

Stimulation of Human Lymphocytes by Herpes Simplex Virus Antigens

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Lymphocytes from individuals with laboratory evidence of prior infection with herpes simplex virus (HSV) type 1 or type 2 demonstrated transformation (as measured by [H^3]thymidine incorporation) when incubated with heat-inactivated HSV antigens. Higher stimulation indexes were obtained when lymphocytes were incubated with the homologous as compared with the heterologous antigen. Higher mean lymphocyte stimulation indexes were also demonstrated in seropositive as compared with seronegative individuals. Lymphocytes from children with HSV-1 stomatitis usually became responsive to HSV-1 antigen within 2 to 6 weeks after the onset of illness. Lymphocytes from infants with neonatal HSV-2 infection were stimulated by HSV-2 antigen.

A number of clinical and experimental animal observations suggest that cellular mechanisms play an important role in immunity to herpes simplex virus (HSV) infection (4). To permit more finite studies, several *in vitro* assays for cell-mediated immunity to HSV, including assays for lymphocyte transformation, are currently being developed. Stimulation by inactivated HSV antigens of spleen lymphocytes obtained from HSV-inoculated rabbits was demonstrated by Rosenberg and co-workers (6, 7). Several investigators have also reported that HSV-1 antigens stimulate peripheral blood lymphocytes obtained from adults with recurrent HSV-1 infections (5, 8, 9).

The purpose of the present study was to characterize further the transformation of lymphocytes obtained from seronegative individuals and from those with primary, as well as recurrent, HSV-1 infections. In addition, the response to HSV-2 antigens was studied in both adults and newborns.

MATERIALS AND METHODS

Preparation of antigens. HSV antigens were prepared from HSV-infected BHK-21 cells. Crude virus pools were partially purified by two 1-h centrifugations at $100,000 \times g$. The pelleted viruses were resuspended in pH 7.2 phosphate-buffered saline containing penicillin, streptomycin, and fungizone, and were placed in a 56 C water bath for 1 h. Before heat inactivation, the HSV-1 (HE newborn strain) and HSV-2 (FLO newborn strain) preparations contained 3×10^8 and 8×10^7 plaque-forming units per ml, respectively; inactivated preparations contained

no infectious virus. BHK antigen was prepared from uninfected BHK-21 cells treated in the same fashion. All antigens were stored at $-70^\circ C$ before use.

Leukocyte cultures. Peripheral blood was drawn into syringes containing 50 to 100 U of heparin (Panheprin, Abbott Laboratories, North Chicago, Ill.) per ml of blood. Macrodex (6% vol/wt) (Pharmacia Laboratories, Inc., Piscataway, N.J.) was added to give a final concentration of 1%, and the blood was allowed to sediment at $37^\circ C$ for 1 to 1.5 h. The leukocyte-rich plasma was centrifuged, and the cells in the pellet were washed twice with Puck's saline A (Grand Island Biological Co., Grand Island, N.Y.). A suspension containing 5×10^5 viable lymphocytes in RPMI 1640 (Flow Laboratories, Rockville, Md.) with added antibiotics and 20% autologous plasma was prepared. Three-milliliter aliquots were dispensed into plastic tubes (12 by 75 mm; Falcon 2054 tube, Falcon Plastics, Oxnard, Calif.). Antigens at appropriate concentrations were added to triplicate cell cultures in 0.1-ml volumes. The leukocyte cultures were incubated at $37^\circ C$ in a 5% CO_2 incubator for 6 days, the optimal incubation period as determined in preliminary studies.

Thymidine incorporation. For the final 4 h of incubation, [H^3]thymidine (New England Nuclear Corp., Boston, Mass.) (specific activity, 6.7 Ci/mmol) was added, $2 \mu Ci$ per tube. The cells were then washed successively once with saline, once with 10% trichloroacetic acid, and twice with saline. The precipitates were dissolved in NCS tissue solubilizer (Amersham/Searle, Arlington Heights, Ill.). Scintillation fluid (Spectrafluor, Amersham/Searle, Arlington Heights, Ill.) was added, and the samples were counted in a Packard Tri-Carb liquid scintillation counter. Results are expressed either as the mean counts per minute of triplicate cultures or as the stimulation index (SI), defined as the mean counts per minute of cultures

incubated with HSV antigens divided by the mean counts per minute of cultures incubated with BHK antigens. Geometric mean SIs were calculated from the \log_{10} of the SI indices; P values were calculated by use of the Student's t test.

