Effect of Heat-Labile Factors on the Neutralization of Vaccinia Virus by Human Sera

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Received for publication 16 April 1973

The effect of unheated guinea pig and human prevaccination serum on the neutralization of vaccinia virus was studied. Enhancement of neutralization was found in all sera containing antibody and was more marked (20- to 150-fold) in sera obtained in the first week after primary vaccination than in sera from immune adults or cord sera (4- to 25-fold). The enhancing factor was thought to be complement because it was destroyed by heating and ethylenediaminetetraacetic acid treatment. The fresh serum-enhanced neutralization test represents a highly sensitive and specific test which can be applied to measure low levels of vaccinia antibody not detectable by other means.

Of the several serological tests used to detect antibody to vaccinia virus, that which measures neutralizing activity is widely believed to be the most valuable in estimating immunity to smallpox. Neutralizing antibody is customarily determined in serum which has been heated, so that the nonspecific, and often variable, effect of heat-labile serum factors (HLSF) is eliminated. On the other hand, such artificial treatment of serum removes from the virus-antibody interaction elements present in the intact organism and may obscure certain physiological anti-viral processes of importance in vivo. For this reason, neutralization by unheated serum, or with added heat-labile factors, has been studied in many virus systems (1, 3, 5, 11, 14, 17, 19, 21, 23, 25, 26, 29).

The first reports of the enhancement of virus neutralization by HLSF concerned vaccinia and variola viruses (8, 10). The phenomenon was examined in embryonated eggs by McCarthy and Germer in 1952 (19) and then, briefly, in tissue culture by Aksenov and Smorodinstev (1) and Nishimura et al. (21). In spite of this long history, however, there has been no previous detailed study of the role of heat-labile substances in the neutralization of vaccinia virus by human sera. An investigation of this problem forms the substance of this report.

MATERIALS AND METHODS

Cell cultures and media. LLC-MK2 cells were obtained originally from Bernard Lourie, Center for Disease Control, Atlanta, Ga. The basic medium used in their cultivation consisted of one-half medium 199 and one-half Eagle minimal essential medium with Earle salts, supplemented with glutamine, penicillin, and kanamycin. The pH was maintained at 7.4 with 0.75% sodium bicarbonate. Two percent inactivated calf serum was used in maintenance medium, and 10% was used in growth medium.

Screw-capped tubes were planted with 1 ml of cell suspension containing 5 x 10⁴ cells and incubated at 37 C. On the third or fourth day the growth medium was renewed, and the tubes were used on the seventh day.

Vaccinia virus. Commercially obtained smallpox vaccine (Dryvax calf lymph, Wyeth) was passaged 38 times in LLC-MK2 cell cultures for use as antigen in this study. Pilot experiments using several different strains of vaccinia virus demonstrated that antibody titers in postvaccination sera did not vary significantly when antigens obtained from different sources were used in the neutralization tests. A pool of virus was stored in small samples at -70 C, and the contents of a single vial were thawed for each test.

Guinea pig serum. Lyophilized guinea pig serum obtained from Colorado Serum Co. was used as a source of heat-labile factors. Several lots were pooled, tested by standard methods for both hemolytic and vaccinia neutralizing activity, and stored in 0.5 ml amounts at -70 C. Significant neutralizing activity was absent in all lots tested.

Vaccines. The attenuated CVI-78 strain of vaccinia virus (15), lot 17C-00401, was supplied by M. Z. Bierly of Wyeth Pharmaceuticals. Calf lymph vaccine (Dryvax, Wyeth) was obtained commercially.

Human sera. Sera were obtained from children aged 12 months to 5 years as part of a study of several smallpox vaccines. Bleedings were performed routinely 4 weeks and 6 months after primary immunization. After the second bleeding, all children were

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challenged with a revaccination, and a further serum was obtained 4 weeks later. Sera from healthy adults who had been previously vaccinated for smallpox and cord sera from healthy newborns were collected in the Denver area. Some sequential sera were obtained after primary vaccination of two older children and revaccination of two adults. Most sera were stored at \(-20\) C until testing. However, when serum was to be used as a source of HLSF or as a fresh immune sample, the blood was allowed to clot for 1 h at room temperature, and the serum was removed and stored at \(-70\) C.

