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Role of the respiratory burst in co-operative reduction in neutrophil survival by influenza A virus and *Escherichia coli*

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Influenza A virus (IAV)-induced impairment of neutrophil function or survival may be a cause of bacterial superinfection of IAV-infected subjects. This study was performed to determine the mechanism through which the combination of IAV and *Escherichia coli* co-operatively reduces neutrophil survival. Neutrophil binding of annexin-V and caspase-3 activation was significantly increased by either IAV or *E. coli*, supporting the concept that the micro-organisms accelerate neutrophil apoptosis. The anti-apoptotic agent granulocyte-macrophage colony stimulating factor (GM-CSF) did not improve, but further reduced, survival of neutrophils treated with IAV and *E. coli*. As addition of *E. coli* resulted in greater neutrophil uptake of IAV and greater neutrophil respiratory burst responses to IAV, this study tested whether respiratory burst activation by IAV and *E. coli* contributes to reducing neutrophil survival. The cell-permeant NADPH oxidase inhibitor, diphenylene iodonium, significantly increased survival of neutrophils treated with either *E. coli* alone or the combination of IAV and *E. coli*. In contrast, catalase, which is not cell permeant, did not alter survival of *E. coli*- and IAV-treated neutrophils. Azide enhanced neutrophil hydrogen peroxide responses to IAV and *E. coli*, and reduced survival of these cells. These results indicate that co-operative induction of intracellular respiratory burst responses by IAV and *E. coli* mediates the reduced neutrophil survival caused by these pathogens *in vitro*.

Introduction

Bacterial superinfections are a major cause of morbidity and mortality during influenza A virus (IAV) epidemics, particularly among the elderly. IAV infection increases susceptibility to bacterial pneumonia, otitis media and meningitis [1]. The mechanisms through which IAV impairs host defences against bacterial infection have not been fully elucidated, but *in-vitro* and animal studies indicate that IAV-induced depression of neutrophil function is an important predisposing factor [2]. Recent studies also suggest that co-operative effects of IAV and bacteria on neutrophil survival may contribute to the risk of bacterial superinfection. IAV accelerates apoptosis of epithelial cells, neutrophils and monocytes *in vitro* [3–5]. Treatment of neutrophils with both IAV and

Escherichia coli causes a rapid, marked decline in neutrophil viability [3]. This decline in viable neutrophils was much greater than that observed with either IAV or *E. coli* alone. Similar results were obtained with the combination of IAV and *Streptococcus pneumoniae* [6].

This study was undertaken to characterise the mechanisms through which IAV and *E. coli* co-operatively reduce neutrophil viability. First, increased annexin-V binding and caspase-3 activation were shown to occur after infection of neutrophils with IAV or *E. coli*. However, IAV and *E. coli* did not co-operatively increase caspase-3 activation. In earlier studies IAV was shown to increase neutrophil Fas antigen and Fas ligand expression; however, as in the case of caspase-3 activation, it was not possible to clearly demonstrate co-operative increases in Fas antigen or ligand expression by co-infection with IAV and *E. coli* [3]. Hence, this study investigated another mechanism to account for the co-operative effects of IAV and *E. coli* on neutrophil survival and the neutrophil respiratory burst

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response, which has been implicated as a cause of neutrophil apoptosis induced by *E. coli*, immune complexes or phorbol myristate acetate (PMA) [7–9].

Materials and methods

Reagents

Dextran, sodium citrate, RPMI-1640, trypan blue stain, Wright's-Giemsa stain, scopoletin, catalase, horseradish peroxidase type II, fMLP, diphenyleneiodonium (DPI), propidium iodide (PI) and sodium azide were purchased from Sigma. Ficoll-Paque was obtained from Pharmacia Biotech (Piscataway, NJ, USA). Dulbecco's phosphate-buffered saline (PBS) was purchased from Gibco BRL (Grand Island, NY, USA). Purified *E. coli* LPS was kindly provided by Dr Douglas Golenboch (Boston University School of Medicine, Boston, MA, USA).

Neutrophil isolation and culture

Neutrophils from healthy volunteer donors were isolated to >95% purity (based on Wright's-Giemsa stain) by dextran precipitation, followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes [10]. Cell viability after isolation was >98%, as determined by trypan blue staining.

