The Influence of DEAE-dextran on Plain and Synergistic Plaque Formation by Two Poxviruses

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SUMMARY

The plaque titre of rabbitpox and early passage vaccinia viruses was enhanced by diethylaminoethyl-dextran. Both viruses have low initial plaquing efficiency. Later passage vaccinia virus of high plaquing efficiency was little enhanced either before or after exposure to u.v., X or γ rays or to heat at 56°. The dextran made an insignificant improvement in adsorption of the virus particles to the cells. It formed flat, round bodies visible in the electron microscope but these were not found to form complexes with the virus particles nor to induce them to aggregate. This eliminated synergistic infection by aggregation of virus particles as a mechanism for plaque enhancement by dextran but suggested a protective action of the dextran particles upon freshly uncoated DNA within the cell vesicle.

INTRODUCTION

The plaque titre of certain infectious preparations of nucleic acid, both RNA and DNA, from animal viruses has been greatly enhanced (up to 100,000-fold) by diethylaminoethyl-dextran (DEAE-D) (Vaheri & Pagano, 1965; Pagano & Vaheri, 1965; Pagano, McCutchan & Vaheri, 1967; McCutchan & Pagano, 1968; Bachrach, 1966). Addition of this reagent to inocula of whole virus particles has enhanced the plaque titre of some and reduced that of others but the changes have been small compared with those obtained with the nucleic acids. In fact these reported changes are comparable in magnitude with those that we have seen in some preparations of poxvirus when particles aggregate and disaggregate. We have, therefore, suspected that the reported effects of dextran upon the plaquing efficiency of whole virus particles might be another manifestation of multiplicity activation (Dalton, Kim & Sharp, 1967) or synergistic infection (Kim & Sharp, 1968a) and thus attributable to changes in particle aggregation.

In this paper we report experiments with dextran on rabbitpox and early L-cell-passage vaccinia virus, both of which exhibit multiplicity activation, and on high-passage vaccinia virus which does not. Analyses of virus-particle aggregation were made by electron microscopy on all inocula used for the plaque assays.

METHODS

The vaccinia virus used in this work was the WR (mouse neurotropic) strain from the ATCC. This virus, passed 33 times or less in L cells, is called early passage. Virus of
passage 194 and beyond are late passage. Culture methods, plaquing and quantitative electron microscopy (particle counting and analyses of aggregation) have been previously described in detail (Sharp & Kim, 1966). The rabbitpox virus is RPU+ passed 3 to 5 times in L cells. Dextran was obtained from Pharmacia, Uppsala, Sweden, (mol. wt = 2 × 10⁶). In expressions of plaquing efficiency, frequent use will be made of the term active unit. If aggregation exists in a virus preparation not all the particles will be free to act independently as potential plaque forming units. The number of mechanically free or active units will then be just the number of single particles plus the number of aggregates, irrespective of size, that are counted by electron microscopy. Plaquing efficiency is the ratio, p.f.u./active units.

The degree of aggregation has been manipulated upward, when necessary, by sedimenting the particles and gently resuspending them in the same fluid. Decreases in aggregation were made by brief applications of 20 kcyg. waves from a Branson Sonifier.

Adsorption of virus to monolayers was measured by putting 3 × 10⁷ virus particles in the usual volume (0.1 ml.) of inoculum on cell monolayers in bottles containing 3 × 10⁶ L cells. After initial spreading and periodic tipping during the 3 hr adsorption period the unadsorbed virus was washed off with 3 ml. of phosphate buffered saline. The virus particle content of wash fluids was determined directly by counting in the electron microscope.

Partial inactivation of virus with soft X-rays and with ¹³⁷Cs γ-rays was the same as previously described (Kim & Sharp, 1968b).

RESULTS

Degree of enhancement

Dextran in concentrations ranging from 3 to 1000 µg./ml. was added to virus inocula for plaque titration on monolayers of L cells. With rabbitpox virus the plaque number was increased fourfold at 3 µg./ml. and about 12-fold at all concentrations from 5 to 1000 µg./ml. Vaccinia virus of 33rd L cell passage was enhanced in titre about 3.5-fold in the range 5 to 30 µg./ml. but the degree of enhancement decreased at higher concentrations (Fig. 1); plaque size seems not to be changed by dextran.