Neutralization test. A neutralization test using added fresh guinea pig serum was developed by modifying the procedure described by Downie and Kempe (9). After inactivation at 56 C for 30 min, the sera were diluted in twofold increments in two parallel series. The virus inoculum was diluted to contain 70 to 100 plaque-forming units (PFU) per tube by using basic medium plus 0.5% bovine serum albumin. Vaccinia virus was stable at 37 C for 18 h in this diluent. The reaction mixture consisted of two parts serum dilution, one part virus suspension, and one part either basic medium or unheated, diluted guinea pig serum. Neutralization tests were carried out with and without HLSF by using the separate serum dilution series as noted above. In most experiments, a dilution of fresh serum providing 6 hemolytic units of complement was used. Since the addition of unheated guinea pig serum reduced the final plaque count by 5 to 10%, separate virus controls were included for each dilution series. The reaction mixtures were shaken vigorously and incubated overnight at 37 C. Growth medium was removed from tissue culture tubes prepared as noted above, and the cell monolayers were inoculated with 0.2 ml of the reaction mixture. Maintenance medium (1.5 ml) was added immediately, and the tubes were incubated for 40 to 44 h at 36 to 37 C. Two or three tubes were used for each serum dilution. Plaques were counted with a hand lens after formalin fixation and staining with 0.13% crystal violet.

Neutralization titers were calculated at 50% plaque reduction after points representing arithmetic means of counts from replicate tubes were plotted on probit paper. The average number of plaques in the virus control tubes was taken as 100% infectivity. Known positive and negative sera were included in each test as controls.

RESULTS

Effect of unheated guinea pig serum on vaccinia virus neutralization by antibody. The effect of unheated guinea pig serum on vaccinia virus neutralization is shown in Fig. 1. One milliliter of vaccinia virus suspension (2 \(\times\) 10^8 PFU/ml) was incubated with 2 ml of undiluted inactivated immune adult serum at 37 C and either 1 ml of unheated guinea pig serum (diluted 1:15) or 1 ml of basic medium. At various times thereafter small amounts of the reaction mixture were removed from each sys-

![Figure 1](https://via.placeholder.com/150)  
**FIG. 1. Effect of unheated guinea pig serum on the neutralization of vaccinia virus by antibody.** A, virus control; B, surviving virus in reaction mixture containing virus and antibody; C, surviving virus in reaction mixture containing virus, antibody, and unheated guinea pig serum; D, with unheated guinea pig serum added to reaction mixture in curve B after incubation for 1 h.
then measured in each mixture, as in the previous experiment.

The results are presented in Fig. 2. The neutralization potentiating effect of unheated guinea pig serum (curve D) is in clear contrast to the lack of activity of heat-inactivated serum (curve B) and EDTA-treated serum (curve C). EDTA alone had no effect on the virus titer. The neutralization-enhancing effect of fresh serum was evident within 5 min, when 90% of the infectious fraction was neutralized. Maximal neutralization occurred within the following 20 min. Chelation with EDTA significantly reduced the effect of unheated guinea pig serum.

Because heating and EDTA treatment destroyed or significantly reduced the effect, it is likely that the factor in fresh guinea pig serum responsible for the potentiation of neutralization was complement.

**Optimal concentration of guinea pig serum.** In order to determine the optimal amount of HLSF for neutralization enhancement, the concentration of fresh guinea pig serum was varied while the quantities of virus and antibody in the reaction mixture were held constant. In this experiment, serum from an immune adult was diluted so that 40% plaque reduction was achieved in a neutralization test without added guinea pig serum. In Fig. 3, curve C represents the contribution of heat-labile factors (A minus B) to the neutralization of virus. Unheated guinea pig serum diluted 1:11 to 1:22 (containing 4 to 8 hemolytic units of complement) was sufficient for maximal enhancement of neutralizing activity in immune serum. No further potentiating effect was observed when more concentrated serum was used. On the basis of these results, 6 hemolytic units were used routinely in the neutralization test described in the following sections.

**Effect of HLSF on the neutralization of vaccinia virus by sera obtained after smallpox vaccination.** The neutralizing antibody titers in 40 sera obtained 4 weeks after primary vaccination and in 40 sera from immune adults were tested as described in Materials and Methods in the presence and absence of HLSF (Fig. 4).

The presence of HLSF in the reaction mixture increased the neutralizing antibody titer of adult immune sera 4- to 25-fold (the enhancement ratio), with a geometric mean of 10.5-fold. In contrast, the enhancement ratio of sera obtained 4 weeks after vaccination was 20 to 150, with a geometric mean of 51.0. Sera in which antibody was not detectable with HLSF added were always negative by the traditional neutralization test, whereas the opposite was not always the case.