Virus preparation

Influenza A virus (Phillipines 82 H3N2 strain) was grown in the chorio-allantoic fluid of 10-day-old embryonated hen's eggs and purified on a discontinuous sucrose density gradient, as described previously [10]. Virus stocks were dialysed against PBS divided into small volumes and stored at -80°C until used. Haemagglutination titres were determined by dilution of virus samples in PBS followed by addition of thoroughly washed human type O red blood cells. After thawing, the virus stock contained $c. 5 \times 10^8$ plaque-forming units/ml and had a protein concentration of $3.34 \mu\text{g/ml}$ by protein assay (BioRad).

Bacterial preparations

E. coli (K12 strain) was obtained from the American Type Culture Collection, grown in this laboratory and fixed with formalin for use in the experiments. *S. pneumoniae* suspensions in PBS were washed three times and sonicated (for three 20-s intervals) to eliminate bacterial aggregates before each experiment. *S. pneumoniae* was pre-incubated with specific anti-pneumococcal IgG $32 \mu\text{l}/100 \mu\text{l}$ for 30 min at 37°C and rewashed with PBS to remove residual sodium azide.

Assessment of neutrophil viability and percentage of hypodiploid neutrophils

Assessment of neutrophil viability was made after incubation of the cells for 15 min with trypan blue dye. The numbers of neutrophils that did and did not take up the dye were counted with a haemocytometer. Neutrophils that did not take up trypan blue were counted as viable. The percentage viability of neutrophils was obtained by dividing the number of viable cells by the total number of cells (i.e., both those which did and did not take up trypan blue). The percentage of cells with hypodiploid nuclei was assessed by permeabilising the cells and then exposing them to the DNA-binding dye PI. The method was performed with a slight modification of the method described by Nicoletti *et al.* [11]. Neutrophils were incubated with IAV or bacteria, or both, as described previously [3]. Briefly, neutrophils were incubated with either *E. coli* (or *S. pneumoniae*), IAV or a combination of IAV and the bacteria. The final approximate ratio of bacteria: neutrophil was 30:1. After incubation for 1 h at 37°C neutrophils were washed, resuspended in RPMI 1640 supplemented with autologous human serum 4%, 20 mmol/L HEPES buffer, L-glutamine 1%, penicillin 50 U/ml and streptomycin $50 \mu\text{g/ml}$, at pH 7.4, and incubated at 37°C . After incubation for 5, 10, 18 and 28 h, a fixed volume of cell suspension was removed from the culture and centrifuged at $200 g$ for 10 min. The pellet was fixed in 1 ml of ice-cold ethanol 70% at 4°C for at least 60 min. Fixed cells were then washed with cold PBS with Ca^{2+} and Mg^{2+} and gently resuspended in 1 ml of PI solution ($40 \mu\text{g/ml}$ in PBS with Ca^{2+} and Mg^{2+}). The cells were finally incubated in the dark at room temperature for 15 min and analysed on a FACScan 2 flow cytometer (Becton Dickinson, San Jose, CA, USA). Forward and side scatter of neutrophils were simultaneously acquired. The red fluorescence due to PI staining of individual cells was collected under FL-2 and plotted against forward scatter. The FL-2 data were registered on a logarithmic scale. Cell debris was excluded from the analysis by appropriate raising of the forward scatter threshold. Five thousand cells of each sample were counted. All measurements were done under the same instrument settings and evaluated with the Lysis II programme.

Measurement of annexin-V binding

Phosphatidylserine exposure was measured by binding of annexin-V-FITC by the protocol outlined in the annexin-V assay kit (Pharmingen, San Diego, CA, USA). The assay was performed as described by Fadeel *et al.* [9]. Briefly, this involved staining the cells with annexin and PI ($5 \mu\text{g/ml}$) before analysis with a FACScan flow cytometer (Becton Dickinson). Five thousand events were collected and analysed with the Cell Quest software (Becton Dickinson). Low fluorescence detritus was gated out before analysis. The

percentage of neutrophils that had positive fluorescence and the mean neutrophil fluorescence were measured for each sample.