Six independent experiments with well adapted (194 to 199th L-cell passage) vaccinia virus gave only a 1½ virus average enhancement of plaque titre by dextran. Without dextran, this virus produced 1 plaque for about 15 active units of virus inoculated. Vaccinia of 33rd passage gave 1 plaque for 30 active units and rabbitpox gave 1 for 200 active units. The increase in titre by dextran is apparently related in some inverse manner with the initial plaquing efficiencies of these three viruses. Since previous work has shown that the titre of suspensions of both rabbitpox (Kim & Sharp, 1966a) and early passage vaccinia (Dalton et al. 1967) can be increased by aggregating the particles, we have examined the effect of DEAE-dextran on the physical state of the virus suspensions.

Effect of particle aggregation

A suspension containing 2 × 10⁹ virus particles/ml. of 33rd passage vaccinia with 85% single particles was diluted with an equal volume of phosphate buffered saline containing 600 µg./ml. dextran. A similar dilution was made with phosphate buffered
Plaques of poxvirus with DEAE-dextran

saline alone and both were held at room temperature for 2½ hr. Under these conditions slow reaggregation is regularly observed. This can be seen as a change in slope in the log.-log. group frequency chart (Fig. 2). The percentage of single particles in the control dilution decreased to 46 in 2½ hr while the frequencies of the various aggregates

Fig. 1. Enhancement of plaque titre of rabbitpox (●—●) and early passage vaccinia virus (○—○) by DEAE-dextran.

Fig. 2. Group frequency vs. group size plotted from electron micrographs. They show the change in particle aggregation (reaggregation) of a well dispersed suspension of vaccinia during 2½ hr at room temperature with and without DEAE-dextran.
increased as expected (Kim & Sharp, 1966b). Dextran did not increase the rate of aggregation. In fact there was slightly less reaggregation in the dextran sample.

Possibly aggregates existing at a dextran concentration of 300 μg./ml. could have been dispersed in the 1:100 dilution that was necessary before sedimenting the virus upon the agar surface for pseudoreplication and electron microscopy. However, we have found that poxvirus once aggregated is difficult to disperse. It seems quite unlikely that any high degree of aggregation could have existed at 300 μg./ml. dextran and that this was reduced by simple dilution to almost exactly the same level as that in the control (Fig. 2).

**Material (bodies) sedimented from dextran suspensions**

Electron micrographs for analysis of aggregation have all been made by sedimenting the virus upon agar and examining collodion pseudoreplicas from this surface. In all cases involving dextran there has been a deposit of approximately round, exceedingly flat bodies in the pictures. At 3 μg./ml. dextran (Pl. 1a) these are small. Many of them are about the size of a poxvirus particle and they are few in number. At 10 μg./ml. dextran the particle size is much greater and irregularities are more obvious (Pl. 1b). In Pl. 1c, at 100 μg./ml. dextran virus particles were added and they are clearly visible because of their greater contrast although the dextran deposits now practically cover the picture.

Small particulate material of high electron scattering power can be seen (Pl. 1c) attached to the dextran deposits but the virus particles have never been observed in any particular configuration relative to them either in this or many other pictures covering a wide range of concentrations. If the dextran has no effect upon the distribution of the virus in the pictures it is unlikely that they determine it in suspension.

**Enhancement of titre of partially inactivated virus**

The results presented so far might imply that enhancement of plaque titre would be expected with all virus preparations with low plaquing efficiency. This is not the case. Passage 194 vaccinia virus was treated 20 sec. at an incident energy flux of 33 μw/cm.² with essentially monochromatic 2537 Å rays from a low pressure mercury lamp (Sharp & Kim, 1966). One sample was quite thoroughly dispersed (80% singles); another was aggregated (31% singles). Both were titrated with and without 100 μg./ml. dextran. The plaquing efficiency of the more dispersed sample was reduced 60-fold by the rays while that of the aggregated one fell only threefold. This is consistent with previous experience with irradiated virus (Kim & Sharp, 1968a, b) showing a high degree of multiplicity reactivation. The plaquing efficiency of these samples was only slightly enhanced (1.4-fold) in the presence of 100 μg./ml. dextran. This is indistinguishable from the effect on the fresh virus and the dextran has not altered the degree of multiplicity reactivation which is exceedingly sensitive to particle aggregation.

