Specific Removal of Host-cell
or Vaccinia-virus Antigens from Extracts of Infected Cells by
Polyvalent Disulphide-linked Immunosorbents

By T. H. BIRKBECK and J. STEPHEN
Department of Microbiology, The University of Birmingham, Birmingham 15

(Accepted 16 April 1970)

SUMMARY
Disulphide-linked immunosorbents prepared from rabbit antisera against extracts of uninfected HeLa (ERK) cells specifically removed host-cell antigens from extracts of cells which had been infected with vaccinia virus. Similarly, disulphide-linked immunosorbents prepared from rabbit anti-vaccinia sera specifically removed vaccinia-specific antigens from infected cell extracts. Disulphide-linked preparations derived from unrelated sera adsorbed neither host nor virus-specific antigens.

INTRODUCTION
Recent attempts (Cohen & Wilcox, 1966, 1968; Marquardt, Falsen & Lycke, 1967, 1969; Williamson & Rondle, 1968) to purify vaccinia antigens by conventional means have not been wholly successful, especially in removing host-specific material from isolated fractions. In this paper we indicate how host-specific antigens, present in extracts of vaccinia-infected cells and detectable by immunodiffusion with antisera against uninfected host cells, may be specifically removed. In addition, attempts were made to estimate directly the content of virus-specific antigens in such extracts, using immunosorbents derived from rabbit anti-vaccinia sera.

This work is part of current studies on the purification of vaccinia-specific antigens as a prelude to further attempts to characterize the determinants of cytotoxicity (Bablanian, 1968) as discussed elsewhere (Stephen & Birkbeck, 1969).

METHODS
Virus. The strain of vaccinia virus described by Westwood et al. (1966) was used; the titre of the stock suspension of virus prepared from rabbit dermal pulp was \(7 \times 10^8\) p.f.u./ml.

Cell line and growth media. HeLa (ERK) cells (Appleyard & Westwood, 1964) were grown and passaged in calf serum medium (calf serum 10%, tryptic digest meat broth 5%, yeast extract (Oxoid L21) 0.1%, NaHCO₃ 0.22%, crystamycin 0.01%, all in Earle’s saline), gassed with 5% CO₂ in air immediately after seeding. Inocula of \(2 \times 10^6\) cells produced confluent monolayers (\(5 \times 10^7\) cells) in Roux bottles after 6 days.

Production of vaccinia-specific antigens. Growth medium was removed from Roux bottles containing confluent monolayers and replaced with 10 ml. vaccinia virus suspension (stock diluted in calf serum medium to 25 p.f.u./cell). The virus was allowed to adsorb at 37°C for 15 min. (with rocking) and then calf serum medium (75 ml.) was added to each Roux bottle. After 48 hr the cells were dislodged by shaking, pooled, centrifuged (1800 g for 15 min. at 4°C) and the pellet resuspended in phosphate-buffered saline (PBS1, Gallop et al. 1966;
0.5 ml./Roux bottle equivalent). The cells were disrupted by ultrasonic treatment (Dawe Soniclean 500 w, for 20 min. at 4°) followed by grinding in a mortar prechilled to –70°. The thawed preparation was centrifuged (12,000 g for 30 min. at 4°) and the supernatant fluid irradiated with ultraviolet light. Preparations were stored at –20° until required; upon retrieval they were treated with ultrasound (M.S.E. ultrasonic disintegrator, 1 min. at 0°), centrifuged (12,000 g for 20 min. at 4°) and the supernatant fluid passed through a Millipore filter (220 nm.) which removed all traces of infective virus and then dialysed against phosphate-buffered saline 2 (PBS 2, Gallop et al. 1966). These preparations were designated HVSA, batches 3, 4, 5 and 8 being used for the experiments described below.

The ultrasonic treatment was necessary to maximize the amount of soluble material in the HVSA preparations used in these experiments. However, it was impossible to control this step absolutely and this gave rise to some variation in protein concentration in different samples of the same HVSA preparation, as well as between different preparations. Moreover, as shown below, the numbers and intensities of lines in separate immunodiffusion tests varied presumably for the same reason.

