Influenza virus enhancement of membrane leakiness induced by staphylococcal $\alpha$ toxin, diphtheria toxin and streptolysin S

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Release of $\alpha$-amino[14C]isobutyric acid from ferret Mpf cells was promoted by staphylococcal $\alpha$ toxin, diphtheria toxin and streptolysin S. This release was enhanced to a significant extent if the cells had been previously infected with influenza virus strain A/Puerto Rico/8/34 (PR8, H1N1), although infection with virus alone did not increase the release of radiolabel as compared with that from untreated cells; inactivated virus had a similar enhancing action. The mechanism of enhancement is unclear but it occurs between 0-5 and 2 h post-inoculation and viral membrane/endosome membrane fusion is essential. Endotoxin had no effect on membrane permeability, either alone or with PR8. The relevance of these in vitro observations to the previously observed enhancement of toxin lethality by influenza virus in vivo is discussed.

Introduction

In an investigation of the possible role of enhancement of bacterial toxicity by influenza virus in the sudden infant death syndrome, the lethality of staphylococcal $\alpha$ and $\gamma$ toxins, endotoxin and diphtheria toxin for 5 day old ferrets was shown to be enhanced by infecting the neonates at 1 day old with influenza virus (A/Puerto Rico/8/34) (PR8) (Jakeman et al., 1991). The mechanisms involved in virus enhancement of toxin lethality are probably manifold and different for each toxin. However, because two of the four toxins (staphylococcal $\alpha$ and $\gamma$ toxins) showing enhanced lethality in PR8-infected neonates are known to be cytolytic, acting primarily on the cytoplasmic membrane, and because influenza virus also enhances the permeability of cells (Patel & Pasternak, 1985), we have investigated whether influenza virus can enhance toxin-induced leakiness of membranes. The toxins examined were staphylococcal $\alpha$ toxin (cytolytic and toxic for ferret neonates), streptolysin S (cytolytic and non-toxic for ferret neonates), endotoxin (non-cytolytic and toxic for ferret neonates), and diphtheria toxin (non-cytolytic and toxic for ferret neonates) (Jakeman et al., 1991). Effects on cell membranes were quantified by measuring the release of radioactively labelled $\alpha$-aminoisobutyric acid (AIB) (Thelestam & Molby, 1975). This non-metabolizable amino acid accumulates in the cytoplasm and is released spontaneously at low levels only; it has been used in preference to labelled nucleotides and $K^+$ and $Rb^+$ ions as a more sensitive and readily quantifiable measure of membrane permeability changes induced by toxins (Thelestam & Molby, 1975).

Methods

Cells. Ferret Mpf cells, kindly supplied by Dr R. S. Trowbridge, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A., were derived from the brain of a 6 week old ferret. They were cultured in Eagle’s minimal essential medium containing 14% heat-inactivated lamb serum, 0.11% sodium bicarbonate, 2 mM-L-glutamine, 100 units (U) penicillin/ml and 100 $\mu$g streptomycin/ml (growth medium) (Trowbridge et al., 1982). Madin Darby canine kidney (MDCK) cells were grown in the same growth medium as Mpf cells except that the heat-inactivated lamb serum was replaced by 10% heat-inactivated newborn bovine serum.

Viruses and their assay. PR8 was prepared as described previously (Matsuyama et al., 1980) and assayed by the egg or egg-bit technique (Matsuyama et al., 1980); titres are expressed as 50% egg infectious doses or 50% egg-bit infectious doses (EBID 50) respectively. Inactivated virus stocks were u.v.-irradiated as described previously (Coates et al., 1986) and shown to lack infectivity after double passage in eggs.

