Origin of the Vaccinia Virus Hemagglutinin

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The relationship between the vaccinia virus hemagglutinin and hemadsorption was examined. Hemagglutinin synthesis was temporally related to the appearance of the hemadsorption reaction. Only chicken erythrocytes, which reacted with hemagglutinin, hemadsorbed to infected cells, and both of these reactions were inhibited by Ca\textsuperscript{2+}. The distribution of the vaccinia hemagglutinin and 5'-adenosine monophosphatase, a plasma membrane marker enzyme, in sucrose gradients was similar. Plasma membrane ghosts derived from infected cells hemadsorbed erythrocytes and yielded hemagglutinin upon sonic disruption. These data suggest that the majority of vaccinia hemagglutinin is derived from the plasma-membrane of the infected cell.

The origin of poxvirus hemagglutinins has remained obscure since the early work of Chu (7) which suggested that the vaccinia virion could be separated from its hemagglutinin. During the intervening years, the vaccinia hemagglutinin has been the object of numerous investigations which attempted to characterize this particle. This task was further complicated by the finding that vaccinia-infected chloroallantoic membranes contain both virus-specific and normally-occurring hemagglutinins (24). Detailed information on the chemical composition of the hemagglutinin is lacking; however, the studies of Stone (20) suggested that phospholipids were essential components. Gausch and Youngner (10) further characterized the vaccinia hemagglutinin lipids and showed that definable changes occurred in the lipid complement of chicken chloroallantoic membranes following infection by the virus. More recently, Neff et al. (15) concentrated and partially purified the vaccinia hemagglutinin from a variety of infected cells. They found that the density of the hemagglutinin varied as to the tissue or cell of origin. This finding would suggest that, although the hemagglutinin is synthesized under the direction of the viral genome, the host cell plays an important role in the biosynthesis of the particle.

Despite the studies cited here and the others concerned with poxvirus hemagglutinins (12), the origin of the vaccinia hemagglutinin has not been elucidated. Consequently, we reexamined this problem by exploring a possible relationship between the vaccinia hemagglutinin and the hemadsorption of chicken erythrocytes (19) and the vaccinia-infected cells cultured in vitro. This approach yielded data which suggest that the vaccinia hemagglutinin is derived from the plasma membrane of the vaccinia-infected cell.

MATERIALS AND METHODS

Cell cultures and virus. HEp-2 cells were grown in suspension culture (2). The H7D strain of vaccinia virus was obtained from the American Type Culture Collection (Rockville, Md.) and passed eight times through HEp-2 cells before preparation of a single virus pool used throughout this study. Infectivity titrations were carried out as previously described (5), except that plaques were counted 60 hr after infection. Newcastle disease virus (California strain 11914) was propagated in embryonated hen's eggs (13).

Hemadsorption. To 0.1 ml of a 0.5% suspension of fresh, washed chicken erythrocytes in phosphate-buffered saline (PBS) was added an equal volume of vaccinia-infected HEp-2 cells. The concentration of infected cells was adjusted to a number appropriate for enumeration and observation under high-power magnification. Cell suspensions were thoroughly mixed on a microscope slide for 5 min prior to observation, and only those infected cells which adsorbed at least two chicken erythrocytes were counted as hemadsorption-positive cells. The fraction of cells adsorbing erythrocytes was determined by counting a minimum of 200 cells.

Hemagglutination titrations. Hemagglutination titrations were carried out in test tubes (13 by 100 mm) in 0.5-ml volumes. Twofold dilutions of a hemagglutinin preparation were prepared in 0.25 ml of Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-free PBS (pH 7.2), or as dictated by a particular experiment. An equal volume of washed chicken eryth-
rocytes (0.5% suspension in the same diluent) was added to each tube, and the test was read by conventional means after 1 hr of incubation at 37 C. Newcastle disease virus (NDV) hemagglutination tests were carried out in a similar manner, but were incubated at room temperature. Hemagglutination titers are given as the reciprocal of the highest dilution yielding complete shield formation.