Antisera against soluble HeLa antigens. HeLa cells (7 days old), harvested from 20 Roux bottles by the addition of trypsin + EDTA solution (10 ml./Roux bottle) were pooled, centrifuged, washed in PBS 1 and finally resuspended in 20 ml. PBS 1. After ultrasonic treatment (Dawe Soniclean 500 w, 40 min. at 4°), debris was removed by centrifugation (1800 g for 5 min.); the supernatant fluid was held at 4° overnight and mixed with an equal volume of Freund’s complete adjuvant. Each of 19 rabbits was inoculated intramuscularly with 2 ml. of the mixture; a similar dose was administered by the same route 7 and 14 days later. Blood samples taken during several weeks yielded anti-HeLa sera which were used in preliminary experiments only. The rabbits then received a second course of injections after a period of 26 weeks. Each animal received three intravenous injections at 2 day intervals; fresh HeLa extract was used, without adjuvant, and the dose was equivalent to ½ of that used initially. The rabbits were bled from the ear 2 and 5 weeks after the last injection and individual sera pooled to provide anti-HeLa 2A and anti-HeLa 2B sera, respectively. Four months later the intravenous injection and bleeding schedules were repeated to yield anti-HeLa 3A serum.

Anti-vaccinia sera. The immunization schedule was based on that of E. A. Boulter (personal communication). The stock virus suspension was diluted 70- or 700-fold and mixed with an equal volume of Freund’s complete adjuvant. Each of ten rabbits was inoculated intramuscularly with 2 ml. of the most dilute mixture and each of another ten rabbits inoculated intradermally with 0.2 ml. of the least dilute mixture. Small blood samples taken at various intervals up to 18 weeks were shown by immunodiffusion to contain precipitating antibodies to components of infected HeLa extracts. All rabbits were then bled, and since no qualitative difference in the intramuscular and intradermal sera was observed in immunodiffusion tests, they were combined to provide anti-vaccinia 1B serum. Two months later the rabbits were restimulated with three intravenous injections of virus (3 x 10⁸ p.f.u.) without adjuvant at 2 day intervals and bled 2 and 5 weeks later to provide anti-vaccinia sera 2A and 2B respectively. This course of intravenous injections was repeated after a further 5 months, the rabbits being bled after 2 weeks to yield anti-vaccinia 3A serum; at 18 weeks the animals were bled to yield anti-vaccinia 4 serum. Little or no qualitative differences between sera could be demonstrated by immunodiffusion against extracts of infected cells.

γ-Globulin concentrates were prepared as described by Smith et al. (1962).

Immunosorbents. Disulphide-linked immunosorbents were prepared and used in general
Immunosorption of vaccinia-infected cell extracts

as described by Stephen, Gallop & Smith (1966). In some experiments, however, the eluates from columns were recycled using a Recychrom apparatus (LKB Produkter AB Stockholm) at least five times before bleeding off the unadsorbed material for analysis. An additional step of recycling columns an equivalent number of times before loading with antigen was necessary to measure the background of soluble column-protein which accumulated in contrast to the steady state conditions for a single pass operation described by Stephen et al. (1966).

Concentration of eluates was best achieved by placing them inside 'Visking' dialysis tubing which was then immersed in dry Sephadex G-200 (Pharmacia, superfine grade). In preliminary experiments other methods of concentration, e.g. 'dialysis' against polyvinyl pyrrolidone or polyethylene glycol and freeze-drying were tried and discarded because of failure to recover all the antigenic material in the concentrates.

Immunoelectrophoresis followed the method of Scheidegger (1955) using 1% Ionagar (Oxoid) (2 mm. deep) on microscope slides. Electrophoresis was continued for 1½ to 2 hr in barbiturate buffer pH 8.6, 10-05, by the application of 100 v producing 2-5 mA/microscope slide.

Specificity and efficiency of antigen adsorption by polyvalent immunosorbents. The immunodiffusion test described by Crowle (1958) was used. Crude extract and the column eluate after concentration to a volume equal to that of the HVSA originally applied to the column were tested against appropriate reference sera. The specificity of antigen adsorption was judged by the degree of symmetry in the precipitin pattern obtained when crude extract and the concentrated eluate were diffused against a common well containing either anti-vaccinia or anti-HeLa serum. Efficiency of antigen adsorption was assessed by immunodiffusion of the concentrated eluate and the crude extract against antiserum of the same specificity as that of the immunosorbent.

Protein was estimated by Kjeldahl digestion followed by nesslerization or the method of Lowry et al. (1951), or by $E_{280}$ measurements where appropriate.

