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Cell-Mediated Immunity to Herpes Simplex in Humans: Lymphocyte Cytotoxicity Measured by ^{51}Cr Release from Infected Cells

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We assessed cell-mediated immunity to herpes simplex virus type 1 antigen in patients suffering from recurrent cold sores and in a series of healthy controls. Paradoxically, all those subject to recurrent herpetic infections had, without exception, evidence of cell-mediated immunity to herpes antigens. This was demonstrated by lymphocyte transformation and specific ^{51}Cr release from infected human amnion cells after incubation with peripheral blood mononuclear cells. Where performed, skin tests with herpes antigen were also positive. In addition, serum from these patients specifically sensitized herpesvirus-infected cells to killing by nonimmune, control mononuclear cells. These tests were negative in the control patients except in a few cases, and it is suggested that these latter may be the asymptomatic herpesvirus carriers previously recognized or that they may have experienced a genital infection.

Recurrent circumoral infection with herpes simplex virus (HSV) is virtually confined to people who have demonstrable serum-neutralizing antibodies to the virus (2, 34). This inconsistency with previous ideas of virus-host interaction was first commented upon by Andrewes and Carmichael in 1930 (2). We have recently added to this paradox by demonstrating that all such patients can also be shown to have cell-mediated immunity (CMI) to HSV antigens (36, 37); submitted for publication. Since the techniques used (lymphocyte transformation and inhibition of leukocyte migration) are only indirect indexes of the cell-mediated immune response, we have looked for more direct manifestations of CMI and examined the ability of mononuclear cells from patients with recurrent herpes simplex infections to kill virus-infected amnion cells. This may be a more reliable measure of CMI than lymphocyte transformation alone. Some of the antigens synthesized under the direction of viral deoxyribonucleic acid are expressed in the membrane of infected cells, and it is likely that these are more relevant in vivo than the several capsid proteins that were present in our initial viral extracts. There is thus less danger of examining the response to a functionally inappropriate antigen (22, 26, 39). This technique depends on the release of ^{51}Cr from damaged target (i.e., amnion) cells after direct interaction with specifically sensitized mononuclear cells. Using blood from the same volunteers, we also examined lymphocyte stimulation in response to a preparation of herpes simplex antigen. In addition, intradermal skin tests with viral proteins were performed in some of the subjects.

MATERIALS AND METHODS

Volunteers were recruited from university students and staff and were divided into two groups: (i) those who were susceptible to circumoral herpes and had had at least two attacks in the previous year; and (ii) those who had never had any form of circumoral herpetic lesion. No volunteers were accepted if their history was uncertain or did not fit clearly into either of these two groups. None of the subjects had an active lesion at the time of testing.

HSV type 1 cultured from a patient with active circumoral lesions was grown in tissue culture and passaged in a continuous line of HAE cells (human amnion Edmonton 70) provided by J. M. S. Dixon, Provincial Laboratories of Public Health, Edmonton, Alberta. The stock virus was titrated and frozen in portions at −70 C.

Two Roux bottles were seeded with 10^6 HAE cells in minimal essential medium with 2% fetal calf serum. Twenty-four hours later one of the bottles was infected with 4×10^5 plaque-forming units of stock virus, and the cells were reincubated in growth medium. After a further 48 h, the supernatant and dead cells were decanted and the adherent cells were removed from both bottles by using 0.25% trypsin and calcium- and magnesium-free Hanks balanced salt solution. The cells were then washed and labeled with ^{51}Cr according to the method of Perlmann and Perlmann (28). Before labeling, the viability of both the uninfected and infected cells that remained (determined by trypan blue exclusion) was usually over 95%. Blood was taken from a patient with a clear

