Proteolytic Activation of Influenza WSN Virus in Cultured Cells is Performed by Homologous Plasma Enzymes

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SUMMARY

The effect of chick embryo allantoic fluid, porcine plasma or canine plasma on virus progeny was studied in cultured chicken, porcine and canine cells infected with influenza WSN virus. Cells incubated either without plasma or with heterologous plasma produced virions which had uncleaved haemagglutinin and low infectivity. Cells incubated with homologous plasma produced highly infectious virions with cleaved haemagglutinin. Little increase of progeny virus infectivity was observed in canine cel–porcine plasma and porcine cel–canine plasma host systems. The addition of protease inhibitors to culture containing homologous plasma, in particular e-aminon-caproic acid (an inhibitor of plasminogen activation), suppressed cleavage of haemagglutinin, and virions which had uncleaved haemagglutinin and low infectivity were produced by the cells. It therefore follows that haemagglutinin cleavage and activation of influenza WSN virus infectivity in cultured cells is most efficiently performed by homologous plasma proteolytic enzyme(s). The mechanism of selective plasma-mediated influenza virus proteolytic activation in homologous cells is discussed.

The major influenza virus glycopolypeptide, the haemagglutinin, is located outside the virion lipid bilayer as 'spikes'. The haemagglutinin spike consists either of the precursor polypeptide (HA) with a mol. wt. of 75000 or of the disulphide-linked fragments HA1 and HA2, with mol. wt. 50000 and 25000 respectively (Laver, 1971; Skehel & Waterfield, 1975). Proteolytic cleavage of HA to HA1 + HA2 is essential for the biological behaviour of the virus since it is a prerequisite for full infectivity of virions (Klenk et al., 1975; Lazarowitz & Choppin, 1975).

Cleavage of the haemagglutinin in different host–virus systems depends on virus strain as well as host cell type. There are strain-specific variations in the susceptibility of HA to cleavage (Klenk et al., 1972, 1975, 1977). These differences correlate with the pathogenicity of the virus strains: the highly pathogenic strains possess HA more susceptible to host proteases (Bosch et al., 1979, 1981; Nakajima & Sugiura, 1980; Rott et al., 1980; Vallbracht et al., 1980). The cellular proteases are involved in the process of HA cleavage, and, depending on the presence of appropriate enzymes in a given cell, virions with cleaved or with uncleared haemagglutinin may be formed (Klenk et al., 1975, 1977; Lazarowitz et al., 1973a, b; Stanley et al., 1973). The mechanisms which control selective cleavage of virus HA in different host cells are as yet unclear.

Lazarowitz et al. (1973a, b) have shown that the cleavage of influenza WSN virus HA in cultured cells can be induced by the addition of serum to the culture medium. The cleavage in this host system was performed by serum plasminogen converted to the active enzyme, plasmin, by cellular activators (Lazarowitz et al., 1973a). In the present paper, we have shown that serum-induced cleavage of influenza virus haemagglutinin can be most efficiently performed by enzymes of serum homologous to the host cell used.

The effect of chick embryo allantoic fluid (CAF), porcine plasma or canine plasma on the infectivity of progeny virions in chicken embryo fibroblast (CEF), canine (MDCK) and porcine (SPEV) cells infected with influenza WSN virus was studied. Since CAF contained plasminogen at concentrations 5 to 10 times lower than in blood plasma (O. P. Zhirnov, A. V. Ovcharenko & A. G. Bukrinskaya, unpublished results) it was used as an equivalent to chicken blood plasma in higher concentrations. The incubation period used for the plasmas for these experiments was 12 to 13 h after infection, since a cytopathic effect was not detected until this time of infection and, therefore, any non-specific influence on progeny virus of enzymes released
Table 1. *Effect of heterologous or homologous plasma on progeny virus infectivity in influenza WSN virus-infected cultured cells and the effect of protease inhibitors*

