

Antibody-Dependent Cell-Mediated Cytotoxicity Against Varicella-Zoster Virus-Infected Targets

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Antibody-dependent cell-mediated cytotoxicity (ADCC) against cryopreserved varicella-zoster virus-infected human foreskin fibroblasts was detected in a ^{51}Cr release assay. Target cells, samples of seropositive or seronegative sera, and mononuclear cells obtained by Ficoll-Hypaque centrifugation of human peripheral blood were added to microtiter plate wells and allowed to incubate at 37°C for 4 h. Fibroblasts infected for 48 to 96 h were susceptible to ADCC. Effector cells from seropositive and seronegative normal children were equally active in the assay. Antibody titers were determined by testing serial dilutions of sera in the ADCC assay. Zoster immune globulin had a titer of 204,800. Sera from 40 naturally seropositive individuals were compared by assays for ADCC and fluorescent antibody to membrane antigen. All sera that were negative by fluorescent antibody to membrane antigen (<2) were also negative by ADCC (<20). All sera that were positive by fluorescent antibody to membrane antigen were also positive by ADCC, but titers of individual sera were frequently 5 to 20 times higher in the ADCC assay.

Clinical observations suggest the importance of immune mechanisms in recovery from varicella-zoster virus (VZV) infections. Primary VZV infections may be severe in individuals with deficiencies in cellular immunity, although individuals with deficiencies in antibody production usually have no difficulty in handling infections. These observations suggest that cellular immunity is more important than humoral immunity in recovery from primary VZV infection (9). On the other hand, administration of large amounts of antibody to VZV in the form of zoster immune globulin may prevent or modify primary VZV infection (3). However, cellular immune mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), may play a role in this effect. Finally, reactivation infection (herpes zoster) tends to occur more frequently and to be more severe in certain immunocompromised individuals, but it is not known whether immune mechanisms contribute to prevention of reactivation infections.

In vitro assays for cellular immunity to VZV have been developed for studies of host responses to VZV infections. VZV-specific lymphocyte proliferation has been widely used to assess cellular immunity (9). Lymphocyte-macrophage-mediated inactivation of VZV has also been described (4) but is a somewhat cumbersome

assay for clinical studies. Cytotoxic activity against VZV-infected targets has been described, but the type of killing was not fully defined (11). Recently a preliminary report appeared on ADCC against target cells infected with VZV (Cross et al., *Fed. Proc.* 40:988, 1981). In the present study we confirmed the existence of ADCC to VZV by using cryopreserved target cells, and we compared antibody titers detected in the ADCC assay to those obtained in the commonly used assay for fluorescent antibody to membrane antigen (FAMA; 12).

MATERIALS AND METHODS

Cell cultures and cell-free virus pool. Human embryonic foreskin fibroblasts, FS-4 strain, were stored at -190°C in 10% dimethyl sulfoxide. Thawed cells were grown in Eagle minimum essential medium with 7.5% heat-inactivated fetal calf serum (FCS). Ellen strain VZV was originally obtained from the American Type Culture Collection, Rockville, Md., and was multiply passaged in MRC-5 cells in our laboratory. Monolayers of FS-4 cells in 75-cm² flasks were infected with cell-free Ellen strain VZV at a multiplicity of infection of approximately 0.01. When 20 to 30% of the cell sheet showed cytopathic effect, the cells were detached by exposure to 0.1% EDTA-0.25% trypsin and used to infect fresh flasks of FS-4 cells. When 70 to 80% of these cells showed cytopathic effect, the culture fluid was discarded, and the infected cells were washed three times with phosphate-buffered saline free of Ca²⁺ and Mg²⁺. The cells were then detached

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TABLE 1. Susceptibility of VZV-infected targets to ADCC^a

Time postinfection (h)	Membrane fluorescence (% positive cells)	% ⁵¹ Cr release in presence of serum ^b :		ADCC
		Antibody negative	Antibody positive	
Uninfected	Not determined	6.2 ± 4.0	5.0 ± 2.3	-1.2
24	9	11.1 ± 1.5	10.9 ± 1.7	-0.2
48	71	9.9 ± 0.5	26.8 ± 2.4	16.9
72	60	6.7 ± 2.9	23.0 ± 3.3	16.3
96	86	3.6 ± 4.5	16.9 ± 7.9	13.3

^a Effector cells were obtained from a seropositive adult and were tested against targets at an E:T ratio of 50:1.