In view of the consistent difference observed between 4-week convalescent sera and apparently hyperimmune adult sera, it was of interest to follow the effect of HLSF on sera from individuals after primary immunization and “boosting”. Therefore, a series of individual children’s sera were studied, which had been obtained 4 weeks after successful primary vaccination (at
almost always weeks HLSF-enhanced neutralizing antibody that measurable in the absence (Fig. 4), but tended dose of geometric mean antibody measured cent level which time a booster was administered), and 4 weeks after this. The sera were tested for neutralizing capacity both with and without added HLSF. The results (Fig. 5 and 6) show that HLSF-enhanced neutralizing antibody titer declined during the 5 months after the primary convalescent serum specimen (from a geometric mean of 1,740 to a geometric mean of 890), but tended to rise again with a booster dose of vaccine. The final level, however, was almost always lower than the primary convalescent level (geometric mean 1,480). Neutralizing antibody measured in the absence of HLSF, on the other hand (Fig. 6), usually increased over the 5-month interval (from a geometric mean of 34 to a geometric mean of 66), and then either remained the same or increased again with revaccination (geometric mean 102). The mean enhancement ratio was 51.2 in the 4-week serum, declined to 13.5 in the 6-month serum, and then increased only slightly to 14.5 in the serum obtained after revaccination.

Development of neutralizing antibody after vaccination. Closely spaced sequential sera were obtained from two older children after primary vaccination with the CVI-78 strain and from two adults after revaccination with Dryvax. HLSF-enhanced neutralizing antibody first appeared on day 8 after primary vaccination, 7 days before neutralizing antibody was measurable in the absence of HLSF (Fig. 7). Even the hemagglutination inhibition test (9), which is widely regarded as the most sensitive test for early antibody after primary vaccination, did not detect antibody until day 13. After revaccination of adults (Fig. 8), the HLSF-enhanced neutralization test, although more sensitive, detected a change in antibody titer only 1 day earlier than did the traditional neutralization test.

It is important to note that in the eight sera obtained before or immediately after primary vaccination, no neutralizing activity was present, even in the presence of fresh serum. Thus, the HLSF-enhanced neutralization test appeared specific.

Neutralizing antibody titer after vaccination with attenuated vaccine. Neutralizing antibody levels after vaccination with the attenuated CVI-78 strain are low, and often un-
measurable, even though a clinical “take” has occurred (20). The results described here suggested that antibody might be more easily measured in sera obtained after vaccination with CVI-78 vaccine if HLSF were added to the reaction mixture. Ten sera obtained 4 weeks after primary immunization with the CVI-78 strain contained little or no neutralizing antibody when tested in the absence of HLSF (Table 1). Four of the 10 children had responded to percutaneous inoculation of virus with a small but definite Jennerian vesicle (clinical “take”). The addition of fresh serum to the reaction mixture significantly increased the neutralizing capacity of eight of the sera. If only the traditional neutralization test were used, six children would be considered nonresponders even though they had formed neutralizing antibody measurable by the more sensitive test.

Potentiating effect of fresh human serum on vaccinia neutralizing activity. The results described above suggested that the enhancement of virus-neutralizing activity by HLSF might occur in the human host and play a significant role in the defense against natural disease. In order to test this hypothesis, fresh prevaccination human serum was added to the vaccinia neutralization test in place of guinea pig serum. Furthermore, in certain human sera neutralizing antibody activity was measured without previous heat-inactivation and without the addition of extraneous fresh serum.

The results of these tests are summarized in Tables 2 and 3. The addition of fresh prevaccination human serum (diluted 1:10) produced a marked potentiating effect on vaccinia neutralization by sera from the same or different individuals. The titers measured were close or identical to the antibody titers measured with the addition of guinea pig serum (Table 2).

High neutralizing antibody titers were also noted when unheated, fresh, immune sera were used (Table 3). These titers were, however, not as high as those measured after the addition of exogenous serum. It appears likely that this diminished effect was due to the disappearance of endogenous complement action at high dilutions of serum.