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Additions to Medium 199†</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Modified</td>
<td>Standard</td>
<td>Modified</td>
<td>Standard</td>
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<tr>
<td>CEF</td>
<td>None</td>
<td>$2.2 \times 10^4$</td>
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<td>$6.8 \times 10^3$</td>
<td>$3.1 \times 10^6$</td>
</tr>
<tr>
<td>CEF</td>
<td>None (trypsin)§</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>CAF</td>
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<td>$5.1 \times 10^6$</td>
</tr>
<tr>
<td>CEF</td>
<td>Canine</td>
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<td>$1.7 \times 10^7$</td>
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<td>ND</td>
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<td>Porcine</td>
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<td>$1.3 \times 10^7$</td>
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<td>ND</td>
</tr>
<tr>
<td>CEF</td>
<td>CAF + ε-AcA</td>
<td>ND</td>
<td>ND</td>
<td>$4.1 \times 10^3$</td>
<td>$2.0 \times 10^6$</td>
</tr>
<tr>
<td>CEF</td>
<td>CAF + Contrical</td>
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<td>ND</td>
<td>$3.2 \times 10^3$</td>
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<td>ND</td>
<td>$7.7 \times 10^6$</td>
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<td>$1.0 \times 10^6$</td>
<td>$5.8 \times 10^6$</td>
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<td>None (trypsin)§</td>
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<td>ND</td>
<td>$1.9 \times 10^6$</td>
<td>$3.5 \times 10^6$</td>
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<tr>
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<td>$4.5 \times 10^7$</td>
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<tr>
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<td>ND</td>
</tr>
<tr>
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<td>Porcine</td>
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<td>$0.9 \times 10^6$</td>
<td>$2.5 \times 10^6$</td>
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<tr>
<td>SPEV</td>
<td>Porcine + ε-AcA</td>
<td>ND</td>
<td>ND</td>
<td>$3.2 \times 10^3$</td>
<td>$1.4 \times 10^6$</td>
</tr>
<tr>
<td>SPEV</td>
<td>Porcine + Contrical</td>
<td>ND</td>
<td>ND</td>
<td>$2.1 \times 10^3$</td>
<td>$2.5 \times 10^6$</td>
</tr>
</tbody>
</table>

* Primary cultures of CEF, MDCK and SPEV cells were grown as monolayers in Medium 199 supplemented with 10% bovine calf serum. Confluent monolayers were infected with chick embryo-propagated WSN virus at 1 to 10 p.f.u./cell. After 30 min adsorption the medium was replaced at hourly intervals during the first 4.5 h post-infection to remove unadsorbed virus. The cells were then radio-labelled with 14C-labelled algal hydrolysate (40 μCi/ml) for 1.5 h and afterwards incubated in Medium 199 containing either CAF (final concn. 15%), or canine plasma (final concn. 2%) or porcine plasma (final concn. 2%) for 7 h. Contrical or ε-AcA were added at final concentrations of 5 trypsin-inhibiting units/ml and 10 mg/ml respectively. Samples of culture medium were clarified at 10000 g for 15 rain and aliquots were taken for infectivity assay. For polypeptide analysis the virus was additionally purified (see Fig. 1).

† Whole blood (canine and porcine) was collected in the presence of 0.35% sodium citrate. CAF was used as an equivalent to chicken plasma. The samples were centrifuged at 25000 rev/min for 15 min and the supernatant (plasma) was used.

‡ The infectivity of the samples was determined by plaque assay on CEF. The modified plaque assay employed consisted of a double agar overlay, with trypsin being included in the second layer added 24 h after infection. In the unmodified standard method, trypsin was included in the agar from the beginning. The exact experimental details are as described by Zhirnov et al. (1982).

§ The culture medium from cells incubated with Medium 199 alone was treated in vitro with trypsin (5 μg per 128 haemagglutinating units for 30 min at 37°C) before plaque titration.

|| ND, Not done.

From damaged cells was excluded. The infectivity of progeny virus in different plasma-cell systems was determined by two different plaque assay methods: standard and modified (Zhirnov et al., 1982). The modified method allows the real infectivity of the virion population to be determined (i.e. infectivity of virions with a cleaved haemagglutinin), whereas the potential infectivity (i.e. that provided by the combined effect of virions with a cleaved haemagglutinin and virions with an uncleaved haemagglutinin) is measured by the standard method. It is evident that potential infectivity reflects the total virus yield in the virus-host cell system examined. As can be seen from Table 1 (expt. 1), in the cell-plasma systems analysed the potential infectivity and, consequently, the virus yield are approximately equal, while the real infectivity of the same samples differs greatly. The virus produced by the cells with homologous plasma had the highest infectivity, which was close to its potential level. In contrast, the real
infectivity of the virus produced by the cells either without plasma or with heterologous plasma was significantly lower than its potential infectivity. Less significant enhancement of the progeny virus infectivity was observed in the MDCK–porcine plasma and the SPEV–canine plasma systems.

