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Cell-Mediated Cytotoxicity in Recurrent Herpes Simplex Virus Infections in Man (39853)

RICHARD C. REICHMAN, RAPHAEL DOLIN,[1] MONROE M. VINCENT, AND ANTHONY S. FAUCI

Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Herpes simplex virus (HSV) is a common cause of mucocutaneous infections in man. Up to 20–40% of population groups in the United States suffer from "fever blisters" (herpes labialis) (1), and approximately 300,000 cases of herpes genitalis are estimated to occur annually in the United States (2). Of the two distinct antigenic types of HSV, type 1 is generally associated with orofacial lesions, while type 2 is most commonly found in genital infections (3). Disease manifestations of HSV infection occur in both primary and recurrent forms. The recurrent form tends to be less severe and of shorter duration than the primary form, but, because of the occurrence of repeated episodes in the same patient, it is responsible for the major portion of morbidity associated with these infections.

The alterations of host-virus interaction which result in recurrent HSV infections are presently unknown. Because these lesions are known to recur in the presence of high levels of serum-neutralizing antibody against the homologous virus (4), recent investigations have sought possible deficiencies in other host defense mechanisms which might result in the activation of latent infection (5). Particular interest has been directed toward evaluation of cell-mediated immunity (CMI) in patients with HSV infections (6, 7), and some studies have suggested possible defects in this arm of the immune response in patients with recurrences with HSV-1 (8–10), although studies of CMI in patients with HSV-2 infections have not previously been reported. The present investigations were undertaken to examine one parameter of specific CMI in patients with HSV-1 or HSV-2 infections, namely, the peripheral blood mononuclear cell-mediated cytotoxic activity against target cells acutely infected with HSV. Cytotoxic activity was measured in patients during as well as as in between recurrences. This activity was compared to that found in sero-positive individuals without histories or evidence of recurrent lesions in an attempt to detect a possible immune defect in the patient group.

Materials and methods. Patient populations. Patients with recurrent HSV infections were referred to the Out Patient Department of the Clinical Center of the NIH. Except for the presence of mucocutaneous lesions during active infections, all patients had normal physical examinations and normal complete blood counts. Whenever possible, individual patients were studied both when lesions were present and when lesions were absent. Patients in the study had no known underlying diseases.

The characteristics of patients with type 1 (orofacial) infections are depicted in Table I. Thirteen normal volunteers with serologic evidence of infection, as defined by the presence of neutralizing antibody to HSV type 1, but without histories or evidence of recurrent infections are also described. In addition, seven adult donors, mean age 25.0 ± 3.9 years (mean ± SEM), with neutralizing antibody titers to HSV-1 of <4 were also studied.

The characteristics of patients with type 2 (genital) infections are shown in Table II. Seven volunteers with serologic evidence of infection but without histories or evidence of recurrent infections are also described. Five adult sero-negative donors, mean age 34.2 ± 3.8 years, were also studied. Serologic evidence of infection with HSV type 2 was defined by the criteria of Rawls *et al.* (11).

Patients with active infection were seen

[1] To whom all correspondence should be addressed at: National Institutes of Health, Building 10, Room 11N-214, Bethesda, Maryland 20014.

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TABLE I. CHARACTERISTICS OF PATIENTS WITH HSV-1 INFECTIONS

Patient group	No. of Patients	Age[a]	Male/female	Onset of recurrences prior to study	No. of recurrences per year	Serum antibody[b]
Recurrences						
Lesions present	6	34.8 ± 3.6	2/4	> 10 years	5.2 ± 1.4	91.5
Lesions absent	15	32.5 ± 2.4	5/10	> 10 years	4.5 ± 0.6	78.3
No recurrences	13	30.2 ± 1.7	8/5	–	–	34.1

[a] Mean ± SEM in years.
[b] Geometric mean of neutralizing antibody titers.

