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**Research Article**

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# Inhibition of Neutrophil Lysosome-Phagosome Fusion Associated with Influenza Virus Infection In Vitro

## ROLE IN DEPRESSED BACTERICIDAL ACTIVITY

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ABSTRACT The present study examined the effect of various unopsonized strains of influenza A virus on release of myeloperoxidase (MPO) and acid phosphatase in polymorphonuclear leukocytes (PMNL). These results were correlated with the effect that these same viruses had on bactericidal activity in PMNL. Several strains of virus inhibited the fusion of azurophil granules with phagosomes containing *Staphylococcus aureus*. These same strains inhibited the extracellular release of MPO from PMNL (39–59%) and caused depressed killing (42–77%). In contrast, one of the influenza viruses (X-47a) did not inhibit PMNL MPO release or killing. The data indicate a close relationship between the ability of influenza virus to ablate normal intracellular lysosome-phagosome fusion with subsequent depression of bactericidal functions of PMNL.

## INTRODUCTION

Influenza A viral infections can cause increased susceptibility to bacterial and fungal superinfections coincident with depressed metabolic and bactericidal activities of circulating and alveolar phagocytic cells (1–4). The mechanism by which influenza virus causes phagocyte dysfunction has not been determined. We have recently reported that influenza virus inhibits human polymorphonuclear leukocytes (PMNL)[1] as monitored by luminol enhanced chemiluminescence (5). Although this assay has been used to assess cellular oxidative metabolism, it is also dependent on normal

delivery of myeloperoxidase (MPO) (6, 7). Studies have shown that Sendai virus, which is similar to influenza virus in many of its physical components, can inhibit fusion of lysosomal granules with phagosomes in alveolar macrophages of mice (8, 9); however, the capacity of viruses to inhibit lysosome-phagosome fusion in human cells has not been examined. In this study we have investigated the effect that different influenza A viruses have on the release of two lysosomal enzymes found in the azurophil granules of PMNL (i.e., MPO and acid phosphatase) in conjunction with the effect of these viruses on bactericidal activity in the cell. The results indicate that virus or virus-associated products inhibit the fusion of azurophil granules with phagosomes containing bacteria. The inhibition of lysosome-phagosome fusion directly correlates with depressed bactericidal activity in the cell.