^b Serum pools were used at final dilutions of 1:100. Values show mean ± standard deviation.

with 0.1% EDTA-0.25% trypsin and were suspended in SPGA medium (1) with 10% FCS. The cell suspension was sonicated at 40 W for 30 s and then was centrifuged at 3,000 rpm for 15 min. The supernatant contained 10^{3.5} PFU/ml as determined by plaque formation in FS-4 cells. Samples of the virus were stored at -70°C.

Preparation of target cells. Monolayers of FS-4 cells were infected with cell-free VZV prepared as described above at a multiplicity of infection of approximately 0.01. When 85 to 90% of the cells exhibited cytopathic effect (usually in 6 to 7 days), the cells were detached with 0.1% EDTA-0.25% trypsin, suspended in minimum essential medium with 7.5% FCS, and added to monolayers of FS-4 cells in 75-cm² flasks at a ratio of one flask of infected cells to five flasks of uninfected cells. After 24 to 96 h of incubation at 37°C, infected cells were removed by exposure to EDTA-trypsin and suspended in 10 ml of minimum essential medium containing 2% FCS. Cell suspensions were centrifuged at 1,500 rpm for 10 min. Cell pellets were suspended in 2 ml of minimum essential medium with 10% FCS per flask, and dimethyl sulfoxide was added to give a final concentration of 10%. Samples of 6 × 10⁵ cells were placed in vials, stored at -70°C overnight, and transferred to a nitrogen freezer (-190°C) the following day. Uninfected target cells were also prepared and stored at -190°C.

Effector cell preparation. Mononuclear cells were obtained by buoyant density centrifugation of human peripheral blood on Ficoll-Hypaque gradients (2). Cells were washed three times with and suspended in RPMI 1640 supplemented with 20% FCS.

Membrane fluorescence assay. VZV membrane antigens on infected FS-4 cells were detected by indirect immunofluorescence as previously described (12).

Sera. Seropositive and seronegative serum pools were prepared, each with sera of three individuals whose immune status was previously determined by the FAMA assay. The seropositive pool had a FAMA titer of 128. These serum pools and individual sera were de complemented by heat inactivation at 56°C for 30 min before use in the cytotoxicity assay. Unless otherwise stated, the serum pools were used in the cytotoxicity assay at final dilutions of 1:100.

ADCC assay. Frozen uninfected and infected target cells were thawed rapidly by immersing vials in a 37°C water bath and were washed twice with 10 ml of Hanks balanced salt solution with 5% FCS. The cells were suspended in 0.5 ml of Hanks balanced salt solution with 5% FCS, and 50 μC of Na⁵¹CrO₄ (New England Nuclear Corp., Boston, Mass.) was added. The cells

were then incubated at 37°C in a 5% CO₂ incubator for 1 h, during which time the tubes were gently agitated at intervals of about 15 min. The labeled target cells were washed four times with cold Hanks-balanced salt solution with 5% FCS and were suspended at a concentration of 5 × 10⁴ cells per ml in RPMI 1640 with 20% FCS. Labeled target cells were distributed into round-bottomed microtiter plate wells (Flow Laboratories) with 5 × 10³ cells per well in a volume of 0.1 ml. Samples (10 μl) of appropriate dilutions of seropositive or seronegative individual sera or serum pools were added, and the plates were incubated for 30 min at 4°C.

Effector cell suspensions were added in 100-μl portions to give the desired effector-to-target cell (E:T) ratios. Each determination was performed in triplicate. The microtiter plates were then centrifuged at room temperature for 3 min at 800 rpm to facilitate cell-to-cell contact and were then incubated for 4 h at 37°C in a 5% CO₂ incubator. After incubation, 100 μl of medium was removed from the top of each well with an Eppendorf pipette and was transferred to a counting tube. ⁵¹Cr release was counted in a gamma scintillation counter (LKB Wallac, Turku, Finland). Total releasable activity was determined by the addition of 1% Triton X-100. Spontaneous release from target cells was determined in the absence of effector cells. The percentage of ⁵¹Cr release in the presence of effector cells and either antibody-positive or antibody-negative serum was calculated as (counts per minute experimental release - counts per minute spontaneous release)/(counts per minute total release - counts per minute spontaneous release).

