Lymphocyte-Derived Chemotactic Factor Synthesis in Initial Genital Herpesvirus Infection: Correlation with Lymphocyte Transformation

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Lymphocyte transformation and production of lymphocyte-derived chemotactic factor in response to herpes simplex virus antigen were studied in 15 patients with initial genital herpes and 10 controls. The patients underwent frequent genital examinations, viral cultures, and weekly immunological studies for a period of 11 weeks. The production of lymphocyte-derived chemotactic factor was maximal in week 1 of the disease and declined to control levels by week 6. In contrast, lymphocyte transformation was lowest in week 1, reached a maximum by week 4, and declined to control levels by week 11. Production of lymphocyte-derived chemotactic factor in week 1 was significantly lower in nine patients who developed signs or symptoms of systemic herpes infection than in six who had localized disease. In addition, a marked but transient decline in the production of this lymphokine was observed in patients at the time of clinical recurrence. Virus-specific lymphocyte transformation correlated inversely with the duration of genital pain and lesions and did not correlate with the presence of systemic signs or symptoms. These findings indicate that during initial genital herpes infection the dynamics of lymphocyte transformation and those of lymphocyte-derived chemotactic factor production are different, and that the generation of this lymphokine is an early component of the cellular immune response in this disease. Furthermore, adequate produce of lymphocyte-derived chemotactic factor may be important in restricting herpes simplex virus to the genital area and preventing disease recurrence.

The host response to herpes simplex virus (HSV) infection includes humoral and cellular immunity; however, the relationship between specific immunity and the severity of clinical disease is incompletely understood. Recurrent infections develop in the presence of high titers of virus-specific neutralizing antibody, and severe herpetic infections occur in individuals with impaired cell-mediated immunity (15). For these reasons, investigators have examined various aspects of cellular immunity in patients with HSV infections (17, 20, 21, 24, 25); however, most previous studies have focused on recurrent disease, and there is little information available regarding cell-mediated immunity in initial HSV infections. Recently, we demonstrated that the proliferative response of lymphocytes from patients with initial genital HSV infection reached a maximum during the 4th week of the disease and that the magnitude of the response was inversely correlated with the duration of viral shedding and the persistence of symptoms (4). The present study was initiated to evaluate the development and evolution of lymphokine syn-

thesis during initial genital HSV infection and to examine the relationship of this response to that of lymphocyte transformation and the clinical manifestations of disease. For this purpose, we performed weekly clinical examinations, viral cultures, and measurements of lymphocyte-derived chemotactic factor (LDCF) production and lymphocyte transformation in 15 patients. Our studies show that maximal LDCF production occurs in the 1st week of infection in advance of lymphocyte transformation and that adequate production of this mediator may be important in controlling the severity of initial herpetic infection and the development of recurrent disease.

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MATERIALS AND METHODS

Study design. The study population consisted of 15 adults (6 men and 9 women) with culture-proven

genital HSV infection. All had symptoms for 10 days or less, denied having a history of previous genital HSV infection, and had intact vesicular or pustular lesions on initial examination. The course of the disease was followed by means of genital examinations and viral cultures at 2- to 3-day intervals while lesions were present and then weekly until 11 weeks after the onset of the disease. In addition, a standardized questionnaire regarding the signs and symptoms of genital HSV was administered at each visit. Ten healthy subjects with no history of oral or genital HSV infection served as a control group.

Virus isolation, typing, and serological testing. Specimens from penile and vulvar lesions and the cervix were taken with calcium alginate swabs (Inolex, Glenwood, Ill.) and placed into viral transport medium (2-sucrose-phosphate gelatin broth). If no lesions were seen, the surface of the penis or vulva was swabbed. Duplicate tubes of fetal tonsil cells were inoculated with 0.25 ml of transport medium and were examined weekly for cytopathic effect. Viral isolates were obtained from the supernatants of infected cells, stored at -70°C , and subsequently typed by an indirect immunoperoxidase method (2).

Titers of complement-fixing antibody to HSV and neutralizing antibody to HSV type 1 (HSV-1) and HSV-2 were measured in serum specimens taken at the initial and 4th-week visits, using previously described methods (5, 14, 30).