**Plasma membrane isolation.** Intact plasma membrane ghosts were isolated from uninfected and vaccinia-infected HEp-2 cells by a modification of the method of Boone et al. (3). Cells were removed from the suspending culture fluid by centrifugation at 300 X g for 10 min, resuspended in an equal volume of the supernatant fluid from the preceding centrifugation and 0.001 M MgCl₂. This procedure was repeated three times. After the last centrifugation of the series, the cells were resuspended in 40 ml of 0.001 M MgCl₂ and transferred to a loose Dounce homogenizer. The cell suspension was then gently homogenized until 90 to 95% of the cells were disrupted (usually 10 to 15 strokes). Ghosts could be easily distinguished from intact cells and free nuclei by phase-contrast microscopy. Sucrose (60%, w/v, in water) was added to the homogenate to yield a final concentration of 8%. Ghosts were recovered from the homogenate by centrifugation through discontinuous sucrose gradients as described by Boone et al. (3).

**Electron microscopy.** Membrane preparations were washed twice with PBS and were fixed in 3% gluteraldehyde in 0.2 M phosphate buffer, pH 7.4. The fixed materials were then exposed to 0.5% osmium tetroxide prior to staining with uranyl acetate, embedded in Epon, and sectioned.

**Experimental procedures.** For all experiments, HEp-2 cells were seeded into fresh culture medium 18 hr prior to infection. Cells were exposed to a vaccinia virus multiplicity of 20 plaque-forming units (PFU) per cell for 1 hr at 37 C with occasional shaking. Routinely, 10⁶ cells were placed in an adsorption volume of 50 ml. After virus adsorption, the cells were washed twice with PBS containing 0.2% methocel, resuspended in culture medium at a concentration of 10⁶ cells per ml, and incubated on a gyratory shaker (2) for the time dictated by the experimental plan. Samples for virus assay were removed and stored at -20 C until assayed for infectivity.

Samples for hemadsorption assays were removed and examined as described above. Hemagglutination titrations were performed on specific cellular fractions as described in the text or on cell samples which had been sonically disrupted for 30 sec at setting no. 4 on a Branson sonifier (model 125; Branson Instruments, Inc.) fitted with a microtip horn.

**RESULTS**

**Kinetics of virus and hemagglutinin synthesis as related to hemadsorption.** It was of immediate interest to examine the temporal relationship between virus and hemagglutinin synthesis and the appearance of hemadsorption activity in vaccinia-infected HEp-2 cells. Synthesis of progeny virus was first detectable at approximately the seventh hour after infection (Fig. 1). Under our conditions of suspension culture, maximal virus titers (14 hr after infection) ranged between 1 × 10⁶ and 5 × 10⁶ PFU/ml. The appearance of vaccinia hemagglutinin was coincidental with the capacity of infected cells to adsorb chicken erythrocytes and subsequent to the synthesis of progeny virus. The close temporal association between hemagglutinin synthesis and hemadsorption suggested that these virus-directed events may be related phenomena.

That the IHD strain of vaccinia virus was separable from its hemagglutinin is clear from the data in Table 1. In this experiment, vaccinia virus

![Figure 1](http://jvi.asm.org/)  
**FIG. 1. Relationship of vaccinia virus and hemagglutinin synthesis to hemadsorption.** Approximately 10⁶ HEp-2 cells were infected as described in Materials and Methods. At various times, samples were collected, sonically disrupted for 15 sec, and assayed for virus and hemagglutinin content. Undisrupted cells were tested for hemadsorption.

| TABLE 1. Removal of hemagglutinin from purified vaccinia virus suspensions by absorption with chicken erythrocytes<sup>a</sup> |
|-----------------|-----------------|------------------|
|                  | Before absorption | After absorption |
|                  | PFU<sup>b</sup>/ml | HA titer<sup>c</sup> | PFU/ml | HA titer |
| 1.3 × 10⁷        | 8                | 8.3 × 10⁶         | 0      |

<sup>a</sup> A 1-ml amount of a 10% suspension of washed chicken erythrocytes was added to 5.0 ml of purified vaccinia virus. The mixture was incubated at 37 C for 1 hr with frequent agitation. Erythrocytes were removed from the mixture by centrifugation at 1,000 × g for 10 min. The supernatant fluid was collected and assayed for virus and hemagglutinin content.

<sup>b</sup> PFU, plaque-forming units.

<sup>c</sup> HA, hemagglutinin.
grown in HEp-2 cell suspension cultures was purified by the method of Zwartouw et al. (26). The purified virus material, however, contained a small amount of residual hemagglutinin. This was completely removed by adsorption of the virus suspension with chicken erythrocytes without materially affecting the virus titer.