Fluorometric analysis of caspase-3 activity

Caspase-3 activity was measured *in vitro* by cleavage of the fluorogenic substrate DEVD-AMC (amino-4-methylcoumarin) with a fluorometric assay kit (Pharmingen). The caspase-3 aldehyde inhibitor Ac-DEVD-CHO was used as a negative control. For preparation of cytoplasmic extracts after incubation for 5 and 18 h, neutrophils were lysed in lysis buffer (Pharmingen). Cell lysates and substrate were combined in a reaction buffer (Pharmingen). AMC release was monitored in a spectrofluorometer at an excitation wavelength of 380 nm and emission wavelength of 440 nm. Relative AMC fluorescence was expressed as the percentage of fluorescence of control cells.

Assays of neutrophil H_2O_2 production and intracellular calcium release

H_2O_2 production by neutrophils was measured by the fluorescent scopoletin assay as described previously [12]. Intracellular calcium release was measured with Fura-2 AM (Molecular Probes, Eugene, OR, USA) as described previously [10]. Briefly, neutrophils were loaded with Fura-2AM, washed and resuspended in fresh buffer. Baseline and stimulated intracellular calcium values were obtained by measuring the ratio of fluorescence at 340 and 380 nm with a spectrofluorometer.

Measurement of viral uptake by neutrophils

Neutrophils were pre-treated with bacteria or bacterial products. Viral uptake was then measured by incubating FITC-labelled IAV with neutrophils, followed by evaluation of cell-associated fluorescence by flow cytometry, as described previously [13]. Bacterial preparations were washed just before use to remove unbound FITC. Fluorescence was measured in the presence of trypan blue to quench extracellular fluorescence.

Statistical methods

Statistical significance was determined by Student's paired *t* test. A *p* value ≤ 0.05 was considered to indicate a statistically significant difference.

Results

Effects of IAV and *E. coli* on annexin-V binding by neutrophils

As shown in Table 1, the combination of IAV and *E. coli* significantly increased the mean fluorescence of neutrophils stained with annexin-V-FITC after exposure

Table 1. Effect of IAV and *E. coli* on neutrophil binding of annexin-V-FITC

Treatment group	Mean (SEM) percentage of annexin-V-positive cells	Mean (SEM) annexin-V fluorescence
Control	6.6 (2.2)	27.6 (5)
IAV	16 (7.4)	76.6 (19)
<i>E. coli</i>	27.8 (5.8)*	58 (20)*
IAV + <i>E. coli</i>	42 (6.7) [†]	152.5 (44) [†]

Neutrophils were incubated under the indicated conditions for 5 h, washed and labelled with annexin-V-FITC as described in *Materials and methods*. The percentage of neutrophils with fluorescence higher than unlabelled cells and the mean fluorescence of neutrophils were measured by flow cytometry. Results are mean and SEM of at least four experiments.

*The percentage of positive neutrophils or mean neutrophil fluorescence was significantly greater than for cells treated with control medium alone.

[†]The percentages of annexin-V-positive cells and mean annexin-V fluorescence were significantly higher in neutrophils treated with the combination of IAV and *E. coli* than in neutrophils treated with control medium, IAV or *E. coli* alone.

for 5 h. In these samples, 42% of neutrophils were positive for annexin-V binding, compared with 16% or 28%, respectively, for IAV or *E. coli* alone. Hence, a large proportion of these cells were undergoing early changes of apoptosis (i.e., phosphatidylserine exposure on extracellular membrane).

Effects of IAV and *E. coli* on caspase-3 activation

Caspase-3 activity, an indicator of apoptosis, increased in neutrophils treated with control buffer alone (i.e., from 6.4 (SEM 1.2) after incubation for 5 h to 15 (SEM 3.7) after 18 h; *n* = 8; *p* < 0.05), but treatment with either IAV or *E. coli* alone resulted in further significant increases (Table 2). Maximal caspase-3 activation appeared to occur after exposure for 10 h to IAV or *E. coli*, or both. Then caspase-3 activity declined so that after 18 h the level was no longer higher than that of buffer-treated neutrophils. Caspase-3 activity in neutrophils treated with the combination of IAV and *E. coli* was not significantly greater than activity in neutrophils treated with IAV or *E. coli* alone (Table 2).