The addition to homologous plasma of the protease inhibitors, ε-amino-n-caproic acid (ε-AcA), an inhibitor of plasminogen activation (Alkjaersig et al., 1959; Brockway & Castellino, 1971), and Contrical, an inhibitor of plasmin (VEB, Arzneimittelwerk, G.D.R.; a 6000 mol. wt. polypeptide prepared from bovine lungs and widely used in medical practice as an inhibitor of trypsin-like proteases; this compound is an analogue of Trasylo), to infected cells suppressed the plasma-induced activation of progeny virus (Table 1, expt. 2). Since the total virus yield in the cells incubated with and without inhibitors was approximately equal (Table 1, expt. 2, standard method) it appears that neither Contrical nor ε-AcA interfere with virus intracellular synthesis and have no direct virucidal effect on mature virions. Thus, the activation of progeny virus infectivity in cultured cells is induced by proteolytic enzyme(s) and is provided most efficiently by enzyme(s) of homologous plasma.

These observations suggest that activation of virus infectivity by plasma enzymes is most probably realized via the cleavage of virus HA. To test this hypothesis directly, we examined the protein patterns of the viruses produced in different plasma–cell systems. The analysis of polypeptides by SDS–polyacrylamide gel electrophoresis (Fig. 1a) showed that all three types of cells incubated without plasma produced virions with uncleaved haemagglutinin. This observation indicated the failure of HA cleavage by cellular proteases during at least a 13 h period of infection in the host cell types used. It is also seen (Fig. 1a) that HA electrophoretic mobility varies with the host cell types, most probably due to host-specific glycosylation–sulphation (Collins & Knight, 1978; Compans & Pinter, 1975; Nakamura & Compans, 1977, 1979; Schwarz et al., 1977). The host cells incubated with heterologous plasma (Fig. 1b, c) produced virions with uncleaved haemagglutinin. In some experiments minor amounts of cleaved haemagglutinin were revealed in the progeny virus produced in the SPEV–canine plasma and MDCK–porcine plasma systems. The cells with homologous plasma produced virions containing both HA and HA1 + HA2 (Fig. 1b, c). In this case, the amount of HA1 + HA2, as estimated by gel scanning, varied in different experiments from 50 to 90%. Thus the cleavage of progeny virus HA is most efficiently performed by homologous plasma enzymes. The addition of a protease inhibitor, ε-AcA or Contrical, with homologous plasma to culture medium effectively suppressed the plasma-mediated cleavage of the WSN virus HA in cultured cells and virions with uncleaved haemagglutinin were produced (see Fig. 1d, e).

The data presented here show that homologous plasma added to influenza virus-infected cells induces the cleavage of virus HA. As a result, highly infectious virions with cleaved haemagglutinin are produced in the host cell–homologous plasma system. The cleavage of HA in cultured cells incubated in serum-containing medium has been shown by Lazarowitz et al. (1973a) to be performed by serum plasminogen converted into the active enzyme, plasmin, by cellular activators. Our finding that ε-AcA, an inhibitor of plasminogen activation (Alkjaersig et al., 1959; Brockway & Castellino, 1971), effectively prevents plasma-mediated cleavage of virus HA supports this result.

The cleavage of WSN HA by plasma enzymes could be performed both in the cell plasma membrane and in the virus particles. The serum plasminogen is in contact with the cell surface where the virus HA molecules (Lazarowitz et al., 1971; Stanley et al., 1973; Hay, 1974) and cellular plasminogen activators (Quigly, 1976; Loskutoff & Edginton, 1977) are accumulated. Virus envelope originating from host cell plasma membrane can also contain cellular plasminogen activators. The data on the presence of proteases within myxoviruses (Holland et al., 1972; Zhirnov & Bukrinskaya, 1977) are in agreement with this suggestion. Therefore, serum plasminogen can be converted into active enzyme (plasmin) by virus-associated proteases providing the cleavage of HA at the virion surface. Activation of plasminogen and proteolytic cleavage HA into HA1 + HA2 by plasmin are seemingly coupled events.

