Cell-Mediated Immunity to Varicella-Zoster Virus Measured by Virus Inactivation: Mechanism and Blocking of the Reaction by Specific Antibody

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The process whereby varicella-zoster (V-Z) virus is inactivated in vitro by immune human peripheral blood leukocytes stimulated with V-Z antigen was examined. It was found that stimulation of leukocytes by V-Z antigen, but not by other viral antigens, was required for inactivation of V-Z virus to occur. Viral inactivation could be blocked by addition of V-Z antiserum to either the stimulation phase of the reaction or the inactivation phase, further demonstrating the specificity of the reaction. In addition these blocking experiments suggested that modulation of V-Z membrane antigen by antiserum occurred with an accompanying loss of immunological recognition of virus-infected cells. Inactivation of V-Z virus in vitro in this study appeared not to be dependent upon the secretion of interferon or upon antibody-dependent cellular cytotoxicity. The specific cells required for V-Z inactivation were T lymphocytes and monocytes (macrophage precursors).

Cell-mediated immunity (CMI) is believed to play a major role in recovery from varicella-zoster (V-Z) virus infections. Thus, persons with defects in humoral immunity alone recover normally from these infections, whereas persons with defects in CMI may not (13). However, the precise immune mechanisms used by the infected host to eliminate V-Z virus are unknown. Recently we described an immune reaction in which peripheral leukocytes (WBC) from persons immune to varicella inactivate V-Z virus in vitro. WBC from individuals susceptible to varicella do not do this (7). Ability of WBC to inactivate V-Z virus could conceivably play an important role in recovery from V-Z infections in vivo. The present investigation was designed to determine the nature of the cellular process involved in eradication of this virus from infected tissue culture by immune WBC.

MATERIALS AND METHODS

Virus and cell cultures. V-Z virus (Ellen) was propagated in human embryonic lung fibroblasts (HELF) using modified Eagle medium containing 10% fetal calf serum for cell growth and 2% fetal calf serum for maintenance of infected cultures. Cell-free virus was prepared by sonication of infected cells (22).

V-Z stimulating antigen for WBC was prepared by exposing cell-free virus to ultraviolet light 20 cm from the source, for 1 h, after which no infectivity remained. Control stimulating antigen was prepared similarly from the same concentration of uninfected cells (7). The complement fixation titer of the V-Z antigen was 1:16; a volume of 0.1 ml per culture (1 ml) was used.

Other stimulating antigens were prepared by infecting HELF with herpes simplex virus (HSV) type I (VR1) or cytomegalovirus (AD 169). These antigens were prepared by methods similar to those used for V-Z antigen with regard to handling, ultraviolet light treatment, and concentration.

Isolation of human WBC. Blood was obtained from consenting human volunteers. Heparinized whole blood was diluted 1:3 in Hanks balanced salt solution, layered over an equal amount of Ficoll-Hypaque, and centrifuged for 40 min at 100 x g at room temperature. Mononuclear leukocytes (WBC) were removed from the resulting central cloudy band, washed three times with Hanks balanced salt solution, and counted in a standard cell-counting chamber. Macrophage-enriched cells were prepared by allowing the monocytes (macrophage precursors) present in the mononuclear cell suspension to adhere to glass or plastic (3). WBC rich in T lymphocytes were prepared by rosetting WBC, previously adsorbed to remove monocytes, with sheep erythrocytes (5, 8). The mixture of rosetted T cells and the remaining free mononuclear cells was then placed on another Ficoll-Hypaque gradient and centrifuged for 40 min at 100 x g. The T rosettes fell to the bottom of the tube. The supernatant was removed, and the erythrocytes were lysed by addition of distilled water. NaCl (2 N) was then quickly added to restore isotonicity. The remaining WBC were rich in T lymphocytes. Cells rich in B lymphocytes were prepared by rosetting adsorbed WBC with sheep erythrocytes treated with complement and antibody to sheep erythrocytes (12). The B-cell rosettes were then subjected to a second Ficoll-Hypaque gradient, followed by lysis of the erythrocytes in the resulting...
pellet as described above, leaving a population of WBC rich in B lymphocytes.

