Sequential Changes in Cell-Mediated Immune Responses to Herpes Simplex Virus After Recurrent Herpetic Infection in Humans

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Received for publication 17 March 1977

Lymphocyte responses to herpes simplex virus (HSV) were studied in 23 patients with recurrent herpes labialis and in 19 control subjects. Lymphocytes of seropositive, but not seronegative, controls responded to HSV by increased thymidine incorporation, and the supernatant fluids inhibited the migration of guinea pig macrophages. Lymphocytes from patients with a recurrent herpetic lesion responded to HSV by significantly greater thymidine incorporation than seropositive controls, but supernatants did not show an increased macrophage migration inhibition response. During the 28 days after the onset of a lesion, the thymidine incorporation to HSV fell to the level of the seropositive controls, and supernatants then induced an increased inhibition of macrophage migration. Lymphocyte responses to Candida albicans, purified protein derivative, or phytohemagglutinin did not fluctuate according to the presence of a lesion and did not differ from those of the controls. Lymphocyte responses to HSV were unaffected by culture in the presence of serum from seronegative or seropositive controls, or from patients with or without a herpetic lesion. It is suggested that in patients with recurrent herpes labialis a periodic defect of the migration inhibition response might have allowed the recurrent infection to develop, and that the increased thymidine incorporation stimulated by HSV in vitro is a result of antigenic stimulation from the lesion.

Individuals prone to recurrent infection by herpes simplex virus (HSV) have circulating HSV antibody, and their lymphocytes respond to HSV by increased thymidine incorporation (21, 29). An immune defect was postulated by Wilton et al. (29), who reported that supernatants of HSV-stimulated lymphocytes from patients with a herpetic lesion failed to inhibit migration of guinea pig macrophages. This was confirmed by the finding that leukocyte migration was not inhibited by HSV when the patient had a herpetic lesion (7). However, in the absence of lesions in susceptible subjects, inhibition of leukocyte migration was found to be intact (23). In the present study sequential observations were made on the thymidine incorporation and macrophage migration inhibition responses to HSV of lymphocytes from patients prone to recurrent herpes labialis (RHL). In addition, the influence of serum factors on the lymphocyte response was studied by culture in the presence of sera from different phases of RHL and controls.

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MATERIALS AND METHODS

Patients. A series of 23 patients with RHL was studied; the age range was 18 to 46 years (mean 27), and there were 9 males and 14 females. All were in good health and not receiving any drugs. Immunological investigations were performed as soon as possible after the onset of a recurrent lesion and again 2 and 4 weeks later. The diagnosis of RHL was made on the basis of the patients’ history, the appearance of the lesion, and virological cultures. As controls, a group of 19 healthy individuals, matched for age and sex, was studied.

Complement-fixing antibody. Serum antibody to HSV was assayed by the method of Bradstreet and Taylor (2). Titers were converted to logs, and the geometric mean titer was calculated.

Lymphocyte cultures. Approximately 50 ml of blood, of which 10 ml was allowed to clot, was obtained from each individual, and the serum was separated. The remaining 40 ml was anticoagulated with heparin, layered onto Ficoll-Triosil (Pharmacia, Nyegaard), and centrifuged at 900 × g for 20 min (1). The cells at the interface of Ficoll-Triosil and plasma were removed, washed twice in Hanks balanced salt solution, and suspended at 10⁶/ml in medium 199. Cultures were established in disposable tissue culture tubes (Falcon 3035) and consisted of 1 ml of lymphocyte suspension, 0.1 ml of serum, and 0.1 ml of either saline,
antigen, or phytohemagglutinin (PHA). Sera were fresh autologous, except where stated otherwise. Homologous sera had been stored at −20°C for up to 4 weeks. Either two or three replicates of each culture were used and were maintained at 37°C in an atmosphere of 5% CO₂ for 4 days.

For the last 4 h of culture, 1 μCi of [³H]thymidine (5 Ci/mmol) (TRA 120, Amersham) in 0.1 ml of medium was added to each culture. Cultures were terminated by filtration onto Whatman GFA paper, using a multiple automated sample harvester. Filters were washed with saline and dried, and radioactivity was assayed by scintillation counting. The mean counts per minute of replicate cultures stimulated by each antigen, or PHA, was divided by that of the unstimulated control cultures; this value was recorded as the "stimulation index" (SI).

Antigens. (i) HSV. HSV-1 complement-fixing antigen was kindly supplied by C. M. P. Bradstreet, Public Health Laboratory Service, Colindale, London. A confluent monolayer of Vero tissue culture cells was infected with a recent isolate of HSV-1 from a patient with RHL and incubated at 37°C until full cytopathic effect was present. The infected cells were harvested mechanically and centrifuged at 900 × g for 10 min. The pellet was resuspended in peptone water to 10% of the original volume and disrupted by sonication in an MSE ultrasonic disintegrator at 20 kc/s. The virus was inactivated by addition of 0.3% β-propiolactone, but no preservative was used. In one experiment only, a similar preparation of a genital isolate of HSV-2 was used. (ii) PPD. Preservative-free purified protein derivative (PPD) was obtained from the Ministry of Agriculture, Fisheries and Food and was dissolved in saline. (iii) Candida albicans. A sample of saliva from an oral carrier of C. albicans was inoculated onto Sabouraud agar and incubated at 37°C until a confluent growth was obtained. The yeast cells were harvested in the minimum possible volume of saline and disrupted by vibration in a Mickle glass-bead disintegrator. The suspension was centrifuged at 900 × g for 20 min, and the supernatant was used.

