An Antigenic Difference between Intracellular and Extracellular Rabbitpox Virus

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SUMMARY

Extracellular rabbitpox virus released naturally from infected cells differed antigenically from intracellular virus released by artificial disruption of cells. Intracellular virus was neutralized by antiserum prepared against live rabbitpox virus and by antiserum against inactivated vaccinia virus. In contrast, extracellular virus was neutralized only by rabbitpox antiserum. The antibodies responsible for the neutralization of intracellular and extracellular virus could be absorbed separately from rabbitpox antiserum. Morphologically, extracellular virus differed from intracellular virus in possessing an outer envelope. This envelope was probably the site of the virus antigen characteristic of extracellular virus, and fluorescent antibody staining of infected cells suggested that it was derived from the modified host cell membrane. Antibody directed against extracellular virus was responsible for the ability of rabbitpox antiserum to control the spread of rabbitpox virus in tissue culture and probably for its ability to protect rabbits from rabbitpox infection. Extracellular virus should therefore be used as the test virus in titrations of neutralizing antibody if these are to assess the protective activity of an antiserum.

INTRODUCTION

Vaccinial antiserum or γ-globulin is of value for the treatment of some of the complications of vaccination and for the prophylaxis of smallpox in contacts (Peirce et al. 1958; Kempe, 1960). Using rabbitpox as an experimental model, Boulter, Westwood & Maber (1960) showed that convalescent antiserum had both protective and therapeutic effects. However, antisera produced by immunization of rabbits with large doses of inactivated vaccinia virus failed to protect against infection, despite neutralizing antibody titres ten to one hundred times higher (Boulter, 1969). A similar difference was found in the ability of the two types of antisera to prevent the spread of rabbitpox virus in tissue culture. The lack of correlation between the neutralizing and protective activities of antisera led Boulter (1969) to seek an antigenic difference between intracellular poxvirus, which forms most of the yield from an infected cell, and naturally released extracellular virus, which is probably more important for the spread of infection. Preliminary studies indicated that, whereas intracellular virus was neutralized by both types of antiserum, extracellular virus was not neutralized by antiserum prepared against inactivated virus.

This paper gives further information on the differences between intracellular and extracellular virus and between antisera prepared against live and inactivated poxviruses.
Methods

Viruses. The Utrecht strain of rabbitpox virus was grown in monolayers of HeLa cells cultured in Earle's saline plus tryptic meat broth, yeast extract and 5% calf serum (Appleyard & Westwood, 1964). To harvest intracellular virus, the infected cells were washed with phosphate-buffered saline, suspended in fresh medium and then disrupted by ultrasonic vibration. To obtain extracellular virus, the virus growth medium was centrifuged to remove free cells, and the supernatant fluid was taken. When extracellular virus was to be used in neutralization tests, the virus growth medium was supplemented with 0.1% antiserum prepared against inactivated vaccinia virus; this antiserum neutralized the fraction of virus that behaved as intracellular virus in neutralization tests. Vaccinia virus for the absorption of rabbitpox antiserum was grown in rabbit skin and purified and concentrated by density gradient centrifugation (Zwartouw, Westwood & Appleyard, 1962). Virus infectivity was titrated as p.f.u. in HeLa cell monolayers under a liquid overlay that contained rabbitpox antiserum to prevent the formation of secondary plaques (Appleyard & Westwood, 1964).

Antisera. Rabbitpox antiserum was obtained from rabbits that had recovered from a rabbitpox infection and then received a single intravenous inoculation of 2 × 10^8 p.f.u. live virus grown in rabbit testis. Antiserum against inactivated vaccinia virus was derived from a horse immunized by five intramuscular injections of about 3 mg. purified vaccinia virus inactivated by ultraviolet light and emulsified with Freund's complete adjuvant (Boulter et al. 1971).

Rabbitpox antiserum was absorbed by mixing with either 4 vol. of purified vaccinia virus of infectivity 5 × 10^8 p.f.u./ml. or 40 vol. of extracellular rabbitpox virus concentrated by centrifugation to 5 × 10^8 p.f.u./ml. After incubation at 36° for 16 hr, the mixtures were centrifuged at 40,000 g for 15 min. and the supernatant fluids were heated at 56° for 1 hr to inactivate residual infectivity.

