In Vitro Dissociation and Reconstitution of Poxvirus Haemagglutinin

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SUMMARY

Poxvirus haemagglutinin has been separated into two components by ether/ethanol extraction and also by column chromatography after treatment with 2-chloroethanol. One component, lipid in nature, carried the haemagglutinating activity. The other, a protein termed the antibody blocking component, carried the virus specificity.

By the use of techniques applied by others to the study of lipoprotein membranes, poxvirus haemagglutinin of high specificity was reconstituted from the two components. The reconstituted material reacted with antibody to haemagglutinin but not with antibody to a non-haemagglutinating poxvirus. Reconstitution did not take place when either of the two components was replaced by fractions prepared from uninfected tissues or from tissues infected with a non-haemagglutinating poxvirus. Mixed haemagglutinins could be prepared from fractions prepared from different tissues or from different haemagglutinating poxviruses.

INTRODUCTION

Extracts of tissues infected with most members of the vaccinia-variola subgroup of poxviruses will agglutinate the erythrocytes of certain fowls. Such haemagglutination is inhibited by virus antiserum, but not by normal serum. The same range of fowl cells is also non-specifically agglutinated by various tissue lipids; such non-specific agglutination is inhibited by normal serum (Burnet, 1946; Stone, 1946a). Burnet (1946) suggested that poxvirus haemagglutinin (VHA), probably a lipoprotein, was a combination of a virus antigen which conferred both serological specificity and the haemagglutination specificity described above, and a lipid which was responsible for attachment to the red cell.

The red-cell binding capacity of VHA has been used to detect VHA and to study many of its physico-chemical properties (Stone, 1946b; Chu, 1948; Briody, 1951; Neff, Ackermann & Preston, 1965; Blackman & Bubel, 1972). At the time our work was started little was known about the properties of the virus antigen, and Burnet's model for the complex had not been confirmed by separation of VHA into distinct components. Work by Marquardt (1971) suggested that this may be achieved by ether/ethanol extraction.

We have confirmed Marquardt's finding and have also separated the components of VHA by column chromatography after treatment with 2-chloroethanol, a method used by Zahler & Wallach (1967) to separate the components of erythrocyte membranes.

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We have also used methods available for the in vitro reconstitution of Mycoplasma laidlawii membranes (Terry, Engelman & Morowitz, 1967) to reconstitute poxvirus haemagglutinin. The results obtained support Burnet’s model for VHA and also suggest probable factors affecting in vivo production of poxvirus haemagglutinin.

METHODS

**Virus strains.** Most experiments were done with the BRIGHTON strain of cowpox, some with the LISTER strain of vaccinia virus. Both produce VHA in infected chick embryos (Chu, 1948; Fenner, 1958) and in RK 13 cells (Baxby & Rondle, 1968). Control experiments were done with the UTRECHT strain of rabbitpox which does not produce VHA and induces no antibody to it in infected animals (Fenner, 1958).

**Antisera.** Antisera to infective virus (Baxby, 1972) were used as a source of antibody to poxvirus haemagglutinin. An antiserum to UTRECHT rabbitpox was kindly provided by Dr E. A. Boulter of the Microbiological Research Establishment, Porton.

**Production of virus haemagglutinin.** Heavily infected chorioallantoic membranes (CAM) and RK 13 cells were used as sources of VHA. Infected cells were disrupted by shaking with glass beads and treatment with a MSE/Mullard Ultrasonic disintegrator. Gross debris was deposited by centrifugation at 10000g for 10 min and the bulk of the virus removed by centrifuging at 100000g for 30 min. The VHA was then deposited at 1000000g for 1 h, resuspended in saline and stored either at −70 °C or at 4 °C after freeze drying. Control samples were prepared from uninfected tissues or tissues infected with UTRECHT rabbitpox.

