Antigens from Vaccinia Virus Particles

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SUMMARY

Soluble material extracted from purified vaccinia virus particles produced eight precipitin lines with antiserum by immunodiffusion. The material contained about 20 % of the viral nitrogen and was obtained by extraction with alkaline buffers up to pH 10.5, by prolonged autolysis or, more conveniently, by digestion with trypsin. It appeared to originate mainly from the virus surface but, as it also contained about 5 % of the viral DNA and a small proportion of the virus particles were converted to ghosts, some of the precipitin lines probably represented internal components of the virus. When more material (up to 75 % of the viral-N) was extracted no further precipitin lines were detected but some immunospecificities may have been destroyed. Serial extractions at increasing alkaline pH values separated the antigens from most of the viral DNA and indicated that the 'nucleoprotein antigen' of earlier workers was probably an artifact.

INTRODUCTION

The difficulty in studying the constituents of vaccinia virus was considered by Smadel & Hoagland (1942) as due to 'its extreme insolubility in all but the most drastic reagents'; 0.04 N-NaOH at 56° dissolved about half the weight of purified virus particles. The extracted material, which included the viral DNA, formed precipitates with vaccinia virus antiserum and was named 'nucleoprotein antigen' (Smadel, Rivers & Hoagland, 1942). No other method of obtaining antigens in solution from the virus particles has been reported apart from some early observations by Craigie & Wishart (1936), who detected that small amounts of antigen slowly dissociated from the particles in suspension. The effect of proteolytic enzymes was investigated by Hoagland, Ward, Smadel & Rivers (1940). Pepsin and papain partially digested the virus but trypsin, chymotrypsin and ficin did not. Later, Dumbell, Downie & Valentine (1957) noted that trypsin could decrease the infectivity of vaccinia virus.

The relatively large size and complex morphology of vaccinia virus suggest the possibility of disrupting the particles by mechanical methods. We have subjected virus suspensions to prolonged ultrasonic treatment, repeated freezing and thawing, grinding at -60°, treatment in the Hughes press and osmotic shock. As with earlier attempts by other workers, we could detect little or no effect on the virus and no antigens were released. Thus it appeared necessary to attack the virus with chemical reagents and to find conditions which caused minimal loss of antigen specificity. The principal chemical constituents of the virus are protein,

DNA and lipid. Small amounts of other substances reported in the early chemical investigations (Smadel & Hoagland, 1942) are probably not true components of the virus (Zwartouw, 1964).

METHODS

Virus purification. Vaccinia virus was obtained from the skin of infected rabbits and purified by centrifugation in a sucrose density gradient as described by Zwartouw, Westwood & Appleyard (1962). Samples of purified virus were stored as frozen aqueous suspensions at -80° .

Assay methods. Nitrogen, DNA and virus infectivity were determined as described previously (Zwartouw, 1964).

Immunodiffusion. For most tests the double-diffusion technique of Ouchterlony (1948) was used with a 2 mm. depth of gel prepared from 1% Ion-agar (Oxoid) in 0.9% NaCl solution. Reactant wells were 7 mm. diameter with 14 mm. between centres. Precipitin lines were recorded after 4 days at room temperature. The more sensitive microtechnique of Crowle (1958) was used to detect the maximum number of precipitin lines.

Antiserum. After an initial dermal vaccination, rabbits were hyperimmunized with four intravenous injections of 5×10^9 pock-forming units of vaccinia virus. The sera from six rabbits were pooled.

General extraction procedure. Purified virus was suspended in the required reagents so that the final virus concentration was 1 mg. (dry weight)/ml. After treatment, a sample was removed for infectivity assay and then the mixture centrifuged at 30,000 g for 15 min. to obtain a clear extract. The extract was tested for precipitin antigens by immunodiffusion and the amounts of viral-nitrogen (viral-N), and DNA determined and expressed as a percentage of the total viral-N or DNA.

Electron microscopy. Specimens were negatively stained with potassium phosphotungstate solution (0.2%, pH 6.8) as described in the text and examined in a Siemens Elmiskop 1 electron microscope operating at 80 kV.