Bacterial toxins. Escherichia coli endotoxin (product number L2637) and streptolysin S (product number S2888) were purchased from Sigma. Endotoxin was prepared from E. coli strain 055. B5 and purified by phenol extraction and chromatography, and contained <1% protein and <1% RNA (Westphal & Jann, 1965); streptolysin S was prepared from Streptococcus pyogenes and had an activity of between 2000 and 10000 haemolytic units (HU)/mg protein (Ginsburg, 1970). Staphylococcal $\alpha$ toxin was kindly supplied by Dr H. Birkbeck, University of Glasgow, U.K., and was prepared from strain Wood 46 of Staphylococcus aureus; it was purified by chromatography on controlled-pore glass followed by ion-exchange chromatography (Freer & Arbuthnott, 1986).
Diphtheria toxin, kindly supplied by Dr A. J. Sheppard, Wellcome Research Laboratories, Beckenham, U.K., was purified by two cycles of ammonium sulphate precipitation. We have not ascertained the purity of these preparations other than that those obtained from colleagues were free of likely contaminating toxins, in particular, all toxin preparations (except endotoxin) lethal for ferrets were free of contaminating endotoxin (≤64 ng endotoxin/1 HU of staphylococcal α toxin; <0.04 ng endotoxin/ng diphtheria toxin) when tested by the sensitive amoebocyte lysate test (Jakeman et al., 1991). For the purposes of the present work it was not necessary to utilize completely characterized toxin preparations.

Haemolysis assay of haemolytic toxins. This was performed for staphylococcal α toxin and streptolysin S as described by Bernheimer (1988). Results are expressed in HU.

Toxin and virus treatment of cells: assay of toxin-induced cell leakage. Leakage was measured essentially as described by Thelestam & Mollby (1975). A uniform suspension of freshly trypsinized cells containing 1×10⁵ Mpf cells/ml (or 5×10⁴ cells/ml for MDCK cells) of culture medium was prepared and dispensed in 1 ml amounts into 6×4 multiwell tissue culture plates. The plates were incubated at 37 °C in a humid atmosphere containing 5% CO₂ for 24 h or until the cell monolayer became confluent. Cells were then washed three times with Hanks’ balanced salt solution (HBSS, pH 7.4) at 22 °C and 0.3 ml of an AIB solution was added to each well. The AIB solution contained 1 μCi/ml [¹⁴C]AIB (NEN Dupont) in HBSS with 10 mM-TEA buffered to pH 7.4 with 1 M-NaOH; it was preincubated at 37 °C for at least 30 min before addition to the cells. The cultures were then incubated for 60 min at 37 °C for uptake of the labelled AIB by the cells’ sodium-coupled active transport system. The AIB solution was removed, replaced with 1 ml of growth medium pH 7.4 and the cultures were incubated for a further 30 min at 37 °C in a 5% CO₂ atmosphere to remove cell surface-associated label and to stabilize intracellular label. Cells were rinsed rapidly three times with HBSS at 22 °C and 1 ml toxin, diluted in TBS (0.1 M-Tris, 0.85% NaCl pH 7.2), was added to each well and the cells were incubated for 30 min at 37 °C. Culture supernatants were then transferred to 1.5 ml Eppendorf tubes and centrifuged at 10,000 g for 5 min to remove cells and debris. Supernatants (0.1 ml) were transferred to 10 ml scintillation fluid (Aquasol-2; NEN Dupont) and the radioactivity was quantified in a Beckman LS7500 liquid scintillation counter.

The maximal release of cytoplasmic radioactivity was determined by incubation of cells in 1 ml of 0.06 M-sodium borate buffer pH 7.8 for 60 min at 37 °C, followed by cell disruption with a rubber policeman. Cell lysates were centrifuged at 10,000 g for 5 min to remove cell debris and the radioactivity was measured as above. The spontaneous release of labelled AIB under standard test conditions, i.e. incubation at 37 °C for 30 min in TBS, varied between 13 and 20%. These values are included for all sets of data in each table and values for toxin-induced release of AIB are expressed as a percentage of the maximal release.

