ATP added) at a concentration of 10^7 cells/ml and exposed to a single 1.45-mV pulse of a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) at a capacitance of 25 μFD. The cells were then placed on ice for 10 min, followed by resuspension in PBS with Ca^{2+} and Mg^{2+} for H_2O_2 assay. Cells treated in this manner were >90% positive for trypan blue uptake and no longer exhibited membrane depolarization in response to fMLP (data not shown). In some experiments, GDPβS or GTPγS (both at a final concentration of 50 μM) was added prior to permeabilization and this concentration was maintained as well during H_2O_2 assay. Addition of GTPγS or GDPβS to nonpermeabilized neutrophils was not found to cause H_2O_2 production or alter responsiveness of the cells to other stimuli (data not shown).

RESULTS

Role of GTP-binding proteins or tyrosine kinase activity in neutrophil respiratory burst activation by IAV or fMLP

In the model of fMLP-induced neutrophil stimulation, activation of pertussis toxin (PT)-sensitive GTP-binding proteins precedes Ca^{2+}_i mobilization. As noted above, however, neutrophil activation by IAV is insensitive to PT [6]. Cholera toxin does inhibit neutrophil H_2O_2 production and Ca^{2+}_i responses to IAV [6]; however, this effect could be secondary to cholera toxin-induced elevation of cAMP. We therefore sought another method of determining whether IAV interacts with neutrophil GTP-binding proteins. A protocol for electropermeabilization of neutrophils was developed which allowed continued detection of H_2O_2 production by virus-stimulated cells despite rendering >90% of the cells permeable to trypan blue. As depicted in **Table 1**, H_2O_2 responses to IAV and fMLP were significantly inhibited in these cells when they were preincubated with GDPβS, whereas responses to PMA were unaffected. It was also possible to prime the H_2O_2 response to IAV and fMLP, but not PMA, by preincubation of the cells with substimulatory concentrations of GTPγS.

We have previously demonstrated that treatment of neutrophils with H-7 or staurosporine inhibits H_2O_2 responses to IAV and fMLP in a similar manner [7, 9]. Whereas both

TABLE 1. Effect of Guanine Nucleotides on H_2O_2 Responses of Electropermeabilized Neutrophils

Stimulus[b]	*n*	Preincubation condition[a] Control	GDPβS	GTPγS
IAV	6	1.2 ± 0.2	0.6 ± 0.15*	
IAV	5	0.7 ± 0.04		2.0 ± 0.7*
FMLP	8	1.0 ± 0.2	0.5 ± 0.15*	
FMLP	6	1.6 ± 0.4		2.6 ± 0.5*
PMA	8	1.8 ± 0.2	1.7 ± 0.16	
PMA	6	2.4 ± 0.3		2.4 ± 0.3

[a]Neutrophils were electropermeabilized as described in Methods in the presence of either control buffer or buffer containing 50 μM GDPβS or GTPγS, followed by stimulation of H_2O_2 production (as measured by the scopoletin assay in presence of 50 μM sodium azide) with IAV, fMLP, or PMA.

[b]Neutrophils were stimulated with either 200 HAU/ml Texas 77 IAV, 10^{-7} M fMLP, or 250 ng/ml PMA and the maximal rate of H_2O_2 production (in nmol/4 × 10^6 cells) over 3 min was calculated. GDPγS significantly prolonged the lag period prior to onset of H_2O_2 production for all stimuli; GTPγS did not consistently affect the lag period (data not shown). Guanine nucleotides did not significantly alter H_2O_2 responses to PMA.

*$P < .05$ compared with control.

inhibitors may exert this effect by blunting protein kinase C activation, staurosporine also inhibits tyrosine kinases. Genistein inhibits tyrosine kinase activity with minimal effect on protein kinase C [16]. As shown in **Table 2,** neutrophil H_2O_2 responses to IAV, fMLP, and PMA were significantly blunted by preincubation of the cells with 25 μg/ml genistein. Inhibition of IAV- and fMLP-induced responses was significantly greater than inhibition of those stimulated by PMA. [Chemiluminescence responses to IAV and fMLP were similarly inhibited by genistein (data not shown).]