Serological testing. All donors of WBC were tested for immunity to varicella by the fluorescent antibody to membrane antigen (FAMA) technique (6, 22). Only persons with V-Z antibody titers of 1:4 or greater were used as donors of immune WBC. For experiments requiring specific V-Z antibody, hyperimmune V-Z globulin was used at a dilution of 1:500. The V-Z FAMA titer of this material was 1:1,024. Convalescent serum from a patient with recent varicella and a V-Z FAMA titer of 1:256 was also used, diluted to a titer of 1:10. For control antiserum, serum taken from the same person prior to development of varicella, with a V-Z FAMA titer of <1:2, was used at a similar dilution.

Assay for inactivation of V-Z virus. This assay has been described in detail previously (7). Briefly, 1,000 immune WBC were added, along with 0.1 ml of ultraviolet light-treated V-Z stimulating antigen (or control antigen), to HELF cultures containing approximately 10^6 cells newly infected with 20 plaque-forming units of V-Z cell-free virus. The cultures were passed after 3 days, and the number of V-Z plaques was counted. WBC from individuals immune to varicella in the presence of V-Z stimulating antigen consistently reduced the number of V-Z plaques by 40 to 90% compared to cultures with WBC in the presence of control stimulating antigen. WBC from persons susceptible to varicella in the presence of either V-Z or control stimulating antigen did not reduce the number of V-Z plaques (7). This assay is diagrammed in Fig. 1.

The inactivation assay was modified in several ways for some of the experiments. If T- or B-enriched lymphocytes alone were used to test for inactivation, 1,000 lymphocytes were used. In experiments when T- or B-enriched lymphocytes were isolated and then recombined with macrophages as either enriched T or B cells plus macrophages, 1,000 lymphocytes were combined with 200 macrophages.

A two-step assay was also performed, in which stimulation of WBC and inactivation of V-Z virus were performed independently (Fig. 1). In this reaction (step one), V-Z antigen (or control antigen) was incubated with the WBC under study for 72 h at 37°C in RPMI 1640 (GIBCO) medium with 10% fetal calf serum. The entire contents were then added (step two) to HELF monolayers, which had been infected 24 h previously with V-Z cell-free virus. In some experiments V-Z antiserum was added either to step one or step two to test the specificity of each phase. After 3 days cultures were passed, and the number of plaques was counted as usual.

Interferon assay. This was performed on supernatants from inactivation experiments. Growth of vesicular stomatitis virus on normal fibroblast tissue culture cells and trisomic fibroblast tissue culture cells (GM 258; Human Genetic Mutant Cell Repository, Camden, N.J.) (20) were used to assay for interferon. The latter assay is 20 times as sensitive as the former assay for interferon. Using twofold dilutions of supernatant, the interferon titer was expressed as that dilution which caused 50% inhibition of vesicular stomatitis virus cytopathic effect.

Supernatants from inactivation experiments prior to passage were also tested for effects on fresh V-Z cultures, another test for an antiviral mediator. This was done by (i) adding the supernatants to uninfected HELF and infecting with V-Z virus 1 day later and (ii) adding the supernatants to HELF infected with V-Z virus 45 min previously. In each case, the number of V-Z plaques was determined 5 days after infection. The supernatants were also assayed for production of V-Z antibody by FAMA.

RESULTS

Specificity of the reaction. When WBC from an individual immune to varicella were stimulated with V-Z antigen, inactivation of V-Z virus occurred. However, when WBC from this same individual were stimulated with antigens prepared from other viruses such as cytomegalovirus or HSV, no inactivation of V-Z virus occurred. This individual was known to have previously been infected with both cytomegalovirus and HSV.

No V-Z inactivation occurred with WBC from a donor susceptible to varicella who had a history of previous HSV infection (Table 1).