Of most interest are the data on the selective effect of homologous plasma enzymes on the virus HA in cultured cells. This selective effect could be provided by either of the following
Fig. 1. Protein patterns of WSN virus produced by CEF, MDCK and SPEV cells incubated with heterologous and homologous plasma and the effect of protease inhibitors. (a) After protein labelling WSN-infected cells (lane 1, CEF; lane 2, MDCK; lane 3, SPEV) were incubated in Medium 199 alone (see also footnote to Table 1). At 13 h after infection the samples of culture medium were clarified (10000 g for 20 min) and virus pelleted through 6 ml 25% sucrose (22000 rev/min for 2 h in a Spinco SW27 rotor) and then analysed by 10 to 15% gradient polyacrylamide gel electrophoresis using Tris-glycine-SDS buffer as described previously (Zhirnov & Bukrinskaya, 1981). (b, c) After protein labelling WSN-infected cells (b, CEF; c, MDCK) were incubated either in Medium 199 alone (lane 1) or in Medium 199 containing CAF (lane 2), canine plasma (lane 3) or porcine plasma (lane 4) at final concentrations of 15%, 2% and 2% respectively, for 7 h. Lane 5 shows culture medium from infected cells incubated with Medium 199 alone and treated in vitro with trypsin (5 μg per 128 haemagglutinating units for 30 min at 37°C). The samples of culture medium were clarified, and virus purified and analysed by polyacrylamide gel electrophoresis followed by autoradiography as described for (a). (d, e) After protein labelling WSN-infected cells (d, CEF; e, MDCK) were incubated either in Medium 199 alone (lane 3), or in Medium 199 containing homologous plasma (15% for CAF and 2% for canine) only (lane 2), or homologous plasma with 10 μg/ml of e-AcA (lane 4) or 5 trypsin-inhibiting units/ml of Contrical (lane 5). At 13 h after infection the culture medium was clarified, and virus purified and analysed by gel electrophoresis followed by autoradiography. Lane 1 shows the polypeptides of WSN virus treated in vitro with trypsin.
mechanisms. Serum plasminogen could be activated more effectively by activators of homologous cells. For example, chicken cells induced higher proteolytic activity in homologous plasma than in plasmas of other origin (Goldberg & Lazarowitz, 1974). The second possibility of selective control may be connected with modification of the HA, a glycoprotein whose glycosylation and sulphation depends on the host cell type (Collins & Knight, 1978; Compans & Pinter, 1975; Nakamura & Compans 1977, 1979; Schwarz et al., 1977) as well as the virus strain (Basak et al., 1981; Nakamura et al., 1980). It follows that the type of glycosylation–sulphation can be important in proteinase–substrate (plasmin–HA) recognition and interaction. There are indirect data that the oligosaccharide component of HA influences the susceptibility of HA to cellular proteases in the processes of cleavage of HA to HA1 + HA2 in a particular cell type (Schulman & Palese, 1977; Nakajima & Sugiura, 1980).

The selective susceptibility of differently derived WSN virus HAs to the plasma enzymes suggests one explanation for host adaptation and cell tropism of influenza viruses. Virus HA in some organs or tissues might be glycosylated and sulphated in such a way that it becomes sensitive to the host proteases and, particularly, to serum plasmin. In this case highly infectious virions would be produced due to HA cleavage and, therefore, the spread of infection would be extensive (Bosch et al., 1979; Rott et al., 1980; Vallbracht et al., 1980) and the host system permissive (Klenk et al., 1975; Nakajima & Sugiura, 1980; Schulman & Palese, 1977). Previously, Lazarowitz et al. (1973a) reported that HA cleavage of WSN virus could be performed by heterologous (chicken) purified plasminogen in cultured (bovine) cells. The lack of selectivity in their system might be explained by the removal from the culture medium of natural plasmin inhibitors and other serum co-factors which could specifically participate in the regulation of plasminogen–plasmin–HA relationships. Furthermore, although we found little activation in heterologous systems, the existence of other heterologous systems with highly effective influenza virus activation cannot be excluded.

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The D.I. Ivanovsky Institute of Virology
Academy of Medical Sciences, Moscow, U.S.S.R.

OLEG P. ZHIRNOV*
ALEXANDER V. OVCHARENKO
ALICE G. BUKRINSKAYA

REFERENCES


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