HSV antigen preparation. HSV antigen was prepared as previously described (4). Briefly, confluent monolayers of HeLa-M cells were infected with HSV-1 and HSV-2 (Robinson and UW-268 strains, respectively). When a 4+ cytopathic effect was observed, the cells were centrifuged at $600 \times g$ for 15 min, and the culture supernatants were removed and saved. The cell pellets were resuspended in minimal essential medium containing 2% fetal calf serum, and the cells were lysed by repeated freeze-thawing and sonication. The supernatants of these cell lysates were then combined with the culture supernatants, and equal portions of the mixture were frozen at -70°C . Control material was prepared by these same methods, using uninfected HeLa-M cells. Antigen and control preparations were thawed and inactivated immediately before use by exposure to ultraviolet light at a distance of 10 cm for 20 min. The same preparations of HSV antigens were used throughout the study period. Preliminary experiments showed the optimal concentrations of antigen for use in both the transformation and LDCF assays to be the undiluted and the 10^{-1} dilutions of HSV-1 and HSV-2, respectively. Inoculation of the ultraviolet light-inactivated antigens into fibroblast cultures was performed periodically throughout the study to confirm that the HSV preparations were not infective.

Preparation of mononuclear cells and generation of LDCF. Mononuclear cells were prepared as previously described (3). Briefly, heparinized peripheral blood was centrifuged (15 min at $200 \times g$), and the buffy coat was removed, diluted 1:2 in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.), and layered on Ficoll-Hypaque density gradients. After centrifugation at $400 \times g$ for 20 min, the mononuclear cells were removed, washed twice, and resus-

pended (1.5×10^6 cells/ml) in RPMI 1640 containing 0.3 mg of L-glutamine, 50 U of penicillin, and 50 μg of streptomycin per ml. Cell viability as determined by trypan blue dye exclusion was $98 \pm 2\%$.

LDCF was produced by culturing 1.5 ml of the mononuclear cell suspensions in serum-free media with 25 μl of the HSV-1 or HSV-2 antigens or phytohemagglutinin (PHA; 10 $\mu\text{g}/\text{ml}$; Burroughs Wellcome Co., Research Triangle Park, N.C.) for 48 h at 37°C in 5% CO_2 in air. Earlier studies have shown these conditions to be optimal for LDCF production (1). The cell suspensions were then centrifuged at $300 \times g$ for 10 min, and the cell-free culture supernatants were frozen at -70°C until they were tested for chemotactic activity. To measure spontaneous or unstimulated LDCF synthesis, cells were cultured for 48 h without HSV or PHA. Twenty-five-microliter portions of PHA or inactivated HSV antigens were added to these control tubes immediately before centrifugation.

Chemotactic assay. Monocyte chemotaxis was measured in modified Boyden chambers, using 5- μm polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) (26). Normal human mononuclear cells at 7.5×10^5 myeloperoxidase-positive cells (monocytes) per ml in Gey balanced salt solution were used as responder cells. These cell suspensions were placed in the upper compartment of Boyden chambers. The culture supernatants to be tested for chemotactic activity were diluted to 33% (vol/vol) with a mixture of Gey balanced salt solution and gelatin Veronal buffer and placed in the lower compartment of the chemotaxis chamber. In all experiments, 10% (vol/vol) bacterial chemotactic factor (BCTX) (29) and phosphate-buffered saline (PBS), pH 7.2, were used as positive and negative controls, respectively. These concentrations of LDCF and BCTX produced approximately half-maximal chemotactic responses (Table 1) and hence were selected to maximize the sensitivity of the chemotactic assay. Cells in the chemotaxis chambers were incubated for 90 min at 37°C . The filters were then removed and stained with Meyer hematoxylin and Wright stains, and leukocyte migration was quan-

TABLE 1. Chemotaxis of monocytes to various concentrations of bacterial and lymphocyte-derived chemotactic factors^a

Concn of BCTX (%)	Response	Concn of LDCF (%)	Response
1.0	5.0 ± 0.9	1.0	3.0 ± 0.6
2.5	39.1 ± 11.0	2.0	11.3 ± 2.0
5.0	54.2 ± 2.0	4.0	21.7 ± 2.8
10.0	73.4 ± 8.0	8.0	33.5 ± 1.5
20.0	97.4 ± 10.2	16.0	51.3 ± 2.3
30.0	160.5 ± 8.5	32.0	72.9 ± 4.6
50.0	158.9 ± 3.5	50.0	119.3 ± 8.1
		66.0	126.6 ± 8.7