Detection of HSV antibodies and typing of virus isolates. Typing of virus isolates was performed by a direct immunofluorescent technique (2), and measurement of HSV type antibodies was by microneutralization assays and the neutralization potency method of analysis (3).

RESULTS

Dose response curves for HSV-1 and HSV-2 antigens. Dose response curves for HSV-1 and HSV-2 antigens were determined by incubating lymphocytes from two individuals with varying doses of homologous antigens. The serum antibody type, as measured by the neutralization potency method (3), in one individual was type 1 (titer to HSV-1 = 1:40 and to HSV-2 <1:10) and in the other individual was type 2 (titers to HSV-1 = 1:40 and to HSV-2 = 1:40). Maximal lymphocyte stimulation was obtained with HSV-1 antigen employed at a 1:5 dilution and with undiluted HSV-2 antigen (Fig. 1). In all studies described below, HSV antigens were used at these concentrations. Corresponding dilutions of BHK antigens were employed, which were found repeatedly to cause less than twofold stimulation or depression of incorporation of [H^3]thymidine, as compared to cultures incubated with no antigen.

When lymphocytes from the above individuals with prior experience with either HSV-1 or HSV-2 were incubated with heterologous antigen, lower SIs were obtained than with homologous antigen (Table 1).

Response of lymphocytes from seronegative and seropositive adults. Lymphocytes

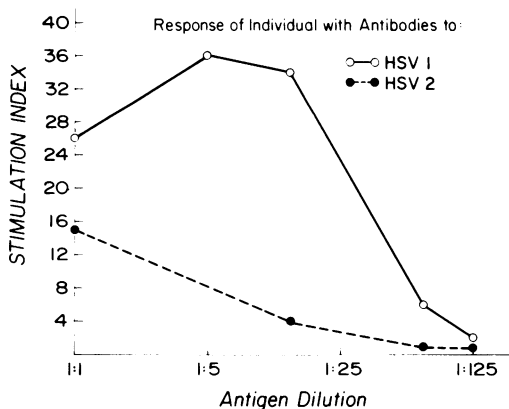


FIG. 1. Stimulation of lymphocytes incubated with various doses of homologous HSV antigen.

were obtained from twelve healthy adults, six with no detectable HSV antibodies and six with HSV-1 antibodies. None of the individuals had herpetic lesions around the time their lymphocytes were tested with the HSV-1 and BHK antigens. The geometric mean SI for seropositive individuals ($1.44 \pm .46$) was significantly higher ($P < 0.01$) than that obtained for seronegative individuals (0.52 ± 0.41). SIs of 11.0, 7.3, and 4.7 were obtained with lymphocytes from three of the seronegative subjects who denied having experienced prior HSV infection. One of these individuals (SI = 4.7) subsequently developed HSV-1 stomatitis; HSV-1 antibodies were detected 10 days after the onset of illness.

Response of lymphocytes from children with HSV-1 stomatitis. Four children, ages 1 to 3 years, with virologically confirmed HSV-1 stomatitis had their lymphocytes assayed at the time of their first clinic visit and 2 to 6 weeks later (Table 2). All of the children demonstrated typical herpetic oral lesions, cervical adenopathy, and fever, and had been symptomatic for 3 to 6 days prior to initial examination. At the time of repeat testing, all showed complete healing of the lesions. The lymphocytes obtained at the time of the first visit, when incubated with the HSV-1 and BHK antigens, yielded a geometric mean SI of 0.25 ± 0.28 . Two to 6 weeks later, the geometric mean SI (1.16 ± 0.67) was significantly increased ($P < 0.01$). The lymphocytes from one patient (no. 1) failed to respond when tested 22 days postinfection, although serum HSV antibodies were detected at that time. Another child (no. 4) demonstrated a low SI 6 days after onset of clinical manifestations, at which time HSV antibodies were already present in his serum.

Lymphocyte stimulation in infants with neonatal HSV-2 infection. Two infants with neonatal HSV-2 infection documented by viral isolation and typing were studied 3 weeks and 1

TABLE 1. Stimulation of lymphocytes from individuals with neutralizing antibodies to HSV by HSV-1, HSV-2 and BHK antigens

Antigen	Lymphocytes			
	From individual with antibodies against HSV-1		From individual with antibodies against HSV-2	
	Counts/min	SI	Counts/min	SI
BHK	634		302	
HSV-1	16,603	25.2	2,448	8.1
HSV-2	10,154	15.9	6,488	21.4

