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Cell-Mediated Immunity to Varicella-Zoster Virus Demonstrated by Viral Inactivation with Human Leukocytes

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Cell-mediated immunity to varicella-zoster (V-Z) virus in persons immune to varicella has been demonstrated, using a tissue culture technique. Cell-mediated immunity was reflected by the ability of peripheral leukocytes (lymphocytes and monocytes) from human donors to inactivate V-Z virus. Leukocytes were stimulated by the addition of noninfectious V-Z antigen to cultures newly infected with V-Z virus. Several days later, the V-Z virus in these cultures was titered. When leukocytes from donors immune to varicella were used, a significant decrease in V-Z titer, compared with controls, was noted. When leukocytes from donors susceptible to varicella were tested, no decrease in V-Z virus titer was found. A mixed population of lymphocytes and monocytes from immune donors was required to demonstrate inactivation of V-Z virus. The development of specific cell-mediated immunity to V-Z virus may play a role in termination of varicella and in prevention of second attacks of this disease.

It has long been suspected that cell-mediated immunity is important in recovery from infections with varicella-zoster (V-Z) virus (3). However, although humoral immune responses to V-Z virus have been extensively investigated (2, 5, 11), methods to detect cell-mediated immune responses to this virus are still in the developmental stage. Two recent reports of transformation of human lymphocytes by V-Z virus revealed evidence of uptake by these cells of radioactive thymidine after exposure to V-Z antigen (6, 9). We have attempted to investigate cell-mediated immune responses to V-Z virus by another, more direct method. We determined what effect leukocytes from donors immune to varicella would have on the growth of V-Z virus in vitro. We also studied the effect of these cells on the virus if the leukocytes were simultaneously stimulated with noninfectious V-Z antigen. The studies described in this report, therefore, were designed to determine whether cell-mediated immunity to V-Z virus could be demonstrated by inactivation of the virus by human leukocytes.

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MATERIALS AND METHODS

Virus and cell cultures. The Ellen strain of V-Z virus was used throughout. Virus was propagated in monolayers of human embryonic lung fibroblasts (HELF) of the New York University-32 strain. For growth of cells, Eagle medium containing 10% fetal calf serum was used; maintenance medium was similar but contained only 2% fetal calf serum (11). Cell-free virus was prepared by propagating V-Z-infected monolayers in 32-ounce (ca. 1.0-liter) prescription bottles for 48 h at 37 C. The infected cells were disrupted by sonication for 30 s. Inactivated V-Z virus was prepared by centrifugation of cell-free virus at 70,000 × *g* for 60 min. The supernatant was discarded, and the pellet was suspended in Hanks solution (0.5 ml/bottle) by brief sonication. The resulting suspension was exposed for 1 h to ultraviolet light and stored at −20 C. Irradiated antigen preparations were monitored to assure that no infectious V-Z virus was present in the antigen. This antigen titered 1:16 by complement fixation; 0.1 ml was added to each culture. It was used in a dilution of 1:1 and 1:2 in each experiment, since on occasion 1:1 was toxic to cultures. At the same time the irradiated V-Z antigen was prepared, a control antigen was also made from a similar number of uninfected cells. This control antigen was prepared exactly as the V-Z antigen in 2% fetal calf serum-Eagle medium and used in a similar concentration and amount as the V-Z antigen. In the future these two types of antigen will be referred to as VZ-Ag and C-Ag.

Preparation of human leukocytes. Blood was obtained from human volunteers with a heparinized syringe. The unclotted blood was layered over an equal amount of a Ficoll-Hypaque gradient (usually 5 ml). The gradients used contained 50% Hypaque and 9% Ficoll; 24 parts of 9% Ficoll was used with 10 parts of 50% Hypaque. Gradients were centrifuged for 40 min at room temperature at 1,000 × *g*. The mononuclear cells were present as a cloudy band near the center of the gradient. Almost all of the cells were found to exclude trypan blue. The cells were washed three times with Hanks solution. They were then counted in a standard cell counting cham-

ber and adjusted to the appropriate concentration. On Wright stain they were found to consist of lymphocytes and monocytes. These cells are henceforth referred to as WBC. One milliliter of each WBC concentrate as specified was added per culture. For control experiments using inactivated WBC, the WBC were treated exactly as described above, but in addition they were sonicated until most of the cells were disrupted.

Serological testing. Serum from all individuals whose WBC were tested was examined for the presence of antibody to V-Z virus, using the fluorescence antibody to membrane antigen (FAMA) technique (5, 11). With this assay one may identify individuals who are immune to varicella. Immune individuals have FAMA titers of equal to or greater than 1:4. Susceptible persons have FAMA titers of less than 1:2. WBC obtained from immune and susceptible individuals were both studied.