Titration of neutralizing antibody. Samples of the test virus suspension, containing about 1500 p.f.u./ml. of either intracellular or extracellular virus, were added to equal volumes of serial tenfold dilutions of the antiserum to be tested. Mixtures were incubated at 36° for 6 hr and then titrated for residual infectivity. The antibody titre of a serum was taken as the reciprocal of the dilution in the reaction mixture that neutralized 50% of the original virus infectivity.

Titration of antibody preventing the spread of virus in tissue culture ('anti-comet' activity). HeLa cell monolayers in Petri dishes were infected with about 100 p.f.u. rabbitpox virus and overlaid with medium containing serial twofold dilutions of the antiserum to be tested; the medium was supplemented with 0.02 μg./ml. actinomycin D which was found to aid virus spread. The cultures were incubated for 3 days, and the surviving cells were stained with 0.04% methyl violet in saline. The 'anti-comet titre' of a serum was taken as the reciprocal of the highest dilution that prevented any distant spread of virus infection from the primary plaques.

Electron microscopy. Specimens for electron microscopy were prepared by mixing equal parts of virus suspension and 1% phosphotungstic acid and applying the mixture to Formvar-coated specimen grids. After draining and drying, the grids were coated with carbon and examined in a Philips EM 300 electron microscope at 80 kv.

Fluorescent antibody staining. The globulin fractions from rabbitpox and inactivated vaccinia antisera were conjugated with fluorescein isothiocyanate. Infected cell monolayers on coverslips were stained with conjugated antibody either after fixing in acetone, in order
to reveal internal antigen, or before fixing in order to reveal surface antigen. The stained cultures were examined microscopically under dark-ground ultraviolet illumination.

RESULTS

Effect of antisera on the spread of virus in tissue culture

Rabbitpox and inactivated vaccinia antisera, both known to have a high content of neutralizing antibody, were tested for their ability to prevent the spread of rabbitpox virus through HeLa cell monolayers infected with about 100 p.f.u. rabbitpox virus. In the absence of antiserum from the overlay medium, virus spread from the primary foci of infection to produce secondary plaques and diffuse, comet-shaped areas of cell destruction (Fig. 1a). The addition of rabbitpox antiserum, diluted 1/400 or less, completely prevented the distant spread of virus and allowed only the development of localized plaques (Fig. 1 b). In contrast, antiserum against inactivated vaccinia virus did not affect the spread of virus even at a dilution of 1/25.

The media from such cultures were tested for free infective virus. Monolayer cultures were infected with about 75 p.f.u. rabbitpox virus, and groups of four were incubated for three days under medium without antiserum or medium containing 1/200 rabbitpox antiserum or antiserum against inactivated vaccinia virus. Rabbitpox antiserum reduced the infectivity of the overlay media to 0.01% of the control value. In contrast, the media from cultures incubated with antiserum against inactivated vaccinia virus contained nearly half as much virus as those without antiserum (Table 1). The subsequent addition of 1/200 antiserum against inactivated vaccinia virus to the media from control cultures incubated without antiserum reduced their infectivity by only 60%. The survival of a large amount of free infective virus in the presence of antiserum against inactivated virus would account for the inability of this antiserum to prevent the spread of virus in tissue culture.
Neutralization of intracellular and extracellular virus

Rabbitpox and inactivated vaccinia antisera were titrated for neutralizing activity against both intracellular and extracellular virus (Fig. 2). The neutralizing antibody titre of rabbitpox antiserum against intracellular virus was 13,000 and that of inactivated vaccinia antiserum more than 1,000,000. When the sera were tested against extracellular virus (grown in the presence of 0.1 % inactivated vaccinia antiserum), rabbitpox antiserum had an antibody titre of 6,000, which was only slightly less than that against intracellular virus, but inactivated vaccinia antiserum showed little or no neutralizing activity. Many tests confirmed the inability of inactivated vaccinia antiserum to neutralize extracellular virus despite its high content of neutralizing antibody against intracellular virus.

