Biochemical Studies on the Cytopathic Effect of Influenza Viruses

By C. SCHOLTISSEK, H. BECHT AND R. DRZENIEK

Institut für Virologie, Justus Liebig-Universität, Giessen, Germany

(Accepted 12 December 1966)

SUMMARY

An influenza virus was inactivated stepwise by ethylene iminoquinone Bayer A 139; a viral product with a target size similar to that of neuraminidase was responsible for the cytopathic effect on cells grown in culture.

INTRODUCTION

It was once thought that a specific virus-induced switch off of cell metabolism was responsible for the cytopathic effect on infected cells in culture. This view was disproved by the studies of Bablanian, Eggers & Tamm (1965a, b) who showed that the switch-off mechanism alone did not cause cell destruction of poliovirus infected cells, but that a virus product was primarily involved in this effect. On the other hand, in spite of the absence of a specific switch-off mechanism in influenza virus infected cells (Scholtissek et al. 1962a), the host cells are finally destroyed.

Before deciding which viral component is primarily responsible for the cytopathic effect, other viral components must be excluded as causative agents. This can be done by showing either that their presence inside the cell does not alter the cell morphology or that cell destruction still occurs in their absence. Fowl plague virus, a member of the influenza A group, is especially suitable for such studies since it can be inactivated stepwise (Scholtissek & Rott, 1964); a short treatment with ethylene iminoquinone Bayer A 139 destroys only the infectivity of the virus particle, but not the capacity to synthesize viral subunits. After longer treatment the virus loses the capacity to synthesize haemagglutinin, followed by the capacity to synthesize neuraminidase. Finally the ability to produce ribonucleoprotein (RNP)-antigen and viral RNA is lost (Fig. 1).

Many different reactions are involved in cell death; it is consequently difficult to define and measure in a single test the cytopathic effect of viral infection on cells in culture. Thus the morphological changes observed by light microscopy might result from different reactions induced by various viral products. However, characteristic changes in cell metabolism might be observed before any morphological changes are recognizable. Therefore we infected cells with partially inactivated fowl plague virus and subsequently made correlative morphological and biochemical observations.

* A short account of this subject was presented at the IXth International Congress of Microbiology, Moscow, 1966.
METHODS

Virus. The Rostock strain of fowl plague virus was used in all experiments. The virus was passaged in chick embryos.

Inactivation of the virus by Bayer A139. The virus samples were purified by centrifugation in the Spinco ultracentrifuge and taken up in 2-amino-2-hydroxymethylpropane-1,3-diol + maleic acid (tris + maleic acid buffer) pH 6.7 (Gomori, 1955). A virus concentrate with a haemagglutination titre of $2^{-11}$ was mixed with an equal volume of a freshly prepared 2% (w/v) solution of Bayer A139 in tris + maleic acid buffer, pH 6.7. At different intervals the reaction was stopped by diluting a sample 1/2 with tris + maleic acid buffer, pH 8. After removal of the Bayer A139 by dialysis against NaCl + phosphate buffer (0.72 % NaCl, 0.02 M-phosphate, pH 7.2) the samples were used for the tests (Scholtissek, Rott & Schäffer, 1962b).

Biological tests. The treated virus samples were added in different dilutions either to primary chick fibroblasts or to HeLa cells in Petri dishes of 5 cm. diameter. After adsorption for 30 min. the cell layers were washed twice and overlaid either by 3 ml. per culture of minimal medium (chick fibroblasts, Eagle & Habel, 1956), or with Eagle's medium (HeLa cells). In the case of chick fibroblasts, rabbit antiserum against fowl plague virus, twice absorbed with embryo homogenate, was added to the incubation medium in order to prevent a second cycle of infection. After 24 and 32 hr the cells were examined by light microscopy for cytopathic changes. In the case of HeLa cells in which only an abortive cycle occurs (Rott, unpublished) the antiserum was omitted.

Biochemical tests. At the times indicated in Figs 2 to 4, 0.25 μC $[^{14}C]$uridine per culture was added as a precursor for RNA synthesis. One hr later the cultures were washed twice with NaCl+phosphate buffer at 4° and 10 ml. of cold 6% (v/v) trichloracetic acid (TCA) was added to each culture. The Petri dishes were shaken briefly and the acid soluble extracts were collected. After neutralization with 4N-KOH a 3 ml. sample of the acid soluble extract was dried on an aluminium planchet and counted in a methane flow counter (Frieseke & Hoepfner, Erlangen-Bruck, Germany). The cell layers were washed again three times with 6% (v/v) TCA, twice with 96% ethanol, and once with ether. The dried cell layers were dissolved in concentrated formic acid, transferred to aluminium planchets, dried and counted. $[^{14}C]$leucine was used for labelling the protein. The procedure for determining the radioactivity in the acid-insoluble material (protein) was the same as described using $[^{14}C]$uridine as the RNA precursor. When $[^{3}H]$uridine was used for incorporation studies, the dried cell layers were overlaid with 1 ml. of m-hyamine in methanol (Packard Instruments, Inc., La Grange, Ill., U.S.A.), heated for 5 min. at 60°, left for 1 hr at room temperature, and transferred to the counting vessels. The contents of each Petri dish were washed once with 1 ml. methanol. Twelve ml. toluol scintillator was added before counting in the Packard scintillation counter. Two ml. of the acid soluble extract were mixed, without neutralization, with 10 ml. of Bray-Scintillator and counted. Corrections were made with the aid of an external standard.