The specificity of V-Z inactivation was also tested by determining whether addition of spe-

![Fig. 1. Diagram showing protocol for experiments demonstrating inactivation of V-Z virus. In the one-step test, (a) (b) and (c) are performed simultaneously. In the two-step test, stimulation of WBC (a + b) is performed prior to addition of (a + b) to (c). In both reactions, a significant (30 to 40%) decrease in V-Z plaques occurs in tests using immune WBC (WBC*) and V-Z stimulating antigen (A) in comparison to tests using control stimulating antigen (B) or susceptible WBC (WBC**) (C). PFU, Plaque-forming units.]
Specific antibody influenced either stimulation or inactivation of WBC. When V-Z antibody was added to WBC and V-Z antigen during stimulation, decreased V-Z inactivation occurred, indicating that specific antibody could block this phase of the reaction. Similarly, when V-Z antibody was added to HELF 24 h after infection and then V-Z-stimulated WBC were added 24 h later, decreased V-Z inactivation also occurred. Thus specific antibody could also block the inactivation phase of the reaction. In contrast, incubation of V-Z-infected HELF with pre-varicella serum did not block the inactivation phase (Table 2).

Mechanism of inactivation. Tests for presence of antiviral mediators including interferon. Supernatants of standard one-step V-Z inactivation assays were tested for the presence of interferon, and none was detected. These supernatants, when added to HELF freshly infected with V-Z virus, also did not restrict the growth of V-Z virus. Even when supernatants were added to HELF cells 24 h before infection with V-Z virus there was no effect on the number of V-Z plaques. V-Z antibody was not detected by FAMA in any of the supernatants.

Interferon was looked for in supernatants from experiments in which WBC at concentrations of 1,000 cells per ml or greater were incubated for 72 h at 37°C in the presence of V-Z or control stimulating antigen. Interferon was found in some of these preparations after stimulation with V-Z antigen, but only if a very large concentration of WBC was employed, 250,000 cells per ml or more (Table 3).

Specific WBC involved in inactivation. Enriched preparations of T lymphocytes, B lymphocytes, or macrophages at a concentration of 1,000 cells per ml were tested individually for their ability to inactivate V-Z virus in the presence of V-Z stimulating antigen. No specific inactivation was demonstrated by any of these populations of cells. However, 1,000 macrophages per ml alone nonspecifically inactivated V-Z virus. Therefore in experiments utilizing combinations of lymphoid cells, macrophages were used at a concentration of 200 cells per ml, which was nontoxic.

Combinations of each type of lymphocyte with macrophages were then studied similarly for their ability to inactivate V-Z virus in the presence of V-Z antigen. In these experiments specific inactivation was best demonstrated in the presence of cells rich in T lymphocytes with macrophages (Table 4).

DISCUSSION

Immunity to the herpesviruses is thought to depend on the complex interplay of a number of immunological factors, including antibody, complement, and various types of WBC. A number of specific CMI reactions to HSV have been identified. These include direct killing of infected cells by lysis, one form specifically mediated by sensitized killer T cells (14) and another nonspecifically mediated by macrophages (11). Antibody-dependent cellular cytoxicity also causes lysis of cells infected with HSV (18).

In this latter reaction, nonadherent lymphoid cells with Fc receptors, termed K cells, in the presence of specific antibody, lyse HSV-infected cells; the K cells do not necessarily have to have come from an animal immune to the virus. Finally, sensitized WBC may secrete antiviral mediators such as immune interferon (19) in response to an antigenic stimulus such as HSV.

In contrast to the situation for HSV, however, factors responsible for eradication of V-Z virus in vitro and in vivo have not yet been well characterized. It was demonstrated many years ago that WBC can be sensitized by prior infection with HSV to lyse HSV-infected cells. In our experiments, WBC were stimulated with V-Z in the presence of specific antibody.

