Characterization and Mixed Infections of Three Strains of Vaccinia Virus: Wild Type, IBT-resistant and IBT-dependent Mutants

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SUMMARY
The characteristics of IBT-dependent (IBT\textsuperscript{D}) and IBT-resistant (IBT\textsuperscript{R}) mutants of vaccinia virus were compared with those of the wild type (wt) strain. The mutants did not differ from the wild type strain by their sedimentation in sucrose gradients. Minor differences in the polypeptide composition of the virus particles and in the neutralization of the three virus strains by anti-vaccinia human immunoglobulin were observed. Mixed infections of the viruses in HeLa cells enabled the growth of the strains under their unfavourable conditions (wt in the presence of IBT and IBT\textsuperscript{D} in the absence of IBT).

INTRODUCTION
Isatin-β-thiosemicarbazone (IBT) is an effective inhibitor of the multiplication of poxviruses (Sheffield, Bauer & Stephenson, 1960). Its mode of action is not fully understood. It was found that IBT does not interrupt early events such as the uncoating of the virus particle, synthesis of early enzymes and early structural polypeptides (Appleyard, Hume & Westwood, 1965; Woodson & Joklik, 1965), DNA replication (Easterbrook, 1962; Woodson & Joklik, 1965) and m-RNA transcription and association with ribosomes (Woodson & Joklik, 1965). Since polyribosomes, which are formed by late m-RNA, are broken down soon after their formation, Woodson & Joklik (1965) suggested that late structural virus polypeptide synthesis is the target for the block caused by IBT.

Mutants of vaccinia virus which are resistant or dependent on IBT may be very useful in the clarification of the mechanism of action of IBT. In the present study we further characterize an IBT-dependent and an IBT-resistant mutant of vaccinia virus and study the interactions between these viruses and the wild type strain during multiplication in mixed-infected cells.

METHODS

Compounds and reagents. [\textsuperscript{3}H]-thymidine (18·9 Ci/m-mol), [\textsuperscript{14}C]-thymidine (520 mCi/m-mol), [\textsuperscript{3}H]-phenylalanine (18 Ci/m-mol), [\textsuperscript{14}C]-phenylalanine (15·3 mCi/m-mol) and [\textsuperscript{35}S]-methionine (144 Ci/m-mol) were obtained from the Radiochemical Centre, Amersham, England; IBT (Mann Research Laboratories, New York, N.Y.) and anti-vaccinia human immunoglobulin (Povite, Poviet Producten N.V. Amsterdam, Holland).

Cell cultures and viruses. HeLa S-3 cells were grown in monolayer cultures in Eagle's medium (Eagle, 1959), supplemented with 10% calf serum. Stocks of WR strain, IBT-resistant mutant (IBT\textsuperscript{R}) and IBT-dependent mutant (IBT\textsuperscript{D}) of vaccinia virus were prepared in HeLa cells and titrated on BSC 1 monolayers, as previously described (Katz et al. 1973).
Infection. Monolayers of HeLa S-3 cells were washed with saline and infected with virus at an input multiplicity of 5 p.f.u./cell. Following incubation for 45 min at 37 °C, the cultures were washed and Eagle's medium containing 2% calf serum was added.

Preparation of labelled virus. Thymidine-labelled virus was prepared by the addition of 0.3 μCi/ml of [14C]-thymidine or 70 μCi/ml of [3H]-thymidine to the infected cultures in Eagle's medium supplemented with 2% calf serum. Phenylalanine-labelled virus was prepared by the addition of 1.2 μCi/ml of [14C]-phenylalanine or 7 μCi/ml of [3H]-phenylalanine in Eagle's medium containing 1/20 the regular concentration of phenylalanine. Methionine-labelled virus was prepared by the addition of 40 μCi/ml of [35S]-methionine in Eagle's medium containing 1/20 the regular concentration of methionine. The cultures were harvested 2 days after infection, and virus was purified as described by Joklik (1962).

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed essentially as described by Summers, Maizel & Darnell (1965). Gels (10 by 0.6 cm) were prepared of 10% acrylamide, 0.27% N,N'-methylene bisacrylamide in 0.1 M-sodium phosphate (pH 7.1) and 0.1% sodium dodecyl sulphate (SDS). Prior to use, excess catalyst was removed from the gels by electrophoresis at 5 mA/gel for 1 h. Purified virus was dissociated with 2% SDS and 1% mercaptoethanol (ME) for 1 min at 100 °C. Sucrose was added to the solubilized virus to 10%, and 200 μl were applied to each gel. Electrophoresis was carried out at 3.5 mA/gel for 17 h. After electrophoresis, the gels were placed in 10% trichloroacetic acid (TCA), stained with 0.1% Coomassie blue in 10% TCA and washed in 75% acetic acid. The gels were sliced into 1 mm slices, dissolved in H2O2, and radioactivity was determined, or were sliced longitudinally, dried and placed in contact with X-ray film (Fairbanks, Levinthal & Reeder, 1965).