TABLE II. CHARACTERISTICS OF PATIENTS WITH HSV-2 INFECTIONS

Patient group	No. of Patients	Age[a]	Male/female	Onset of recurrences prior to study[a]	No. of recurrences per year	Serum antibody[b]
Recurrences						
Lesions present	13	30.4 ± 2.8	3/10	2.4 ± 1.0	6.7 ± 0.9	93.1
Lesions absent	19	31.5 ± 2.1	10/9	2.5 ± 1.0	7.6 ± 0.7	78.1
No recurrences	7	32.0 ± 3.5	3/4	–	–	71.3

[a] Mean ± SEM in years.
[b] Geometric mean of neutralizing antibody titers.

2–7 days after the onset of skin lesions. Seventy-four percent of the cultures from these patients were positive for HSV, while no positive cultures were obtained when lesions were absent. Patients with type 1 infections had a longer history of recurrent lesions prior to study (onset > 10 years) than did patients with type 2 infections (2.5 ± 1.0 years). There were no significant differences among patient groups in age or socioeconomic status.

Viral isolations. Swabs of lesions, or swabs of oral or genital mucous membranes when lesions were absent, were placed in veal infusion broth, inoculated into human diploid fibroblast (W138) and human embryonic kidney (HEK) tissue culture tubes, and observed daily for evidence of characteristic cytopathic effect (CPE). As determined by direct immunofluorescence (12), all isolates from patients with recurrent orofacial lesions were HSV type 1, and all isolates from patients with genital infections were type 2.

Antibody determinations. Serum specimens were assayed for antibody to HSV types 1 and 2 by standard neutralization techniques in WI38 monolayers (13). The HSV type 1 virus (21063) used for antibody determinations was originally isolated from a patient with primary gingivostomatitis and was then passed twice in HEK cells. The HSV type 2 virus was a reference strain (VR-540) obtained from the American Type Culture Collection and was passed once in HEK cells. Antibody titers were expressed as the reciprocal of the maximum serum dilution which resulted in a 50% reduction of CPE produced by the homologous virus.

Cytotoxicity assay: Effector cells. Effector cells were peripheral blood mononuclear cells obtained by Hypaque–Ficoll separation of heparinized venous blood (14). The cells were washed three times in 50-ml volumes with Eagle's minimal essential media modified for suspension culture (MEM-S, HEM Research Inc., Rockville, Md.) and supplemented with 0.02 *M* L-glutamine, 25 μg/ml of tetracycline, 250 μg/ml of streptomycin, and 250 U/ml of penicillin. After washing, cells were resuspended in MEM-S.

Target cells. Human prostate adenoma tissue culture cells (MA 160) (15) were used as target cells in the assay. Forty-eight hours prior to assay, MA 160 cells were infected with HSV type 1 or 2 at a multiplicity of infection of 1. Virus strains used were those described above under Antibody determinations. This procedure routinely resulted in the appearance of CPE in 60–75% of the cells and of HSV-specific immunofluorescence in 85–90% of the cells at the time of assay. On the day of assay, infected MA 160 monolayers were trypsinized using a solu-

tion containing 0.5 g/liter of beef trypsin and 0.2 g/liter of EDTA (NIH Media Unit). The cells were then washed twice with MEM-S supplemented with 10% heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N. Y.). One million targets were then incubated with 100 μCi of ^{51}Cr (Amersham/Searle, Arlington Heights, Ill.) for 1 hr in a total volume of 0.5 ml. Cells were then washed three times in MEM-S supplemented with 10% FCS and were resuspended to a concentration of 2×10^5/ml in MEM-S.

Assay. All assays were carried out in duplicate in 12 × 75-mm plastic tubes (Falcon, Oxnard, Calif.). Effector cell concentrations of 4×10^6, 2×10^6, 1×10^6, and 5×10^5 cells/ml were employed to give effector cell/target cell (E/T) ratios of 200/1, 100/1, 50/1, and 25/1, respectively. Five-tenths milliliter of the effector cell suspension, 0.1 ml of the target cell suspension, 0.1 ml of FCS, and 0.3 ml of MEM-S were added together to give a total volume of 1.0 ml/tube. Prior to incubation, the cell suspensions were centrifuged at 50*g* to ensure maximal contact between effector and target cells. The suspensions were then incubated for 4 hr on a rocking platform (Bellco Glass, Inc., Vineland, N. J.) at 37° in 5% CO_2 in air at 100% humidity. Following the incubation period, the cell suspensions were centrifuged at 600*g*, the supernatants were removed, and the samples were counted in an automatic gamma counting system.

