Use of the 51Chromium Release Test to Demonstrate Antigenic Differences Between Extracellular and Intracellular Forms of Vaccinia Virus

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SUMMARY

Complement-dependent antibody lysis of vaccinia-infected cells was examined to demonstrate the antigenic differences between extracellular (ECV) and intracellular (ICV) forms of vaccinia virus. Cytolytic antibodies present in the antisera raised against ECV or live virus (LV) were completely removed by absorption with infected cell membranes or purified ECV but not with purified ICV. Absorption with infected cell membranes also abolished the neutralizing activity of ECV and LV antisera against ECV. On the other hand, antiserum against ICV did not contain cytolytic antibodies against vaccinia-infected cells, even though its neutralizing antibody titre against ICV was high. Moreover, both ECV and ICV antiserum neutralized a small proportion of the heterologous form of virus, despite using purified preparations of ECV and ICV, respectively, for raising these antiserum in rabbits. In contrast, the 51Cr release test only detected the antibodies against ECV and thus can be used to differentiate between the antibody activity of a serum against ECV and ICV.

INTRODUCTION

Two forms of pox viruses, extracellular and intracellular, may be separated on the basis of structural and antigenic differences (Boulter, 1969; Appleyard et al. 1971; Turner & Squires, 1971; Boulter & Appleyard, 1973). The extracellular form (ECV) differs from the intracellular form (ICV) in having a lipoprotein envelope which is acquired during release of virus from infected cells by the process of budding. It bands in a cesium chloride (CsCl) density gradient at 1.23 to 1.24 g/ml. On the other hand, ICV remains 'cell associated' and may be obtained by rupturing the infected cells. This form is heavier than ECV and bands at 1.27 to 1.28 g/ml in CsCl density gradient. Antigenically ECV differs from ICV in having specific antigen(s) on its envelope. The antibodies to these antigens prevent the spread of virus in monolayers of tissue cultures and inhibit the formation of secondary and tertiary plaques on the cell sheets (anti-comet activity). In addition, these antibodies protect the animals against a lethal challenge by live virus. In contrast, antibodies to ICV neither show anti-comet activity nor afford any protection to the animals against a lethal challenge by live virus. Recently, Payne & Norrby (1976) have demonstrated the presence of haemagglutinin activity in the purified preparations of ECV and not in ICV.

Complement-dependent antibody lysis of cells infected with herpes simplex virus has been used to demonstrate virus-specific antigens on the surface of infected cells (Roane & Roizman, 1964; Smith et al. 1972; Yang & Wentworth, 1972). Since ECV envelope antigens are also present on the surface of cells infected with pox viruses (Appleyard et al. 1971),
the present study was undertaken to demonstrate these antigens by complement-dependent antibody lysis of chromium-labelled vaccinia-infected cells (Cr release test). We have also shown that the lysis of infected cells as measured by Cr release can be utilized to assay the antibody directed against ECV.

METHODS

Cells and virus. A continuous line of African green monkey kidney cells (Vero) was employed for the virus production and all virus assays. Baby hamster kidney cells (BHK-21) were used for complement-dependent antibody lysis of vaccinia-infected cells. The Russian vaccine strain (EM-63) of vaccinia virus was used in this study. The stocks of ECV and ICV were raised according to the method described by Turner & Squires (1970) with minor modifications. Briefly, monolayers of Vero cells were infected with the virus at a m.o.i. of 0.1 p.f.u./cell and the virus was allowed to adsorb for 60 min at 37 °C. Thereafter, the cell sheet was washed twice with Hanks' basal salts solution and was covered with Eagle's minimum essential medium (MEM) without any serum. After incubation at 37 °C for 72 h the supernatant fluid was collected gently and clarified at 600 g for 20 min at 4 °C. The clear supernatant was then centrifuged at 10,000 g for 90 min and the pellet, which was resuspended in McIlvaine's phosphate buffer, consisted of ECV.

The infected cell sheet was washed once with phosphate-buffered saline (PBS, pH 7.2) without calcium and magnesium ions and was monodispersed by 0.02% versene. The cells were then lysed with distilled water at room temperature. The lysate was mixed with an equal volume of double strength Eagle's MEM and the pH was adjusted to 7.4. It was clarified at 800 g for 15 min and the supernatant containing ICV was purified by three cycles of differential centrifugation at 800 g and 20,000 g. Finally, the pellet was resuspended in McIlvaine's phosphate buffer and stored at -70 °C in small amounts.

The purity of ECV and ICV preparations was examined under Philips EM 300 electron microscope at 80 kV using the uranyl acetate staining technique. The ECV preparations showed enveloped particles while the ICV preparations consisted of only naked forms.