RESULTS

Alkaline extraction

An extract was prepared by treating virus with 0.04 N-NaOH for 15 min. at 56° as described by Smadel *et al.* (1942) for the preparation of 'nucleoprotein antigen'. The extract contained all the viral DNA and about 60% of the total-N but produced only faint precipitin lines with antiserum in gel diffusion tests. Extracts prepared at less alkaline pH values and lower temperatures produced stronger precipitin lines although they contained smaller amounts of the viral material. Therefore, attempts were made to obtain the best yield of precipitins by first extracting at pH 9 and then at increasingly alkaline pH values. The material remaining insoluble after each extraction was collected by centrifugation and extracted at the next higher pH value of the series. Each extraction was carried out for 30 min. at 37° with 0.1 M- Na_2HPO_4 and sufficient NaOH to give the required pH value. For the final extraction at pH 12.8, 0.1 N-NaOH was used. Table 1 shows the amounts of material extracted from the virus and the precipitin lines produced by the extracts. Similar results were obtained with 0.1 M-glycine + NaOH buffer, but this was less convenient for measuring the extracted viral-N.

Extracts 1–4 (pH 9–10·5, Table 1) contained most of the precipitins and 20 % of the viral-N. Extracts 6 and 7 (pH 11·5–12·0) contained 81 % of the N and 71 % of the viral-DNA but did not produce any precipitin lines. Although a small amount of DNA was obtained in the first two extracts, possibly from virus particles with defective or damaged outer structures, the precipitins were clearly separated from the main part of the DNA.

Comparison by gel diffusion indicated that the weak precipitin lines produced by ‘nucleoprotein antigen’ extract were also present in the lines produced by the pH 9–10·5 extracts. This was confirmed when the pH 10·5 extract, in which DNA was not detected, was treated with NaOH in the way used for ‘nucleoprotein antigen’ extraction. The precipitin line formation was considerably weakened and the residual weak reaction appeared similar to that of ‘nucleoprotein antigen’.

Table 1. *Serial alkaline extraction of vaccinia virus*

Extract no.	pH	Soluble-N (% of total-N)	Soluble DNA (% of total DNA)	Formation of precipitin lines
1	9·0	5	7	++
2	9·5	4	2	++
3	10·0	3	0	+
4	10·5	8	0	++
5	11·0	12	6	+
6	11·5	22	35	—
7	12·0	9	36	—
8	12·5	3	7	—
9	12·8	4	0	—
Residue		26	0	

Table 2. *Tryptic digestion of vaccinia virus*

Digest no.	Trypsin conc. (%)	Infectivity (% of original)	Soluble-N* (% of total-N)	Formation of precipitin lines
1	0	109	3	Trace
2	0·00001	119	6	+
3	0·0001	122	10	++
4	0·001	30	19	+++
5	0·01	9	23	++
6	0·1	0·2	22	++

* After correction for trypsin content.

Digestion with proteases

Several proteolytic enzymes were tested at 0·01 % for their ability to liberate antigens from 0·1 % (dry weight) virus suspensions. Digestion with pepsin at pH 2·5 did not produce any material which formed precipitin lines by immunodiffusion. Trypsin, chymotrypsin, papain and ficin, enzymes which could be used at pH 7–8, all liberated material which formed similar precipitin lines; trypsin was selected for further study. Digestion was carried out with a range of different concentrations of crystallized trypsin (Armour & Co. Ltd.) in 0·01 M-phosphate at pH 8 for 3 hr at 37°. Table 2 shows the amounts of viral-N obtained in solution and the precipitin lines produced by the soluble materials. Both intensity of precipitin lines and amount of N released increased with the trypsin concentration

up to 0.001 % trypsin. Higher concentrations released only a little more N and caused some loss of the precipitin lines. At the lower trypsin concentrations the virus infectivity was apparently enhanced (Table 2) and in a few experiments it was increased to 150 % of the original infectivity titre. This was most likely due to the separation of virus particles from aggregates when the enzyme started to attack the surface of the particles. The higher trypsin concentrations caused loss of infectivity.