Lack of neutrophil degranulation in response to IAV

As depicted in **Figure 1,** IAV did not elicit any detectable neutrophil degranulation as assessed by lysozyme release assay. This assay was done in the presence of cytochalasin B (a maneuver that substantially increases neutrophil H_2O_2 responses to IAV [7]) to optimize granule enzyme detection. fMLP-treated cells exhibited substantial lysozyme release. Similarly, IAV did not provoke any MPO release from cytochalasin B-treated neutrophils, despite substantial release of this enzyme when the cells were stimulated with fMLP. MPO release after a 20-min incubation with Bangkok 79 IAV (100 HAU/ml) was 10 ± 1% (not significantly different from the 6 ± 3% release from neutrophils treated with buffer alone; mean ± SEM, n = 3). fMLP caused a 62 ± 10% MPO release over the same time period ($P \leq .05$ compared with control).

IAV-induced respiratory burst responses occur predominantly at an intracellular site

We have previously reported that addition of azide significantly increases the H_2O_2 release that is detectable by the scopoletin assay after stimulation of neutrophils with IAV (but not with fMLP) [7]. This finding suggests that the respiratory burst response to IAV may occur predominantly at an intracellular location. According to this hypothesis O_2^- is not detected because it cannot traverse the membrane, while H_2O_2 does. Azide increases the H_2O_2 escaping from the cell by diffusing into the cell and preventing metabolism of H_2O_2 by peroxidases or catalase. As shown in **Table 3,** when no azide was added to the assay, less H_2O_2 was detectable over 5 min in response to stimulation with the Bangkok 79 strain of IAV than was measured within 1 min in the presence of azide (see Table 2). However, a substantial

TABLE 2. Effect of Genistein on Neutrophil H_2O_2 Response to IAV or fMLP

Stimulus[a]	Control neutrophils	Genistein-treated neutrophils[b]	% of control
fMLP	1.41 ± 0.28	0.54 ± 0.2	45 ± 6% ($P \leq 0.005$)
IAV	0.43 ± 0.08	0.14 ± 0.09	20 ± 6% ($P \leq 0.01$)
PMA	2.32 ± 0.25	1.84 ± 0.16	80 ± 3% ($P \leq 0.025$)

[a]The concentrations of fMLP and PMA were same as in Table 1. For IAV, 100 HAU/ml of the Bangkok 79 strain was used.

[b]Neutrophils were pretreated with genistein (100 μM final concentration) or its diluent (control neutrophils) for 10 min at 37°C, followed by resuspension in fresh buffer for measurement of H_2O_2 responses to stimulation with the indicated stimuli (as described for Table 1). Values given are mean ± SEM (n = 4) H_2O_2 produced over 1 min per 4 × 10^6 cells. All assays were carried out in the presence of 50 μM sodium azide. To get % of control, the response in genistein-treated neutrophils was divided by that in control neutrophils × 100. Genistein reduced the response to all stimuli, but responses to IAV and fMLP were reduced significantly more than those to PMA ($P \leq .01$).

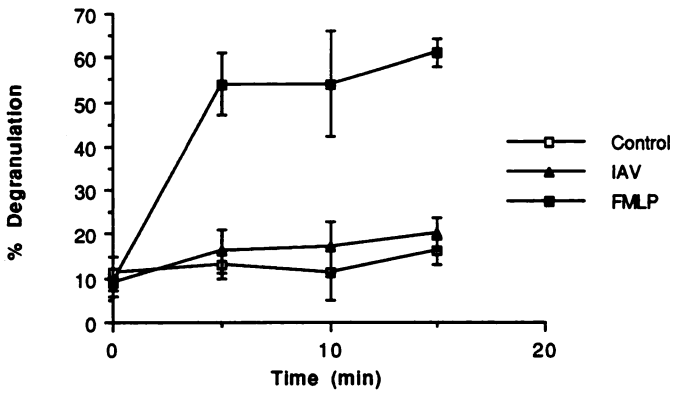

Fig. 1. Human neutrophil lysozyme release in response to IAV, fMLP, or control buffer. Lysozyme release from neutrophil granules was measuring using the *Micrococcus lysodeikticus* assay, as detailed in Methods. Mean ± SEM of five experiments are shown. At time 0, IAV (400 HAU/ml Texas 77 IAV), fMLP (10^{-7} M), or control buffer was added to neutrophils maintained at 37°C. Lysozyme measurements were made immediately after addition (time 0) and at 5, 10, and 15 min. Degranulation was significantly greater ($P < .05$) than the control value for fMLP at 5, 10 and 15 min but not for IAV at any time point.