Immunization of rabbits. Four to 6 month-old rabbits were used for raising antisera. The virus preparations (ECV, titre ~5 × 10^5 p.f.u./ml and ICV, titre 2.3 × 10^5 p.f.u./ml) were inactivated with u.v. light for 5 min. One ml of each virus preparation mixed with an equal volume of Freund's complete adjuvant was given intramuscularly to each rabbit. Two additional injections of similar material were given at weekly intervals. Three weeks after the last injection the rabbits were bled and the sera were stored at -20 °C.

In addition, antiserum to live vaccinia virus was raised in rabbits by intradermal inoculation with 1 × 10^7 p.f.u./ml of live vaccinia virus from lyophilized vaccine vials. Three weeks later, at weekly intervals, the rabbits were given three intravenous injections of similar virus preparations. The blood was collected 21 days after the last injection and the sera were stored at -20 °C.

Absorption of antisera. Antisera raised in rabbits against ECV, ICV and live virus (designated as ECVS, ICVS and LVS, respectively) were absorbed with purified ICV prepared from BHK-21 cells infected with vaccinia virus at an m.o.i. of 5 p.f.u./cell. One ml of ICV preparation containing 6 × 10^5 p.f.u. was mixed with 5 ml of a 1:10 dilution of each antiserum and kept at 37 °C for 3 h with occasional shaking. At the end of the first and second hours, two amounts (1 ml each) of ICV preparations were added to the antiserum. Thereafter, the mixture was transferred to 4 °C for 18 h with constant shaking. The absorbed antiserum was then centrifuged at 20,000 g for 1 h to pellet the virus. The supernatant was separated and heat-inactivated at 56 °C for 30 min.

The antisera were also absorbed with the cell membranes of the infected BHK-21 cells. The membranes were prepared after lysing the cells with distilled water during the
Differentiation between ECV and ICV

preparation of ICV. The preparations were washed twice and tested for residual infectious virus before using for absorption purposes. Five ml of each antiserum diluted to 1:10 were absorbed with cell membranes from 6×10^7 infected BHK-21 cells. The procedure for absorption was similar to the one described above except that after absorption at 4°C, the antisera were clarified at 1500g. All antisera were also absorbed with cell membranes from uninfected BHK-21 cells.

Since large quantities of ECV needed for absorption of all antisera were difficult to obtain, only LVS was absorbed with ECV. Five ml of a 1:10 dilution of LVS was absorbed extensively with 3×10^7 p.f.u. of ECV, first at 37°C for 90 min with constant shaking, followed by 18 h at 4°C. The absorbed serum was centrifuged at 15000 g for 60 min to pellet the virus. The supernatant was heat-inactivated at 56°C for 30 min. The ECV-absorbed LVS was assayed for residual antibody activity only by the ^51Cr release test, whereas antisera absorbed with ICV and cell membranes were tested by all the serological tests described below.

^51Cr release test. The test was performed by a modification of the technique described by Smith et al. (1972). Briefly, confluent monolayers of BHK-21 cells grown in 100 ml milk dilution bottles were infected with vaccinia virus at an m.o.i. of 5 p.f.u./cell. After adsorption for 1 h at 37°C, the cells were covered with Eagle's MEM without serum. The cultures were incubated at 37°C, and 100 μCi of ^51Cr (as sodium chromate, Bhaba Atomic Research Centre, Bombay, India) was added aseptically to each bottle 2 h before harvesting. The labelled cells were harvested after 18 to 20 h incubation using 0.02% versene in buffered saline without calcium and magnesium ions. The monodispersed cells were washed five times with diluent (tris-buffered saline containing 2% heat-inactivated foetal calf serum) and were resuspended in 10 ml of diluent. The cells (of which 90 to 95% were viable by trypan blue exclusion) were enumerated and their concentration was adjusted to 5×10^5 viable cells/ml.

The antisera raised in rabbits were heat-inactivated and diluted from 1:10 to 1:640 in serial twofold steps. One-tenth ml of cells (5×10^4 cells) was mixed with an equal volume of the appropriate serum dilutions and incubated at 37°C for 1 h. Then 0.2 ml of fresh guinea-pig complement diluted to 1:10 was added to each tube and incubated for another 90 min at 37°C. The complement-mediated cytolysis was stopped by adding 2 ml of cold tris diluent to the tubes which were then centrifuged at 600 g for 3 min at 4°C. One ml of the supernatant was carefully transferred to another tube without disturbing the pellet and the transferred supernatant was counted for radioactivity on a Packard automatic gamma spectrometer (Packard Instruments). The controls for each test included: labelled cells only, which were frozen and thawed in distilled water (maximum release); labelled cells and guinea-pig complement but without antisera (spontaneous release) and labelled cells and antisera but no complement (serum toxicity). All dilutions and controls were run in duplicate and their counts per minute were averaged.