**Haemagglutination and haemagglutination-inhibition tests.** These titrations were done as described by McCarthy & Helbert (1960) using 0.25 ml unit vol. in 3 × ½ in tubes. Virus specific haemagglutinin (VHA) was detected by using red cells suspended in 10% normal rabbit serum; the specificity of this test was confirmed by the inhibition of agglutination in titrations using red cells to which 1/40 virus antiserum had been added. Virus-specific haemagglutinin agglutinates red cells to which no serum had been added and it was not possible to distinguish between virus-specific and non-specific haemagglutination unless the latter was in excess. When present alone or in excess, non-specific haemagglutinin could be detected by its agglutination of red cells to which no serum had been added, and by its failure to agglutinate red cells suspended in normal serum.

**Antibody blocking test.** The presence of the virus component of poxvirus haemagglutinin in fractions which did not haemagglutinate specifically was demonstrated by its ability to block the haemagglutination-inhibiting activity of antiserum. Doses of VHA and antiserum were adjusted so that in the absence of antibody blocking activity in the sample, no haemagglutination by the VHA would be detected. The presence of antibody blocking activity reduced the antibody content of the system, and allowed the VHA to initiate haemagglutination. Equal vol. (0.25 ml) of dilutions of sample and antiserum were incubated at 37 °C for 1 h. After addition of 0.2 ml VHA, incubation was continued for a further 1 h, when 0.25 ml of 1% fowl red cells in 10% normal rabbit serum was added. The pattern of haemagglutination was read after further incubation for 90 min, as for the standard VHA titration.

**Treatment of virus haemagglutination with enzymes or detergents.** Suspensions of VHA were stirred with various concentrations of lipase (3.1.1.3, ex wheat germ, Koch-Light), phospholipase A (3.1.1.4, Naja naja venom, Koch-Light), or SDS (BDH), for 1 h at 20 °C. These treatments were carried out in 0.05 M-tris-HCl buffer at pH 7.5. After enzyme treatment, the suspensions were tested for presence of virus-specific and non-specific haemag-
Components of poxvirus haemagglutinin

glutinins and antibody blocking component. Suspensions treated with SDS were dialysed against saline to remove the haemolytic SDS before testing as above.

**Extraction of virus haemagglutination with ether/ethanol.** Freeze-dried VHA was extracted repeatedly with ether/ethanol (3:1 by vol.) at 4 °C (Marquardt, 1971). The suspension was centrifuged at 1000g for 10 min, the lipid-depleted deposit collected, resuspended in 0.05 M-tris-HCl buffer, pH 7.5, and tested for activity as above.

The supernatant fluid was evaporated to dryness in a stream of nitrogen, resuspended in tris-HCl buffer and extracted four times with equal vol. of chloroform/methanol/tris (8:4:3 by vol.). The chloroform/methanol lipid-rich phase was evaporated to dryness in a stream of nitrogen and the residue resuspended in tris-HCl buffer and tested as above.

**Treatment of virus haemagglutinin with 2-chloroethanol.** The method used was essentially that used by Zahler & Wallach (1967) for dissociating the components of red cell membranes. Freeze-dried virus haemagglutinin (50 mg) was dissolved in 15 ml of 90% aqueous 2-chloroethanol (2-CE, BDH) at 0 °C. The vol. was reduced to 5 ml in a stream of nitrogen and the sample applied to a column (2.5 cm diameter, 30 cm long) of Sephadex LH-20 (Pharmacia) previously equilibrated with 90% aqueous 2-CE. The column was developed with 90% 2-CE and 3 ml fractions collected. Protein was detected by extinction at 280 nm, and lipids by thin-layer chromatography of aliquots of fractions on silica gel (Kieselgel G, Merck). The plates were developed with chloroform/methanol/water (60:35:8 by vol.) and lipids were detected by charring after spraying with 5% (w/v) potassium dichromate in 40% (v/v) H₂SO₄. Fractions were dialysed against saline for 48 h to remove 2-CE, then tested for biological activity as above.