RESULTS

Serological analysis of extracts of infected cells

There are large differences in the numbers of virus-specific precipitinogens detected in extracts of vaccinia-infected cells (Williamson & Rondle, 1968). This could be due either to inherent differences in starting materials, e.g. virus strain, host cell differences, or to differences in analytical technique. Since serological methods were the only ones available for quantitative or at least semiquantitative analysis of the efficiency of these immunosorbent systems in removing complex groups of antigens, the effects of several factors on such analyses were determined.

Effect of variation in concentration of HVSA on immunodiffusion and immunoelectrophoretic patterns

Dilutions of HVSA were tested against anti-vaccinia and anti-HeLa sera (Fig. 1a). The extracts contained large numbers of both virus-specific and host-specific antigens; some of these antigens were not easily detected at dilutions of $>$ ½; others were detectable at 1/160; the complexity of the patterns ruled out the possibility of estimating the concentration of individual antigens by dilution null-point techniques.

Immunoelectrophoresis was also done since this had potentially greater resolving power. Analysis of HVSA obtained as described in Methods consistently failed to give as many
distinct lines as had been reported by Rodriguez-Burgos et al. (1966), although there was clearly an anodic region where several precipitating systems were superimposed. However, HVSA concentrated five times with Sephadex G-200 yielded several more discrete precipitation lines and extended (towards the cathode) the region where the superimposition of lines rendered them uncountable (Fig. 1b). Increasing the time of the electrophoresis did not improve the resolution since several of the fainter lines disappeared presumably because of the additional diffusion resulting in a reduced concentration of those antigens in the gel.

Fig. 1 (a). Immunodiffusion analysis of extracts of vaccinia-infected cells. Top row of wells contained anti-HeLa serum 2A. Centre row contained dilutions (1/2, 1/5, 1/10, 1/20, 1/40, 1/80, 1/160) of preparation HVSA 5. Bottom row contained anti-vaccinia serum 2A. (b) Immuno-electrophoretic analysis of extracts of vaccinia-infected cells. Top well contained undiluted HVSA 8 prepared as described in Methods. Bottom well contained HVSA 8 concentrated five times. Centre trough contained anti-vaccinia serum 3A. Conditions: barbiturate buffer pH 8.6, 1.05, 1.5 hr, 100V, 2.5 mA/slide.

Fig. 2. Effect of the presence of traces of host-specific antigens on the pattern obtained between extracts of vaccinia-infected cells and anti-host serum. V, HVSA 5; V/50, HVSA 5 diluted 1/50; AH, anti-HeLa serum 3A.
Immunosorption of vaccinia-infected cell extracts

An experiment was done to compare HVSA with HVSA diluted 1/50, using anti-HeLa as a test serum. The result (Fig. 2) gave clear evidence of some precipitin lines between the diluted antigen well and the antiserum well. This suggested that contamination of column eluates with some unadsorbed homologous antigens would be detectable at levels of 2% of their original concentrations.

Non-removal of HeLa-specific and vaccinia-specific antigens by disulphide-linked preparations derived from ‘normal’ serum

A check on the specificity of adsorption of these immunosorbent systems was made by deriving disulphide-linked preparations from unrelated γ-globulin concentrates (Gallop et al. 1966; Wood, Stephen & Smith, 1968) and passing extracts of infected cells through such columns. In three experiments the average recovery of total protein was 99% and in each case all antigens were demonstrated in the concentrated eluate (Fig. 3).

Removal of host-specific antigens from extracts of infected cells

Experiments with immunosorbents derived from sera taken before anti-HeLa 2A indicated that the uptake of homologous antigens was small. Attempts were therefore made to increase antigen uptake to remove all host-specific antigens from virus-specific antigens in HVSA preparations by using the ‘hyperimmune’ anti-HeLa sera, 2A, 2B and 3A and by recycling column eluates. In several cases columns were reloaded without prior desorption in attempts to define the maximum adsorption capacities of these immunosorbents. The results of representative experiments are summarized in Table 1 and Fig. 4. Comparison of the results obtained with immunosorbent 3A (Table 1) showed that recycling increased the uptake of protein about two-fold.