history of recurrent cold sores and from a normal person. The mononuclear cells were separated by Ficoll-Isopaque flotation using Ficoll (Pharmacia, Uppsala, Sweden) and Isopaque (Nyegaard Co., Oslo, Norway), specific gravity 1.076 to 1.078 (15). After washing, these cells were incubated with the washed amnion cells for 2 h in a shaking water bath at 37 C and for a further 2 h in a 37 C incubator. All the tests were done in plastic tubes (12 by 75 mm) in which 1 ml of the mononuclear, effector cell suspension plus 0.025 ml of HAE target cell suspension were mixed. The optimum ratio of effector to target cells was examined, and after this most studies were performed with a ratio of 100:1. After incubation the reaction was stopped by the addition of 1 ml of ice-cold Hanks balanced salt solution, and the suspension was centrifuged for 8 min at 4 C (4). The supernatant and the cell pellet were counted separately by a gamma counter. The percentage of specific cell lysis was determined as follows: $(I - N/F_I) \times 100$, where I is counts per minute of the supernatant after incubation of infected target cells with test or control lymphocytes minus the counts per minute released by incubation of the target cells alone, N is counts per minute of the supernatant after incubation of the normal target cells with test or control lymphocytes minus the counts per minute released by incubation of the target cells alone, and F_I is counts per minute released from infected target cells by freezing and thawing three times. This was effectively the same as the release from uninfected cells.

The interaction of humoral and cellular immunity in the absence of complement was studied. Dilutions of serum beginning with 0.1 ml of whole serum were added to the above incubation mixtures to assess (i) possible interference with the expression of cellular immunity by humoral factors, i.e., "blocking" antibodies, and (ii) an effect of antibody-mediated cell-dependent immune lysis by lymphocytes with receptors for the Fc portion of immunoglobulin (K cells) (12).

The presence of complement-dependent serum antibodies reacting with membrane antigens from the infected cells was also assessed by the addition of 0.1 ml of fresh guinea pig complement and 0.1 ml of serum to the 0.025 ml of infected and control amnion cells as described by Smith et al. (39).

Lymphocyte transformation in response to HSV antigen was carried out in the same group of patients, as previously described (36, 37).

Complement fixation tests were used to assess serum antibodies to HSV (34), and intradermal skin tests with HSV antigens were performed in 15 subjects in addition to those previously reported (34, 35). A few patients had some induration in response to the control injection of uninfected tissue culture extract. The test was therefore read as positive only if the diameter of induration using the test antigen exceeded the control by 10 mm.

RESULTS

Figure 1. illustrates a typical curve relating the percentage of ^{51}Cr release to the effector/target cell ratio. All of the 13 patients with a clear

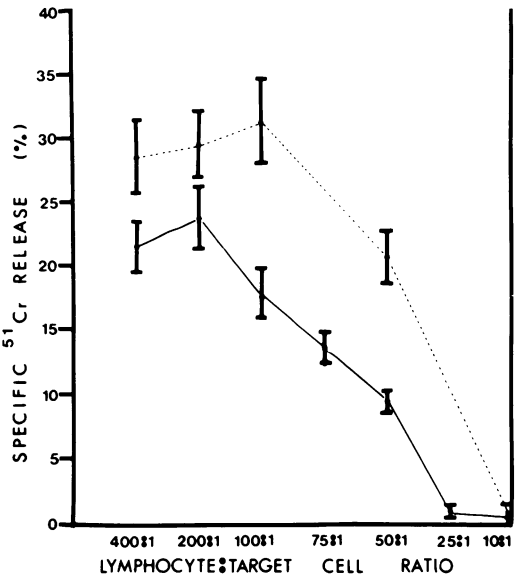

FIG. 1. *Specific ^{51}Cr release from infected HAE cells by sensitized mononuclear effector cells, using varying effector/target cell ratios. Symbols:* ——, *Patient 6;* -----, *patient 7;* I, ± *2 standard deviations.*

history of recurrent cold sores had evidence of ^{51}Cr specific release after incubation of their effector mononuclear cells with infected HAE target cells (Tables 1 and 2). In addition, all had positive lymphocyte transformation, and those eight patients tested had positive skin tests as has been previously demonstrated (35). Serum from these patients had complement-fixing antibodies to HSV antigens and would induce specific ^{51}Cr release from infected HAE cells in the presence of fresh guinea pig complement (Tables 1 and 2), confirming that these antigens are expressed on the surface of the infected cells. In addition, these sera had the property of sensitizing the infected amnion cells to lysis by normal, nonimmune lymphocytes—so-called "K" cells (12). The sera were very potent in this regard, and Fig. 2 shows the specific ^{51}Cr release when serial dilutions of two such sera were added to the infected amnion cells and the mixture was incubated with normal, nonsensitized lymphocytes. In this figure, the cells used were from normal volunteers (no. 18 and 23) and were shown in the same experiment not to induce specific ^{51}Cr release in the absence of serum from group 1 subjects (Table 1). This antibody-mediated cell-dependent immune lysis effect could be demonstrated in all sera of group 1 subjects (herpes positive), but no blocking factors were found on any occasion. Those patients with recurrent infections who were skin tested all had a positive response. Apart