Electron microscopy. Infected cultures were scraped from the glass and the cells were centrifuged at low speed for 5 min. Small pellets were fixed, stained, dehydrated, infiltrated and embedded, according to the technique developed by Hayat & Giaquinta (1970). The blocks were sectioned using an LKB ultratome III and stained with uranyl acetate and lead citrate. The stained samples were examined in a Philips EM 300 electron microscope.

RESULTS
 Physical and chemical characterization of the virus strains
 Sedimentation in sucrose gradients and the polypeptide composition of the virus strains were compared.
 Sedimentation of wt, IBT* and IBT0 in sucrose gradients
 Mixtures of [14C]-thymidine-labelled wt strain of vaccinia virus, with either one of [3H]-thymidine-labelled IBT* or IBT0, were prepared and layered on 25 to 40% sucrose gradients. Fractions were collected after centrifuging in an SW 50.1 rotor at 13000 rev/min for 35 min and radioactivity was determined. IBT* (Fig. 1a) and IBT0 (Fig. 1b) co-sedimented in the two sucrose gradients with the wt strain.
 Comparison of the polypeptides of wt, IBT* and IBT0 by polyacrylamide gel electrophoresis
 Mixtures of [14C]-phenylalanine-labelled wt strain with either one of [3H]-phenylalanine-labelled IBT* or IBT0 mutants were prepared, solubilized and the polypeptides then separated by polyacrylamide gel electrophoresis. No significant differences were observed between the polypeptides of the wt strain and those of IBT* or IBT0.
 An improved resolution of the different polypeptides of vaccinia virus can be obtained...
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Fig. 1. Sedimentation of thymidine-labelled vaccinia virus strains in sucrose gradients. Mixtures of [\textsuperscript{14}C]-thymidine-labelled wt strain (\(\bigcirc\)–\(\bigcirc\)) with [\textsuperscript{3}H]-thymidine-labelled mutant (\(\bullet\)–\(\bullet\)), IBT\textsuperscript{R}; (b), IBT\textsuperscript{D}) were sedimented through 25 to 40 % sucrose gradient in 1 mM-tris, pH 9, in an SW 50.1 rotor at 13000 rev/min for 35 min. Fractions (ten drops), collected after piercing the bottom of the tubes, were dissolved in toluene scintillator solvent containing 40 % Triton X-100 and counted in a TriCarb scintillator counter.

Fig. 2. Autoradiography of the labelled polypeptides of the three virus strains. [\textsuperscript{35}S]-methionine-labelled wt, IBT\textsuperscript{R} and IBT\textsuperscript{D} strains of vaccinia virus were solubilized by SDS and ME. The polypeptides were separated by polyacrylamide gel electrophoresis, as described in Methods. The gels were sliced longitudinally, dried and exposed to X-ray film. Photographs of the autoradiographs are shown. The main virus polypeptides are marked.

by autoradiography of the labelled polypeptides, separated in polyacrylamide gels. When the [\textsuperscript{35}S]-methionine-labelled viruses were compared, a few minor differences, particularly in the relative amounts of several of the polypeptides of the viruses, could be observed (Fig. 2). The lack of the polypeptide migrating in front of 6b of IBT\textsuperscript{R} virus (Fig. 2) is the main qualitative difference observed.

Neutralization of wt, IBT\textsuperscript{R} and IBT\textsuperscript{D} strains of vaccinia virus by immunoglobulin

The degree of neutralization of wt, IBT\textsuperscript{R} and IBT\textsuperscript{D} strains of vaccinia virus by anti-vaccinia human immunoglobulin was compared. Dilutions of the virus suspensions were incubated at 37 °C for 30 min with a constant amount of anti-vaccinia human immunoglobulin. Although all the three virus strains were neutralized by the immunoglobulin, there were minor differences in the degree of the neutralization (Table 1).
**Table 1. Neutralization of wt strain of vaccinia virus and of the mutants IBT<sup>r</sup> and IBT<sup>b</sup> by anti-vaccinia human immunoglobulin**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titre (p.f.u./ml)</th>
<th>Neutralization (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>+ Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>6.2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>98.1</td>
</tr>
<tr>
<td>IBT&lt;sup&gt;r&lt;/sup&gt;</td>
<td>9.1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>99.6</td>
</tr>
<tr>
<td>IBT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>99.8</td>
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</table>