^a Chemotaxis was measured as described in Materials and Methods. Results are expressed as the mean $\pm$ standard error of the mean number of migrating cells in 20 microscopic fields (5 by 5 μm ; $\times 990$). The data are a summary of three representative experiments in which each concentration was tested in quadruplicate.

tified by counting the number of cells on the attractant side of the filter in 20 microscopic fields (5 by 5 μ m; $\times 990$). All samples were tested in triplicate, and the results are expressed according to the following equation: $CI = E - C / (BCTX - PBS) \times 10^{-2}$, where CI is the chemotactic index, E is the mean number of cells migrating in response to supernatants from HSV- or PHA-stimulated cell cultures, C is the mean number of cells migrating in response to supernatants from control (unstimulated) cell cultures, and BCTX and PBS are, respectively, the mean numbers of cells migrating in response to bacterial chemotactic factor and phosphate-buffered saline. This equation was necessary to normalize the data for the day-to-day variability of responder cells.

Lymphocyte transformation assay. Lymphocyte transformation was performed weekly, using conventional microtiter techniques (16). Concanavalin A (ConA; 120 μ g/ml; Calbiochem, La Jolla, Calif.), pokeweed mitogen (PWM; 0.5 mg/ml; GIBCO), PHA (10 μ g/ml), and the undiluted and 10^{-1} dilutions of HSV-1 and HSV-2 antigens were used as stimulants. Incubations were carried out for 72 h with PHA and for 7 days with the other antigens and mitogens. Earlier studies have shown that incubation of lymphocytes with HSV antigens for 7 days produces maximal stimulation (4, 10). Results are expressed as a stimulation index. For the HSV antigens, this index equals the mean counts per minute in cell cultures stimulated with homologous HSV antigen (i.e., HSV-1 antigen in the case of patients from whom HSV-1 was isolated), divided by the mean counts per minute in cell cultures incubated with the HeLa-M cell control suspension. In the case of mitogens, this index equals the mean counts per minute in mitogen-stimulated cell cultures divided by the mean counts per minute in the unstimulated cell cultures. The index reported for HSV stimulation is the highest obtained with either the undiluted or 10^{-1} dilution of antigen.

Statistical methods. All results are presented as mean $\pm$ standard error. Data were analyzed by the Student t and Spearman rank correlation coefficient tests.

RESULTS

Clinical and serological findings. The mean age of the 15 study patients was 25.6 years; 13 were Caucasians and 2 were Blacks. The mean interval from onset of lesions until the first clinic visit was 4.0 days (range, 2 to 7 days). Type 2 HSV was isolated from 14 patients, and type 1 virus was isolated from one patient. All patients had initial HSV infection by clinical and historical criteria; that is, this was their first episode of genital HSV infection. Thirteen patients had neither complement-fixing (CF) nor complement-independent neutralizing antibodies in their acute-phase sera, whereas two patients had CF antibodies in their acute sera, suggesting that they had had prior infection with HSV-1 (4). The LDCF and lymphocyte transformation findings which are presented below are not significantly different if these two patients are excluded from the study. Twelve of

the 13 seronegative patients developed CF antibodies to HSV, and both patients with CF antibodies in their acute-phase sera seroconverted to HSV-2 in their convalescent-phase sera (Table 2). In the control group, 8 of 10 individuals were CF antibody negative to HSV; one subject had a 1:16 and the other a 1:64 CF antibody titer to this antigen. These antibody-positive controls gave no prior history of oral or genital HSV infections, and their mononuclear cells were unresponsive to HSV antigen as determined by LDCF production and lymphocyte transformation.

The mean duration of local symptoms (pain, itching, discharge, and dysuria) was 14.4 ± 2.3 days. The duration of viral shedding from genital lesions (defined as the time from the onset of lesions until the last positive viral culture) averaged 9.5 ± 1.2 days (range, 3 to 18). The mean time until re-epithelialization of lesions was complete was 21.0 ± 1.9 days. These data are similar to those presented in our previous study on the clinical course of initial genital HSV infection (4).