Capacity of selected chicken erythrocytes to react with the vaccinia hemagglutinin and to hemadsorb. Dekking and vanDillen (8) showed that the capacity of erythrocytes from individual chickens to interact with vaccinia hemagglutinin is genetically determined. They reported an almost complete correlation between the vaccinia hemadsorption and hemagglutination reactions. In contrast, Driessen and Greenham (9) found that, while all of the chickens which they tested yielded hemadsorption-positive erythrocytes, only half of these gave positive hemagglutination reactions. It was important, therefore, to reexamine this point and compare the reactivity of erythrocytes from individual chickens in both the hemagglutination and hemadsorption tests. To do this, 12 White Leghorn chickens were randomly selected from a flock and bled, and their cells were used in vaccinia hemagglutination and hemadsorption tests. Parallel hemagglutination tests with NDV were also carried out. Data in Table 2 show clearly that erythrocytes from individual chickens were either positive or negative for the vaccinia hemagglutinin and hemadsorption tests and further supported the idea that the vaccinia hemagglutinin may be related to the hemadsorption reaction. It was interesting to note that chicken erythrocytes which failed to react with the vaccinia hemagglutinin gave uniformly positive results with NDV suspensions. The reactive sites for the vaccinia and NDV hemagglutins on the chicken erythrocyte are apparently unrelated.

Effect of ions on the vaccinia hemadsorption and hemagglutination reactions. The foregoing experiments suggested a relationship between the vaccinia hemagglutination and hemadsorption reactions. To probe further this relationship, use was made of the fact that calcium ions inhibit the vaccinia hemagglutinin (23). Vaccinia hemagglutinin was prepared by the method of Neff et al. (15), and hemagglutination and hemadsorption tests were carried out in isosmotic solutions of varying calcium ion concentrations (Fig. 2). Vaccinia hemagglutinin and the adsorption of chicken cells to vaccinia-infected HEp-2 cells were both markedly inhibited by Ca$^{2+}$ in the range of 0.004 M. Similar results were obtained with Mg$^{2+}$. Hemagglutinin or hemadsorption activity could not be demonstrated in 0.25 to 0.30 M sucrose solutions.

Relationship of the plasma membrane to the vaccinia hemagglutinin. Data already presented stressed the similarity of the requirements for vaccinia hemagglutination and hemadsorption. The latter reaction occurs as the result of the interaction of chicken erythrocytes with surface membranes altered in some manner by the virus infection. It was hypothesized, therefore, that the

<table>
<thead>
<tr>
<th>Chicken</th>
<th>Vaccinia hemagglutinin titer</th>
<th>Vaccinia hemadsorption test</th>
<th>NDV hemagglutinin titer</th>
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<tr>
<td>1</td>
<td>256</td>
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<td>512</td>
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<td>+</td>
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<td>12</td>
<td>0</td>
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FIG. 2. Inhibition of the vaccinia hemagglutinin and hemadsorption reactions by calcium ions. Partially purified hemagglutinin was tested with appropriate chicken erythrocytes in an isoosmolar diluent of varying Ca$^{2+}$ concentrations (19). Eighteen-hour infected cells were tested for the capacity to hemadsorb erythrocytes in the same diluent used in the hemagglutinin assay. (VHA, vaccinia hemagglutinin; HAD, hemadsorption.)
vaccinia hemagglutinin might be associated with, or may in fact be, the altered plasma membrane of the infected cell. To support this view, it would be important to demonstrate that the vaccinia hemagglutinin, under defined conditions of cellular homogenization and sedimentation, behaved as if it were a fixed property of the plasma membrane. Of the many enzyme activities which have been encountered in plasma membrane fractions from a variety of cells, the 5'-adenosine monophosphatase is thought to be an intrinsic enzyme of this structure and has been used as a marker for plasma membranes (1).