Table 2. Caspase-3 activity in neutrophils treated with IAV, *E. coli* or the combination of IAV and *E. coli*

Incubation period (h)	Mean (SEM) percentage of control caspase-3 activity in neutrophils incubated with		
	IAV	<i>E. coli</i>	IAV + <i>E. coli</i>
5	133 (10)*	141 (15)*	147 (27)
10	181 (29)*	247 (69)*	233 (56)*
18	139 (25)	91 (7)	102 (13)

Results shown are mean and SEM of four experiments and are expressed as the caspase-3 activity of neutrophils treated with IAV or *E. coli*, or both (as indicated)/activity in neutrophils treated with control medium alone for the same period of time $\times 100$.

*Results were significantly increased as compared with neutrophils treated with control buffer alone.

Granulocyte-macrophage colony stimulating factor (GM-CSF) and survival of neutrophils treated with the combination of IAV and E. coli

GM-CSF protects neutrophils against constitutive apoptosis, plays an important role in lung and phagocyte physiology [14] and modulates interactions of IAV with neutrophils [15]. As shown in Fig. 1a, treatment of

Fig. 1. Effect of GM-CSF on the survival of neutrophils in samples treated with IAV and *E. coli*. *E. coli* and IAV were incubated with neutrophils (5×10^6 /ml) as described in *Materials and methods*. (a) Mean (bar SEM) number ($n \geq 6$ experiments) of viable neutrophils counted in cultures treated with IAV (○) or *E. coli* (●) alone, the combination of IAV and *E. coli* (◇) or the combination of IAV, *E. coli* and GM-CSF (△) after 5, 18 or 28 h in culture. The results are expressed as percentage of control viable cells (i.e., number of viable neutrophils in cultures treated with IAV, IAV and *E. coli*, or IAV, *E. coli* and GM-CSF divided by viable cells in cultures maintained in control medium alone for the same duration $\times 100$). For cultures treated with the combination of IAV and *E. coli*, viable cell counts were significantly less ($p < 0.05$) than for those treated with control medium, IAV or *E. coli* alone at all time points. (b) Percentage of viable neutrophils defined as percentage of cells able to exclude trypan blue in cultures treated with control medium alone (□), IAV and *E. coli* (◇), or IAV, *E. coli* and GM-CSF (△). The results were obtained by dividing the number of cells that excluded trypan blue by the total number of cells in each culture. Addition of GM-CSF reduced the percentage of viable neutrophils compared with treatment with IAV and *E. coli* alone ($p < 0.05$).

neutrophils with IAV or *E. coli*, or both, caused a marked decline in the number of surviving neutrophils after 18 or 28 h, as compared with neutrophils treated with control buffer alone. As previously reported, the combination of IAV and *E. coli* caused a significantly greater reduction in surviving neutrophils than either IAV or *E. coli* alone. Addition of GM-CSF did not improve survival of neutrophils treated with the combination of IAV and *E. coli*. In fact, after 28 h there were significantly fewer surviving cells in samples treated with IAV, *E. coli* and GM-CSF as compared with those treated with IAV and *E. coli*. The percentage of viable neutrophils also showed that GM-CSF worsened survival of neutrophils treated with IAV and *E. coli* (Fig. 1b).

Effects of E. coli on neutrophil uptake of IAV

An earlier study reported that treatment of neutrophils with IAV increases subsequent uptake of *E. coli* [3]. Treatment of neutrophils with *E. coli* also increased uptake of FITC-labelled IAV by the cells (Table 3). In contrast, *S. pneumoniae* did not significantly increase uptake of IAV by neutrophils. The effect of *E. coli* could not be attributed to *E. coli*-derived LPS alone, as purified LPS did not increase IAV uptake (Table 3). A previous study demonstrated that GM-CSF increases neutrophil uptake of IAV [15]. However, GM-CSF did not cause a significantly greater increase in IAV uptake when added along with IAV and *E. coli* (Table 3).