0.5 ml of dilutions of the three vaccinia virus strains wt, IBT<sup>r</sup> and IBT<sup>b</sup> were incubated with 0.5 ml (=17 mg) of anti-vaccinia human immunoglobulin for 30 min at 37 °C. Virus dilutions without immunoglobulin were incubated as well. The virus was then titrated on BSC<sub>1</sub> monolayers.

**Electron microscopy**

The effect of IBT on the intracellular development of the three virus strains was studied by electron microscopy. Wild type-infected cells, in the absence of IBT (Fig. 3a), contained a great majority of condensed forms with low electron-dense centres which appeared to be fully mature infectious virus (Dales & Siminovitch, 1961). However, in cells exposed to IBT (Fig. 3b) there was a great number of large, almost spherical immature forms, most of which appeared to have complete membranes—findings in agreement with those reported by Easterbrook (1962). When IBT<sup>r</sup>-infected cultures, in the absence (Fig. 3c) or in the presence of IBT (Fig. 3d), were examined, most of the virus particles were mature. In IBT<sup>r</sup>-infected cultures the results seem to be the opposite to that observed with the wt strain; mature particles were very rare in the absence of IBT (Fig. 3c) but, in the presence of IBT most of the forms observed, were mature virus particles (Fig. 3d). It can be concluded that the immature forms which appeared with wt in the presence of IBT look similar to those which appeared with IBT<sup>b</sup> in the absence of IBT, suggesting a similar block in virus development.

**Mixed infections between the different strains of vaccinia virus, in the presence and absence of IBT**

The multiplication of the wild type strain is inhibited in the presence of IBT, while the growth of the mutant IBT<sup>b</sup> depends on a constant supply of IBT in the culture media. The growth of these viruses under their non-favourable conditions, in cells co-infected with another strain of vaccinia virus, was studied in order to find out whether the viruses interact during their multiplication.

**Rescue of wt in the presence of IBT during mixed infection with either IBT<sup>r</sup> or IBT<sup>b</sup>**

The ability of IBT<sup>r</sup> and IBT<sup>b</sup> vaccinia mutants to rescue wt virus in the presence of IBT was studied. Simultaneous infections were carried out with equal amounts of the virus strains in the presence of IBT. The virus titres of wt, IBT<sup>r</sup> and IBT<sup>b</sup> were determined by plaquing in the presence and absence of IBT. The increase in virus titre is shown in Table 2. As expected, when IBT-treated cells were infected with wt alone, no increase in virus titre was detected. However, when either IBT<sup>r</sup> or IBT<sup>b</sup> were used, an increase in virus titre was observed. After a mixed infection with wt and IBT<sup>r</sup> (no. 4), growth of both wt (plaqued in cultures without IBT) and IBT<sup>r</sup> (plaqued in cultures with IBT) occurred. After a mixed infection with wt and IBT<sup>r</sup> (no. 5) the titre in the absence of IBT was more than three times as high as in the presence of IBT. The latter result suggested that the progeny virus from a mixed infection consisted of both genotypes. As proof, eight plaques, formed in the
Fig. 3. Electron microscopy. Infected cells were harvested 22 h after infection and prepared for electron microscopy as described in Methods. (a) wt without IBT; (b) wt with IBT; (c) IBT<sup>B</sup> without IBT; (d) IBT<sup>B</sup> with IBT; (e) IBT<sup>D</sup> without IBT; (f) IBT<sup>D</sup> with IBT.
Table 2. Virus yield after single and mixed infections in the presence of IBT

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Virus inoculum*</th>
<th>Titre</th>
<th>Calculated progeny virus</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>IBT&lt;sup&gt;D&lt;/sup&gt;</td>
<td>IBT&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>—</td>
<td>—</td>
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<tr>
<td>2</td>
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<td>5</td>
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* HeLa cells in 60 mm plastic Petri dishes (Nunc, Denmark) were infected with 5 p.f.u./cell of either wt, IBT<sup>D</sup>, IBT<sup>R</sup> or with combinations of two of these virus strains. Following 45 min of incubation at 37 °C, 5 ml of Eagle's medium, supplemented with 2 % calf serum, and IBT (14 µM), were added.