With 0.001 % trypsin similar results were obtained when the pH was varied between 7.7 and 9.0 but slightly less material was released between 7.0 and 7.4. A second digestion of the insoluble residue with trypsin released only a further 3 % of the total-N. As a standard procedure for obtaining the precipitin antigens, 0.1 % (dry weight) of virus in 0.01 M-sodium phosphate at pH 8.0 was incubated with 0.001 % trypsin for 3 hr at 37°. The material obtained in solution contained about 20 % of the total viral-N and small amounts of DNA (3–7 % of the total). The latter may have originated from damaged virus particles as suggested for the similar amounts of DNA extracted with alkaline buffer at pH 9–9.5.

Table 3. *Autolysis of vaccinia virus at pH 7.5 and 37°*

Sample no.	Time at 37° (hr)	Infectivity (% of original)	Soluble-N (% of total-N)	Formation of precipitin lines
1	4	107	4	Trace
2	8	110	6	+
3	16	102	8.5	+
4	24	93	9.5	++
5	48	87	12	+++
6	72	52	13	++++
7	144	14	15	++++

Autolysis of virus suspensions

Control samples of virus incubated at 37° for 3 hr without trypsin released a small amount of material which produced weak precipitin lines (see Table 2). Prolonged incubation of virus suspended in dilute buffer at pH 7.5–8.0 with 0.1 % sodium azide, to prevent growth of any contaminating organisms, resulted in a slow autolysis. The intensity of the precipitin lines produced increased with the time of incubation up to 72 hr when 13 % of the viral-N was in solution. Incubation for an additional 72 hr released only a further 2 % of N with no detectable increase in the precipitin lines produced (Table 3). The virus infectivity usually decreased slowly during the autolysis, but in some experiments almost no loss was apparent up to 72 hr. This was probably due to disaggregation of virus which masked any loss of infectivity.

Number of precipitin lines detected

Comparison of the precipitin lines produced by the materials released from virus with alkaline buffer up to pH 10.5, tryptic digestion or autolysis showed that they were similar. Some immunodiffusion patterns suggested the presence of six precipitin lines but usually the components did not separate clearly. The line patterns were not improved by altering the distance between reactant wells, adjusting the agar concentration or including buffer in the gel. However, with the more sensitive micro-immunodiffusion method of Crowle (1958) eight precipitin lines were detected.

Morphological changes accompanying the extraction of antigens

Some evidence which indicated that trypsin digested a superficial layer of protein from the surface of vaccinia virus has already been reported (Westwood, Harris, Zwartouw, Titmuss & Appleyard, 1964). Virus negatively stained with phosphotungstic acid (PTA) usually appeared to have a surface consisting of coiled threads. Digestion with trypsin had little effect on the thread layer although it allowed some increased penetration of PTA into the virus. More definite evidence for the digestion of a superficial layer external to the thread layer has been obtained as follows. Untreated virus was allowed to dry on a carbon film and negatively stained by allowing fine droplets ($3-5\mu$ diameter) of PTA solution to fall on to the particles. In areas where the PTA solution dried rapidly so that penetration of the stain was prevented, virus particles had a relatively smooth appearance and the thread layer was not revealed (Pl. 1, fig. 1). When about 20% of the viral-N had been dissolved by digestion with trypsin, no superficial smooth layer could be detected by the same staining technique and the surface of the virus appeared to consist of the thread layer (Pl. 1, fig. 2). Digestion with trypsin also converted a small proportion of the virus particles to empty ghosts. These particles presumably accounted for the 3-7% of the total viral-DNA obtained in the soluble material. Similar ghosts (Pl. 1, fig. 3) were obtained from all virus particles after the serial extraction with alkaline buffers. This final residue, insoluble in 0.1 N-NaOH, contained 26% of the total-N and no DNA.

Attempts to extract further antigens

The extracted material which formed precipitin lines represented only 20% of the total viral-N. Further material dissolved with alkaline buffer above pH 10.5 did not form precipitin lines so some other methods of extraction were examined. The small amounts of DNA obtained with the precipitin components suggested that a defective fraction of the virus particles released internal components and attempts were made to increase the damaged or defective fraction. However, no further precipitin components could be extracted by tryptic digestion or with alkaline buffer from freeze-dried virus (which had lost 90% of its infectivity on drying), virus treated by ultrasonic vibration in anhydrous suspension (freeze-dried virus suspended in carbon tetrachloride), or virus treated with 0.1 N-HCl or 0.1 N-NaOH for 3 min. at 0°.