An experiment was carried out to examine the distribution of 5'-adenosine monophosphatase and vaccinia hemagglutinin following the homogenization of 15-hr vaccinia-infected HEp-2 cells. Figure 3 is a schematic representation of a typical experiment which related 5'-adenosine monophosphatase to vaccinia hemagglutinin activity. There was excellent agreement in the distribution of the two activities in various subcellular fractions which were examined. The initial centrifugation of the homogenate onto a 50% sucrose cushion yielded a heavy band (b) at the 50% sucrose interface and a dense pellet (d). These particulate fractions contained the total 5'-adenosine monophosphatase and vaccinia hemagglutinin activities. Band (b) consisted of the entire postnuclear particulate material of the cells, whereas the pellet (d) consisted of mainly nuclei and some whole cells. When band b was taken up in 45% sucrose, overlaid successively with 40%; and 30% sucrose, and centrifuged, the particulate material was redistributed into two major bands (f, h) and a pellet (i). This last centrifugation was a slight modification of the flotation method which Neville used in isolating plasma membranes from cells (16). When examined by phase-contrast microscopy, bands f and h contained many plasma membrane ghosts and membrane fragments. These were especially numerous in band h, which also exhibited high 5'-adenosine monophosphatase and vaccinia hemagglutinin activities. Fraction e at the top of the tube was slightly turbid and contained microvesicles most likely of membrane origin (3). This fraction exhibited considerable 5'-adenosine monophosphatase and hemagglutinin activity. Fraction i (pellet) contained large bits of dense cellular debris. These experiments suggested that the majority of the vaccinia hemagglutinin is intimately associated with the plasma membrane of the infected cell.

The possibility did exist, however, that internal membranes or other subcellular structures released by the homogenization procedure might give rise to hemagglutinin. An attempt was made to rule this out by the following experiment. HEp-2 cells were infected with vaccinia virus and harvested 18 hr after infection. At this time, all cells strongly adsorbed chicken erythrocytes. A portion of these cells was washed twice with PBS and sonically disrupted, and the treated material was examined for hemagglutinin content. The remaining cells were washed twice with PBS, suspended in PBS containing 1% crystalline trypsin, and incubated for 1 hr at 37 C. The cells were removed from the trypsin solution by centrifugation for 10 min at 1,000 × g, washed twice with PBS to remove residual trypsin, and suspended in PBS. Such treatment rendered the cells hemadsorption-negative. Trypsin-treated cells were then sonically disrupted and titrated for hemagglutinin content. Table 3 shows that trypsin treatment abolished the hemadsorption reaction and significantly reduced the hemagglutinin titer. This experiment indicated that the majority of the vaccinia hemagglutinin was derived from the surface membrane of the infected cell. Whereas the possibility did exist that trypsin entered cells and inactivated intracellular hemagglutinin, this was
TABLE 3. Effect of trypsin on the hemagglutinin and hemadsorption reactions

<table>
<thead>
<tr>
<th>Trypsin treatment*</th>
<th>Percentage of hemadsorption-positive cells</th>
<th>Hemagglutinin titer</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>512</td>
</tr>
<tr>
<td>1 hr</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
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* Washed cells were suspended in 5.0 ml of 1% crystalline trypsin in phosphate-buffered saline (pH 7.2) and incubated at 37°C for 1 hr.

Eighteen-hour vaccinia-infected cells (10⁶) were sonically disrupted and employed as hemagglutinin without further treatment.

unlikely since the majority of cells failed to take up trypan blue. In this regard, Zajac and Crowell (25) found that trypsin treatment of cultured cells under conditions similar to those employed here stabilized cell viability, as tested by trypan blue exclusion and cloning experiments. Furthermore, we have repeatedly examined vaccinia-infected HEp-2 cells (12 to 24 hr) for leakage of intracellular proteins (4) and have found none to occur. This would suggest that the functional integrity of the membrane relative to larger molecules remained intact during the infection. Although indirect, this evidence would argue against trypsin entering the cell and degrading intracellularly bound hemagglutinin.

Vaccinia hemagglutinin derived from isolated plasma membranes. Direct evidence for the association of the vaccinia hemagglutinin with the plasma membrane was derived from experiments in which intact plasma membrane ghosts were tested for hemadsorption and, after sonic disruption, for hemagglutinin activity. intact ghosts isolated from 18-hr vaccinia-infected cells retained the ability to adsorb chicken erythrocytes, whereas ghosts from uninfected cells were hemadsorption-negative. Hemadsorption-positive ghosts were sonically treated for predetermined periods of time and examined for hemagglutinin activity. In Fig. 4 are shown data from such an experiment. Sonic disruption of ghosts for 40 sec produced maximal hemagglutinin titers. one minute of treatment materially reduced the amount of hemagglutinin. The surprisingly rapid reduction of hemagglutinin activity by sonic disruption cannot be satisfactorily explained at this time. Phase-microscope examination revealed that ghosts were rapidly disrupted during the first 30 sec of treatment. It is obvious from these experiments that the integrity of plasma membrane derived from infected cells is important to the hemagglutination reaction.