In the standard procedure for cells pretreated with virus prior to the addition of toxin, 0.01 ml of HBSS containing 1×10⁵ EBID₅₀ PR₈ was added to the cells 30 min into the labelling period; the virus inoculum and label were then removed 30 min after addition of virus, i.e. 60 min after addition of label. Cells were then incubated for 30 min without label before toxin was added as described above. In the experiment in which virus was added at various times from between 0 and 4 h prior to the addition of toxin, this procedure was modified slightly. For times up to 1.5 h prior to the addition of toxin, virus was added according to the standard procedure but at the same time as the label (1.5 h), 15 min after label (1.25 h), 30 min after label (1 h) or at the same time as the toxin (0 h). For the 1.75 h time point, virus was added 15 min prior to addition of label. For times of 2 h or greater, 1 ml of growth medium containing 1×10⁵ EBID₅₀ virus was added to the cells and after 30 min the inoculum was removed and replaced with growth medium. Label and toxin were then added at appropriate times as in the standard procedure.

NH₄Cl acidification of the cytosol to prevent vesicle formation during coated-pit endocytosis. The process described in the previous section was modified as follows. The AIB was added to the monolayers in HBSS with 10 mM-TEA buffered to pH 7.4 and containing 20 mM-NH₄Cl. Cultures were then incubated for 60 min at 37 °C for uptake of AIB and for acidification of cellular contents. The AIB solution containing NH₄Cl was then removed and replaced with 1 ml of Na⁺-free phosphate-buffered KCl pH 7.2; cultures were incubated for 30 min at 37 °C. Cells were then rinsed rapidly three times with Na⁺-free phosphate-buffered KCl and toxin, either alone or together with virus, was added in the Na⁺-free buffer. The remainder of the assay was as described above. The low intracellular pH induced by the pulse of NH₄Cl is maintained for the period of the test because Na⁺/H⁺ exchange is inhibited or blocked in Na⁺-free medium (Sandvig et al., 1987).

Prevention of endosomal fusion with coated vesicles. This assay was performed as in the standard assay except that 20 mM-NH₄Cl was present in all the media throughout and was not used in a pulse process. Under these conditions NH₄Cl enters the cells rapidly and associates with H⁺ to generate NH₃ leading to rapid cellular alkalinization of all low pH intracellular compartments and inhibiting endosomal acidification (Frelin et al., 1988).

Results

Standardization of test conditions

Preliminary experiments established the optimal amount of influenza virus PR₈ for the test. A series of twofold concentrations from 10⁵ to 10⁷ EBID₅₀/ml established that 1×10⁶ EBID₅₀/ml produced 100% haemadsorption in 2×10⁵ cells 24 h after inoculation of virus, indicating that this dose infected all cells in the monolayer; this inoculum was used in all subsequent experiments. The optimum time for addition of toxin after inoculation with PR₈ was established using staphylococcal α toxin. Addition of toxin at the same time as PR₈ (time 0 h) or from 1 to 2 h after PR₈ inoculation showed enhanced release of AIB compared to the addition of toxin alone (Fig. 1). Later addition of toxin decreased the enhancement and, if it was delayed until between 3 and 4 h post-infection, there was a partial suppression of toxin-mediated release of AIB. In subsequent experiments, staphylococcal α toxin was added 1 h post-infection with influenza virus and, for comparative purposes, all other toxins were added similarly.

Enhancement of toxin-mediated membrane permeability of Mpf cells by influenza virus