Chemicals. Bayer A139 was kindly provided by the Bayerwerke, Leverkusen, Germany. $[^{3}H]$uridine (G) (2-18 c/m-mole), $[^{2-14}C]$uridine (42-3 μC/μ mole), and $[^{1-14}C]$ leucine (8.5 μC/μ mole) were obtained from the Radiochemical Centre, Amersham, England.
RESULTS

Changes in cell morphology after infection with partially inactivated virus

To show which viral component caused morphological changes after infection of cells in culture, fowl plague virus was inactivated by Bayer A139 for different times (Fig. 1). Different dilutions of inactivated virus were added to tissue cultures.

Fig. 1. Synthesis of viral components by cells infected with stepwise inactivated fowl plague virus (taken from Scholtissek & Rott, 1964).

Δ = plaque forming units; × = haemagglutinating units; O = neuraminidase units; □ = units of RNP-antigen (complement fixation test). The curve representing the synthesis of viral RNA follows that of the RNP-antigen (Scholtissek & Rott, 1963). Multiplicity of infection = 6.

Table 1. Cytopathic effect on Hela-cells* infected with fowl plague virus inactivated with Bayer A139 for different times

<table>
<thead>
<tr>
<th>Period of inactivation (hr)</th>
<th>Vol. of virus sample per tissue culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0-1 ml.</td>
</tr>
<tr>
<td>0</td>
<td>++ + +</td>
</tr>
<tr>
<td>1½</td>
<td>++ + to + + +</td>
</tr>
<tr>
<td>3</td>
<td>+ + to + + +</td>
</tr>
<tr>
<td>4½</td>
<td>+ to + +</td>
</tr>
<tr>
<td>6</td>
<td>±</td>
</tr>
<tr>
<td>7½</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
</tr>
</tbody>
</table>

= like the uninfected control; + = many cells show a marked granulation with some cell rounding; ++ = many cells rounded and appear in clusters; +++ = many cells afloat in the medium, monolayer no longer intact; ++++ = total destruction of cell layer.

* Similar results were obtained when chick fibroblasts were investigated.
† 0-02 ml. virus sample per tissue culture corresponds to a multiplicity of infection of about 1.

The cultures were examined 24 hr after infection.
Cells infected with untreated virus at a multiplicity of about 5 showed the first signs of granulation at about 12 to 15 hr after infection. The morphological changes proceeded to complete destruction of the cell layer at about 24 hr. The cytopathic effect decreased with increasing time of inactivation of the virus samples (Table 1). After 6 hr inactivation there was only slight alteration of cell morphology. This diminishing cytopathic effect with increasing time of inactivation nearly parallels the loss of the capacity to synthesize neuraminidase (Fig. 1).

Fig. 2. Changes in the uptake of [14C]uridine into RNA and the trichloroacetic acid soluble pool after infection. Chick fibroblasts were infected with fowl plague virus. Every hour after infection a 1 hr [14C]uridine pulse was given.

Alterations in the labelling of RNA and its precursors with radioactive uridine in cells infected with fowl plague virus

Synthesis of viral RNA was previously shown to be maximal between 2 and 3 hr after infection and superimposed on the synthesis of cellular RNA. After 6 hr the production of viral RNA is nearly complete; at this time, therefore, the inhibition of the labelling of RNA with 32P is mainly concerned with cellular RNA (Scholtissek & Rott, 1961).

In the present experiments we measured the incorporation of labelled uridine into RNA and also into the acid-soluble pool of infected chick fibroblasts as compared to uninfected cells. There was a marked change in the uptake of labelled uridine during the infectious cycle (Fig. 2). The increased incorporation of the isotope into the RNA of infected cells with a maximum at 3 hr was not only due to an increased synthesis of RNA, but also to an increased labelling of the acid soluble pool. Later, the decline in the labelling of the RNA was partially due to a decreased labelling of the acid...
Cytopathic effect

soluble pool. The latter might be taken as the first sign of a general inhibition of cellular metabolism, recognizable earlier than the degenerative changes in cell morphology.