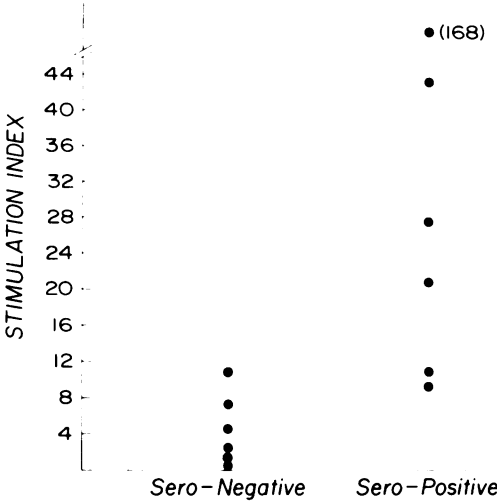


FIG. 2. Stimulation of lymphocytes from seronegative and seropositive individuals by HSV1 antigen.

TABLE 2. Immunological responses in patients with herpetic stomatitis

Patient no.	Age (years)	Days after onset of illness	MN ^a	SI
1	1	3	<1:8	1.1
		22	1:32	2.2
2	2	3	<1:8	3.2
		40	1:16	10.1
3	2	3	<1:8	0.8
		17	1:32	68.0
4	3	6	1:8	2.4
		25	1:16	32.0

^a Microneutralization antibody titer.

year, respectively, after the onset of symptoms. The lymphocytes from both infants were stimulated to a greater extent by HSV-2 than by HSV-1 antigens (Table 3). Not shown are results obtained with lymphocytes from three cord blood specimens which demonstrated no response to HSV antigens.

DISCUSSION

The concentrations of HSV antigens employed in this study were established by dose response determinations in two individuals. Lymphocytes from every individual tested could not be incubated with multiple concentrations of antigens or with mitogens as amounts of blood which could be easily obtained, particularly from infants, were not sufficient. We are currently adapting the assay to a micromethod which would permit testing the

TABLE 3. Lymphocyte stimulation in individuals with neonatal HSV-2 infection

Subject	Antigen				
	BHK	HSV-1		HSV-2	
	Counts/min	Counts/min	SI	Counts/min	SI
J.L.	1,303	2,503	1.9	7,779	6.0
D.F.	3,079	33,749	11.0	65,249	21.2

response of lymphocytes from all individuals with mitogens and at least two antigen doses.

The dose response curve obtained with HSV-2 antigen suggests that higher SIs might be obtained if higher-titered virus were used to prepare the antigen. Nevertheless, lymphocytes from adult or newborn individuals with HSV-2 antibodies were stimulated to a greater extent when incubated with HSV-2 antigen than with HSV-1 antigen. Similarly, lymphocytes from an individual with HSV-1 antibodies demonstrated a greater response to homologous antigen. These results, if confirmed with more human data, would parallel those obtained by Rosenberg et al. in rabbits (7).

The stimulation of lymphocytes from individuals with no detectable HSV antibody by HSV antigens has been infrequently noted (5, 8, 9). In our study, lymphocytes from three of the seronegative individuals demonstrated some stimulation by HSV-1 antigen. One of these individuals, who had a SI of 4.7, subsequently developed HSV-1 stomatitis, which appeared to be a primary infection. The detection of these apparent nonspecific responses to HSV-1 antigen may be due to the nature of the antigen employed in our assays or to cross-reactions with other yet undefined antigens. It has been suggested that lymphocyte transformation may in some cases be a more sensitive indicator of prior exposure to HSV than the presence of neutralizing antibodies (5). However, in our study, lymphocytes from two children failed to demonstrate transformation at a time when serum HSV-1 antibodies were detected. Since lymphocytes were not incubated with mitogens, we cannot rule out the possibility that transient diminished lymphocyte responsiveness, which has been described with some viral infections, was responsible for these findings.

As has been noted in previous studies (5, 8, 9), lymphocytes from individuals with HSV-1 serum antibodies demonstrated transformation when incubated with HSV-1 antigen. We have recently shown that lymphocytes from healthy individuals possessing serum antibodies to

HSV-2 demonstrate transformation when incubated with HSV-2 antigen (S. E. Starr et al., unpublished data).

The lymphocyte stimulation assay may assist in the retrospective diagnosis of herpetic infections in newborns which are caused primarily by HSV-2, particularly if the greater reactivity obtained with the HSV-2 antigen is a consistent phenomenon. This neonatal infection is difficult to diagnose when skin lesions are absent or when virus is not recoverable from accessible sites (1). The lymphocyte assay may therefore prove to be useful for early detection of neonatal HSV infection.

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