The cytolytic activity of the antiserum was expressed as percent specific ^51Cr release according to the formula described by Brunner et al. (1968).

Percent specific ^51Cr release is:

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\text{Percent specific } ^{51}\text{Cr release} = \frac{\text{maximum release} - \text{Spontaneous release}}{\text{maximum release} - \text{Spontaneous release}} \times 100.
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Neutralization test. The procedure described by Appleyard et al. (1971) was followed, with minor modifications. Virus suspensions containing 1000 p.f.u./ml of either ECV or ICV were added to an equal volume of doubling dilution of antisera and incubated at 37°C for 2 h. The mixture was assayed for residual infectivity on Vero cells. Fresh preparations of ECV were prepared whenever the test was performed.
Haemagglutination inhibition (HI) test. The method described in the pox viruses laboratory procedures (C.D.C. manual, 1968) was followed. Four units of haemagglutinin were mixed with equal volumes of different serum dilutions. After incubation for 1 h at 37 °C, 1% fowl erythrocytes were added and the mixture was further incubated for 1 h at room temperature. All the sera were absorbed with 50% fowl erythrocytes prior to the test.

RESULTS

Cytolysis of vaccinia-infected cells

The ability of antisera raised in rabbits to produce cytolysis of vaccinia infected BHK-21 cells in the presence of complement was examined initially. Only LVS and ECVS produced cytolysis (Fig. 1). The percentage of specific 51Cr release varied with serum concentration and 50% end-point titres of the cytolytic antibodies in LVS and ECVS were 1·92 and 2·10 log_{10}, respectively. The antiserum against ICV did not produce any lysis of infected BHK-21 cells. All the experiments were done four times and consistent results were obtained on all occasions. The coefficient of variation of antibody titres was about 6%.

The cytolysis of vaccinia-infected cells by ECVS and LVS was specific, as these sera failed to produce any significant lysis of either uninfected or HSV-infected 51Cr-labelled BHK-21 cells (data not shown). Moreover, pre-immunization sera from all the rabbits did not produce any lysis of vaccinia-infected cells (data not shown).

Effect of absorption on cytolysis

Absorption of LVS with either purified preparations of ECV or vaccinia-infected cell membranes removed the cytolytic antibodies completely (Fig. 2). Similarly, absorption of ECVS with membranes from vaccinia-infected BHK-21 cells abolished its cytolytic activity (Fig. 2). On the other hand, absorption with either purified ICV preparation or membranes from uninfected BHK-21 cells did not affect the cytolytic pattern of these antisera.
Differentiation between ECV and ICV

Fig. 3. Neutralization of extracellular virus by (a) LVS and (b) ECVS. ●, Unabsorbed LVS and ECVS; ▼, LVS and ECVS absorbed with ICV; ■, LVS and ECVS absorbed with infected cell membranes.

Fig. 4. Neutralization of intracellular virus by (a) LVS and (b) ECVS. ○, Unabsorbed LVS and ECVS; ▼, LVS and ECVS absorbed with ICV; ▽, LVS and ECVS absorbed with infected cell membranes.

Antibodies measured by neutralization test

The plaque reduction test was done to measure the neutralizing antibodies against ECV and ICV in the antisera before and after absorption with virus preparations and infected cell membranes. Neutralizing antibodies against ECV present in LVS and ECVS were completely absorbed by the infected cell membranes only and not by ICV (Fig. 3a, b). Unabsorbed LVS had neutralizing antibodies against ICV which were completely removed by purified ICV preparations and not by infected cell membranes (Fig. 4a). Antiserum raised against purified ECV had neutralizing antibody activity against ICV, although in low titres, and this was removed by absorption with ICV and not by infected cell membranes (Fig. 4b). Similarly, ICVS contained neutralizing antibodies which not only inhibited the plaques produced by ICV but also neutralized a small proportion of purified ECV preparation (Fig. 5a, b). The neutralizing activity of ICVS against ICV and ECV was completely
abolished by absorption with purified ICV preparations only and not with infected membranes.

**Haemagglutination inhibiting antibodies**

Antisera to live virus and ECV had HI antibodies (titres of 2.5 and 1.9 log₁₀, respectively) which were completely absorbed by infected cell membranes, while ICV absorption had no effect on HI antibody titres (Table 1). No HI antibodies were detected in ICVS.