Electron microscopy of hemadsorbing cells and

membrane ghosts. To support the foregoing experiments, it would be desirable to identify, at the ultrastructural level, any morphological changes which might be associated with the hemadsorption-positive state. Vaccinia-infected cells and membrane ghosts were examined by conventional electron microscope methods of fixation and staining during various stages in the infectious cycle. No unusual morphological features were observed.

DISCUSSION

The experiments which are presented here show that the vaccinia hemagglutinin originates in the plasma membrane of the infected cell. The evidence for this may be briefly summarized as follows. (i) The adsorption of chicken erythrocytes to the surface of vaccinia-infected cells and the agglutination of such cells by hemagglutinin have similar requirements. That is, divalent cations inhibited both reactions, and only chicken erythrocytes which had adsorbed to infected cells reacted positively with the vaccinia hemagglutinin. (ii) The kinetics of hemagglutinin synthesis and the development of the hemadsorption
reaction occurred as concomitant phenomena. (iii) Infected cells rendered hemadsorption-negative by trypsin treatment yielded little hemagglutinin upon disruption. (iv) Plasma membrane ghosts isolated from hemadsorption-positive cells were themselves capable of adsorbing chicken erythrocytes, and such ghosts yielded hemagglutinin upon sonic disruption. (v) The distributions of 5'-adenosine monophosphatase, a plasma membrane marker, and the vaccinia hemagglutinin were similar in discontinuous sucrose gradients. The latter experiments raised the possibility that some hemagglutinin activity may have been derived from internal cell structures. Also, the evidence that the infected cells, disrupted after being rendered hemadsorption negative by trypsin treatment, contained some residual hemagglutinin activity supports this interpretation. Evidence presented by Marcus (14) suggests that the Golgi complex is involved in the transport of NDV hemagglutinin to the cell surface. In the absence of any definitive information on this point, it may be speculated that the vaccinia hemagglutinin becomes associated with the plasma membrane of the infected cell by a similar mechanism. Future experiments will examine this idea in detail.

That the vaccinia hemagglutinin is derived from the plasma membrane of the infected cell is also inferred from previous studies. Thus, Chu (7) concluded that the vaccinia hemagglutinin is a lipoprotein, and more recently Gaus and Youngner (10) clearly established the lipid nature of this particle. Furthermore, Oda (17) showed that the treatment of vaccinia-infected cells with antivaccinia hemagglutinin antibody abolished hemagglutinin production, thus suggesting that the hemagglutinin was of cell surface origin. Taken as a whole, these studies support our contention that the disrupted or fragmented plasma membrane, altered in some manner by virus infection, is the vaccinia hemagglutinin.

Two important points concerning the vaccinia hemagglutinin remain unresolved at this time. These concern the exact chemical nature of the hemagglutinin and the manner by which its synthesis is directed by vaccinia virus. Our cursory electron micrograph examination of hemadsorption-positive cells failed to reveal any outstanding morphological features which might hint of an alteration in the structure of the plasma membrane. Gross structural rearrangement of the plasma membrane, however, may not be a requirement for hemadsorption (and hemagglutination, in the case of fragmented membranes) to occur. A subtle chemical modification of the plasma membrane surface may be all that is required to promote binding to the erythrocyte surface. In this regard, earlier studies from this laboratory (H. C. Bubel and P. W. Lambert, Bacteriol. Proc., p. 169, 1967) demonstrated that vaccinia-infected cells accumulate acidic mucopolysaccharides on their surfaces. Also, Ueda et al. (21) recently showed by immunofluorescence antibody techniques that a new specific vaccinia antigen appeared on the surface of infected cells. It is quite possible that such materials, which accumulate on the plasma membranes of infected cells, could be involved in the hemadsorption and hemagglutination reactions. Our experiments showed that trypsin rendered infected cells hemadsorption-negative (Table 3); this could be interpreted to indicate that the material involved in the reaction is protein or perhaps glycoprotein in nature. The surfaces of Rous sarcoma-transformed cells appeared to be enriched in large-molecular-weight glycopeptides when these were compared with untransformed cells of the same type (6). These glycopeptides were removable by trypsin treatment.

Finally, the second question, of considerable biological interest, concerns the mechanism by which the vaccinia virus genome directs the synthesis of the substance(s) involved in hemagglutination and hemadsorption and how these are transported to, and become associated with, the plasma membrane. Future experiments will attempt to clarify these points.

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LITERATURE CITED