The effect of influenza virus on staphylococcal α toxin-mediated membrane permeability of Mpf cells is shown in Table 1 (control column). Spontaneous release of AIB
Staphylococcal α toxin alone produced dose-dependent AIB release, increasing from 43 to 57% maximum release over a four-fold increase in toxin concentration. Although PR8 alone did not affect membrane permeability, there was a statistically significant (P < 0.05) enhancement of toxin-induced AIB release; approximately 1.4-fold compared to toxin alone (Table 1). A similar enhancement of toxin-mediated membrane permeability was evident for streptolysin S (Table 1; control column). Again, the effect of the toxin alone was dose-dependent and PR8 only produced significant enhancement of release for doses above 100 HU, by 1.7- to 2.3-fold in a dose-dependent manner. Diphtheria toxin also caused release of AIB in large doses (1-0 ng) (Table 1; control column) and, again, PR8 significantly (P < 0.02) enhanced the release of AIB by 1.6- to 1.8-fold in a dose-dependent manner (Table 1). Endotoxin did not show enhanced release of AIB at any dose from 0.0001 to 100 μg relative to untreated cells, nor was any effect observed with PR8 infection (data not shown).

Table 1. The effect of cytosol acidification (NH₄Cl pulse) and inhibition of viral/endosome membrane fusion using NH₄Cl on the enhancement of staphylococcal α toxin-, streptolysin S- and diphtheria toxin-mediated release of [¹⁴C]AIB by PR8 from Mpf cells

<table>
<thead>
<tr>
<th>Toxin dose</th>
<th>Control†</th>
<th>NH₄Cl pulse‡</th>
<th>Continuous NH₄Cl treatment§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No PR8</td>
<td>PR8</td>
<td>No PR8</td>
</tr>
<tr>
<td>Staphylococcal α (HU)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.3 (0.6)</td>
<td>14.7 (0.1)</td>
<td>13.7 (0.4)</td>
</tr>
<tr>
<td>0.5</td>
<td>43.0 (0.9)</td>
<td>59.1 (2.2)</td>
<td>48.4 (1.6)</td>
</tr>
<tr>
<td>1.0</td>
<td>49.5 (1.3)</td>
<td>69.2 (1.7)</td>
<td>52.2 (1.2)</td>
</tr>
<tr>
<td>2.0</td>
<td>56.9 (2.7)</td>
<td>79.2 (1.9)</td>
<td>57.4 (0.8)</td>
</tr>
<tr>
<td>Streptolysin S (HU)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>12.8 (0.7)</td>
<td>14.5 (0.4)</td>
<td>13.7 (0.8)</td>
</tr>
<tr>
<td>10</td>
<td>13.0 (1.6)</td>
<td>22.3 (2.8)</td>
<td>13.1 (0.4)</td>
</tr>
<tr>
<td>100</td>
<td>16.9 (1.7)</td>
<td>39.5 (1.2)</td>
<td>15.9 (1.1)</td>
</tr>
<tr>
<td>1000</td>
<td>29.3 (1.4)</td>
<td>52.8 (2.8)</td>
<td>29.2 (1.3)</td>
</tr>
<tr>
<td>Diphtheria toxin (ng)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.5 (0.9)</td>
<td>15.0 (0.5)</td>
<td>21.1 (0.7)</td>
</tr>
<tr>
<td>0.1</td>
<td>16.1 (1.4)</td>
<td>28.2 (0.4)</td>
<td>20.3 (0.4)</td>
</tr>
<tr>
<td>1.0</td>
<td>34.0 (1.6)</td>
<td>53.1 (1.5)</td>
<td>20.7 (0.3)</td>
</tr>
</tbody>
</table>

* Results are the mean of two experiments of four replicate tests each. Statistical analysis (analysis of variance) showed that the two experiments were not significantly different.
† No NH₄Cl treatment.
‡ Cells were given a 60 min NH₄Cl pulse to prevent vesicle formation during coated-pit endocytosis (see Methods).
§ Cells were incubated continuously with 20 mM-NH₄Cl to prevent endosomal fusion with coated vesicles (see Methods).
¶ PR8 given 1 h prior to toxin.
¶¶ ND, Not determined.
Table 2. The effect of infectious PR8 on staphylococcal α toxin-mediated release of AIB from MDCK cells