Treatment of passage 194 virus with unfiltered 50 kv X-rays from a tube with a beryllium window, with heat (56°) or with 137Cs γ-rays yielded virus comparably reduced in plaque titre and equally incapable of substantial enhancement by dextran.
In the sedimentation process for preparing poxviruses for count and aggregation analysis, small amorphous bodies are deposited from dilute dextran suspension. (a) 3 μg./ml.; (b) 10 μg./ml.; (c) 100 μg./ml. mixed with a few vaccinia virus particles.

K. S. KIM AND D. G. SHARP

(Facing p. 508)
Enhancement and multiplicity activation

Rabbitpox virus (5th L cell passage) was titrated with and without dextran in two states of aggregation. Without dextran the aggregated sample (31% singles) was $12 \times$ higher in plaquing efficiency than the dispersed sample (75% singles). Enhancement by dextran was greatest for the dispersed sample (Fig. 3). It was much greater than that seen with partially inactivated vaccinia virus and the slope of the graph shows that some synergistic response remains after dextran enhancement of the titre.

Fig. 3. Plaquing efficiency of rabbitpox virus showing synergistic effect of particle aggregation (lower line). Enhancement by DEAE-dextran (upper line) reduces but does not eliminate the multiplicity activation.

Fig. 4. Yield of rabbitpox virus particles from cultures inoculated at low multiplicity (lower line). Similar cultures receiving the same inoculum with DEAE-dextran (upper line).

Growth of virus

In order to observe the enhancing effect of dextran on the cell-infecting power of the virus quite apart from plaque formation, growth experiments were made. Cultures of L cells were inoculated by sedimentation with rabbitpox virus at several multiplicities to determine a value such that less than half the maximum yield for the cells would occur in 6 days. Such a curve is the lower one of Fig. 4. The upper curve was produced in cultures that received the same multiplicity of virus together with 10 µg./ml. dextran. Dextran-bearing supernatant fluid was removed immediately after the virus was sedimented upon the cells and fresh growth medium was added. Growth was detectable by particle count 24 hr sooner and virus yield remained two- to sixfold higher in the cultures inoculated in the presence of dextran. The effect is quite like that expected from a larger inoculum.

Miscellaneous observations

Heparin, which is reported to counteract the enhancing effect of dextran on the plaque titre of some infectious nucleic acids (Pagano et al. 1967), was used in combination with dextran for plaque titration of rabbitpox virus. Inocula (Table 1) contained several combinations of heparin and dextran. From these and appropriate controls it appears that heparin does indeed counteract the enhancing effect of dextran. In fact,
1000 μg. heparin nullified the effect of 100 μg. dextran. Only at concentrations of 100 μg. and less did 100 μg. dextran produce titres greater than controls. Heparin alone (1000 μg./ml.) reduced the titre to 1/10 of the control value.

Maximum enhancing effect was observed when dextran and virus were mixed and applied immediately to cell monolayers. Mixtures that were allowed to stand at room temperature 0, 10, 30 and 60 min. (100 μg. dextran per ml.) before inoculation gave slowly falling enhancement factors (6.9-6.1-4.4- and 4.1-fold). No enhancement in the number of plaques was observed when dextran was used in the overlay after adsorption of the virus.

### Table 1. Combined effects of dextran and heparin on plaque titre of rabbitpox virus

<table>
<thead>
<tr>
<th>Condition</th>
<th>p.f.u./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbitpox control virus*</td>
<td>1800</td>
</tr>
<tr>
<td>Rabbitpox + 100 g./ml. dextran</td>
<td>9300</td>
</tr>
<tr>
<td>Rabbitpox + 100 g./ml. dextran + 1000 μg./ml. heparin</td>
<td>201</td>
</tr>
<tr>
<td>Rabbitpox + 100 g./ml. dextran + 100 μg./ml. heparin</td>
<td>2060</td>
</tr>
<tr>
<td>Rabbitpox + 100 g./ml. dextran + 10 μg./ml. heparin</td>
<td>3240</td>
</tr>
<tr>
<td>Rabbitpox with no dextran and 1000 μg./ml. heparin</td>
<td>106</td>
</tr>
</tbody>
</table>

* 78% single particles in all samples.