Nine of the 15 patients had fever, headache, malaise, myalgias, or photophobia in the 1st week of disease. The mean duration of these systemic signs and symptoms was 7.0 ± 2.1 days. The mean duration of local symptoms was longer (itching, 13.9 ± 0.9 day; pain, 17.1 ± 1.2 days) in the patients with systemic signs or symptoms than in those with localized disease (8.4 ± 3.6 and 10.7 ± 1.2 days, respectively; $P < 0.05$).

Recurrent infection developed in 12 patients. The mean time between initial and recurrent infection was 49 ± 7 days (range, 29 to 81), and the average period for which patients were studied was 186 ± 22 days (range, 30 to 307).

Production of LDCF in response to inactivated HSV antigen and PHA. The production of LDCF by patients' lymphocytes stimulated with homologous HSV antigens varied during the course of infection. The greatest production occurred in the 1st week of disease (29.0 ± 7.5) and declined thereafter (24.0 ± 7.2 , $21.0 \pm$

TABLE 2. *Virus type and antibody status of patients with genital herpes infection*

Antibody in acute-phase serum	No. of patients	No. with viral isolate		Seroconversion ^a	
		HSV-1	HSV-2	CF	Neu- tralizing
Negative	13	1	12	12	8
Positive	2	0	2	1	2
All patients	15	1	14	13	10

^a Development of a titer of $\geq 1:8$ or a fourfold increase in titer in convalescent sera.

6.8, 19.5 ± 5.4 , 15.8 ± 5.8 , and 6.1 ± 0.3 in weeks 2 through 6, respectively; week 1 versus week 6, $P < 0.05$). In contrast, LDCF production by cells from controls was significantly lower and did not vary over the course of the investigation (Fig. 1). Production of LDCF in response to heterologous HSV antigen (i.e., HSV-1 when HSV-2 was the patient's isolate) fluctuated without a significant trend. Furthermore, LDCF generation in response to PHA did not differ between patients and controls, and the production of this lymphokine by PHA-stimulated patients' lymphocytes did not vary over the course of the investigation.

Production of LDCF and clinical disease.

The production of LDCF in response to homologous HSV antigen was significantly lower in week 1 of disease in the nine patients who experienced systemic signs or symptoms than in the six whose disease was localized to the genitalia (15.3 ± 4.8 and 49.6 ± 14.0 , respectively; $P < 0.05$; Fig. 2). In weeks 2 through 6, HSV-stimulated lymphokine production was similar in the two groups of patients. In contrast, no significant correlations were observed between the magnitude of the LDCF response to HSV antigen at week 1 and either the duration of viral shedding ($r = -0.23$) or the persistence of lesions ($r = 0.04$), itching ($r = -0.22$), or pain ($r = 0.50$). In those patients whose disease recurred, LDCF production was significantly lower during the week of recurrence than in the weeks immediately preceding or following, although maximal LDCF production was similar in patients who developed recurrent infection and in those who did not (34.6 ± 3.5 and 54.9 ± 14.3 , respectively; not significant) (Fig. 3).

Relationship between lymphocyte transformation response and clinical disease. The mean transformation response of patients'

FIG. 1. Temporal course of HSV-stimulated LDCF production in patients with initial genital HSV infection (●—●) and in controls (●---●). The value for patients at week 1 is significantly different from that at week 6 and from that for controls at week 1, both $P < 0.05$.

FIG. 2. Temporal course of HSV-stimulated LDCF production in patients with systemic signs and symptoms of HSV infection (●—●), those with disease localized to the genitalia (●····●), and in controls (●---●). The value at week 1 for patients with localized disease is significantly different from that at week 1 for patients with systemic disease and controls, $P < 0.05$.

lymphocytes to homologous HSV antigen was 5.3 ± 2.5 at week 1, 28.6 ± 8.0 at week 2, 34.3 ± 8.5 at week 3, 44.6 ± 13.5 at week 4, and 24.4 ± 6.1 at week 6. By week 11, the response of patients' lymphocytes had declined to 7.7 ± 3.2 (Fig. 4). Although our intention was to study only patients with initial HSV infections, 12 of the 15 patients we followed developed recurrences, and lymphocyte transformation generally increased in the week after the onset of a recurrence. However, the time of peak lymphocyte transformation, i.e., week 4, was unaffected by these recurrent infections since they all occurred between weeks 4 and 11. In contrast to the temporal rise and fall in transformation of patient lymphocytes, the proliferation of control lymphocytes to HSV was low and did not fluctuate over the course of the study. Furthermore, the transformation of patients' lymphocytes to PHA, ConA, and PWM did not vary with the course of disease and did not differ from the response of control lymphocytes to these mitogens.