† The virus yield (p.f.u./ml) was determined from the increase in p.f.u. between 1 and 22 h after infection. The plaque titre was measured in BSC 1 cells, in the absence and in the presence of IBT (14 µM) in the agar overlay. In sample no. 4 the progeny IBT<sup>D</sup> virus is the plaque titre with IBT in the overlay while the progeny of wt is the plaque titre without IBT in the overlay. In sample no. 5 the progeny IBT<sup>R</sup> is the plaque titre with IBT in the overlay and the progeny wt is the difference between the titres without and with IBT in the overlay.

Table 3. Virus yield after single and mixed infections in the absence of IBT

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Virus inoculum*</th>
<th>Titre</th>
<th>Calculated progeny virus</th>
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<tbody>
<tr>
<td></td>
<td>wt</td>
<td>IBT&lt;sup&gt;D&lt;/sup&gt;</td>
<td>IBT&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>1</td>
<td>5</td>
<td>—</td>
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<td>5</td>
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</table>

* As in Table 2 except for the absence of IBT in the medium added following the 45 min incubation at 37 °C.

† As in Table 2. In sample no. 5 the progeny IBT<sup>R</sup> is the plaque titre without IBT in the overlay and the progeny IBT<sup>D</sup> is the difference between the titres with and without IBT in the overlay.

absence of IBT (no. 5), were picked and replaqued in the presence and absence of IBT. Of the eight, seven grew only in the absence of IBT, indicating that they were wt, and the remaining one grew in both the presence and absence of IBT, indicating that it was an IBT<sup>R</sup> mutant. Not only does IBT<sup>R</sup> rescue wt, but wt also inhibits the growth of IBT<sup>R</sup> by 90 % (nos. 3 and 5) as previously was noted by Appleyard & Way (1966).

Rescue of IBT<sup>D</sup> in the absence of IBT during mixed infection with either wt or IBT<sup>R</sup>

The ability of wt and IBT<sup>R</sup> vaccinia virus strains to rescue IBT<sup>D</sup> virus in the absence of IBT was tested. The increase in virus titre is shown in Table 3. As expected, when the cells were infected with IBT<sup>D</sup> alone, only a slight increase in virus titre was detected; when wt or IBT<sup>R</sup> were used alone, an increase in virus titre was observed. After a mixed infection with IBT<sup>D</sup> and wt (no. 4), growth of both IBT<sup>D</sup> (plaqued in cultures with IBT) and wt (plaqued in cultures without IBT) was observed. Following a mixed infection with IBT<sup>D</sup> and IBT<sup>R</sup> (no. 5), the plaque titre was more than twice as high in the presence of IBT. The latter result suggested that the progeny virus from a mixed infection consisted of the two genotypes. As proof, nine plaques which formed in the presence of IBT (no. 5) were picked
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and replaqued in the presence and absence of IBT. Of the nine, eight grew only in the presence of IBT, indicating that they were IBT⁰, and the remaining one grew in both the absence and presence of IBT, indicating that it was an IBT⁸.

DISCUSSION

The two mutants of vaccinia virus differ from the wild type strain in their resistance or dependence on IBT, which is an effective inhibitor of the wild type strain. Physical and biochemical characteristics of the mutants, morphology of the virus particle (revealed by electron microscopy – our unpublished results), and sedimentation in sucrose gradient are similar to those of the wild type strain. All three strains were neutralizable to approximately similar extents by the antiserum used. The proportion of several polypeptides of the virus strains are different and the apparent absence of one of them in the IBT⁸ strain was noted.

Depending on the target of IBT in the vaccinia-infected HeLa cells and the extent to which the products of the different genomes are capable of interacting in double-infected cells, the following possibilities can be considered: (1) the effect of IBT on an event in the development of one virus strain might interfere with the production of all particles; (2) a product coded by the genome of one of the strains might enable all particles to be made; (3) the gene products of the two virus strains might not interact at all, so that some particles would form while the formation of others would still be blocked. Measurements of the growth of vaccinia virus strains showed that the second possibility exists, indicating assistance between either IBT⁰ or IBT⁸ towards the growth of wt strain, in the presence of IBT, and also assistance between either wt or IBT⁰, to the growth of IBT⁰ in the absence of IBT. These findings suggest that there are common events in the multiplication of these three virus strains and that they are capable of sharing an event or a factor(s) involved in their multiplication.

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REFERENCES


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