chemiluminescence response was evident in the absence of azide as shown in Table 3. Note that the CL and H_2O_2 assays presented in Tables 2 and 3 were carried out under identical conditions (i.e., same concentrations of cells and virus) apart from the deletion of azide in the case of Table 3. Hence, the CL assay is apparently more sensitive than the H_2O_2 assay for detecting a respiratory burst response to IAV. Since luminol can diffuse into neutrophils [17, 18], an intracellular response may be more evident on the CL assay. Alternatively, excitation of the IAV particle itself may contribute to the CL response [19]. Further assessment of oxidative metabolism was therefore sought.

To ascertain the amount of H_2O_2 released into the extracellular medium, in contrast to that remaining in the cell's interior, CL experiments were conducted with catalase, an enzyme too large for cellular incorporation. In preliminary experiments (data not shown) we determined that 200 μg/ml catalase fully inhibited the detection of H_2O_2 in response to either fMLP or IAV. In **Figure 2,** the effects of azide and catalase on CL generation in response to IAV or fMLP are shown. For these experiments (in contrast to those shown in Table 3), the mixture of neutrophils, luminol, and stimuli was not diluted at the time of scintillation counting (method II). This was done in an effort to maximize detection of an extracellular component of the CL response. Note that using this method, a substantial increase in light emission was detected in response to IAV compared with results shown in Table 3. Catalase did not significantly alter the CL response to IAV, although the CL response to fMLP was markedly inhibited. As previously reported [20], the CL response to fMLP appears to consist of an early phase (from 1-4 min after stimulation) in which the CL is produced largely outside the cell, followed by a later phase in which intracellular CL production is more evident. The latter component of the fMLP response resembles that triggered by IAV. Azide essentially eliminated the CL response to both IAV and fMLP, as expected [19].

Preincubation of neutrophils with cytochalasin B has been found to enhance extracellular release of O_2^- in response to *Histoplasma* [21] or *Neisseria gonorrhoeae* [18]. This maneuver enhances the H_2O_2 response to IAV, although not leading to any detectable O_2^- release [6]. As shown in **Figure 3,** we tested the effect of cytochalasin B on the CL response to

TABLE 3. Comparison of Neutrophil H_2O_2 and Chemiluminescence Responses upon Stimulation with IAV in Absence of Azide

Response	Control buffer[a]	IAV
H_2O_2	0.17 ± 0.03	0.30 ± 0.03*
CL	408 ± 155	58,900 ± 17,690*

[a]Neutrophils were treated either with 100 HAU/ml Bangkok 79 IAV (as in Table 2) or control buffer and H_2O_2 (n = 3) or chemiluminescence (n = 5) responses were measured using the scopoletin and luminol assays, respectively. No azide was added for these assays. The maximal CL response (in counts per 30 s) was obtained at 5 min after addition of IAV or fMLP. The results given are mean ± SEM counts/30 s taken at this time. The H_2O_2 results are mean ± SEM produced over the first 5 min after addition of IAV. The CL assay for these experiments was performed using method I (see Methods).

*$P \leq .05$ compared with control response.

fMLP and IAV. The CL response to both stimuli was significantly enhanced by preincubation of neutrophils with cytochalasin B. In the case of fMLP this resulted in a much larger and more prolonged extracellular component of the CL response (as judged by the inhibitory effect of catalase). Again, however, catalase had no significant effect on the response to IAV.

We have previously reported that opsonization of IAV with the serum lectin mannose-binding protein (MBP) or conglutinin [22, 23] significantly enhanced the H_2O_2 (but not O_2^-) response to IAV. Similarly, preincubation of IAV with conglutinin significantly enhanced the CL response to IAV (mean CL response after 4 min was 91,250 ± 47,524 cpm/30 s for IAV alone vs. 231,000 ± 94,249 for IAV opsonized with 20 μg/ml conglutinin; $P \leq .05$, n = 4). Catalase did not significantly inhibit the CL response to IAV opsonized with conglutinin (data not shown). We also tested the ability of various priming agents including interferon-γ, tumor necrosis factor α, and GM-CSF to enhance neutrophil CL responses to IAV. While preincubation of neutrophils with each of these agents increased the CL response to IAV, GM-CSF was most potent in this regard, leading to a greater than tenfold enhancement of the CL response in four experiments (data not shown). Despite this, GM-CSF-treated neutrophils still did not release O_2^- in response to the virus in three experiments.