There were inverse correlations between the magnitude of lymphocyte transformation to HSV antigen and (i) the duration of pain and (ii) the persistence of genital lesions (Table 3). The maximal proliferative response to HSV antigen was 45.9 ± 11.8 in the eight patients who had pain for more than 15 days and 83.2 ± 14.9 in the seven who had pain for less than 15 days ($P < 0.05$). Similarly, the maximal response to HSV antigen was significantly lower in the four pa-

FIG. 3. Production of LDCF by HSV-stimulated lymphocytes from patients with recurrent infection. Weeks -1, 0, and +1 are the week before recurrence, the week of recurrence, and the week after recurrence, respectively. The values at weeks -1 and +1 are both significantly different from those at week 0 at $P < 0.05$ and < 0.001 , respectively.

tients in whom the healing of lesions was delayed (≥ 25 days) than in those whose healing was more rapid (mean stimulation index of 27.9 ± 10.0 versus 76.3 ± 16.3 ; $P < 0.05$). In contrast, the duration of viral shedding from genital lesions did not correlate with lymphocyte transformation. Although this finding differs from that of our previous study (4), the discrepancy may in part be explained by the fact that we studied fewer patients in the present study.

DISCUSSION

The most significant findings of the present study are that (i) virus-specific lymphokine,

LDCF, production in initial genital HSV infection was highest during the 1st week of disease and declined to control levels by week 6; (ii) HSV-stimulated lymphocytes from patients with signs or symptoms of systemic HSV infection (fever, malaise, myalgias, headache, or photophobia) produced significantly less LDCF in week 1 than did cells from patients with local disease; and (iii) a transient decline in LDCF generation occurred in patients at the time of clinical recurrence. Taken together, these findings indicate that LDCF production is an early component of the cell-mediated immune response to HSV infection and suggest that normal generation of this lymphokine may be important in controlling the severity of primary infection

FIG. 4. Temporal course of HSV-stimulated lymphocyte transformation to HSV antigen in patients with initial genital HSV infection (●—●) and in controls (○---○).

TABLE 3. Relationship between lymphocyte transformation to HSV antigen and duration of initial genital disease

Disease state	No. of patients	Mean duration (days)	Stimulation index
Pain			
<15 days	7	7.2 $\pm$ 1.3	83.2 $\pm$ 14.9 ^a
≥ 15 days	8	20.8 $\pm$ 2.4	45.9 $\pm$ 11.8
Lesions			
<25 days	11	18.3 $\pm$ 1.2	76.3 $\pm$ 16.3 ^b
≥ 25 days	4	31.8 $\pm$ 2.6	27.9 $\pm$ 10.0
Viral shedding			
<10 days	12	7.9 $\pm$ 1.1	66.4 $\pm$ 15.1
≥ 10 days	3	17.0 $\pm$ 1.1	51.5 $\pm$ 12.5

^a P versus ≥ 15 days < 0.05 .

^b P versus ≥ 25 days < 0.05 .

with this virus. In addition, the finding that a transient deficiency in herpes-specific LDCF production occurred at the time of recurrence suggests that this mediator may play a role in preventing the development of recurrent HSV infection. Alternatively, recurrent disease may cause a transient decline in LDCF production because the cells which produce this mediator are temporarily sequestered at the site of the genital lesions. The present study also shows that the transformation of lymphocytes from patients with initial HSV infection was lowest in the 1st week of disease, increased to a peak at week 4, and returned to control levels by week 11. The magnitude of the transformation response to HSV antigens was found to correlate inversely with the duration of local symptoms and the persistence of lesions. That is, patients who experienced pain for more than 15 days or had lesions that persisted for more than 25 days had significantly lower lymphocyte transformation responses to HSV antigen than did patients whose pain and lesions resolved in less time. These findings verify our previous observations (4).