<table>
<thead>
<tr>
<th>Toxin dose (HU)</th>
<th>No PR8</th>
<th>PR8†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.4 (0.2)</td>
<td>20.4 (0.2)</td>
</tr>
<tr>
<td>0.5</td>
<td>26.9 (0.7)</td>
<td>33.2 (0.9)</td>
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<tr>
<td>1.0</td>
<td>33.2 (0.8)</td>
<td>43.8 (0.9)</td>
</tr>
<tr>
<td>2.0</td>
<td>38.2 (0.2)</td>
<td>54.3 (0.9)</td>
</tr>
</tbody>
</table>

* Results are the mean of two separate experiments each with four replicate tests. Statistical analysis (analysis of variance) showed that the two experiments were not significantly different.
† PR8 given 1 h prior to toxin.

Table 3. Effect of u.v.-inactivated PR8 on staphylococcal α toxin-mediated release of AIB from Mpf cells

<table>
<thead>
<tr>
<th>Toxin dose (HU)</th>
<th>No PR8</th>
<th>PR8†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.8 (0.4)</td>
<td>14.1 (0.7)</td>
</tr>
<tr>
<td>0.5</td>
<td>35.6 (0.6)</td>
<td>58.3 (0.8)</td>
</tr>
<tr>
<td>1.0</td>
<td>43.9 (1.2)</td>
<td>68.2 (1.6)</td>
</tr>
<tr>
<td>2.0</td>
<td>51.2 (2.5)</td>
<td>79.0 (1.4)</td>
</tr>
</tbody>
</table>

* Results are the mean of two separate experiments each with four replicate tests. Statistical analysis (analysis of variance) showed that the two experiments were not significantly different.
† PR8 given 1 h prior to toxin.

Enhancement of staphylococcal α toxin-mediated membrane permeability of MDCK cells by influenza virus

To demonstrate that the effect was not confined to ferret brain-derived Mpf cells, the experiments were repeated for staphylococcal α toxin with the epithelial-like MDCK cells (Table 2). While the maximum percentage release was less than that for Mpf cells, the response to toxin was dose-dependent and prior PR8 infection significantly (P < 0.05) enhanced AIB release by 1.2- to 1.5-fold.

Investigation of the stage of cell interaction at which virus enhances toxin-mediated AIB release

Viral enhancement of toxin-mediated permeability occurs very rapidly (Fig. 1); PR8 enhanced AIB release when virus and toxin were given together to the cells so the effect must have occurred within 0.5 h, when supernatants were removed for measurement of the AIB released into the medium. The effect is also short-lived and is reversed if toxin is given 3 to 4 h post-infection (Fig. 1). Indeed, viral replication is not necessary for the enhancement because u.v.-inactivated virus significantly enhanced staphylococcal α toxin-mediated AIB release by approximately 1.5-fold (Table 3).

Cytosol acidification with a pulse of NH₄Cl prevents vesicle formation from coated pits (Sandvig et al., 1987) and interferes with receptor-mediated uptake of influenza virus. It completely prevented PR8 enhancement of toxin-mediated membrane permeability but did not affect the activity of the toxin itself in the case of staphylococcal α toxin and streptolysin S (Table 1; NH₄Cl pulse column). Not surprisingly, the inherent activity of diphtheria toxin was blocked; it is known to enter cells by receptor-mediated endocytosis (Table 1). The effect of incubating cells with NH₄Cl throughout the experiment was then investigated. The inherent effect of staphylococcal α toxin on AIB release was aborted but not that of streptolysin S. However, the lack of endosomal acidification completely abrogated PR8 enhancement of streptolysin S-mediated membrane permeability (Table 1; continuous NH₄Cl treatment column).

Discussion

Clearly influenza virus enhances cell membrane leakiness in vitro induced by staphylococcal α toxin, streptolysin S and diphtheria toxin (Table 1), all membrane-active toxins, but has no effect on endotoxin's lack of membrane activity. Two questions arise from this. Firstly, how do influenza virus and toxin cooperate to enhance membrane permeability, and secondly, does the enhanced membrane permeability have any relevance to the enhanced lethality of some of these toxins in influenza virus-infected newborn ferrets?