TABLE 1. *^{51}Cr Specific release from herpes simplex-infected human amnion cells after incubation with lymphocytes, or serum and complement from patients with (group 1) and without (group 2) recurrent herpetic infections*[a]

Patient	Serum antibody[b] (reciprocal titer)	Lymphocyte transformation (stimulation ratio)	^{51}Cr specific release from infected cells[c]		Skin test[d]
			By lymphocytes	By serum plus complement	
Group 1					
1	32	43.0	13.3	24.7	40
2	16	8.7	27.4	26.5	11
3	8	46.7	23.5	19.1	14
4	16	16.2	21.6	17.5	13
5	16	14.6	25.9	73.0	ND
6	16	14.1	26.0	21.8	ND
7	8	84.2	30.1	26.3	ND
8	32	38.0	19.2	28.9	12
9	16	31.0	16.1	39.0	20
10	16	74.4	4.5	8.6	15
11	32	24.4	14.0	12.1	10
12	8	8.5	22.4	21.1	ND
13	16	76.0	38.4	23.6	ND
Group 2					
14	—	21.0	1.7	1.2	
15	—	59.0	2.7	0.9	12
16	16	11.8	16.7		10
17	—	3.3	−1.6	1.4	ND
18	—	0.4	1.9	−0.1	ND
19	—	1.0	26.0	19.7	12
20	—	0.5	2.4	−2.0	
21	—	0.6	−3.8	−1.6	
22	—	0.8	0.8	0.9	
23	—	0.5	5.1		2
24	—	0.9	2.6	1.9	ND
25	—	0.6	−1.2	0.6	ND
26	—	1.2	−1.9	−2.2	ND

[a] The mean (range) background ^{51}Cr release from the infected cells was 3.7% (2.3 to 8.0) and from uninfected cells 4.9% (3.1 to 9.1). This correction has already been included in the calculation of the percent specific release (see footnote *c*).

[b] −, Negative at the first serum dilution used (36).

[c] $I - N/F_1$, expressed as percentage.

[d] Induration with herpes simplex antigen (diameter in millimeters) minus induration with the control antigen, where relevant. ND, Not done.

from patient no. 19, those with negative lymphocyte transformation tests had negative skin tests.

DISCUSSION

We have demonstrated that all subjects susceptible to circumoral herpes have CMI against virus-directed antigens expressed on the surface of infected human cells. In addition, their serum contains antibody usually in very high titer that will sensitize these infected cells to specific killing by nonsensitized lymphocytes (antibody mediated cell dependent immune lysis; 19). No blocking effect of antibody was found, but this effect is notoriously difficult to

TABLE 2. *Immune response to herpes simplex virus type 1 in subjects with (group 1) and without (group 2) a history of cold sores*

Group[a]	No. with:				
	Serum CFA	Significant lymphocyte stimulation	Specific ^{51}Cr release by mononuclear cells alone	Specific ^{51}Cr release by patients serum and unsensitized normal mononuclear cells	Positive skin tests
1	13	13	13	13	8/8
2	1	4[b]	3[b]	3[b]	3[b]/7

[a] Both groups had 13 subjects.

[b] Includes the same three subjects.