Calculations. Ri, or the percentage of ^{51}Cr released from infected target cells when incubated with mononuclear effectors, was calculated as follows: $Ri = [(Li - Si)/(Mi - Si_0)] \times 100$, where *Li* represents the percentage of ^{51}Cr released from infected target cells incubated with lymphocytes, *Si* represents the percentage of ^{51}Cr released spontaneously from infected target cells alone, *Mi* represents the maximum percentage of ^{51}Cr released with target cells suspended in distilled water and subjected to four cycles of freezing and thawing, and Si_0 represents the percentage of ^{51}Cr released from target cells in the interval between the last wash and the beginning of incubation. The percentage of ^{51}Cr released from uninfected target cells when incubated with mononuclear effectors (*Ru*) was calculated as follows: $Ru = [(Lu - Su)/(Mu - Su_0)] \times 100$, where the terms represent analogous values obtained using uninfected target cells. Percentage of specific immune release (SIR) was calculated by subtracting *Ru* from *Ri*.

The mean value of *Si* was 14.31 ± 0.95 and that of *Su* was 11.98 ± 0.62. The mean value of *M*, the maximum percentage of ^{51}Cr released after freezing and thawing of target cells, was 88.16 ± 0.58. The mean value of *So,* the percentage of ^{51}Cr released prior to incubation, was 4.33 ± 0.21.

Results. Antibody levels. Serum-neutralizing antibody levels in patients with recurrent HSV type 1 infections are shown in Table I. Geometric means were compared by application of a Student's *t* test to the mean $\log_{10}$ of the serum-neutralizing antibody titers. Among patients with recurrences, no significant differences were noted between antibody levels measured when lesions were present and those levels measured when lesions were absent. Individuals with serologic evidence of infection with HSV type 1, but without histories of recurrent infection, had significantly lower antibody titers than did patients subject to recurrences either when lesions were present ($P < 0.05$) or when lesions were absent ($P < 0.01$).

Antibody levels in patients with recurrent HSV type 2 infections are shown in Table II. There were no significant differences in neutralizing antibody titers between any of the groups.

Effect of varying effector cell target cell (E/T) ratios on cytotoxicity. Figure 1 depicts the effect of varying E/T ratios on cytotoxicity in representative experiments employing HSV-1 infected target cells and mononuclear effectors from a single immune donor. Maximum percentages of SIR were noted at E/T ratios of 100 to 1 and 200 to 1 for both HSV-1- and HSV-2-infected target cells. Subsequently, cytotoxicity was calculated using an E/T ratio of 100/1.

Cytotoxicity in patients with type 1 infections. The levels of cytotoxicity observed in patients with recurrent type 1 infections are depicted in Table III. The mean percentage of SIR in patients with active lesions was greater than that observed when lesions

were absent ($P < 0.001$) and greater than that measured in sero-positive individuals without recurrences ($P < 0.02$). Cytotoxicity during periods when lesions were absent was not significantly different from that seen in the sero-positive group without recurrences. Cytotoxicity directed against uninfected target cells was not statistically different between any of the groups. The mean percentage of SIR observed in the seven sero-negative controls was -0.30 ± 0.49, which is significantly less than that measured in sero-positive individuals without recurrences ($P < 0.05$). Four patients with recurrent type 1 infections were studied both when lesions were present and when lesions were absent. In every instance, cytotoxicity was higher when active lesions were present than when lesions were absent (mean difference of 14.25%).