The maximum capacity of the immunosorbents was difficult to assess accurately but may be estimated from the data in Table 1 to be about 2 to 4 mg. protein/g. immunosorbent protein. The columns could be regenerated by elution with glycine-HCl buffer pH 2.2, followed by PBS 2 (Chidlow, Stephen & Smith, 1970a) and reused several times without apparent loss of specificity. Not all of the adsorbed antigens could be demonstrated in the eluate, since they had different degrees of stability towards the desorbent. In many experiments, immuno-
sorption of about 53% of the applied protein resulted in the recovery of all virus-specific precipitinogens in the concentrated eluates and little or no detectable host-specific antigens. A typical result is shown in Fig. 4. In this experiment HVSA was passed through a column of immunosorbent derived from anti-HeLa serum 3A; virus antigens were not removed (Fig. 4a) but host antigens were (Fig. 4b).

Table 1. Use of disulphide-linked immunosorbents prepared from antiserum to HeLa cells to remove host antigens from extracts of cells infected with vaccinia virus

<table>
<thead>
<tr>
<th>Anti-HeLa γ-globulin concentrate (mg.)</th>
<th>-s-s-protein</th>
<th>HVSA preparation</th>
<th>HVSA applied (ml.)</th>
<th>Protein (%)</th>
<th>Protein adsorbed (% load)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>1600</td>
<td>3</td>
<td>0.30</td>
<td>2.3</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.35</td>
<td>2.7</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.50</td>
<td>11.4</td>
<td>13</td>
</tr>
<tr>
<td>3A</td>
<td>700</td>
<td>8</td>
<td>0.50</td>
<td>7.0</td>
<td>20</td>
</tr>
</tbody>
</table>

Recycling experiments

<table>
<thead>
<tr>
<th>Anti-HeLa γ-globulin concentrate (mg.)</th>
<th>-s-s-protein</th>
<th>HVSA preparation</th>
<th>HVSA applied (ml.)</th>
<th>Protein (%)</th>
<th>Protein adsorbed (% load)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B</td>
<td>750</td>
<td>4</td>
<td>0.25</td>
<td>2.7</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.25</td>
<td>2.7</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.25</td>
<td>2.7</td>
<td>35</td>
</tr>
<tr>
<td>3A</td>
<td>600</td>
<td>8</td>
<td>0.37</td>
<td>4.5</td>
<td>53</td>
</tr>
</tbody>
</table>

* Successive loadings were applied without regeneration.

Fig. 4. Immunodiffusion analyses of extracts of vaccinia-infected cells after passage through an immunosorbent column derived from anti-HeLa serum 3A. V, HVSA 8; EH, concentrated eluate from column; AV, anti-vaccinia serum 3A; AH, anti-HeLa serum 3A.

Estimation of vaccinia-specific antigen content in extracts of infected cells

These experiments provided a direct estimate of the quantity of vaccinia-specific proteins in the extract of infected cells and an additional check on the specificity of these immunosorbent systems. The experiments were similar to the single-pass experiments described for the removal of HeLa antigens. The results of representative experiments are summarized in Table 2.
Immunosorption of vaccinia-infected cell extracts

The maximum capacity of these immunosorbents was assessed from the data in Table 2 to be about 1.7 mg. protein/g. immunosorbent protein. The immunosorbent could be re-used as for the anti-HeLa system, but again not all of the adsorbed antigens were demonstrable in the desorbed material presumably for the same reasons as given above for failure to recover host antigens.

Table 2. Use of disulphide-linked immunosorbents prepared from antiserum to vaccinia virus to remove virus-specific antigens from extracts of cells infected with vaccinia virus

<table>
<thead>
<tr>
<th>Anti-vaccinia γ-globulin concentrate</th>
<th>HVSA applied</th>
<th>Protein adsorbed (%) load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loading*</td>
<td>Protein (mg.)</td>
</tr>
<tr>
<td>1 B</td>
<td>450</td>
<td>3 1 0.3 4.1 15</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.3 4.1 5 5</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.3 3.1 17</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>2.0 2.0 1.5 1.5</td>
</tr>
</tbody>
</table>

* Successive loadings were applied without regeneration.
† Illustrates difference between preparations.
‡ Illustrates differences within preparations.