Because these data indicate that the respiratory burst response to IAV occurs predominantly at an intracellular site, we tested whether electropermeabilized neutrophils can release O_2^- in response to IAV. Despite extensive permeabilization as assessed by trypan blue dye uptake, and substantial H_2O_2 production, no O_2^- was detectable (data not shown).

DISCUSSION

Treatment of neutrophils with IAV leads to rapid and irreversible depression of the responses of these cells to subsequent stimulation with a variety of agonists [4]. This IAV-induced deactivation is characterized not only by impaired respiratory burst responses but also by impaired chemotaxis, degranulation, phagolysosome fusion, and bacterial killing [1, 2]. In the process of studying the mechanisms of these deactivating effects of IAV, we noted that the virus in its own right caused activation of the neutrophil as assessed by a variety of assays [4, 6, 7, 9]. Neutrophil stimulation by the virus was found to be anomalous in that a respiratory burst response was triggered in which H_2O_2, but no O_2^-, was released. Other features that distinguished the response to IAV from that to fMLP included (1) failure of pertussis toxin to inhibit responses to IAV and (2) an intracellular calcium response mainly derived from release of calcium from intracellular stores. We have attempted to further characterize the distinctive features of IAV's interactions with the neutrophil with the ultimate goal of clarifying why the virus depresses neutrophil function. Other neutrophil stimuli, including fMLP and concanavalin A, do not cause similar depression ([5] and our unpublished data).

As noted, one feature of neutrophil activation by IAV that is distinctive from stimulation by chemoattractants is the insensitivity of the former to pertussis toxin. Despite this difference, the findings reported in this paper suggest that ac-