Changes of RNA and protein metabolism by partially inactivated fowl plague virus

Virus samples inactivated for different times were added to tissue cultures and a pulse of radioactive uridine was given at different intervals after infection (Figs. 3, 4). If the cells were left in contact with the isotope between 2 and 3 hr after infection an enhanced incorporation into the acid-soluble pool was found in all of those cells infected with virus inactivated from 0 to about 7½ hr (Fig. 3). Virus samples inactivated for longer than 7½ hr did not enhance uridine incorporation. Similarly the incorporation of the isotope precursor into RNA was also enhanced in those cells which were infected with virus inactivated up to about 7½ hr (Figs. 3, 4). Part of this increased labelling of the RNA in the infected cells compared to the control might represent synthesis of viral RNA. This would agree with previous results, in which it was shown that the capacity to synthesize viral RNA is lost after treatment for about 8 hr with Bayer A139 (Fig. 1).
When the pulse with radioactive uridine was given between 6 and 7 hr after infection a general inhibition of the RNA metabolism in normally infected cells was apparent (Fig. 2). If, however, the virus used for infection was inactivated for 4½ hr, the inhibitory capacity was almost lost (Figs. 3, 4). In experiments in which the pulse was given between 8 and 9 hr after infection, the effect was even more pronounced. But at this time the amount of radioactivity even after infection with totally inactivated virus was never as high as that of the uninfected controls. This might have been due to some toxic components found in influenza viruses (Henle & Henle, 1944).

Corresponding experiments were made with [14C]leucine as precursor for protein synthesis. The pulse was given between 6 and 7 hr after infection when incorporation of [14C]leucine into the protein of cells infected with uninactivated fowl plague virus has already been inhibited (Scholtissek, 1965). The inhibition of the incorporation of [14C]leucine can also be overcome with virus inactivated for 4½ hr or longer (Fig. 4).

DISCUSSION

With fowl plague virus inactivated by Bayer A 139 for 4½ hr, a nearly tenfold increase in virus concentration was needed to obtain the degree of cell destruction found with uninactivated virus (Table 1). Under these conditions the capacity to synthesize viral haemagglutinin fell to less than 1% of untreated control virus (Fig. 1). Haemagglutinin was therefore apparently not responsible for the morphological changes occurring after cell infection. On the other hand, at this degree of viral inactivation the synthesis of RNP-antigen and viral RNA was not significantly impaired (Fig. 1); these components could therefore also be excluded as the cause of morphological changes after infection. With virus inactivated for 4½ hr, only the capacity to synthesize viral neuraminidase was reduced to about 10% of cells infected with uninactivated virus. With chemical inactivation the target size for a viral product is measured. Therefore the only firm conclusion can be that a viral product with a target size similar to that of neuraminidase was associated with the morphological changes. We conclude tentatively that neuraminidase might be this product.

Degenerative changes in cell morphology are apparently due to a severe change or breakdown of cellular metabolism. Figure 2 shows that a marked reduction in the labelling of the acid soluble pool using radioactive uridine occurs at 6 to 8 hr after infection. This is about the time of virus maturation (Breitenfeld & Schäfer, 1957). There seems to be an additional effect on RNA synthesis. Since most of the viral RNA has already been synthesized by 6 hr (Scholtissek & Rott, 1961), the subsequent decrease in RNA synthesis in the infected as compared to the uninfected cells is primarily a decrease in cellular RNA. There is almost no inhibition in the labelling of the acid soluble pool and of RNA when the infecting virus is inactivated for 4½ hr (Figs. 3, 4). Corresponding results were obtained with [14C]leucine as precursor for protein synthesis (Fig. 4). Thus, the metabolic changes of the cells infected with partially inactivated virus correspond to the morphological alterations after infection. In both cases it must be assumed that a viral product with a target size similar to neuraminidase causes these changes which by definition are called the cytopathic effect. If this product is neuraminidase the enzyme might act indirectly, perhaps by attacking the lysosomal membranes and releasing lysosomal enzymes. The only viruses which contain and induce synthesis of neuraminidase are myxoviruses. Therefore, if neuraminidase is
responsible for the cytopathic effect in myxovirus-infected cells, the cytopathic effects of other viruses must have a different explanation. It has been shown that lysosomes are somehow involved in the cytopathic effect of other viruses (Wolf & Bubel, 1964; Allison & Mallucci, 1965; Mallucci & Allison, 1965).

We wish to thank Professor R. Rott for his stimulating discussions and interest in this work. The assistance of Mrs L. Groh, Miss I. Drebes, Miss E. Wiegand and Mr E. Ayad is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES


(Received 2 December 1966)