In many experiments immunosorption of 12 to 17% of the applied HVSA protein resulted in recovery of all host-specific antigens in the concentrated eluates and little or no detectable virus-specific antigens. This is illustrated in Fig. 5. HVSA was passed through a column of immunosorbent derived from anti-vaccinia serum 4. Virus antigens were removed (Fig. 5a), but host antigens were not significantly absorbed (Fig. 5b).
DISCUSSION

Our results indicate that polyvalent disulphide-linked immunosorbents derived from anti-HeLa or anti-vaccinia sera can specifically remove their respective groups of homologous antigens from crude cell extracts. However, this claim must be qualified. In model systems described by Stephen et al. (1966) and Chidlow, Stephen & Smith (1970b) specificity of adsorption was determined by demonstrating quantitative recoveries of characterized heterologous antigens from immunosorbent columns which adsorbed their characterized homologous antigens. In this study, the complex nature of the system permitted only semi-quantitative estimation of antigen recovery. However, total protein recoveries obtained in the experiments with disulphide-linked polymers derived from unrelated γ-globulin strongly suggest that non-specific absorption was not occurring. This, together with the fact that some antigens would have been detected at levels of 2% of their original concentration, supports the interpretation of the immunodiffusion analyses in Figs. 4 and 5 that, under appropriate conditions, it is possible specifically to remove host-specific or virus-specific antigens from a mixture containing both. One further comment is necessary on the apparent difference in the line patterns obtained between, e.g. V and AV (Figs. 3a, 4a, 5a). This difference could not be readily explained on the basis of our initial observations on the factors which determined the qualitative line pattern. The main difference between the system described in Figs. 3a, 4a on the one hand and 5a on the other was the absence of vaccinia-specific precipitinogens in the well marked EV. Substitution of V for EV, however, did result in the production of a picture similar to that in Figs. 3a and 4a.

Although specificity of adsorption was high, the adsorption capacities of these immunosorbents were low, particularly when the total removal of host antigens from vaccinia antigens was attempted. The factors governing the uptake of homologous antigen by monovalent disulphide-linked immunosorbents have been discussed by Chidlow et al. (1970b). With polyvalent immunosorbents an additional factor is operative in limiting the efficiency with which the total removal of one group of antigens from another can be achieved. This concerns the ratio of each antigen in the mixture to its homologous immunosorbent antibody. Clearly the system for which the antigen/immunosorbent antibody ratio is highest will determine the capacity of that immunosorbent for the total removal of the mixture of homologous antigens.

Our results indicate that the problem of removing host-specific antigens from preparations containing virus-specific antigens can now in principle be solved by the application of immunosorbent techniques. The removal of host-specific antigens will best be attempted after preliminary fractionation by conventional means has decreased the host-specific antigen content in a particular subfraction. The immunosorbents may be of the polyvalent type described here or derived from sera against the appropriate subfraction of uninfected host-cell material (cf. the detection of host-cell antigens by Pizer & Cohen (1962) in T-even phage protein preparations derived from Escherichia coli).

The fact that the anti-vaccinia immunosorbents specifically removed their homologous antigens is encouraging in that production of monoprecipitin antisera comparable to those produced against herpes antigens (Watson & Wildy, 1969) would permit the preparation of immunosorbents which would selectively remove their homologous antigens from infected cell extracts. Antigens might then be recovered by techniques similar to those described by Chidlow et al. (1970a).

Finally, it was possible to estimate from the data presented in Tables 1 and 2 that the protein content of infected cell extracts comprised 12 to 17% vaccinia antigens, about 53%
Immunosorption of vaccinia-infected cell extracts

HeLa antigens, leaving about 35% unaccounted for as protein not adsorbed by these immunosorbents. The nature of this fraction was not investigated but it can be accounted for in several ways. It could be non-antigenic. Or, it is not unreasonable to suppose that some host-specific, or virus-specific proteins, might induce only a weak antibody response or that antibody may not be formed at all due to antigenic competition (Ben-Efraim, 1969). The use of subfractions of uninfected cells might yield information on the possible involvement of antigenic competition in suppressing a potential antibody response to constituents of the host cell. Alternatively, host-specific materials present in cell extracts prepared 48 hr after virus infection may contain virus-modified host proteins, and these might not be detected by the serological analysis used here. Immunization of rabbits with extracts of virus-infected cells might help to solve the problem of the identity of this fraction.

We thank the Director Dr C. E. Gordon Smith, Dr E. A. Boulter and Dr J. Keppie of the Microbiological Research Establishment, Porton for help in the production of antisera, and virus antigen preparations.

REFERENCES


*(Received 23 July 1969)*