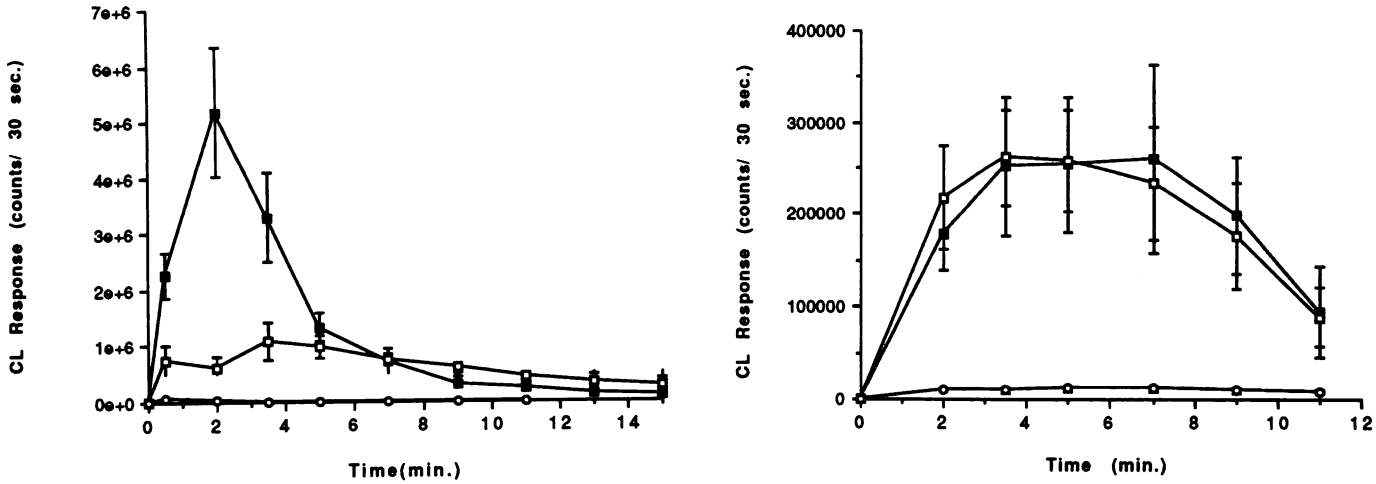

Fig. 2. Effect of catalase on neutrophil chemiluminescence responses to fMLP (left) or IAV (right). Neutrophils (10^7 cells in 10 ml) were treated with either 100 HAU/ml Bangkok 79 IAV or 2.5×10^{-7} M fMLP at time zero in the presence of luminol (1.1×10^{-5} M). At the time points indicated, 1-ml aliquots of these stimulated neutrophil preparations (containing 10^5 neutrophils/ml) were tested for light emission by scintillation counting (method II). Results represent mean ± SEM (n = 5 except for assays in sodium azide, where n = 3). Open squares indicate duplicate runs of these assays in which 200 μg/ml catalase (final concentration) was incorporated into the buffers. Catalase significantly reduced the CL produced between 1 and 4 min after fMLP exposure ($P \leq .05$), but had no significant effect on the response to IAV. Azide (final concentration 50 μM) essentially eliminated the CL response to either fMLP or IAV (open circles).

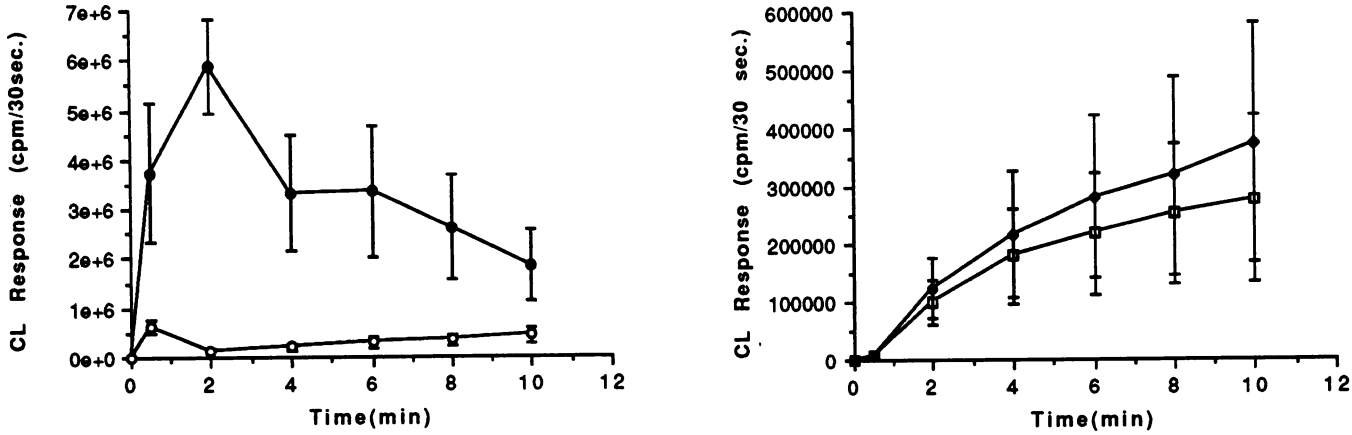

Fig. 3. Effect of catalase on chemiluminescence response to fMLP (left) or IAV (right) in cytochalasin B-treated neutrophils. The assay was performed as described in the legend of Figure 2 with the exceptions that neutrophils were pretreated for 5 min with 5 μg/ml cytochalasin B prior to stimulation with fMLP or IAV, and neutrophils were stimulated with 50 (rather than 100) HAU/ml IAV. Means ± SEM of four or five experiments are shown. Concurrent assays were performed in the absence of cytochalasin B (not shown). Cytochalasin B significantly increased the CL response to both IAV and fMLP ($P \leq .05$). Catalase significantly reduced ($P \leq .05$) the CL response to fMLP at all time points studied but had no significant effect on the response to IAV.

tivation of the neutrophil respiratory burst response by IAV does involve mediation of GTP-binding proteins. This conclusion is based on the inhibitory or enhancing effects, respectively, of GDPβS or GTPγS in electropermeabilized cells. Although one could argue that these guanine nucleotides are not necessarily acting on receptor-associated signal-transducing G proteins but possibly on low-molecular-weight proteins directly associated with NADPH oxidase assembly, this appears unlikely because responses to PMA were unaffected, while those to fMLP and IAV were altered in a very similar manner. The relatively gentle permeabilization procedure we employed may have allowed only limited penetration of guanine nucleotides into the cell so that only GTP-binding proteins associated with the plasma membrane were significantly affected. The identity of membrane receptor sites for IAV and which (possibly distinctive) GTP-binding proteins these receptors are associated with are important topics for future study.