### Table 1. Specificity of V-Z inactivation

<table>
<thead>
<tr>
<th>WBC donor</th>
<th>Antigen used</th>
<th>Mean no. of V-Z plaques*</th>
<th>Percent plaque reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune to varicella*</td>
<td>V-Z</td>
<td>24 ± 8</td>
<td>68 ± 11</td>
</tr>
<tr>
<td>Immune to varicella*; previous HSV infection</td>
<td>HSV</td>
<td>55 ± 5</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>Immune to varicella*; previous CMV infection</td>
<td>CMV</td>
<td>58 ± 7</td>
<td>68 ± 11</td>
</tr>
<tr>
<td>Susceptible to varicella*; previous HSV infection</td>
<td>V-Z</td>
<td>43 ± 3</td>
<td>35 ± 5</td>
</tr>
</tbody>
</table>

*a Ability of WBC to inactivate V-Z virus following exposure of WBC to V-Z, HSV, and cytomegalovirus (CMV) stimulating antigens. Data from representative donors are shown.

* Average of two to four determinations ± standard error of the mean.

* 100 − [(plaques with V-Z stimulating antigen/plaques with control stimulating antigen) × 100].

* Serum V-Z FAMA titer, 1:16.

* Serum V-Z FAMA titer, <1:2.
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### Table 2. Blocking of V-Z inactivation by specific antibody

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Inactivation</th>
<th>Mean no. of V-Z plaques</th>
<th>Percent plaque reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC + V-Z Ag</td>
<td>V-Z HELF</td>
<td>44 ± 6</td>
<td>80 ± 0</td>
</tr>
<tr>
<td>WBC + V-Z Ag + V-Z Ab</td>
<td>V-Z HELF</td>
<td>26 ± 8</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>WBC + V-Z Ag</td>
<td>V-Z HELF + V-Z Ab</td>
<td>28 ± 4</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>WBC + V-Z Ag</td>
<td>V-Z HELF + V-Z Ab</td>
<td>58 ± 8</td>
<td>75 ± 9</td>
</tr>
</tbody>
</table>

*a* The specificity of V-Z inactivation by immune WBC was examined by studying the influence of specific antibody on each phase. Ag, Antigen; Ab, antibody.

*b* Average of two to four determinations ± standard error of the mean.

*c* 1,000 WBC, from one individual immune to varicella, used in each assay.

*d* A 75% loss of V-Z membrane antigen as long as specific antibody was present in the medium; V-Z antibody titer was 1:10.

### Table 3. Interferon levels in supernatants from V-Z immune WBC

<table>
<thead>
<tr>
<th>Conc of WBC per ml</th>
<th>Interferon geometric mean titer&lt;sup&gt;b&lt;/sup&gt; of supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control stimulating antigen</td>
</tr>
<tr>
<td>1,000</td>
<td>4</td>
</tr>
<tr>
<td>125,000</td>
<td>4</td>
</tr>
<tr>
<td>250,000</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>500,000</td>
<td>347 ± 1</td>
</tr>
</tbody>
</table>

*a* WBC were incubated for 72 h at 37°C with either V-Z or control stimulating antigen.

*b* Reciprocal; based on two to four determinations, ± standard error of the mean.

*c* Constant amount of stimulating antigen, 0.1 ml with V-Z CF titer of 1:16, per 1 ml of culture.

### Table 4. Ability of different types of WBC and combinations of WBC to inactivate V-Z virus in the presence of V-Z stimulating antigen

<table>
<thead>
<tr>
<th>WBC employed</th>
<th>Mean no. of V-Z plaques&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent V-Z plaque reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>All WBC (1,000/ml)</td>
<td>13 ± 3</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>T lymphocytes (1,000/ml)</td>
<td>21 ± 3</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>+ macrophages (200/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B lymphocytes (1,000/ml)</td>
<td>25 ± 4</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>+ macrophages (200/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T lymphocytes (1,000/ml)</td>
<td>41 ± 3</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>+ macrophages (1,000/ml)</td>
<td>7 ± 7</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>+ macrophages (200/ml)</td>
<td>24 ± 7</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

*a* Average of two to four determinations, ± standard error of the mean.

appear. If antibody is removed from the medium, however, the number of plaques increases significantly. Thus antibody can limit the spread of V-Z virus in vitro but it cannot eradicate the virus (21).