Lipid was removed from virus by adding a 1% (dry weight) suspension to 100 volumes of acetone at -60° and the treated virus was recovered by centrifugation. The weight of lipid obtained after evaporating the acetone (5% of the original virus weight) showed that extraction of the lipid was almost complete. Although the lipid-free virus was not more soluble in alkaline buffer, it was digested to a greater extent by trypsin. The soluble digest contained 43% of the viral-N but the pattern of precipitin lines produced was not more intense and did not appear to contain any different components when compared with the digest from untreated virus. A similar result was obtained when 75% of the viral-N was dissolved by tryptic digestion of lipid-free virus which had been treated with 2-mercaptoethanol (2% for 1 hr at 20°).

After digestion of native virus with trypsin, 0.1 M-KCN (pH 11) at 37° for 1 hr extracted a further 42 % of the viral-N compared with 16 % extracted by 0.1 M- $\text{Na}_2\text{CO}_3 + \text{NaHCO}_3$ at the same pH. The materials extracted by either reagent appeared to contain a little of the same precipitin components which had already been obtained with trypsin.

DISCUSSION

The earlier observation of Craigie & Wishart (1936) that antigen was slowly released from vaccinia virus suspensions has been confirmed. At 37° the material released contains up to 15 % of the viral-N after 144 hr. Similar material was obtained more conveniently by suitable digestion with trypsin, which rapidly released about 20 % of the viral-N. Serial extraction with alkaline buffers up to pH 10.5 also dissolved the same material from the virus. However, extraction above pH 10.5 dissolved the viral-DNA with further amounts of protein which did not produce precipitin lines. Thus the 'nucleoprotein antigen' extracted with NaOH by Smadel *et al.* (1942) was probably a mixture of nucleic acid and protein with partially degraded precipitin components.

Eight precipitin lines were produced by the extracted material, although only about 20 % of the viral-N was dissolved. When more than 20 % was obtained in solution no further precipitin lines were detected but some antigens may have been destroyed. Indirect evidence for one further antigenic component of the virus is reported in the accompanying paper (Westwood, Zwartouw, Appleyard & Titmuss, 1964), which describes a comparison of the virus particle antigens with the soluble antigens found in vaccinia virus infected tissue. Most of the immunospecific material appeared to be extracted from a surface layer of the virus. However, the conversion of a small proportion of the virus particles to empty ghosts by tryptic digestion and the presence of about 5 % of the total viral-DNA in the soluble material indicated that some virus particles were completely dissolved except for the residual ghosts. Thus it seems likely that some of the eight precipitin lines represented internal components of the virus. Some experiments suggested that the surface layer could be dissolved away to leave virus which was still infective. However, the results were inconclusive since partial loss of infectivity might have been masked by the increased infectivity due to disaggregation of virus. Some observations of Morgan, Rifkind & Rose (1962) indicate a definite surface layer of antigens on vaccinia virus. These authors found that antibody labelled with ferritin combined with the surface of mature vaccinia virus but not with immature virus. The surface layer of antigens appears to be present as an envelope around the 'thread layer', and the latter is probably the most insoluble part of the virus which constitutes the ghosts left after alkaline extraction. Reagents which allow more material to be extracted from the virus, such as 2-mercaptoethanol and KCN, probably dissociate disulphide bonds in the insoluble thread layer.

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EXPLANATION OF PLATE 1

Figs. 1-3. Electron micrographs of vaccinia virus.

Fig. 1. Untreated virus with minimal penetration of PTA stain showing the smooth surface ($\times 190,000$).

Fig. 2. Virus after digestion with 0.001 % trypsin and stained with PTA as in Fig. 1; showing the thread layer at the surface ($\times 240,000$).

Fig. 3. Residual ghosts left after extraction of virus with alkali ($\times 135,000$).