**Reconstitution of virus haemagglutinin.** The method used was that of Terry et al. (1967). Separate fractions containing either high levels of antibody blocking activity or non-specific haemagglutinin were mixed and dissolved in 0.02 M-SDS in 0.05 M-tris-HCl buffer, pH 7.5, and then dialysed against various concentrations of Mg²⁺ in 1/20 β-buffer: β-buffer (Terry et al. 1967) contains 0.156 M-NaCl, 0.05M-tris, 0.01 M-2-mercaptoethanol, pH 7.4. Dialysis was continued until the haemolytic SDS was removed, usually 4 days. Extracts of uninfected tissues and tissues infected with UTRECHT rabbitpox virus were used as sources of non-specific haemagglutinin and antibody blocking component for control experiments.

**RESULTS**

**Enzyme or detergent treatment of virus haemagglutinin**

In preliminary experiments designed to detect the antibody blocking component we attempted to inactivate the specific haemagglutinating activity of VHA. A number of enzymes and detergents were used; unequivocal results were obtained with lipase, phospholipase A and SDS (Table 1). Treatment considerably reduced the haemagglutinating activity of virus-specific haemagglutinin but did not affect the antibody blocking activity. With SDS and phospholipase A there was an increase in the amount of non-specific haemagglutinin present. These results suggest that the antibody blocking component may be released from the virus-specific haemagglutinin thus releasing non-specific haemagglutinin. Experiments were then designed to achieve physical separation of antibody blocking component and non-specific haemagglutinin.

**Separation of the components of virus haemagglutinin**

Ether/ethanol extraction separated VHA into two fractions (Table 1). The ether/ethanol-soluble, lipid fraction showed high non-specific haemagglutinating activity, but no VHA or...
antibody blocking activity was detected. The lipid-depleted fraction had high antibody blocking activity with very little non-specific or virus-specific haemagglutination.

Separation of VHA into two components was also obtained by column chromatography on Sephadex LH-20 following treatment with 2-CE (Fig. 1). After treatment with 2-CE the specific haemagglutinin was not detected. Antibody blocking activity was detected and eluted as a single peak containing protein but no lipid. Non-specific haemagglutinin also eluted as a single peak containing lipid but very little protein. These results suggested that VHA was composed of a lipid haemagglutinating component and a virus protein component carrying serological specificity, as suggested by Burnet (1946).

**In vitro reconstitution of poxvirus haemagglutinin**

*Reconstitution of cowpox virus haemagglutinin from fractions obtained from infected CAM*

The antibody blocking and non-specific haemagglutinin components obtained by ether/ethanol extraction of VHA from cowpox-infected CAM combined to produce a specific haemagglutinin of high activity (Table 2). The presence of both 2-ME and Mg$^{2+}$ was necessary for reconstitution as noted by Terry *et al.* (1967). Reconstitution occurred over the range from 5 to 50 mm-Mg$^{2+}$ and was optimal at 20 mm-Mg$^{2+}$; the results below were obtained with this optimal concentration. Reconstitution also occurred with components separated by 2-chloroethanol treatment. However, the ether/ethanol method was both quicker and less hazardous and so was used in the experiments below.

Table 3 shows typical results and necessary controls which provide some information about factors determining in vivo production of VHA. The haemagglutinin produced was active in the presence of normal rabbit serum. That it behaved as VHA was confirmed by the ability of antivirus serum to block its activity completely. Anti-rabbitpox serum, which does not contain anti-HA but which cross-reacts with other cowpox antigens, did not inhibit the activity of the reconstituted material. This indicates that the lipid material was combined specifically with antibody blocking component and not randomly with other virus antigens.

As might be expected, specific haemagglutination was not detected when antibody blocking component was replaced by protein fractions prepared from uninfected CAM. The reconstitution of VHA did not occur when the lipid fraction was replaced by fractions from uninfected CAM, suggesting that components synthesized before infection were not
Components of poxvirus haemagglutinin

Fig. 1. Column chromatography of the components of cowpox VHA on Sephadex LH-20 after treatment with 2-chloroethanol. Extinction at 280 nm, ○—○; non-specific haemagglutination subject to inhibition by normal rabbit serum, □—□; antibody blocking component, ●—●. The top panel indicates the detection on silica gel of lipid material in the fractions.