Vaccinia neutralization in cord sera. In order to assess the immunity to smallpox in newborn infants and examine the effect of HLSF on the neutralizing capacity of serum containing antibodies largely of the immuno-

![Figure 7. Development of serum antibody after primary vaccination. Antibody titers were measured simultaneously in sequential sera obtained from a 15-year-old girl (O) and a 14-year-old boy (●) after primary vaccination with the CVI-78 strain of vaccinia virus.](image)

![Figure 8. Antibody response after revaccination. Antibody titers were measured simultaneously in sequential sera obtained from two adults (O or ●) after revaccination with commercial vaccinia (Dryvax).](image)

Table 1. Vaccinia neutralizing antibody after primary vaccination with the CVI-78 strain

<table>
<thead>
<tr>
<th>Child no.</th>
<th>Reciprocal neutralizing antibody titers</th>
<th>Vesicle</th>
<th>With HLSF</th>
<th>Without HLSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>360</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>320</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>320</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>600</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>180</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>−</td>
<td>120</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>80</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>−</td>
<td>16</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>−</td>
<td>&lt;10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>−</td>
<td>&lt;10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*Heat-labile serum factors (fresh guinea pig serum).
Table 2. Enhancement of vaccinia neutralizing antibody by fresh human serum

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Basic medium</th>
<th>Fresh guinea pig serum*</th>
<th>Fresh human serum 1*</th>
<th>Fresh human serum 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>320</td>
<td>400</td>
<td>350</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>320</td>
<td>320</td>
<td>1,600</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>2,000</td>
<td>1,600</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>1,600</td>
<td></td>
<td>1,600</td>
</tr>
</tbody>
</table>

* Diluted 1:15.
* Serum obtained from subject 1 or 2 before primary vaccination and diluted 1:10 for use in the test.

Table 3. Vaccinia neutralizing antibody activity in unheated sera from previously vaccinated adults

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Reciprocal neutralizing antibody titers under designated conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unheated</td>
</tr>
<tr>
<td>1</td>
<td>450</td>
</tr>
<tr>
<td>2</td>
<td>320</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
</tr>
<tr>
<td>5</td>
<td>240</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
</tr>
</tbody>
</table>

* Fresh guinea pig serum.

globulin (Ig)G class, 10 cord sera, including five where maternal serum was also available, were tested. Enhancement of neutralizing antibody activity was clearly observed in cord sera (Table 4). Enhancement ratios were, with one exception, 25 or less, as seen in immune adult sera. The antibody levels and the degree of enhancement were (with one exception) similar in maternal and cord sera. In case no. 5, the considerable disparity between antibody activity in cord and maternal sera may have been attributable to a recent maternal vaccination.

DISCUSSION

The investigation reported here has indicated that unheated guinea pig serum enhances the neutralizing effect of human anti-vaccinia virus antibody, and that this enhancement is most marked in sera obtained in the first weeks after primary vaccination. Enhancement is probably due to complement, since the effect can be destroyed both by heating and EDTA.

Neutralization potentiation by fresh serum has been previously demonstrated for antibodies to vaccinia virus (1, 8, 10, 21), Newcastle disease virus (NDV) (18), respiratory syncytial virus (3), rubella virus (23), adenovirus (25), mumps virus (17), arboviruses (5, 14), cytomegalovirus (CMV) (22), herpes simplex virus (7, 12, 27, 29), and the T-even bacteriophages (26). Complement has also been found to enhance virus hemagglutination inhibition by antibody (14, 24). In many of these studies (1, 3, 8, 12, 14, 21, 22, 27, 29), the nature of the factor in fresh serum has not been examined beyond the demonstration of its heat lability.

In the herpes virus system, HLSF increased both the rate and titer of neutralization in rabbit sera obtained early (8 days) after primary infection; in hyperimmune sera only the rate, and not the titer, was increased (12). Antibodies sensitive to HLSF were either IgM or IgG, although the former were more consistently affected. Early IgM antibodies to both herpes simplex (7) and NDV (18) have been activated to neutralize by exposure to only the first four components of the complement system. Because late components were apparently not necessary for the full expression of complement enhancement, it was postulated that neutralization was effected by the piling up of C1, C4, C2, and C3 on the surface of the virion. On the other hand, aggregation of herpes simplex virus has been shown to occur with the addition of fresh serum factors to early immune rabbit serum, and represents at least one mechanism of enhancement (27). Consistent with both of

Table 4. Comparison of vaccinia neutralizing antibody titer in cord and maternal sera, as measured in the presence and absence of heat-labile serum factors

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cord serum</th>
<th>Maternal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With HLSF*</td>
<td>Without HLSF</td>
</tr>
<tr>
<td>1</td>
<td>420</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>360</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>640</td>
<td>16</td>
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<tr>
<td>7</td>
<td>64</td>
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<td>8</td>
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<tr>
<td>9</td>
<td>960</td>
<td>120</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>10</td>
</tr>
</tbody>
</table>

* Fresh guinea pig serum.