The process of enhancement is rapid, occurring within 30 min of virus infection, and short-lived, being maximal 1 to 2 h after virus infection. Productive viral replication is not required. A stage in the virus infection cycle after attachment is important because interruption of coated-pit endocytosis by prevention of vesicle formation blocked the enhanced membrane leakiness. It appears that, at least in the case of streptolysin S, fusion of the viral membrane with the endosomal membrane at low pH is essential because alkalinization of the endosome prevented virus enhancement of release. How the process of receptor-mediated uptake of influenza virus affects staphylococcal α toxin or streptolysin S is unclear because the toxins act via the plasma membrane by inducing pore formation (Stephen & Pietrowski, 1981). One possibility is that following uptake of virus and viral/endosome membrane fusion, viral membrane may...
be recycled back to the plasma membrane where it enhances toxin-induced membrane permeability. This is possible as some ligands taken up by receptor-mediated endocytosis have been shown to recycle back to the plasma membrane in as little as 8 min (Wileman et al., 1985). Also, influenza virus can fuse with the plasma membrane at pH 5-3 (Patel & Pasternak, 1985) and, when it does, it has been shown to enhance membrane leakiness (Patel & Pasternak, 1985). However, if this were the case it would be expected that influenza virus alone might induce membrane leakiness in the present experiments and this clearly did not occur (Tables 1 to 3).

Unexpectedly, diphtheria toxin induced membrane leakiness despite its uptake being receptor-mediated and pH-dependent (Parker et al., 1990), and its principal effect being the inhibition of protein synthesis by ADP-ribosylation of elongation factor 2 (Stephen & Pietrowski, 1981). Nevertheless, it does induce pore formation in artificial membranes (Parker et al., 1990). B fragment insertion into the endosomal membrane allows access of toxin A fragment to the cytosol and so produces the pores. Endosomal membrane incorporating B fragment could be recycled to the cell surface to affect cellular permeability. This may be manifest in the present experiments because of the very large dose (1 ng) used. Another unexpected finding was that continuous treatment with NH₄Cl to neutralize endosome acidity abolished staphylococcal α toxin activity despite the fact that this toxin acts directly on cell membranes. However, haemolysis induced by this toxin has been reported to require low pH (Parker et al., 1990).

The relevance of the enhancement of cell leakiness to the exacerbation of lethality of these toxins in influenza virus-infected newborn ferrets is an interesting question. Clearly, enhancement of cell leakiness cannot play a part in enhancement of endotoxin lethality for the neonates because this toxin does not cause changes in membrane permeability. It is also irrelevant to the situation for streptolysin S, which was not lethal for the neonates either alone or together with influenza virus (Jakeman et al., 1991). The mechanisms may, however, be relevant to virus exacerbation of the lethality of staphylococcal α and diphtheria toxins in vivo. Influenza virus may allow increased or more rapid uptake of the toxins, resulting in cell death by the same mechanism but at lower external concentrations. This seems unlikely because the available evidence suggests that receptor-mediated endocytosis is constitutive and thus would not be enhanced by virus infection. Also, it is unlikely that toxin is binding to virus and passively being taken up by the cells when virus enters because enhancement of toxin activity by influenza virus is maximal when cells have been infected 1 to 2 h previously. A more likely reason for the increase in lethality is that cellular permeability leads to the release of physiologically active materials from cells. In this context it is interesting that newborn ferrets treated with staphylococcal α toxin and influenza virus showed inflammation in the upper respiratory tract, lung oedema and collapse, and early bronchopneumonia, whereas animals treated with toxin or virus alone did not (Jakeman et al., 1991). This inflammatory response may have resulted from production and increased release of histamine or other inflammatory mediators such as interleukin-1, tumour necrosis factor and platelet activating factor as a result of the increased membrane permeability.

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References


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