Evidence for a role of respiratory burst activation in co-operative induction of apoptosis by IAV and E. coli

Previous studies have also demonstrated that the combination of IAV and *E. coli* triggers a greater respiratory burst response than either agent alone [3]. Hence, agents that inhibit or enhance the respiratory burst were tested for their ability to affect the survival of neutrophils treated with IAV or *E. coli*, or both. Pre-treatment with DPI, a cell-permeant inhibitor of NADPH oxidase activation, fully inhibited H_2O_2 responses and reduced intracellular calcium responses

Table 3. Effect of *E. coli*, *S. pneumoniae*, LPS and GM-CSF on neutrophil uptake of FITC-labelled IAV

Stimulus	Mean (SEM) IAV binding (%)
Control	100
<i>E. coli</i>	209 (8)*
<i>E. coli</i> + GM-CSF	251 (37)*
LPS	101 (7.5)
<i>S. pneumoniae</i>	116 (24)
<i>S. pneumoniae</i> + GM-CSF	124 (13)

Results represent mean $\pm$ SEM ($n = 3$) of neutrophil fluorescence after treatment with FITC-labelled IAV and are expressed as percentage of IAV binding (as compared with binding of FITC-labelled IAV to untreated neutrophils).

* $p < 0.05$ compared with binding of FITC-labelled IAV alone.

of neutrophils treated with IAV and *E. coli* (Table 4). DPI significantly reduced the development of hypodiploid neutrophils in cultures treated with *E. coli* alone or the combination of IAV and *E. coli* (Fig. 2) such that these cultures were identical to controls. DPI did not

Fig. 2. The effect of the NADPH oxidase inhibitor DPI on viability of neutrophils treated with *E. coli* alone (a) or the combination of IAV and *E. coli* (b). Neutrophils (5×10^6 /ml) were incubated with IAV or *E. coli*, or both, in the absence or presence of DPI ($10 \mu\text{mol/L}$). DPI was added 10 min before IAV or *E. coli*. The percentage of cells displaying hypodiploid DNA was measured by flow cytometry. Δ , *E. coli* alone; $\blacktriangle$, *E. coli* + DPI; $\square$, control medium; $\blacklozenge$, *E. coli* + IAV + DPI; $\diamond$, *E. coli* + IAV. Results represent mean (SEM) of four separate experiments. (*) indicates instances in which DPI significantly improved the viability of neutrophils ($p < 0.05$).

significantly alter the viability of control neutrophils or neutrophils treated with IAV alone (data not shown).

Although catalase blocks detectable H_2O_2 in the extracellular media of neutrophils stimulated with IAV, it does not inhibit chemiluminescent responses to the virus [16]. Thus, the IAV-induced respiratory burst response largely originates at an intracellular location and is not inhibited by catalase because catalase is not cell permeant. Catalase also did not inhibit the generation of hypodiploid neutrophils in cultures treated with IAV and *E. coli*. The mean percentages of hypodiploid neutrophils after 18 and 28 h were 32 (SEM 14) and 55 (SEM 18), respectively, in the absence of catalase and 30 (SEM 11) and 63 (SEM 15), respectively, in the presence of catalase ($n = 4$).

Azide, a cell-permeant inhibitor of catalase and myeloperoxidase which enhances detectable H_2O_2 release from IAV-treated neutrophils [16], also increased H_2O_2 release after stimulation with *E. coli* or the combination of IAV and *E. coli*. The response to *E. coli* or the combination of IAV and *E. coli* was increased by 67 (SEM 14)% and 50 (SEM 7)%, respectively ($n = 4$; $p < 0.005$ for each). Azide also significantly increased the percentage of hypodiploid cells in samples treated with IAV and *E. coli* (Fig. 3) but not in samples maintained in control buffer alone (Fig. 3).

Discussion

In previous studies, incubation with IAV or *E. coli* alone caused an acceleration of neutrophil apoptosis [3, 7], and this was increased on incubation with both IAV and *E. coli*. Also, initial studies at this laboratory demonstrated that either IAV or *E. coli* increased Fas antigen expression on neutrophils and IAV caused significantly increased Fas ligand expression on neutrophils [3]. However, the combination of IAV and *E. coli* did not clearly increase Fas antigen or Fas ligand expression to a greater extent than either pathogen alone.

In searching for the mechanisms of the co-operative adverse effect of IAV and *E. coli* on neutrophil

Table 4. Effect of DPI on neutrophil H_2O_2 production or intracellular calcium rise in response to IAV or the combination of IAV and *E. coli*

Stimulus	Mean (SEM) H_2O_2 production		Mean (SEM) calcium response	
	DPI–	DPI+	DPI–	DPI+
IAV	1.0 (0.3)	0	76.5 (9)	60 (7)
IAV + <i>E. coli</i>	2 (0.4)	0.04 (0.04)	91 (17)	46 (12)
fMLP	2 (0.4)	0.2 (0.04)	...	...