ADCC was defined as the percentage of ⁵¹Cr release in the presence of antibody-positive serum minus the percentage of ⁵¹Cr release in the presence of antibody-negative serum.

Determination of titer of ADCC antibodies against VZV. Twofold dilutions of test sera from 1:1 to 1:256 were prepared. Samples (10 μl) of each dilution were added in triplicate to the cytotoxicity assay as described above. The ADCC titer of a serum was defined as the reciprocal of the highest final serum dilution which resulted in ⁵¹Cr release which was more than two standard deviations above the mean ⁵¹Cr release detected in the presence of antibody-negative serum.

RESULTS

Susceptibility of VZV-infected targets to ADCC. FS-4 cells infected with VZV for 24 to 96 h and uninfected FS-4 cells were tested for

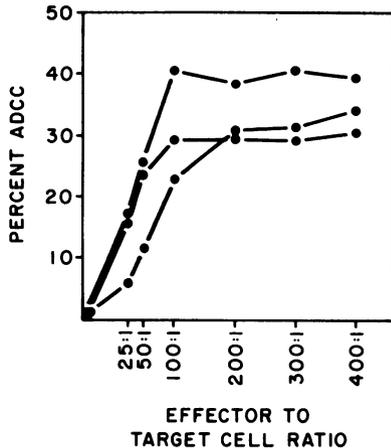


FIG. 1. Effect of E:T ratio on ADCC against VZV-infected targets. Curves show results for three donors.

susceptibility to ADCC. Mononuclear effector cells were obtained from a seropositive adult. Spontaneous ^{51}Cr release was less than 20% in 4 h for each of the target cells. Cytotoxicity against uninfected FS-4 cells was similar in the presence of either antibody-negative or antibody-positive pooled sera (Table 1). In contrast, cytotoxicity against cells infected for 48, 72, and 96 h was augmented in the presence of the antibody-positive serum. Positive membrane fluorescence was demonstrated for 71, 60, and 86% of infected target cells from these three groups, respectively (Table 1). For the purpose of uniformity, subsequent experiments were done with the batch of target cells which had been infected for 48 h and then cryopreserved.

E:T ratios. Mononuclear cells of three seropositive adults produced a linear rise in ADCC with increasing E:T ratios from 25:1 to 100:1 (Fig. 1). With higher E:T ratios, the magnitude of ADCC did not increase. E:T ratios of 50:1 were used in subsequent experiments.

Effect of donor immune status on ADCC. To determine whether immune status influenced the magnitude of ADCC, effector cells from seropositive and seronegative normal children were tested in the ADCC assay. The mean ages of the children in the two groups were similar: 4.8 years for the seronegative group and 4.5 years for the seropositive one. The magnitude of ADCC varied greatly among individuals in each group (Fig. 2), but mean ADCC was not significantly different ($P > 0.01$ by Student's *t* test). There was no correlation between age and the magnitude of ADCC (data not shown).

Determination of antibody titer by ADCC. The titers of 10 sera obtained from naturally seropositive donors were determined in the ADCC assay. ADCC usually declined with increasing serum dilutions, except for one serum which

demonstrated an apparent prozone phenomenon (Fig. 3). The percentage of cytotoxicity with pooled seronegative sera was 1.7 ± 1.6 in this experiment (data not shown). Therefore, the ADCC titer for each tested serum was the reciprocal of the highest dilution which produced ^{51}Cr release of $>4.9\%$, two standard deviations above the mean obtained with pooled seronegative sera. The 10 sera had the titers of 320 (2 sera), 1,280 (6 sera), and 5,120 (2 sera). Zoster immune globulin with a FAMA titer of 4,096 had a titer of 204,800 in the ADCC assay (data not shown).

Comparison of antibody titer by ADCC and FAMA assays. To determine the relative sensitivity of the ADCC and FAMA assays for detection of VZV antibodies, 40 sera from normal children or adults with no history of recent varicella were studied by both procedures (Fig. 4). Sera with FAMA titers of <2 were also negative in the ADCC assay. All sera with FAMA titers of >2 were positive in the ADCC assay. Antibody titers determined by ADCC were frequently 5 to 20 times higher than those obtained by FAMA, and there was significant positive correlation between titers obtained in these two assays ($r = 0.67$, $P < 0.005$; Spearman rank correlation).