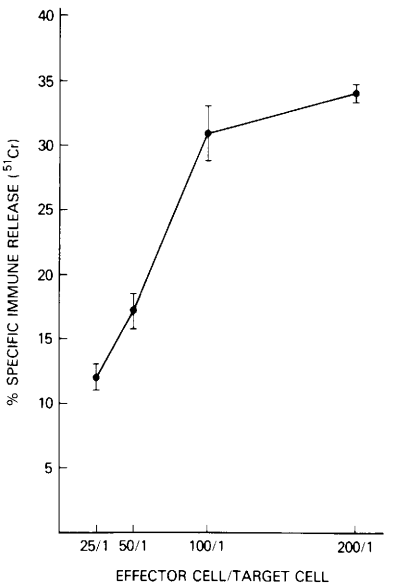

FIG. 1. Effect of varying effector cell/target cell (E/T) ratios on cytotoxicity in representative experiments employing HSV-1-infected target cells and mononuclear effectors from a single donor. Points represent means ± SEM of percentage of specific immune release plotted against E/T ratios.

Cytotoxicity in patients with type 2 infections. Table IV depicts the levels of cytotoxicity observed in patients with recurrent type 2 infections. In the patients with active lesions, the mean percentage of SIR was greater than that observed when lesions were absent ($P < 0.001$) and greater than that seen in sero-positive individuals without recurrences ($P < 0.002$). Cytotoxicity observed during periods when lesions were absent was not significantly different from that seen in the sero-positive group without recurrences. The cytotoxicity directed against uninfected target cells was not statistically different between any of the groups. The mean percentage of SIR observed in the five sero-negative controls was -2.20 ± 1.83, which is significantly less than that measured in sero-positive individuals without recurrences ($P < 0.05$). Five patients with recurrent type 2 infections were studied both when lesions were present and when lesions were absent. In every instance, cytotoxicity was higher when active lesions were present than when lesions were absent (mean difference of 14.20%).

Discussion. Cytotoxic activity against target cells acutely infected with HSV, expressed as the percentage of SIR, was present in circulating mononuclear cells from patients with recurrent HSV infections, as well as in serum antibody-positive individuals without recurrences. When lesions were absent, mean levels of cytotoxicity were not significantly different between

TABLE III. CYTOTOXICITY IN PATIENTS WITH HSV TYPE 1 INFECTION

Patient group	No. of patients	^{51}Cr released from infected cells[a] (%)	^{51}Cr released from uninfected cells[b] (%)	Specific immune release[c] (%)
Recurrent infections (lesions present)	6	42.50 ± 6.20[d]	12.33 ± 1.50	30.17 ± 5.92
Recurrent infections (lesions absent)	15	25.53 ± 3.11	16.07 ± 3.55	9.47 ± 2.33
No recurrences	13	30.00 ± 3.34	18.77 ± 4.22	11.38 ± 3.50

[a] Percentage of ^{51}Cr released from infected target cells when incubated with mononuclear effectors.
[b] Percentage of ^{51}Cr released from uninfected target cells when incubated with mononuclear effectors.
[c] Percentage of ^{51}Cr released from uninfected target cells subtracted from percentage of ^{51}Cr released from infected target cells.
[d] Mean ± SEM.

TABLE IV. CYTOTOXICITY IN PATIENTS WITH HSV TYPE 2 INFECTION

Patient group	No. of patients	^{51}Cr released from infected cells[a] (%)	^{51}Cr released from uninfected cells[b] (%)	Specific immune release[c] (%)
Recurrent infections (lesions present)	13	33.08 ± 4.42[d]	9.62 ± 1.38	23.38 ± 3.62
Recurrent infections (lesions absent)	19	17.47 ± 2.64	11.84 ± 2.12	5.68 ± 1.50
No recurrences	7	12.86 ± 2.63	8.29 ± 1.29	4.71 ± 1.78

[a] Percentage of ^{51}Cr released from infected target cells when incubated with mononuclear effectors.
[b] Percentage of ^{51}Cr released from uninfected target cells when incubated with mononuclear effectors.
[c] Percentage of ^{51}Cr released from uninfected target cells subtracted from percentage of ^{51}Cr released from infected target cells.
[d] Mean ± SEM.

these two groups. However, the percentage of SIR measured when lesions were present was significantly elevated above those levels found when lesions were absent and was also higher than levels found in sero-positive individuals without recurrences. Non-virus-specific cytotoxicity, as measured by cytotoxic activity directed against uninfected target cells, was not different between any of the patient groups whether lesions were present or not. These patterns of cytotoxicity were observed in patients with HSV-1 (oral) and in patients with HSV-2 (genital) infections.