Table 1. Virus infectivity in overlay media containing antiserum

<table>
<thead>
<tr>
<th>Antiserum in medium</th>
<th>Treatment before assay</th>
<th>Mean infectivity of four cultures (log p.f.u./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>Nil</td>
<td>5.68 ± 0.14</td>
</tr>
<tr>
<td>Rabbitpox, 1/200</td>
<td>Nil</td>
<td>1.41 ± 0.08</td>
</tr>
<tr>
<td>Inactivated vaccinia, 1/200</td>
<td>Nil</td>
<td>5.21 ± 0.36</td>
</tr>
<tr>
<td>Nil</td>
<td>Inactivated vaccinia, 1/200</td>
<td>5.24 ± 0.19</td>
</tr>
</tbody>
</table>

Neutralizing antibody titres are commonly expressed as International Units/ml., derived by comparison with the W.H.O. international standard smallpox antiserum which is defined as containing 1000 i.u./ml. (Anderson & Skegg, 1970). By our method, the antibody titre of the international standard against intracellular virus was 20,000 whereas against extracellular virus it was only 400.
Intracellular and extracellular poxvirus

Absorption of neutralizing antibodies from rabbitpox antiserum

Since the above results suggested that intracellular and extracellular virus were neutralized by different antibodies, attempts were made to absorb each antibody separately from rabbitpox antiserum.

Absorption of antibody against intracellular virus. Rabbitpox antiserum absorbed with purified vaccinia virus was titrated, in parallel with untreated antiserum, for neutralizing activity against both intracellular and extracellular virus (Fig. 3). More than 99% of antibody against intracellular virus was removed by absorption, the titre falling from 56,000 to 300. In contrast, the antibody titre against extracellular virus fell by only 50%, a doubtfully significant change.

Absorption of antibody against extracellular virus. Absorption of rabbitpox antiserum with concentrated extracellular rabbitpox virus reduced the titre of neutralizing antibody against extracellular virus from 6000 to 160, but resulted in no significant fall in the titre against intracellular virus (Fig. 4).

Anti-comet titrations on absorbed antisera. The antisera absorbed with intracellular or extracellular virus in the above experiments were titrated for anti-comet activity (Table 2). Absorption of rabbitpox antiserum with intracellular virus did not affect its anti-comet titre, but absorption with extracellular virus eliminated detectable activity. The ability of
rabbitpox antiserum to prevent distant virus spread was associated, therefore, with the presence of antibody directed against extracellular virus and was unrelated to neutralizing activity against intracellular virus.

Table 2. Anti-comet titrations

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Absorbed with</th>
<th>Antiserum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbitpox</td>
<td>Nil</td>
<td>1/25   1/50 1/100 1/200 1/400 1/800 1/1600</td>
</tr>
<tr>
<td>Inactivated vaccinia</td>
<td>Nil</td>
<td>+      +      +      +      +      –      –</td>
</tr>
<tr>
<td>Rabbitpox</td>
<td>Intracellular virus</td>
<td>+      +      +      +      +      –      –</td>
</tr>
<tr>
<td>Rabbitpox</td>
<td>Extracellular virus</td>
<td>+      +      +      +      +      –      –</td>
</tr>
</tbody>
</table>

+, complete inhibition of distant virus spread.
- , incomplete or no inhibition of distant virus spread.

Fig. 5. Extracellular rabbitpox virus stained with phosphotungstate. The virus particles are enclosed in partial or complete envelopes.

Electron microscopy

Having demonstrated an antigenic difference between intracellular and extracellular virus, we looked for a morphological difference by electron microscopy. Intracellular virus was examined both as a crude suspension, from which only the larger cell debris had been removed by centrifugation, and after purification on a sucrose density gradient. Extra-
Intracellular and extracellular poxvirus

Intracellular virus was concentrated one hundred-fold by centrifugation, but was not further purified. Particles of extracellular virus were seen to be surrounded by well-defined envelopes, 5 to 10 nm. in thickness (Fig. 5). Envelopes were generally applied closely to their virus particles, but some separation was common. Although in some samples more than 90% of extracellular virus particles possessed at least a partial envelope, this structure was not seen in crude or purified preparations of intracellular virus.