Respiratory burst responses to IAV and fMLP were similarly reduced by the tyrosine kinase inhibitor genistein. Taking these findings in conjunction with our prior data, many similarities emerge in the post-G protein signal transduction cascade elicited by IAV and fMLP. On the other hand, the stoichiometry of the respiratory burst response to IAV is clearly distinctive from that to fMLP. We believe this discrepancy may provide clues to IAV-induced deactivation. We report here that treatment of neutrophils with IAV fails to cause any extracellular release of either lysozyme (a component of both specific and azurophilic granules) or myeloperoxidase (a component of azurophilic granules). Suchard et al. [24] have reported that exocytosis of a distinct population of lactoferrin-containing specific granules is a prerequisite for H_2O_2 production by adherent neutrophils. It remains possible such a population of granules is mobilized in IAV-treated neutrophils.

Using the CL assay, we also show that the respiratory burst response to IAV occurs largely at an intracellular site. The CL assay is capable of detecting respiratory burst products generated in the cell's interior, but the H_2O_2 assay is not. The marked increase in sensitivity of the CL assay compared with the H_2O_2 assay for detecting an IAV-induced respiratory burst response in the absence of azide provided an initial indication that this response occurs largely at an intracellular site. The marked inhibitory effect of azide on IAV-induced CL responses (as well as its enhancing effect of H_2O_2 responses) suggested that an intracellular peroxidase was involved in catalyzing respiratory burst products generated by IAV in neutrophils. An alternative explanation of the discrepancy between results of these CL and H_2O_2 assays was that ingested IAV particles themselves participated in light emission in a manner akin to excitation of ingested zymosan [19]. This appears unlikely in view of the marked inhibitory effect of azide on the CL response to IAV. However, it remains possible that part of inhibitory effect of azide in this system results from effects on viral membrane fluidity (e.g., resulting in reduced light emission from IAV particles) rather than from inhibition of peroxidases. We, therefore, proceeded to demonstrate that the IAV-induced CL response occurs predominantly at an intracellular site by use of catalase. This enzyme is too large to gain access to the cell's interior and hence inhibits CL production only outside the cell [17, 18]. Even when we used neutrophils pretreated with cytochalasin B, no inhibitory effect of catalase on IAV-induced CL responses was found.

Maneuvers that substantially increase the intensity of the neutrophil H_2O_2 or CL response to IAV (e.g., opsonization with conglutinin or priming of cells with GM-CSF) do not lead to generation of O_2^-. Hence, the failure to detect O_2^- is not likely to be due to lower sensitivity of the O_2^- assay. We have also previously shown that IAV does not quench O_2^- [25].

We cannot conclude, however, that intracellular respiratory burst activation provides the entire explanation for the failure of IAV to trigger O_2^- release, because electropermeabilized neutrophils also did not release O_2^- upon stimulation with IAV. It remains theoretically possible that IAV triggers H_2O_2 production directly without intermediate production of O_2^-. Further studies with cell-free NADPH oxidase preparations may help to resolve this issue. However, given the strong similarities in signals elicited by IAV and fMLP, it appears unlikely that a qualitatively different oxidase activity is involved. One intriguing possibility is that the virus binds directly to and perturbs membrane sites closely associated with, or actually including components of, the NADPH oxidase itself.

We conclude from these studies that determination of the mechanism through which IAV causes neutrophil deactivation could most profitably be achieved by (1) more direct studies of neutrophil surface binding sites for the IAV hemagglutinin molecule and (2) establishment of how, and

into which compartments, IAV is internalized. Characterization of which G proteins are involved in IAV-induced stimulation and the manner in which they are activated may also be revealing. However, IAV-induced neutrophil deactivation affects responses to PMA as much as to other stimuli [4]. Therefore, deactivation is unlikely to be attributable mainly to alterations in membrane receptor-associated G proteins.

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