Previous studies of lymphocyte cytotoxicity in patients with HSV infections have been limited to type 1 infection and have produced variable results. Wilton *et al.* (8) reported depressed levels of non-virus-specific cytotoxicity directed against chicken erythrocyte target cells in patients with HSV infections regardless of whether lesions were present or absent. Russell *et al.* (16), employing human amnion cells acutely infected with HSV as target cells, detected substantial virus-specific cytotoxicity in patients subject to recurrent oral lesions and none or very low levels of cytotoxicity in most sero-negative individuals. Steele *et al.* (9) and Thong *et al.* (10), using a target cell chronically infected with HSV-1, described depressed levels of virus-specific cytotoxicity in patients with recurrent lesions compared to sero-positive individuals without histories of recurrences, although Thong *et al.* (10) described heightened levels of cytotoxicity associated with active lesions. Differences in the results of the above studies suggest that cytotoxic responses vary according to the type of target cells employed. When infected target cells are used, responses to acutely infected cells may differ from those to chronically infected cells.

Our findings indicate that levels of virus-specific cytotoxicity in patients with recurrent infections fluctuate with activity of the underlying infection and that, during the presence of active infection, higher levels of cytotoxicity are observed. Although we were unable to measure serially the cytotoxic activity in individual patients after active infection, the presence of lower percentages of SIR when lesions were absent suggests that the elevated or "boosted" levels of cytotoxic activity may be of short duration.

The precise nature of the effector cell in our studies and in those discussed above has not been defined. Preliminary studies suggest that the effector cell in our assay is a nonadherent, nonphagocytic, non-T lymphocyte.[2] Further studies to characterize more precisely the nature of the effector cell and to determine the possible role of cytophilic antibody in this system are in progress.

The current studies have demonstrated that a single *in vitro* parameter of cell-mediated immunity, cell-mediated cytotoxicity against acutely infected target cells, does not appear to be depressed in patients with recurrent HSV infection. Previous studies have shown that other parameters of cell-mediated immunity, such as antibody-dependent cell-mediated cytotoxicity (17) and blastogenic responses of peripheral blood mononuclear cells (6, 8, 9), also appear to be intact in patients with recurrent infections. In addition, the patients with recurrent infections studied here maintained high

[2] R. Reichman, R. Dolin, and A. S. Fauci, unpublished observations.

levels of circulating antibodies to HSV, as has been previously noted (4). While it remains possible that eliciting even higher levels of immunity may prevent recurrences, attempts at augmentation of immune responses should take into account the fact that patients with recurrences already have appreciable levels of cellular and humoral immunity to the virus. The absence of a demonstrable deficit in immunity in patients with recurrent HSV infections in these studies suggests that examination of other aspects of host–virus interaction will be required to delineate the pathogenesis of recurrences and to design appropriate therapeutic measures.

Summary. Cell-mediated cytotoxic activity in circulating mononuclear cells from patients with recurrent herpes simplex virus (HSV) infections with types 1 and 2 was examined employing target cells acutely infected with HSV in a ^{51}Cr-release assay. Cytotoxicity was determined both when lesions were present and when lesions were absent in otherwise normal patients and was compared to levels of cytotoxicity in sero-positive individuals without histories of recurrences. When lesions were present, patients with recurrences had significantly higher levels of cytotoxicity than when lesions were absent. In the absence of lesions, cytotoxicity in patients with recurrences was not significantly different from levels in sero-positive individuals without recurrences. These patterns of cytotoxicity were observed in patients with both HSV-1 and HSV-2 infections. These studies indicate that patients with recurrent HSV infections have intact cell-mediated immune responses, as measured by this assay; and that although the levels of response vary with activity of the underlying infection, the responses are equal to or greater than those observed in sero-positive individuals without recurrences.

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