Lymphokine production and lymphocyte transformation in HSV infection have been examined in earlier investigations. However, these studies have focused primarily on recurrent disease. Shillitoe and co-workers (24) found that lymphocyte transformation to HSV-1 was higher during the 1st week of infection in patients with recurrent herpes labialis than in seropositive controls. They also found that production of migration inhibition factor (MIF) was lowest in the 1st week after a recurrence, which led them to speculate that a transient decline in MIF synthesis may have permitted the reactivation of the latent viral infection. In a later study, the same investigators reported that both MIF production and lymphocyte proliferation in response to HSV antigen were functions of T cells and that lymphocytes from patients with primary herpes labialis manifested a peak proliferative response within 2 weeks after symptom onset (25). O'Reilly and co-workers (17) found that HSV-specific transformation could be regularly detected in lymphocyte cultures from patients with recurrent herpes labialis and herpes progenitalis, irrespective of the clinical stage of their infection. In contrast, they found transient declines in the HSV-stimulated production of leukocyte MIF (LMIF) and interferon at the time of and immediately before HSV recurrence. The synthesis of these mediators was found to increase significantly during convalescence. Rasmussen and co-workers, in a study of patients with recurrent HSV infection, found that interferon production was maximal between weeks 2 and 6 after disease onset and that patients whose

lymphocytes produced high titers of interferon had less frequent recurrences (19). Furthermore, these authors observed that lymphocyte transformation was unrelated to disease activity and appeared to reflect prior immune status. Rosenberg et al. (22) reported that peripheral lymphocytes from individuals who had antibodies to HSV produced LDCF in response to HSV antigen, whereas cells from antibody-negative individuals did not. Of interest, we found that two of our controls were CF antibody positive to HSV, although their lymphocytes were unresponsive to this antigen as determined by LDCF production and lymphocyte transformation. This observation, although not consistent with some earlier studies, such as that of Rosenberg et al. (22), is in keeping with our previous work (4) in which we showed that lymphocyte responsiveness correlated more closely with disease activity than with serological status. That is, we found that patients may remain seropositive to HSV and have lymphocytes which fail to respond *in vitro*. It is evident from the above studies that our understanding of the development of cellular immunity during the course of HSV infection is incomplete. It is interesting to note, however, that Shillitoe et al. found a transient decrease in MIF synthesis and that O'Reilly et al. found a similar abnormality in leukocyte MIF and interferon production coincident with disease recurrence. These observations are comparable to our finding that LDCF production was significantly lower in patients with recurrent HSV infection at the time that the recurrence first developed.

A number of lines of evidence suggest that normal monocyte-macrophage accumulation and function may be important in host defense against herpetic infections. These include the present study and the work of Shillitoe et al., which indicate that there are abnormalities in the synthesis of lymphokines which affect monocyte-macrophages in HSV infections (24). In addition, studies by Zisman et al. (32) and McGeorge and Morahan (11) have shown that the dissemination of HSV in mice was enhanced and the mortality increased when the animals had been pretreated with macrophage-inhibiting agents such as silica, trypan blue, dextran sulfate, and antimacrophage serum. Other studies (8, 9, 13) in humans and mice have suggested that macrophages with Fc receptors may mediate HSV resistance via antibody-dependent cellular cytotoxicity in the presence of small amounts of antiviral antibody.

The fact that LDCF production and lymphocyte transformation reach a maximum and then return to control levels during initial HSV infection suggests that suppressor mechanisms may regulate cellular immunity in this disease. Pre-

viously described inhibitors of cellular immunity include soluble immunoglobulins (18), immune complexes (12), macrophages (6, 7), and both antigen-specific and nonspecific suppressor T cells (23, 28, 31). Prior studies have shown that sera containing anti-HSV antibodies do not suppress lymphocyte transformation to HSV antigen (27), and preliminary experiments in our laboratory indicate that peripheral monocytes are not involved in suppression of the cellular immune response to HSV. However, the role of T cells in regulating HSV-induced lymphocyte proliferation and lymphokine synthesis has not been examined.

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