## METHODS

*Preparation of influenza virus.* Influenza A viruses were harvested from allantoic fluid (10) and purified using a sucrose gradient (11). The following unopsonized strains of virus were used in this study: (a) a naturally occurring $H_3N_2$ A/Texas/77 virus (Texas 77), (b) a naturally occurring $H_1N_1$ A/PR/8/34 virus (PR8), (c) a recombinant virus (X-31) containing the internal proteins of the PR8 virus and the surface glycoproteins of A/Aichi/2/68 ($H_3N_2$) and (d) a recombinant virus (X-47) containing some of the internal proteins of PR8 and the surface glycoproteins of A/Victoria/3/75 ($H_3N_2$) (12). Most of the X-47 virus that was grown inhibited fusion of lysosomes with phagosomes in PMNL. However, several harvests of this virus did not inhibit fusion of these vacuoles and this nondepressing virus was designated X-47a. To date, determinations of egg infectivity dose, 50% ($EID_{50}$), hemagglutinin, and neuraminidase content (11), protein content (13) and SDS gel electrophoresis (14) have not established the difference between X-47 and X-47a. The Texas 77, PR8, X-31, X-47 and X-47a viruses had hemagglutination titers
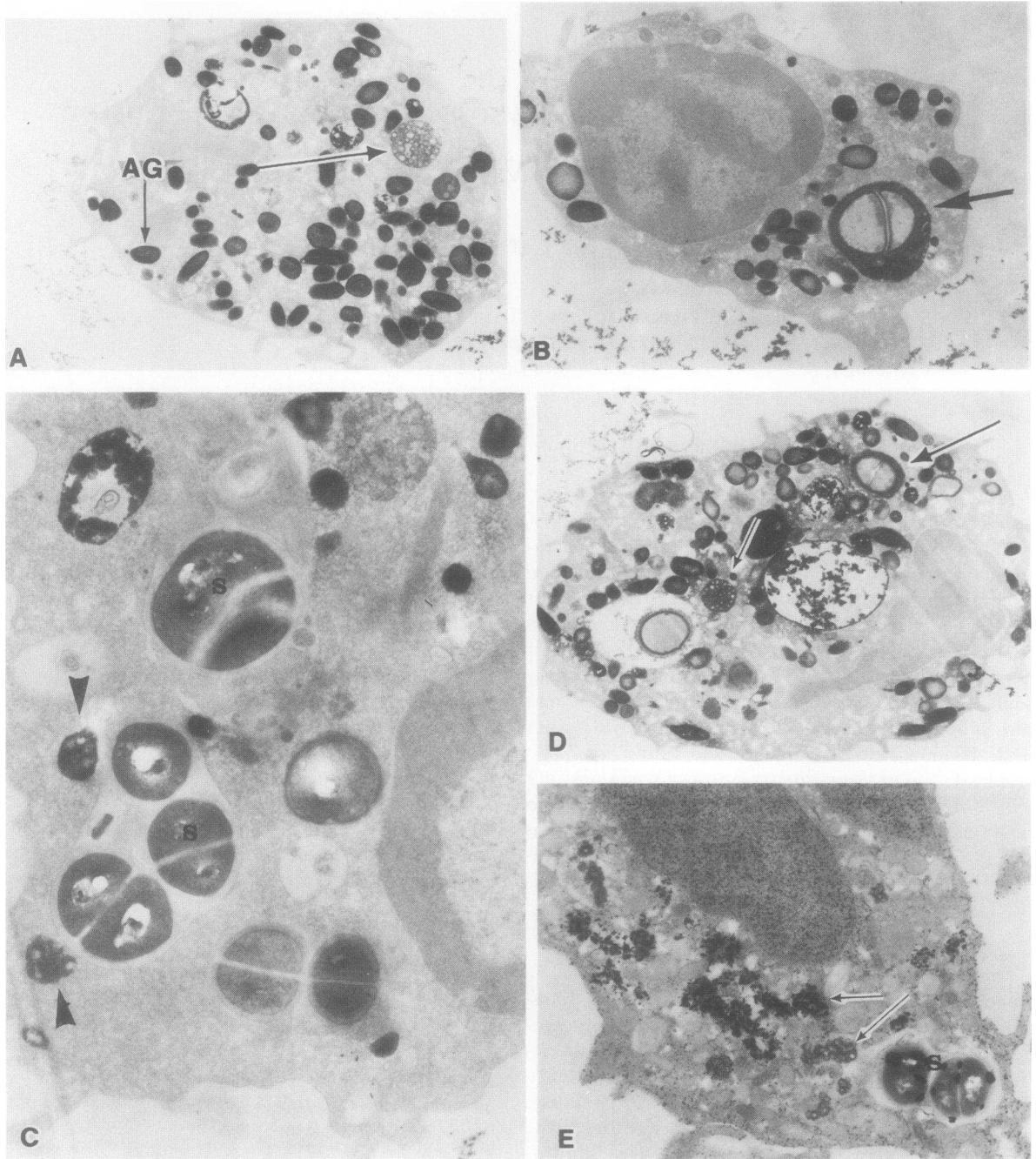
FIGURE 1   The deposition of lysosomal enzymes in PMNL challenged with influenza virus and/ or *S. aureus*. Electron micrographs are all of unstained sections (i.e. no lead or uranyl acetate used) in order to highlight the cytochemical reactions. (A) PMNL exposed to X-47 virus for 30 min. MPO is located in azurophil granules (AG) and in vacuoles containing virus particles (arrows). ×9,500, (B) PMNL exposed to staphylococci for 30 min. MPO is found both in azurophil granules and phagosomes containing the bacteria (arrow). ×13,500, (C) PMNL exposed to X-47 virus for 30 min followed by staphylococci for 30 min. MPO is present in phagosomes containing virus particles (arrows), whereas very little MPO is seen in phagosomes containing staphylococci (S). ×20,500, (D) PMNL exposed to X-47a virus for 30 min followed by staphylococci for 30 min. MPO is in phagosomes containing virus particles (small arrow) as well as staphylococci (large arrows) ×9,500, (E) PMNL exposed to X-47a virus for 30 min followed by staphylococci for 30 min. Acid phosphatase is present in phagosomes containing virus par-