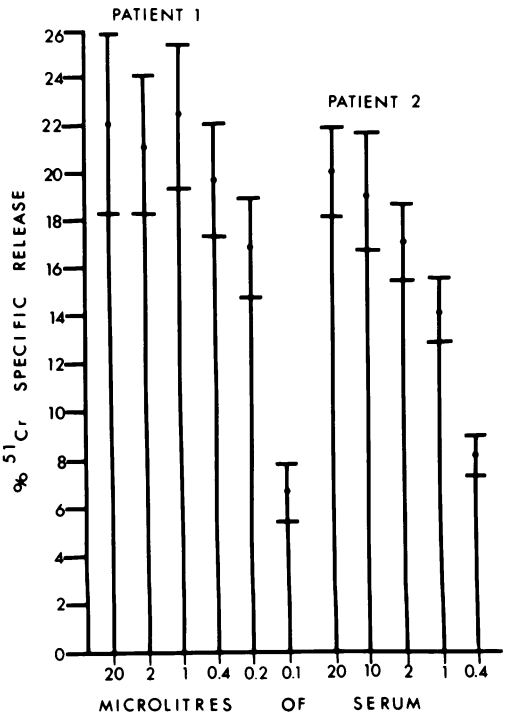

FIG. 2. *Specific ^{51}Cr release from infected HAE cells by nonsensitized, normal lymphocytes in the presence of serial serum dilutions of patients 1 and 2, beginning with a final serum dilution of 1:50, i.e. 20 μliters of serum.* $\bar{I}$, ± *2 standard deviations.*

detect in ^{51}Cr release assays. The same patients, where tested, all had positive skin tests to herpes antigen and their lymphocytes were stimulated by in vitro incubation with the same antigen. The results with ^{51}Cr release agree very closely with the results of lymphocyte proliferation and the skin tests and reinforce our earlier findings with these parameters (34, 35).

Most people not suffering from recurrent herpes labialis do not show any immune response to the virus (Tables 1 and 2), but as found earlier there are a few exceptions (34, 35). Some of these persons may be the asymptomatic virus carriers already described (18, 20) or perhaps have had a genitourinary infection with HSV type 2, a virus possessing many antigens in common with the type 1 virus studied (22). A previous report (41) suggested a specific generalized defect of migration inhibition factor production in patients subject to recurrent herpetic infections. There were no in vivo studies, and the results are difficult to interpret because the control population had evidence of a high incidence of previous infection with the virus. We have previously shown in a large number of persons that those patients with no lymphocyte transformation to HSV antigen have negative skin tests and, as in our present study, those patients tested who have recurrent clinical infections have positive tests (Tables 1 and 2).

The herpesviruses have an unusual propensity to induce latent infections in humans, with clinical recurrences at intervals in some patients (11). The form in which HSV remains latent is not known (33). Nahmias and Roizman (25) have advocated the use of the term "recurrent infections" to include the asymptomatic viral reactivation that may occur (8, 18, 20) and used the term "recrudescent infection" to describe the recurrence of specific clinical lesions. Rasmussen et al. (30) have demonstrated that interferon production in vitro by patients' lymphocytes in response to HSV antigen wanes some weeks after a recrudescent infection. Lymphocyte transformation was essentially unchanged. This type of dissociation between the synthesis of various products of lymphocyte activation has already been described (6, 32). It has also been suggested that this waning of the interferon response may account for the development of recrudescent infection (30). Since the role of interferon in recovery from any virus infection is still uncertain (3, 37) and in experimental HSV infections in animals interferon would seem to play no part in the recovery process (7, 10, 17, 21, 27, 37, 38), this extrapolation, although attractive, does not yet seem fully justified. Moreover, only recrudescent infections were included by Rasmussen et al.; thus the virus reactivation occurring during asymptomatic infection is not considered. It is often stated that CMI is the principal defense against HSV (9, 10, 23, 37), but on the basis of our results we suggest that conventional CMI may not be important in resistance to recrudescent HSV lesions. This gives a theoretical basis for the therapeutic inefficacy of the various forms of immunotherapy that have been used (1, 3, 13, 16). On the other hand, CMI may be of major importance in maintaining the usual localized nature of the herpetic lesions (35). Diseases or drugs associated with cellular immune deficiencies in man are associated with an increased frequency of herpes zoster infections (5, 14, 31, 40), and we have previously demonstrated a probable defect in CMI to account for this (36). The more severe deficiencies of CMI are also associated not with recurrent cold sores but with persistent and disseminated HSV infections, usually primary (5, 24; C. Lopez, W. D. Biggar, and R. E. Kissling, Fed. Proc. **31**:635, 1972), but sometimes a secondary infection may spread widely. Thus localization of the viral lesions and recovery from them may indeed be a function where CMI has a definite role.

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