Investigations of CMI to V-Z virus have been few in number and have in the main been concerned with measurement of proliferative responses of sensitized lymphocytes to V-Z antigen (9, 15, 16, 17). Such studies are of great interest, but they do not provide information concerning possible effects of WBC on host cells infected with the virus. In contrast, the V-Z CMI assay utilized in this study measures inactivation of V-Z virus and thus determines the effect of WBC on infected tissue culture cells. This assay may reflect events that occur in vivo, and the responsible mechanism may represent at least a component of the host's process of elimination of virus.

In the present study we have demonstrated that inactivation of V-Z virus by immune WBC appears to be specifically initiated by V-Z antigen. Thus, stimulation of immune WBC with other viral antigens did not result in inactivation of V-Z virus. Similarly, V-Z inactivation could be blocked by specific antibody both in the phase of stimulation of immune WBC by V-Z antigen and in the phase of inactivation of V-Z virus. Presumably, during the inactivation phase, sensitized WBC are alerted to the presence of V-Z virus by the appearance of V-Z membrane antigens on HELF cells. When V-Z virus is propagated in vitro in the presence of specific antibody, a marked decrease in the number of cells displaying membrane antigen occurs, and despite the presence of stimulated WBC the virus is protected from inactivation.

The ability of V-Z antibody to block the inactivation phase is of special interest because of the process termed antigenic modulation. This phenomenon has been described for both tumor...
and viral antigens in the presence of specific antibody (10). For example, when cells infected with measles virus are propagated in the presence of specific antibody, the virus-induced surface antigen is shed or ingested and antigenic modulation is said to have occurred. CMI reactions to measles-infected cells cannot be demonstrated in vitro during modulation. However, when the antibody is removed, measles viral antigens reappear, infectious virus is again produced, and CMI to infected cells may again be demonstrated. Since antigenic modulation is a mechanism by which a virus may evade immunological recognition and thus persist, it has been suggested that this process may be important in the maintenance of viral latency (10). Therefore, it is of interest that antigenic modulation with loss of immunological reactivity has now been demonstrated for V-Z virus, another agent which, like measles virus, causes latent infections.

It appears that T lymphocytes and macrophages together mediate the reaction whereby V-Z virus is inactivated in our CMI assay. Interferon could not be demonstrated to be present in supernatants of the CMI assay, and therefore it seems unlikely that interferon plays a role in this reaction. However, interferon was demonstrated when much higher concentrations of WBC were stimulated with V-Z antigen. V-Z virus is sensitive to interferon (2), and, although it is not involved in the assay we are studying, interferon probably does play a role in recovery from V-Z infections (1). Antibody-dependent cellular cytotoxicity also does not appear to play a role in the reaction under study, since antibody is not detectable in supernatants from inactivation experiments and since specific antibody blocks the CMI assay rather than enhances it. Moreover, the reaction occurs in the absence of B cells, which would have to synthesize the necessary antibody. Again, however, antibody-dependent cellular cytotoxicity may play a role in elimination of virus in vivo.

In the present experiments V-Z inactivation was attributable to the actions of T cells and macrophages. It is likely that one of these cells acts as an afferent cell and one as an effector cell. We have no data as yet to indicate which cell is acting in each role or if either cell has only one role. However, if T cells recognize V-Z virus and arm macrophages, one would expect to find stimulation of CMI to be specific but the inactivation phase of the reaction to be nonspecific (4). In our assay, stimulation appeared to be specific. For example, when immune WBC were stimulated with HSV antigen, V-Z virus was not inactivated. However, it remains to be deter-

mined whether inactivation is specific or nonspecific. Further experiments are planned to determine whether WBC, stimulated appropriately to act against V-Z virus, also inactivate other viruses. These experiments will help to determine which cells are afferent and which are efferent in the reaction against V-Z virus.

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