of 1:640, 1:5120, 1:5120, 1:10,240, and 1:10,240, respectively, and all had an egg infectivity dose, 50% of $10^8$. Uninfected allantoic fluid did not inhibit lysosome-phagosome fusion.

*Leukocyte preparation.* A purified population of PMNL (≥97%) was obtained from heparinized whole blood as previously described (5). The PMN were resuspended in HBSS with gelatin (0.1% gelatin in Hanks' balanced salt solution without phenol red or $NaHCO_3$; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) to the desired final concentration.

*Electron microscopy.* PMNL ($5 \times 10^6$/ml) were preincubated with 0.4 ml of virus or buffer for 30 min at 37°C. In some experiments opsonized *Staphylococcus aureus* (American Tissue Type Culture 29213) was prepared as previously described (5), and incubated with these preincubated PMNL at a 20:1 bacteria/cell ratio for an additional 30 min at 37°C. Cells for cytochemical studies were fixed at 1–3°C with 0.1 M cacodylate-buffered (pH 7.4) glutaraldehyde (1.25%) in the presence of 2% sucrose. Cells were thrice washed over a period of 1 h in cold 0.1 M cacodylate buffer containing 2% sucrose. MPO was demonstrated by the diaminobenzidine technique of Graham and Karnovsky (15) as applied to human leukocytes (16, 17). Acid phosphatase cytochemistry was done using the (tris)-maleate buffered lead precipitation technique as previously reported for peripheral blood cells (16).

*MPO release.* PMNL ($1.3 \times 10^7$/ml) were preincubated with 0.5 ml of virus or buffer for 30 min at 37°C. They were centrifuged at 300 $g \times 10$ min and the pellet was resuspended in half the original volume. The PMNL were then stimulated for 15 min with opsonized zymosan (17 mg/ml, opsonized as described previously [5]) in a 1:1 vol:vol ratio, centrifuged at 300 $g \times 10$ min and the supernatant assayed for MPO content. Extracellular MPO release was measured by a modification of the method using *o*-dianisidine DiHCl (Sigma Chemical Co., St. Louis, MO) as substrate (18). MPO was determined in a volume of 3 ml containing 0.003% hydrogen peroxide and 0.25 mg *o*-dianisidine in 10 mM phosphate-buffered saline with addition of 50 ml of supernatant to be assayed. Absorbance at 460 nm was determined on a Beckman spectrophotometer (model 35, Beckman Instruments, Inc., Fullerton, CA). MPO activity was expressed as nanomoles per $10^7$ PMNL per minute, using an extinction coefficient of 11.3 for *o*-dianisidine.

*Bactericidal activity.* Prior to exposure of PMNL to bacteria the cells were preincubated with 0.4 ml of virus or buffer for 30 min. Preopsonized *S. aureus* was then incubated with $5 \times 10^6$ PMNL at 37°C for 0, 15, and 60 min at a 20:1 ratio using a previously described bactericidal assay (19). Triplicate 20-$\mu$l samples were taken using several dilutions of the incubation mixture and put on sheep blood agar plates that had been prewarmed at 37°C for 2 h. The percentage of bacteria killed was calculated as previously described (5).

Statistical evaluation was done using the paired Student's *t* test.