Table 2. Conditions affecting reconstitution of VHA from separated lipid and protein components obtained by ether/ethanol extraction of VHA prepared from cowpox-infected CAM

<table>
<thead>
<tr>
<th>Dialysis medium: tris-HCl buffer solution supplemented with 2-ME (0.01 M)</th>
<th>Mg²⁺</th>
<th>Haemagglutinating activity in the presence of</th>
<th>No serum</th>
<th>NRS*</th>
<th>AVS†</th>
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<tbody>
<tr>
<td>Nil</td>
<td>2048</td>
<td>8</td>
<td>&lt; 5</td>
<td></td>
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</tr>
<tr>
<td>+ 5 mm</td>
<td>2048</td>
<td>32</td>
<td>&lt; 5</td>
<td></td>
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<tr>
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<td>2048</td>
<td>512</td>
<td>&lt; 5</td>
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</tr>
<tr>
<td>+ 20 mm</td>
<td>2048</td>
<td>1024</td>
<td>&lt; 5</td>
<td></td>
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<td>+ 50 mm</td>
<td>2048</td>
<td>2048</td>
<td>&lt; 5</td>
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<tr>
<td>- 20 mm</td>
<td>1024</td>
<td>160</td>
<td>&lt; 5</td>
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*, †, As for Table 1.

Table 3. Reconstitution of cowpox VHA from separated protein and lipid components

| Source of | Haemagglutinating activity of reconstituted material when titrated in presence of |
|-----------|-----------------------------------------------|---------|------|
| Lipid     | Protein | No serum | NRS* | AVS† |
| Cowpox CAM| Cowpox CAM | 1280  | 1280 | < 2  |
| Uninfected CAM | Cowpox CAM | 320  | < 5  |
| Cowpox CAM | Uninfected CAM | 1280  | < 5  | Not tested since samples failed to haemagglutinate in NRS |
| Rabbitpox CAM | Cowpox CAM | 1280  | < 5  |
| Cowpox CAM | Rabbitpox CAM | 1280  | < 5  |

*, †, As for Table 1.
incorporated into VHA (Table 3). Specific haemagglutination did not occur when antibody blocking component was replaced by components prepared from rabbitpox-infected CAM, or when the lipid component from cowpox VHA was replaced by the lipid component from rabbitpox-infected tissue. This suggested that the lipid was synthesized only in response to infection by a haemagglutinin-producing poxvirus.

**Reconstitution of virus haemagglutinin from components prepared from different tissues**

VHA prepared from cowpox-infected RK 13 cells was separated and reconstituted as for components prepared from CAM (Table 4). In addition, the lipid component from cowpox-infected CAM could be combined with the antibody blocking component from infected RK 13 cells, and the lipid from infected RK 13 cells with the antibody blocking component from infected CAM (Table 4). Thus, no difference was detected between the components obtained from VHA produced in different tissues, and the results support the specificity of reconstitution of VHA.

**Reconstitution of virus haemagglutinin from components prepared from vaccinia and cowpox-infected tissues**

VHA prepared from vaccinia-infected tissue was separated and reconstituted as for components prepared from cowpox-infected tissue (Table 5). Moreover, the lipid from vaccinia VHA combined with the antibody blocking component from cowpox VHA, and the lipid from cowpox VHA combined with vaccinia antibody blocking component (Table 4). That such hybrid VHA were inhibited by antisera to either virus emphasizes the close serological relationship between their haemagglutinins (McCarthy & Helbert, 1960).
DISCUSSION