After incubation with or without DPI ($10 \mu\text{M/L}$) neutrophils were stimulated with IAV (Phil82H3N2) or *E. coli* (K12), or both, or fMLP (10^{-7} M). Results are mean (SEM) maximal rate of H_2O_2 production in $\text{nmol/min}/4 \times 10^6$ cells ($n = 3$) and maximal rise in intracellular calcium as measured by Fura-2AM ($n = 3$). DPI significantly reduced H_2O_2 and calcium responses to all stimuli tested ($p < 0.05$).

Fig. 3. Effect of sodium azide, a blocker of intracellular catalase and peroxidases, on IAV- and *E. coli*-induced generation of hypodiploid neutrophils. Neutrophils (5×10^6 /ml) were incubated with IAV and *E. coli* in the absence ($\square$) or presence ($\blacksquare$) of sodium azide (1 mM) for indicated times. The percentage of cells displaying hypodiploid DNA was measured by flow cytometry. Results represent mean (SEM) of three separate experiments. (#) indicates where addition of sodium azide significantly increased generation of hypodiploid neutrophils ($p < 0.05$).

survival, this study demonstrated that the combination of these micro-organisms increased annexin-V expression significantly more than either IAV or *E. coli* alone after incubation for 5 h. These results indicate that IAV and *E. coli* modify neutrophil membrane phosphatidylserine expression relatively rapidly *in vitro* and support the concept that these micro-organisms co-operatively induce neutrophil apoptosis. IAV or *E. coli* also increased caspase-3 activation in neutrophils after 5 h. Therefore, increased annexin-V binding and caspase-3 activation preceded the marked decline in neutrophil viability observed in cultures treated with IAV and *E. coli* (Fig. 1b). However, the caspase-3 assay (like the Fas ligand and Fas antigen assays noted above) did not show co-operative effects of IAV and *E. coli*. Hence, mechanisms other than Fas signalling or caspase-3 activation appear to be involved in the co-operative induction of apoptosis by IAV and *E. coli*.

The study next attempted to modulate survival of neutrophils treated with IAV and *E. coli* with the anti-apoptotic agent GM-CSF, but it caused a further reduction in survival of IAV- and *E. coli*-treated neutrophils with greater evidence of cell necrosis. Although the mechanism of this effect of GM-CSF is unclear it may relate to its ability to increase neutrophil respiratory burst responses to IAV [15] (see below).

The present study evaluated whether the effects of *E. coli* on neutrophil uptake of IAV may contribute to co-operative reduction in neutrophil survival caused by IAV and *E. coli*. A previous study found that IAV increases neutrophil uptake of and respiratory burst responses to *E. coli* [3] and, as shown in Table 3, there

is also a reciprocal effect through which *E. coli* increases neutrophil uptake of IAV. Also, it has been shown that the respiratory burst response triggered by IAV occurs mostly at an intracellular location [16] and recent studies have indicated that neutrophil respiratory burst responses (especially those occurring intracellularly [17]) are an important trigger for neutrophil apoptosis. Therefore, it was speculated that the ability of IAV and *E. coli* to reciprocally increase their uptake or co-operative enhancement of respiratory burst responses could account for co-operative increases in apoptosis.

The use of several biochemical modulators provided strong evidence for a critical role of intracellular respiratory burst products in mediating the reduction in neutrophil survival caused by the combination of IAV and *E. coli*. The cell-permeant respiratory burst inhibitor DPI significantly improved the viability of neutrophils treated with *E. coli* or the combination of IAV and *E. coli*, without affecting the viability of neutrophils maintained in control buffer. Furthermore, azide, an inhibitor of catalase and peroxidases which is also cell permeant and potentiates H_2O_2 production in response to these stimuli, markedly potentiated IAV- and *E. coli*-mediated reduction in neutrophil survival (again without altering survival of control neutrophils). Finally catalase, which catalyses the breakdown of H_2O_2 but which is not able to permeate the cell membrane, did not alter the effects of IAV and *E. coli* on neutrophils. These results indicate that the ability of *E. coli* alone or in combination with IAV to reduce neutrophil survival *in vitro* involves generation of respiratory burst products within the neutrophil.