## RESULTS

*Electron microscopy.* Prior to challenge with virus or bacteria the DAB reaction product indicative of MPO was localized to numerous large azurophil gran-

ules. Following ingestion of either virus (Fig. 1A) or staphylococci (Fig. 1B) PMNL degranulation occurred and electron opaque reaction product was observed in the respective phagosome of each microbe. When PMNL were preincubated with Texas 77, PR8, X-31, or X-47 and then exposed to staphylococci, apparently normal phagocytosis of bacteria occurred; however, there was a striking decrease in the deposition of MPO within phagosomes containing bacteria (Fig. 1C). The lack of normal granule-phagosome fusion (and intraphagosomal degranulation) was noted in >90% of the PMNL even though MPO-containing granules were observed immediately adjacent to the phagosomes using stereo electron microscopy (data not included). In contrast to this pattern, PMNL incubated with X-47a and then exposed to staphylococci had MPO in bacterial as well as viral-containing phagosomes (Fig. 1D). Similar studies of intraphagosomal deposition of acid phosphatase demonstrated identical results (Fig. 1E).

*MPO release.* PMNL stimulated with buffer for 30 min did not release any detectable MPO. All virus preparation (including X-47a) caused a modest stimulation of extracellular release of MPO ranging from one-twelfth to one-third that seen after zymosan ingestion (Table I). When PMNL were preincubated with Texas 77, PR8, X-31, or X-47 virus for 30 min and then stimulated with opsonized zymosan, there was a significant decrease in the extracellular release of MPO as compared with cells initially treated with buffer ($P \leq 0.05$). In contrast, preincubation of PMNL with X-47a virus did not depress MPO release induced by phagocytosis of zymosan (Table I).

*Bactericidal activity.* The ability of PMNL to kill *S. aureus* was significantly decreased after 15 and 60 min in cells pretreated with Texas 77, PR8, X-31, and X-47 ($P \leq 0.05$), but not with X-47a (Table II).

## DISCUSSION

The mechanism by which influenza virus enters PMNL has not been studied, but this virus has been shown to enter canine kidney cells by endocytosis and then to rapidly fuse with lysosomal membranes (20). In the present study, ultrastructural cytochemistry of PMNL incubated with Texas 77, PR8, X-31, and X-47 virus revealed azurophil granule enzymes within vacuoles containing these viruses. Upon subsequent exposure of virus incubated cells to *S. aureus*, enzyme product release into phagosomes containing the bacteria was markedly decreased. Stereo electron microscopy demonstrated MPO-containing granules adjacent to these phagosomes but fusion of these granules with phago-

ticles (arrows), and is seen in phagosomes containing staphylococci (S). ×9,000. Similar experiments done with X-47 virus and staphylococci showed acid phosphatase in the phagosomes containing virus but not in phagosomes containing bacteria (data not shown).

TABLE I

Extracellular MPO Release from PMNL Preincubated with Virus or Buffer prior to Zymosan Stimulation*

| Preincubation | Phagocytic stimulant | MPO release‡ | | % depression§ |
|---|---|---|---|---|
| | | Preincubation | After phagocytic stimulant | |
| | | nmol/10¹ PMNL/min | | |
| HBSS | Zymosan | 0 | 1.07 | |
| Texas 77 | Zymosan | 0.39 | 0.65 | 39 |
| HBSS | Zymosan | 0 | 6.75 | |
| PR8 | Zymosan | 0.57 | 2.78 | 59 |
| HBSS | Zymosan | 0 | 2.51 | |
| X-31 | Zymosan | 0.29 | 1.08 | 57 |
| HBSS | Zymosan | 0 | 2.6 | |
| X-47 | Zymosan | 0.45 | 1.6 | 38 |
| HBSS | Zymosan | 0 | 2.51 | |
| X-47a | Zymosan | 0.65 | 2.27 | 10 |

* PMNL were preincubated with buffer or virus for 30 min and cells were then stimulated with opsonized zymosan for 15 min.
‡ Results are given as the mean of closely agreeing duplicate determinations from a representative example of three or more experiments. In all instances the range was <10% of the mean.
§ Percent depression equals 1-(MPO released from virus preincubated cells in response to zymosan ÷ MPO released from control cells in response to zymosan) ×100.