The lipoprotein model for poxvirus haemagglutinin proposed by Burnet (1946) was based chiefly on the results of enzyme action on VHA (Stone, 1946b) and on the non-specific haemagglutination by tissue lipids (Burnet & Stone, 1946; Stone, 1946a). In our investigation the effects on VHA of lipase, phospholipase A or the ionic detergent SDS were to reduce the specific haemagglutinating activity without affecting the ability of the virus component to block antibody to haemagglutinin. Treatment with phospholipase A or SDS led to the appearance of large amounts of non-specific haemagglutinin, suggesting that their action was to remove and degrade the lipid component of VHA and release the antibody blocking component. The separation of these two components after treatment of VHA with 2-chloroethanol, and the correlation of their properties with their lipid and protein composition, supported Burnet’s lipoprotein model.

An alternative method for separation of these components involved repeated extraction of VHA with the lipid-solvent mixture ether/ethanol, as described recently by Marquardt (1971). This method, although producing a less complete separation than 2-CE, nevertheless resulted in a lipid fraction with high non-specific haemagglutinating activity, and a lipid-depleted fraction with very low specific or non-specific haemagglutinating activity, but a high antibody blocking activity.

It has been reported that lipoprotein membranes can be reconstituted from previously separated lipid and protein fractions (Terry et al. 1967; Zahler & Weibel, 1970). Our attempts to reconstitute VHA from the haemagglutinating and antibody blocking components were successful. The presence of 2-ME and at least 20 mM-Mg$^{2+}$ were necessary for optimal recombination. These conditions were similar to those used in the reconstitution of lipoprotein membranes of mycoplasma (Terry et al. 1967; Cole et al. 1971) and provide further evidence for the lipoprotein nature of VHA.

We attach particular significance to the observation that haemagglutination by the reconstituted complex was inhibited by an antiserum containing antibody to VHA, but not by an antiserum which lacked antibody to VHA but contained antibody to other virus antigens. This indicated that the lipid component combined specifically with the antibody blocking component and not randomly with other virus proteins.

Although there is no evidence that VHA has a typical lipoprotein membrane structure, Blackman & Bubel (1972) have shown that the bulk of vaccinia VHA is derived from the plasma membrane of the infected cell. They suggest that VHA is disrupted plasma membrane altered in some way by virus infection.

As fowl red cells are agglutinated non-specifically by certain tissue lipids such as lecithin (Stone, 1946a), and as the specific haemagglutinating activity of VHA is destroyed by lecithinase (phospholipase) (Stone, 1946b), Burnet (1946) suggested that VHA consisted of a virus-specific protein combined with a tissue lipid. However, our results indicate that pre-existing lipids are not incorporated into VHA, and further that infection with a haemagglutinin-producing virus is essential for the stimulation of the synthesis of the active lipid.

Differences have been detected in the buoyant density of VHA produced by infection of different tissues (Neff et al. 1965) but these differences are apparently insufficient to prevent the specific recombination of lipid and protein components prepared from VHA obtained from different infected tissues.

Various references have been made to the polydisperse nature of VHA (Briody, 1951; Neff et al. 1965; Blackman & Bubel, 1972). However, Gillen, Burr & Nagler (1950) have demonstrated the presence of two distinct virus haemagglutinins, a high-density, heat-stable
form which sedimented at 35,000g for 1 h, and a low-density, heat-labile form which remained in the supernatant fluid. Similar results were reported by Anthony et al. (1970). Our results (E. C. Smith, unpublished) show that lipid and protein components prepared from such high-density VHA would recombine with components prepared from low-density VHA. This indicates that the components incorporated into these distinct forms are not so different as to prevent their recombination. These results are consistent with the hypothesis that VHA is polydisperse.

The purification of VHA has so far been unsuccessful, probably because of its polydisperse nature, and this has hindered attempts at chemical analysis. Preliminary experiments on the separation and characterization of SDS-treated lipid and protein components suggest that recombination methods may be used to produce pure VHA which can then be subjected to further chemical and serological examination.

REFERENCES


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