Furthermore, as azide inhibits myeloperoxidase, the effect does not involve products formed by myeloperoxidase (e.g., HOCl⁻).

It remains to be determined whether the ability of IAV and bacteria to co-operatively reduce neutrophil survival *in vitro* is relevant to the clinical observation that IAV predisposes to bacterial superinfections. Although *E. coli* is not an organism that is commonly isolated from IAV-infected patients who develop bacterial pneumonia, the more relevant *S. pneumoniae* also markedly reduced neutrophil survival when added along with IAV *in vitro* through a respiratory burst-related mechanism [6]. It has been demonstrated in murine models that measures which reduce the production of oxidants reduce the severity and mortality of influenza pneumonia [18]. Investigations should be done to determine whether similar findings would be obtained in models of co-infection with IAV and bacteria.

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References

1. Hartshorn KL. Etiology of bacterial superinfections complicating influenza viral infection. In: Brown LE, Hampson AW, Webster RG (eds) Options for the control of influenza III. Amsterdam, Elsevier Science. 1996: 499–508.
2. Abramson JS, Mills EL. Depression of neutrophil function induced by viruses and its role in secondary microbial infections. *Rev Infect Dis* 1988; **10**: 326–341.
3. Colamussi ML, White MR, Crouch E, Hartshorn KL. Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria. *Blood* 1999; **93**: 2395–2403.
4. Mori I, Komatsu T, Takeuchi K, Nakakuki K, Sudo M, Kimura Y. In vivo induction of apoptosis by influenza virus. *J Gen Virol* 1995; **76**: 2869–2873.
5. Morris SJ, Price GE, Barnett JM, Hiscox SA, Smith H, Sweet C. Role of neuraminidase in influenza virus-induced apoptosis. *J Gen Virol* 1999; **80**: 137–146.
6. Englich G, White M, Hartshorn KL. Neutrophil survival is markedly reduced by incubation with influenza virus and *Streptococcus pneumoniae*: role of respiratory burst. *J Leukoc Biol* 2001; **69**: 50–56.
7. Watson RWG, Redmond HP, Wang JH, Condron C, Bouchier-Hayes D. Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. *J Immunol* 1996; **156**: 3986–3992.
8. Gamberale R, Giordano M, Trevani AS, Andogenui G, Geffner JR. Modulation of human neutrophil apoptosis by immune complexes. *J Immunol* 1998; **161**: 3666–3674.
9. Fadeel B, Ahlin A, Henter J-I, Orrenius S, Hampton MB. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood* 1998; **92**: 4808–4818.
10. Hartshorn KL, Collamer M, Auerbach M, Myers JB, Pavlotsky N, Tauber AI. Effects of influenza A virus on human neutrophil calcium metabolism. *J Immunol* 1988; **141**: 1295–1301.
11. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; **139**: 271–279.
12. Hartshorn KL, Collamer M, White MR, Schwartz JH, Tauber AI. Characterization of influenza A virus activation of the human neutrophil. *Blood* 1990; **75**: 218–226.
13. Hartshorn KL, Crouch E, White MR *et al.* Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria. *Am J Physiol* 1998; **274**: L958–L969.
14. Dranoff G, Crawford AD, Sadelain M *et al.* Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science* 1994; **264**: 713–716.
15. Little R, White MR, Hartshorn KL. Interferon-alpha enhances neutrophil respiratory burst responses to stimulation with influenza A virus and FMLP. *J Infect Dis* 1994; **170**: 802–810.
16. Kazhdan M, White MR, Tauber AI, Hartshorn KL. Human neutrophil respiratory burst response to influenza A virus occurs at an intracellular location. *J Leukoc Biol* 1994; **56**: 59–64.
17. Lundqvist-Gustafsson H, Bengtsson T. Activation of the granule pool of the NADPH oxidase accelerates apoptosis in human neutrophils. *J Leukoc Biol* 1999; **65**: 196–204.
18. Akaïke T, Noguchi Y, Ijiri S *et al.* Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci USA* 1996; **93**: 2448–2453.