TABLE II

Bactericidal Activity against S. aureus of PMNL Incubated with Virus or Buffer*

| Preincubation | % bacteria killed‡ | | % depression§ | |
|---|---|---|---|---|
| | 15 min | 60 min | 15 min | 60 min |
| HBSS | 71 | 91 | | |
| Texas 77 | 27 | 51 | 62 | 46 |
| HBSS | 47 | 70 | | |
| PR8 | 8 | 16 | 83 | 77 |
| HBSS | 66 | 79 | | |
| X-31 | 36 | 46 | 45 | 42 |
| HBSS | 47 | 70 | | |
| X-47 | 8 | 31 | 83 | 56 |
| HBSS | 66 | 79 | | |
| X-47a | 63 | 81 | 4 | −3 |

* PMNL were preincubated with virus or buffer for 30 min before adding bacteria.
‡ Results are given as the mean of closely agreeing triplicate determinations from a representative example of three or more experiments. In all instances the standard error of the mean was <10% of the mean.
§ Percent depression equals 1-(bactericidal activity of virus preincubated cells ÷ bactericidal activity of control cells) ×100.

somes containing bacteria was noted only rarely. In contrast, PMNL preincubated with X-47a virus and subsequently exposed to staphylococci had enzyme product within phagosomes containing bacteria as well as virus. These data suggest that internalization of influenza virus into the PMNL is not by itself sufficient to inhibit lysosome-phagsome fusion. Additionally, all of the viruses caused a modest release of MPO extracellularly during virus-PMNL preincubation, but upon subsequent exposure of the cells to opsonized zymosan, extracellular MPO release was normal only for PMNL pretreated with X-47a virus. Thus, viral-induced abrogation of PMNL degranulation to subsequent stimuli (e.g., bacteria, zymosan) is due to neither (a) the initial MPO release elicited by the virus, nor (b) the exhaustion of cellular stores of MPO. Other studies from our laboratory examining the effect of influenza virus on PMNL chemiluminescent activity in the presence of luminol, indicate that virus-induced depression of chemiluminescent activity in response to particulate and soluble stimuli is independent of the capacity of the virus to stimulate the cell's respiratory burst (manuscript submitted for publication).

The virus-induced depression of intraphagosomal degranulation and extracellular MPO release from the cell directly correlated with decreased PMNL bactericidal activity; i.e., Texas 77, PR8, X-31, and X-47

viruses inhibited MPO release and killing, whereas X-47a virus did not inhibit these parameters. Influenza virus can cause depressed PMNL bactericidal activity in vivo and in vitro without affecting phagocytic activity (1, 5). Inhibition of MPO release could produce decreased bactericidal activity by inhibiting the formation of peroxidase-dependent microbicidal oxidants (21). Inhibition of intraphagosomal release of other lysosomal enzymes could also cause depressed non-oxidative microbicidal killing (21).

To date, studies involving depressing virus and non-depressing virus of identical parentage (i.e., X-47 and X-47a) have not identified a difference in the physical characteristics of these two viruses. The PMNL dysfunction could be due to a specific component of these viruses, a substance released from viruses or a product associated with the interaction of viruses with eukaryotic cells. Although determining the difference between X-47 and X-47a virus could identify the mechanism by which inhibition of lysosome-phagosome fusion occurs, the data obtained in this study indicates that the disruption of lysosome-phagosome fusion occurring in the presence of influenza virus may lead to depressed bacterial killing by PMNL. Additionally, the inhibition of lysosome-phagosome fusion in conjunction with depressed bactericidal activity of PMNL may help to explain the pathogenesis of secondary microbial disease in patients with influenza virus infections.

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