Assay for viral inactivation. Human WBC from Ficoll-Hypaque gradients were added to tubes containing HELF monolayers. The HELF had just previously been infected with 5×10^{-1} plaque-forming units (PFU) of V-Z cell-free virus. Maintenance medium was added, and after 3 days of growth the cultures were trypsinized and passed to fresh monolayers. After 3 days the number of viral plaques was counted, using a light microscope at a magnification of ×100. Human WBC were also added to uninfected HELF monolayers as a control to monitor for toxicity. In some experiments VZ-Ag was added to the infected cultures along with WBC; this antigen was added to stimulate the WBC. As a control, C-Ag was similarly added to some cultures along with the WBC.

Absorption of WBC to remove monocytes. WBC from Ficoll-Hypaque gradients were allowed to settle on plastic in the presence of growth medium (7). The cells were left for 30 min at 37 C, and the supernatant cells were gently removed; this absorption procedure was performed for a total of three times. The supernatant cells, consisting almost entirely of lymphocytes, were tested for ability to inactivate V-Z virus, as described above. The adherent cells, mainly monocytes, were scraped off and similarly tested for ability to inactivate V-Z virus. Only donors immune to varicella were used as a source of WBC for these experiments.

RESULTS

Toxicity of human WBC. A suspension of 1,000,000 human WBC was added to HELF monolayers, which had been adsorbed with 5×10^{-1} PFU of V-Z cell-free virus. This represents a multiplicity of infection of approximately 1/100. In these preparations the ratio of WBC to fibroblasts in the culture was 1:1. After passage the number of viral plaques was counted. It was found that a 10-fold reduction in the number of PFU occurred. This 10-fold reduction was noted when WBC from all the individuals tested were used, including persons who were susceptible to varicella as well as those who were immune.

Varying numbers of human WBC were then added to V-Z-infected cultures to determine the highest number of WBC that could be added without causing a decrease in V-Z virus titer. One million, 500,000, 10,000, 5,000, 1,000, and 500 WBC were tried. It was found that there was no decrease in the number of V-Z plaques if 5,000 WBC or less were used. In the following experiments, therefore, 1,000 WBC or occasionally 500 WBC were used.

Inactivation of V-Z virus by WBC exposed to VZ-Ag. For these experiments, monolayers infected with V-Z virus were employed and 1,000 WBC plus 0.1 ml of VZ-Ag was added to some cultures. To other infected cultures 1,000 WBC alone were added. Other controls consisted of infected cultures with VZ-Ag alone and infected cultures plus 1,000 WBC and C-Ag. The ratio of WBC to HELF in these cultures was 1:1,000. After passage, the number of viral plaques was counted. A diagrammatic representation of these experiments is shown in Fig. 1.

Studies of WBC from donors immune to varicella revealed the following results. In cultures treated with WBC alone, with VZ-Ag alone, or with WBC plus C-Ag, the number of V-Z PFU was similar. In cultures that received WBC and VZ-Ag, however, the number of V-Z PFU was halved. The difference between the number of PFU in infected cultures treated with WBC and VZ-Ag was significantly less than the number of PFU in cultures treated with WBC and C-Ag (Table 1). WBC from six donors immune to varicella all gave similar results. When WBC inactivated by sonication were used, however, no reduction of V-Z titer occurred.

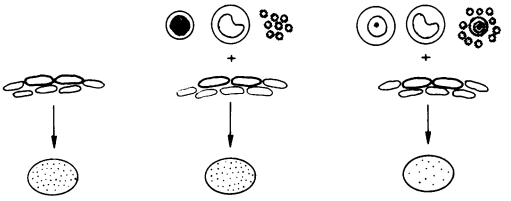

FIG. 1. *Schematic representation of experiment to demonstrate inactivation of V-Z virus by human WBC. (Left) A control; V-Z-infected fibroblasts are titered after 3 days. (Middle) Also a control; human WBC (lymphocytes and monocytes) are added alone with C-Ag to infected fibroblasts and titered after 3 days. (Right) Human WBC and VZ-Ag are added to infected fibroblasts and cultured after 3 days. When WBC from donors immune to varicella were used, significant reduction in the number of plaques was noted in the right-hand panel, compared to the left-hand and middle panels. When WBC from donors susceptible to varicella were used, the number of plaques in all panels were similar.*

Similar tests of WBC from five seronegative donors with no prior history of varicella revealed no such differences. Cultures containing WBC from a susceptible donor plus VZ-Ag yielded a similar number of PFU as cultures containing WBC plus C-Ag (Table 2). The number of PFU in control cultures treated with WBC alone and VZ-Ag alone was also similar. The minor differences noted are not significant. A similar result was observed when the WBC were obtained from the cord blood of a newborn infant.