### Table 2. Adsorption of virus to L cell monolayers

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dextran (No/L)</th>
<th>Dextran (10 μg./ml/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>Expt 2</td>
<td>99</td>
<td>97</td>
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<tr>
<td>Expt 3</td>
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<td>—</td>
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<tr>
<td>Averages</td>
<td>87</td>
<td>99</td>
</tr>
<tr>
<td>Rabbitpox</td>
<td></td>
<td></td>
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<tr>
<td>Expt 1</td>
<td>79</td>
<td>99</td>
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<td>Expt 2</td>
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<tr>
<td>Expt 3</td>
<td>97</td>
<td>—</td>
</tr>
<tr>
<td>Averages</td>
<td>76</td>
<td>99</td>
</tr>
</tbody>
</table>

Adsorption of the virus to monolayers of L cells was slightly improved in the presence of dextran. Table 2 shows the improvement with vaccinia virus to be barely within the range of detectability. After the usual 3 hr adsorption period an average of 12% of the particles inoculated without dextran were found, by direct count, in the wash fluid; 88% remained on the monolayer. With rabbitpox virus the average adsorbed was 76% with no bottles showing less than 50%. With dextran (10 μg./ml.) practically all the virus was adsorbed. Still, the degree of enhancement achieved through improved adsorption was very slight.

### DISCUSSION

Whenever preparations of poxvirus have been examined critically in the electron microscope, aggregation has been observed (Kim & Sharp, 1966b). Complete dispersal of these aggregates is difficult to achieve without treatment of the virus with enzymes or other reagents that are likely to alter their reaction, at least the early phases of their
Plaquing of poxvirus with DEAE-dextran

contact with host cells. Only in very dilute virus preparations (about $10^8$ virus particles/ml.) have suspensions approaching 100% single particles been obtained (Galasso & Sharp, 1964). Ordinary lysates of cells infected by well adapted (high passage) vaccinia virus are regularly enhanced three- to fivefold in plaque titre by treatment with sonic or ultrasonic waves which increase the observed fraction of single particles from roughly 15 to 85%. Irradiated or otherwise damaged virus may produce plaques mainly by multiplicity reactivation, even at low average input multiplicity, by virtue of synergistic infection (Kim & Sharp, 1968a, b). Rabbitpox as well as certain early passage preparations of vaccinia virus also exhibit synergistic infection through clumps which so closely resembles multiplicity reactivation that it has been called multiplicity activation. Virus preparations exhibiting either multiplicity reactivation or multiplicity activation are very low in plaquing efficiency when dispersed but in the aggregated state their efficiency may be increased 100-fold or more. We have, therefore, carefully observed the rate of reaggregation of well dispersed virus suspensions in critical concentrations such that it can be monitored by a series of electron microscopic analyses. Dextran, present in plaque-enhancing concentrations, had no effect on the reaggregation rate.

The particles that appear in electron micrographs of dextran solutions are very flat, apparently amorphous round bodies of diameters ranging from less to more than virus size. Their size and number is related to dextran concentration. In concentrations of plaque-enhancing significance they are present and it seems likely that they enter cell vesicles along with virus particles. There is, however, nothing revealed by many electron micrographs that suggests any form of attachment between dextran and virus particles in suspension.

Adsorption measurements, based upon direct particle count of input and wash fluids, show a slight improvement in the presence of dextran but this is insignificant compared with its enhancement in plaque titre of the rabbitpox and the early passage vaccinia virus preparations. These latter viruses have low plaquing efficiency. They exhibit multiplicity activation. Nevertheless high passage (high efficiency) virus reduced by irradiation with u.v. or soft X-rays to comparable plaquing efficiency is not similarly enhanced by dextran. Thus dextran makes a distinction between multiplicity activation and multiplicity reactivation. Apparently the mechanism of group synergistic infection is not identical for multiplicity activation and multiplicity reactivation although these experiments do not reveal the nature of this difference.

Enhancement of the plaque titre of the multiplicity activation viruses is not sufficient to bring them up to the efficiency level of well adapted vaccinia virus (1 p.f.u. per 10 virus particles). In the enhanced condition there is still some evidence of synergism in aggregated preparations.

We are led in conclusion to recall that dextran has exhibited a marked stabilizing effect upon the infectivity of some purified nucleic acids (Pagano et al. 1967). The particles of dextran, being comparable in size with the virus particles, are probably present at the time of uncoating of the virus in the L cell vesicles. Viruses of low plaquing efficiency, in particular those that show multiplicity activation, may owe all or a part of their inefficiency to an increased vulnerability to cellular DNases acting in the interval between uncoating and transcription. Any delay could have this effect. Protective action of the dextran during this interval could account for the plaque enhancement of these particular preparations of whole poxviruses.
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REFERENCES


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