* Enhancement ratio.

* Where no numbers appear, data were not available.
these mechanisms is the observation that most of the effects of complement or HLSF can be reproduced by using antoglobulin (2, 12, 28). Possible alternative mechanisms are based on the observation that antibody plus HLSF is capable, in certain systems, of altering the surface of membrane-coated viruses so that “holes” are observed by electron microscopy (4). Indeed, C-type ribonucleic acid tumor viruses were lysed with release of internal components when treated with anti-envelope serum plus HLSF (22).

The mechanism by which fresh serum enhances neutralization in the vaccinia system reported here is not clear. Titers of “late” antibody from immune adults, as well as cord sera, were enhanced up to 25-fold by HLSF. It is thus likely that, in man, “late” IgG antibody is sensitive to enhancement, although less so than antibody formed shortly after infection. Extreme HLSF sensitivity of hyperimmune monkey IgG antibodies to human CMV has been recently reported (11), and sera from adults with recurrent herpes simplex infections often demonstrate significant enhancement (13), so the vaccinia system is by no means unique in this respect.

It is clear, however, that the enhancement ratio is highest for antibody produced early after primary vaccinia virus infection. In human viral infection, the time-dependent aspect of HLSF-enhanced neutralization was shown first for herpes simplex virus by Yoshino and Taniguchi (31), but these findings could not be confirmed by Heineman (13). Indeed, attempts to use “complement enhanced antibodies” in man as a tool for the early diagnosis of disease due to herpesvirus have proven disappointing (16). The artificial nature of vaccinia virus infection undoubtedly contributed to the reproducibility of our findings.

The sensitivity of any virus-antibody complex to complement or HLSF is probably a function both of the antibody (its specificity, complement activating properties, and affinity) and the mechanisms by which the virus is neutralized. Early antibody formed against inactivated Japanese encephalitis virus (14), which was highly sensitive to the potentiating action of HLSF, was shown to be directed primarily against host components in the immunizing virus. Such a mechanism seems unlikely in natural vaccinia virus infection, however, where virus replication takes place and anti-host cell antibodies are probably not formed. However, there is evidence that vaccinia virus can be neutralized by inhibition of intracellular uncoating (6), and this action of antibody, in addition to the inhibition of absorption or penetration, may be sensitive to the effect of HLSF. Moreover, virion lysis has not been ruled out as a contributory mechanism.

From our data one aspect of the mechanism can be deduced. As in the herpesvirus system (30), antibody which attaches to the virus in the absence of HLSF is sufficient to produce neutralization after HLSF has been added. In our system it is not clear that this antibody requires HLSF (or complement) to neutralize, although under the conditions of our tests virus-antibody complexes were infectious without them. However, the impotence of these antibody molecules could have been due to events occurring during or after their attachment to the virus (for example, their placement at sites unimportant for neutralization) rather than qualities of the antibodies themselves. Hence, we have avoided the term “complement-requiring antibody” used by others (11, 12, 16, 21, 27, 29, 31).

The sensitivity and also the specificity of the HLSF-enhanced vaccinia virus neutralization test were demonstrated by the appearance of antibody in sera obtained at closely spaced intervals after primary vaccination. Sera obtained before and up to 7 days after vaccination consistently failed to neutralize, even in the presence of HLSF. Furthermore, for a 5-day period (days 8–12 after vaccination), HLSF-enhanced neutralizing antibodies represented the only detectable immune response. Advantage was taken of this demonstrated sensitivity and specificity to measure antibody activity in children after primary immunization with the attenuated CVI-78 vaccine.

It appears likely that the antibody titers measured in the presence of fresh serum reflect more closely the true neutralizing capacity of serum than those measured after heating. Because fresh prevaccination serum was just as effective in enhancing neutralization as fresh guinea pig serum, it may be that these antibodies play a more important part in early recovery from vaccinia infection in the normal human host than was previously thought.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-01632 and contract NIH-70-2264 from the National Institute of Allergy and Infectious Disease.

LITERATURE CITED