WBC from a total of 12 normal persons were tested; six were immune and six were susceptible to varicella. WBC from these individuals were tested on multiple occasions. A summary of the data from all the individuals is presented in Fig. 2. The average number of V-Z PFU seen after addition of WBC from persons immune to varicella plus VZ-Ag was 14. In contrast, the average number of PFU after addition of WBC from persons susceptible to varicella and VZ-Ag was 37. These numbers are significantly different ($P < 0.001$). There was no significant reduction of PFU when WBC from either immune or susceptible donors were exposed to C-Ag.

V-Z inactivation after removal of monocytes by adsorption. Inactivation experiments were performed as above, counting the number of V-Z PFU after treatment of V-Z cultures with WBC plus VZ-Ag, WBC alone, VZ-Ag alone, and WBC plus C-Ag. In these experiments, however, WBC were first treated to remove most of the monocytes by adsorption. No decrease in the number of V-Z PFU was observed when these monocyte-depleted WBC were used. Similarly, when a predominance of monocytes, i.e., adherent cells, were substituted for the WBC, there was no decrease in V-Z titer.

Clinical studies. Experiments were performed to determine the ability of WBC from patients with V-Z infections to inactivate V-Z virus. These WBC were exposed to VZ-Ag or C-Ag and added to cultures newly infected with V-Z CFV. The number of V-Z PFU in cultures containing WBC plus VZ-Ag was compared with the number of PFU in cultures containing WBC plus C-Ag. WBC from one child with varicella did not inactivate V-Z virus on day 2 of his infection, but they did inactivate V-Z virus on day 4 of his illness. WBC from another child with impaired cell-mediated immunity and fatal progressive varicella did not inactivate V-Z virus on day 10 of his infection. WBC from a child with zoster did not inactivate V-Z

TABLE 1. *Titration of V-Z virus in the presence of human WBC and VZ-Ag: WBC from donors immune to varicella*

Donor	V-Z antibody titer[a]	With control stimulating antigen (PFU)[b]	With V-Z stimulating antigen	
			PFU[b]	P
A.G.	1:16	39 ± 5	8 ± 2	<0.001
R.R.	1:16	47 ± 7	6 ± 2	<0.001
Z.K.	1:16	58 ± 6	31 ± 4	<0.01
S.S.	1:8	78 ± 7	34 ± 8	<0.01
P.M.	1:8	30 ± 7	6 ± 2	<0.01
K.B.	1:16	31 ± 6	10 ± 1	<0.01

[a] As measured by fluorescent assay for antibody to V-Z membrane antigen (11).
[b] Average of four to six determinations.

TABLE 2. *Titration of V-Z virus in the presence of human WBC and VZ-Ag: WBC from patients susceptible to varicella*

Donor	V-Z antibody titer[a]	With control stimulating antigen (PFU)[b]	With V-Z stimulating antigen	
			PFU[b]	P[c]
C.G.	<1:2	48 ± 1	40 ± 6	NS
R.L.	<1:2	41 ± 5	36 ± 7	NS
R.K.	<1:2	40 ± 5	45 ± 3	NS
M.F.	<1:2	49 ± 8	27 ± 3	NS
S.M.	<1:2	85 ± 5	87 ± 3	NS
Newborn infant		20 ± 8	29 ± 9	NS

[a] As measured by fluorescent assay for antibody to V-Z membrane antigen (11).
[b] Average of four to six determinations.
[c] NS, Not significant.

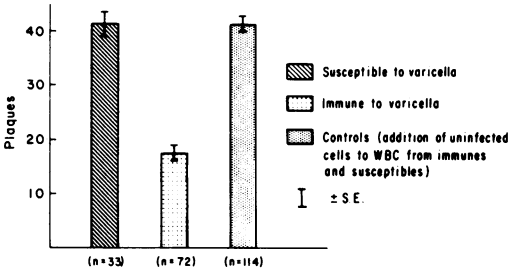

FIG. 2. *Graph of summary of results of experiments comparing the ability of 500 to 1,000 human WBC from persons immune to varicella and persons susceptible to varicella to inactivate V-Z virus. WBC from six donors susceptible to varicella and six donors immune to varicella were studied on many occasions. The mean number of plaques is depicted for (i) WBC from susceptibles exposed to V-Z antigen, (ii) WBC from immunes exposed to V-Z antigen, and (iii) WBC from all donors exposed to control antigen. The ability of WBC from individuals immune to varicella is significantly greater than that from those susceptible to varicella to inactivate V-Z virus. n, Total number of determinations.*

virus on day 4 of her illness, but WBC from yet another child, who had had zoster for 3 weeks, inactivated V-Z virus.