Fig. 6. HeLa cell infected with rabbitpox virus and stained for virus antigen on the cell surface. The infected culture was incubated for 24 hr and stained, before fixation, with fluorescein-conjugated rabbitpox antiserum.

Fluorescent antibody staining of infected cells

Since it was possible that the envelope of extracellular virus was derived by budding through the host cell membrane, infected cells were tested for the presence of a surface antigen capable of reacting with rabbitpox antiserum but not with inactivated vaccinia antiserum. Cell cultures on coverslips were infected by the adsorption for \( \frac{1}{2} \) hr of 0.1 ml. rabbitpox virus of infectivity 10^7 p.f.u./ml. After incubation at 36° for 24 hr the cultures were treated, either fixed or unfixed, with fluorescein-conjugated rabbitpox or inactivated vaccinia globulin. In fixed cultures, the cytoplasmic virus antigen was stained by both types of globulin. In unfixed cultures, rabbitpox globulin produced a bright granular fluorescence on the surface of infected cells (Fig. 6), whereas inactivated vaccinia globulin gave little or
no fluorescence. The surface fluorescence produced by conjugated rabbitpox globulin was blocked by previous treatment of the cultures with unlabelled rabbitpox antiserum but not by pre-treatment with inactivated vaccinia antiserum.

DISCUSSION

Antiserum against live rabbitpox virus passively protects rabbits against lethal infection, but antiserum against inactivated vaccinia virus does not protect, despite an apparently high content of neutralizing antibody (Boulter, 1969; Boulter et al. 1971). The demonstration of a similar difference between the protective abilities of the two antisera in a tissue culture system, the 'anti-comet test', has allowed a closer examination of this phenomenon. The virus important for the spread of infection in an animal or tissue culture is extracellular, whereas the virus used in conventional neutralization tests is predominantly intracellular. These two forms of virus have now been shown to be antigenically distinct. The failure of inactivated vaccinia antiserum to prevent the spread of rabbitpox virus in tissue culture or in animals is associated with inability to neutralize extracellular virus. Rabbitpox antiserum, on the other hand, contains antibodies to both types of virus, and these antibodies can be removed separately by absorption. Anti-comet tests on absorbed sera confirmed that protection by rabbitpox antiserum is mediated by antibody against extracellular virus. These results are consistent with those obtained independently by Turner & Squires (1971).

Electron micrographs demonstrated the presence of envelopes around particles of extracellular virus. It is likely that the envelope contains the antigen specific to extracellular virus and is formed as the virus buds through the modified membrane of the host cell. This possibility was supported by the demonstration that the surfaces of infected cells reacted with fluorescein-conjugated rabbitpox antiserum but not with inactivated vaccinia antiserum.

A difficulty in the titration of neutralizing antibody against extracellular virus is that a variable proportion of extracellular virus resembles intracellular virus in being susceptible to neutralization by inactivated vaccinia antiserum. For use in neutralization tests, extracellular virus should therefore be grown in the presence of inactivated vaccinia antiserum or treated with this antiserum after harvest. The fraction of extracellular virus which can be neutralized by inactivated vaccinia antiserum may arise by escape of intracellular virus from cells without normal passage through the surface membrane, as by the complete rupture of some cells. Alternatively, it is possible that damage to the envelope of extracellular virus allows penetration of antibody directed against intracellular virus; this is supported by the presence, in some preparations, of an envelope on 90% of extracellular virus particles, although less than 50% of the virus infectivity resisted neutralization by inactivated vaccinia antiserum.

We conclude that antibody against extracellular virus is important for protection against poxvirus infections, whereas antibody against intracellular virus has little or no effect. The relevance of this to the use of inactivated poxvirus vaccines has been discussed by Boulter et al. (1971). A further implication is that the results of conventional neutralizing antibody titrations using intracellular virus may bear little relation to the immune status of an individual or to the therapeutic potency of an antiserum. For the assessment of protective antibody, the test virus in neutralization titrations should be extracellular virus treated with inactivated vaccinia antiserum. Alternatively, the examination of an antiserum for anti-comet activity may be a valid test.

We wish to thank Mr W. J. Harris for electron microscopy.
REFERENCES


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