DISCUSSION

The cytotoxic activity measured in our assay appears to be ADCC in view of the requirement for antibody and the ability of effector cells from seronegative as well as from seropositive donors to mediate the reaction. The detection of high titers of antibody in zoster immune globulin suggests that the antibody is largely of the immunoglobulin G class, although on the basis of our data we cannot rule out the participation of other immunoglobulin classes.

We have not yet defined the nature of the effector cells which mediate ADCC against VZV-infected targets, but preliminary experi-

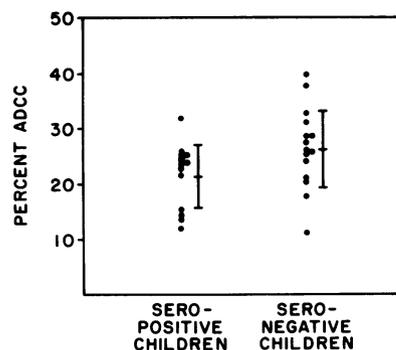


FIG. 2. Effect of donor immune status on ADCC against VZV-infected targets. Bars indicate mean \pm one standard deviation.

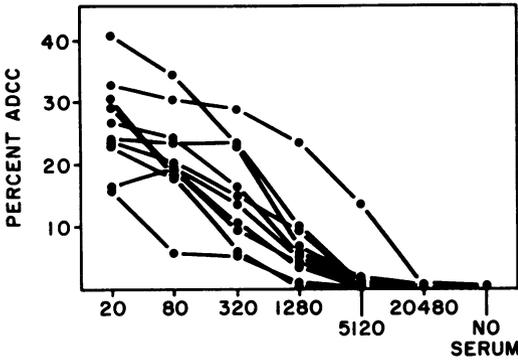


FIG. 3. Determination of serum titer by ADCC. A total of 10 seropositive sera were serially diluted and tested for their ability to augment killing of VZV-infected targets.

ments indicate that most of the activity is in nonadherent cell fractions (data not shown). K cells, monocyte-macrophages, and polymorphonuclear leukocytes have all been shown to mediate ADCC against herpes simplex-infected targets (6, 7, 10). Experiments are in progress to determine the relative contribution of each of these effector cells to ADCC against VZV-infected targets.

Our results are similar to those obtained by Cross et al. (Fed. Proc. 40:988, 1981), who used freshly infected target cells. The cryopreserved target cells used in our study proved to be quite satisfactory and are advantageous for clinical studies since they are always available. We have maintained frozen target cells for up to 1 year without loss of either viability or susceptibility to lysis.

When the ADCC assay was used to quantitate antibodies to VZV, the observed titers were considerably higher than those detected by assays for FAMA. It remains to be determined whether higher titers detected in the ADCC assay will be useful for assessing humoral responses to VZV infections in various clinical

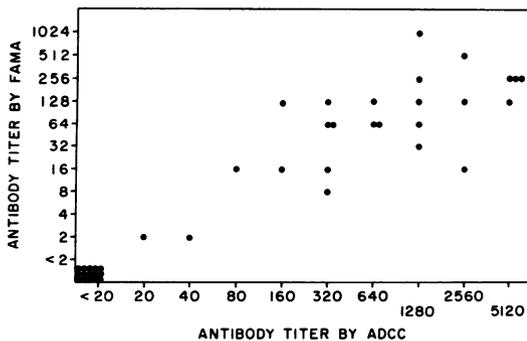


FIG. 4. Comparison of antibody titers as determined by FAMA and ADCC.

settings. Other assays have recently been described which are also considerably more sensitive than FAMA for the detection of antibodies to VZV (8).

Recently, Gershon and Steinberg have described inactivation of VZV by nonadherent peripheral blood mononuclear cells and antibody in a plaque reduction assay (5). This effect appeared to be due to the antibody-dependent killing of infected cells. Although the plaque reduction assay of Gershon and Steinberg and the ^{51}Cr release assay described in the present paper may measure the same immunological function, the ^{51}Cr release assay is easier to perform, particularly if large numbers of specimens are to be tested.

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