**DISCUSSION**

The data presented in this study show that antiserum raised against inactivated ECV produced lysis of the cells infected with vaccinia virus in the presence of complement. This cytolytic activity was effectively removed by absorption of serum with membranes from infected cells. This suggested that similar antigenic determinants for cytotoxic antibodies
were present on ECV and membranes from vaccinia virus-infected cells. On the other hand, ICV seemed to lack these antigen(s) as absorption with this virus preparation did not abolish the cytolytic activity of ECVS. Moreover, ICVS failed to show any cytotoxic antibody against vaccinia-infected cells. This observation confirms the earlier findings that the two forms of vaccinia virus, ECV and ICV, differ antigenically from each other (Payne & Norrby, 1976; Prakash et al. 1977; Turner & Squires, 1971). This is further strengthened by the removal of cytolytic activity of LVS by purified ECV or membranes from infected cells.

We also observed that absorption of ECVS and LVS with infected cell membranes alone removed the neutralizing activity of these antisera against ECV. Since absorption with ICV did not affect the neutralizing antibody titres of these sera, it is evident that similar neutralizing antigenic determinants were present on ECV and membranes of vaccinia-infected cells, but not on ICV particles. However, it is not clear from the present data whether antigenic determinants for cytotoxic antibodies and neutralizing antibodies are the same.

The presence of similar antigenic determinants on ECV and the membranes of infected cells is quite expected. As ECV is released by budding through the infected cells, it gets enveloped by the cell membrane (Boulter & Appleyard, 1973). On the other hand, ICV remains cell associated and is devoid of the envelope. Evidently, the envelope is the only additional structure present in ECV and it accounts for the antigenic differences between the two forms of pox viruses (Boulter & Appleyard, 1973).

Removal of HI antibodies from antisera raised against ECV or live virus by the infected cell membranes suggests that haemagglutinating antigens (HA) are present on the membrane. Moreover, the presence of HI antibodies in ECVS and not in ICVS shows that HA are present in ECV only and not on ICV. In fact, a suspension of vaccinia-infected cell membranes (protein content 3 mg/ml) clarified after sonication at 20 kHz for 5 min had an HA titre of 1:32 (N. Balachandran & P. Seth, unpublished results). Similarly, a purified preparation of ECV containing $5 \times 10^4$ p.f.u./ml had an HA activity of 1:8. No HA activity was detected with purified ICV preparations. This finding agrees with those of Payne & Norrby (1976) who found 10% of HA activity in ECV preparations and none in ICV.

Three additional points are raised by the present study. Firstly, the ECVS had neutralizing antibodies against ICV, although this antiserum had been raised against inactivated purified suspension of ECV particles. These antibodies could only be removed by ICV preparations and not by infected cell membranes. Similar observations have been made recently by Prakash et al. (1977). This could perhaps be due to the presence of antibodies produced against other components of the virion besides the envelope which are common to both the forms of vaccinia virus.

Secondly, antiseraum raised against a purified preparation of ICV also neutralized a small proportion of ECV. This neutralizing ability of the ICVS could be effectively removed by an ICV preparation but not by infected cell membrane. The presence of a small proportion of contaminating ICV particles in the ECV preparations used in the neutralization test as a probable reason for partial neutralization of ECV by ICVS seems to be unlikely as we used only those preparations of ECV which had been checked for purity by electron microscopic examination. However, as noted by Appleyard et al. (1971), it is possible that a break in the envelopes of a small proportion of ECV particles could have made them susceptible to neutralization by antibodies against ICV.

Finally, the $^{51}$Cr release test appears to be the serological test which may be used in future to assess the immunity against pox viruses, since it measures antibodies against ECV only. Extracellular virus is responsible for the generalized form of the disease which can be prevented by antibodies against ECV (Boulter & Appleyard, 1973). Other serological tests, namely, a neutralization test using purified ECV and an 'anti-comet' test are, no doubt, good
but are cumbersome and take 72 to 96 h for completion. Moreover, as noted above, partial neutralization of purified ECV preparation by ICVS may occasionally make the interpretations of the results of neutralization tests difficult. On the other hand, the \( ^{51} \text{Cr} \) release test is very simple and specific and thus can be used more successfully to differentiate between the antibody activity against ECV and ICV than the neutralization test.

Complement-dependent antibody lysis of infected cells may play a major role in the host defence mechanisms against virus infections (Notkins, 1971; Porter, 1971; Rawls & Tompkins, 1975). In addition it has been suggested that in pox viruses, antibody-dependent cell-mediated cytotoxicity may help in restricting the spread of virus (Perrin et al. 1977; Møller-Larsen & Haahr, 1978). Preliminary data from our laboratory have shown that cytotoxic lymphocytes are generated only by the live and inactivated ECV and not by the inactivated ICV. The appearance of cytotoxic lymphocytes precedes cytotoxic antibodies by at least 4 days in rabbits (our unpublished results). Therefore, the protection in pox virus infection appears to be due to a complex interplay between lymphocyte, antibody and complement on one hand and the virus-specific membrane antigens on the other.

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REFERENCES


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