DISCUSSION

The data presented in these experiments indicate that cell-mediated immunity to V-Z virus develops after an attack of varicella. This phenomenon was demonstrated by using lymphocytes and monocytes (macrophage precursors) from peripheral blood of individuals whose immune status with respect to varicella was known. The cell-mediated response was demonstrated by the ability of viable WBC from immune, but not susceptible, individuals to inactivate V-Z virus in vitro. A similar reaction was demonstrated after experimental infection with herpes simplex virus (8), in which large numbers of WBC also inactivated herpes simplex virus non-specifically. Less WBC plus herpes simplex virus-stimulating antigen inactivated herpes simplex virus specifically, as occurred with V-Z virus in our experiments (8).

It is unknown what role inactivation of V-Z virus by WBC plays in vivo, but it is tempting to speculate that it may indeed play a role in termination of an attack of varicella and in prevention of second attacks of varicella. It is well known, for example, that patients with agammaglobulinemia, who are unable to form humoral antibodies, do not develop recurrent varicella. Therefore, it is likely that their WBC protect them from repeated attacks of varicella. This protective effect may reflect the ability of their WBC to inactivate V-Z virus.

There is evidence that inapparent V-Z infection without disease may occur in individuals immune to varicella who are intimately exposed to the virus (4). Again, it is possible that after re-exposure to V-Z virus WBC may be stimulated, thus allowing the WBC to inactivate the virus and prevent disease from occurring despite reinfection. It is likely that the cell-mediated response would work in concert with specific humoral antibodies in a normal individual in this instance.

It will be of interest to examine the ability of WBC to inactivate V-Z virus in patients with zoster and, if possible, prior to the onset of zoster. In the two patients with zoster described in this report, it was found that early in the course of zoster inactivation of V-Z virus by WBC was poor, whereas later in the recovery phase it was active. These observations corroborate the findings of Russell et al. (9), who noted decreased lymphocyte transformation in response to V-Z virus in 14 patients early in zoster. It is also in agreement with the observations of Jordan and Merigan (6), who noted both transformation and an increase in production of interferon after exposure to V-Z antigens of lymphocytes from patients in the recovery phase of zoster. The demonstration of a decrease in cell-mediated immunity soon after zoster is of interest, since the titer of V-Z serum antibody in patients before and soon after the onset of zoster is similar to the titer observed in persons immune to varicella (4). Whether a decrease in cell-mediated immunity to V-Z virus is causally related to the onset of zoster is at this time unknown. Unfortunately, we have not been able to compare results of our assay of cell-mediated immunity to V-Z virus with the frequently used technique of thymidine uptake by WBC after exposure to viral antigen. The latter technique has not been reproducible in our laboratory using V-Z virus as antigen.

The mechanism by which human WBC are able to inactivate V-Z virus remains to be determined. The results of these experiments suggest, however, that macrophage-lymphocyte interaction is necessary since V-Z inactivation did not occur if macrophages were removed by adsorption from the WBC preparations. Similarly, macrophages alone in the presence of V-Z antigen did not cause V-Z inactivation. An attempt to define the precise role of T cells, B cells, and macrophages in the inactivation process will be made in future studies. In addition, it is not known whether the stimulated WBC block V-Z synthesis or kill the target cells, causing a reduction of V-Z virus titer. Experiments to clarify this point are currently in progress.

It is possible that release of interferon from WBC after exposure to V-Z antigen leads to viral inactivation. It has been demonstrated that V-Z virus can be inactivated by interferon (1). Stevens and Merigan have also demonstrated the presence of interferon in zoster skin vesicles, and they noted a correlation between recovery from disease and vesicular interferon (10). In future experiments we plan to determine whether interferon is produced by immune WBC in the presence of V-Z antigen and if it correlates with viral inactivation.

Aside from the observation that the ability of WBC to inactivate V-Z virus is acquired after an attack of varicella, several unanswered questions remain concerning the specificity of the demonstrated viral inactivation. For example, after exposure of immune WBC to V-Z antigen, are the WBC able to inactivate other viruses? Similarly, upon exposure of WBC to another antigen to which they are immune, will V-Z virus be inactivated? These questions will be dealt with in future experiments. Even

without the answers to these questions, however, these experiments support the hypothesis that specific cell-mediated immunity after V-Z infection develops in normal persons. In the normal individual both cellular and humoral immunity probably contribute to the prevention of second cases of varicella.

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