PHA. Reagent-grade PHA was obtained from Wellcome Reagents Ltd., and each vial was reconstituted in 5 ml of distilled water.

Each antigen was stored in 1-ml samples at −20°C for up to 6 months before use, but PHA was freshly reconstituted on each occasion. The optimal dilution of each antigen and PHA was determined by comparison of lymphocyte responses of a number of subjects to several dilutions of the preparation, as described previously (23a).

Macrophage migration inhibition. The indirect test of Thor et al. (27) was used, as described previously (29). The supernatant fluids from the lymphocyte cultures were assayed for macrophage migration inhibitory activity, with oil-induced guinea pig peritoneal cells. Each supernatant was assayed with four capillary tubes of exudate cells, and the migration index was calculated by dividing the mean area of migration in antigen-stimulated supernatant by the mean area of migration in unstimulated supernatant and multiplying by 100.

Statistical analysis. Results were analyzed by the t test for dependent or independent means, as appropriate, or by analysis of linear regression.

RESULTS

HSV antibody titer. Control subjects with HSV antibody titers greater than 1:4 were designated seropositive (11 subjects) and the remainder were seronegative (8 subjects).

All patients had an HSV antibody titer of 1:4 or greater, and no difference was found between the titer when a lesion was present (geometric mean titer ± standard error = 4.31 ± 0.25) and that after the lesion had resolved (4.64 ± 0.27).

Lymphocyte stimulation. Unstimulated lymphocytes of patients showed no variation in thymidine incorporation with time since onset of a lesion, and no difference was seen between control subjects and patients.

HSV. The highest mean response to the HSV antigen was found in lymphocytes of patients with a recurrent herpetic lesion of up to 3 days' duration, and at each subsequent time of examination the mean response had declined (Fig. 1). The SI to HSV when a lesion had been present for up to 3 days was significantly greater than the SI 28 days after the onset of lesions (P < 0.05). The SI of the patients was significantly greater than that of the seropositive controls when less than 3 days (P < 0.001) or 14 to 21 days (P < 0.05) had elapsed since the onset of the lesion. After 28 days had elapsed, the mean SI in response to HSV was still higher than that of the seropositive controls (Fig. 1), but the difference no longer reached the 5% level of significance.

The increase in the lymphocyte response to HSV at the time of a herpetic lesion was repeat-
edly observed during a sequential study of one patient who experienced 12 episodes of RHL during a period of 10 months (Fig. 2). When a herpetic lesion was present, the SI was invariably greater than before or after the onset of a lesion. The maximum number of herpetic lesions present at the same time was six, and this coincided with the maximum SI in response to HSV of 49.0.

The lymphocyte response to HSV at the time of a herpetic lesion was related to the time elapsed since the previous lesion in 16 patients. The shorter the time elapsed since the previous lesion, the higher the SI, although the correlation did not reach the 5% level of significance (Fig. 3). However, if only values for up to 90 days since the previous lesion were considered, the correlation did reach the 5% level of significance \( r = -0.65, n = 10, P < 0.05 \). No significant relationship was observed between the lymphocyte response to HSV at the time of a herpetic lesion and the time that elapsed before the next lesion developed \( r = -0.41, n = 9 \).

The specificity of the lymphocyte response for HSV-1 was assessed by comparing the responses between HSV-1 and HSV-2 of lymphocytes from 15 patients prone to RHL. No significant difference was seen between the mean SI to HSV-1 \( (12.4 \pm 2.6) \) and HSV-2 \( (14.3 \pm 3.5) \), and the correlation between the responses to each antigen was highly significant \( (P < 0.0001; \text{Fig. 4}) \).

PPD. No difference was seen between the lymphocyte responses to PPD of patients with a herpetic lesion of up to 7 days' duration \( (SI = 11.3 \pm 4.2) \) and patients in whom more than 14 days had elapsed \( (SI = 11.9 \pm 7.8) \). The responses of the seronegative and seropositive controls were combined, showing a mean SI of 6.6 ± 2.0, which was not significantly different from either of the patient groups.

![Fig. 2. Relationship between presence of herpetic lesions and response to HSV-1 in one subject.](image1)

![Fig. 3. Relationship between response of lymphocytes to HSV-1 at the time of a herpetic lesion and the interval since the previous lesion in 16 patients.](image2)
**C. albicans.** Patients with a herpetic lesion showed a mean SI to *C. albicans* of 8.3 ± 1.7, which increased slightly to 10.1 ± 3.5 after more than 14 days had elapsed. The responses of the seronegative and seropositive controls were combined, showing a mean SI of 5.9 ± 1.1, which was not significantly different from either of the patient groups.

**PHA.** No difference was seen between the lymphocyte responses to PHA of patients with a herpetic lesion of up to 7 days' duration (SI = 60 ± 11.7) and patients in whom more than 14 days had elapsed (SI = 63.5 ± 13.8). The responses of the seronegative and seropositive controls were combined, showing a mean SI of 87 ± 12.9, which was not significantly different from either of the patient groups.

Unlike with HSV, no relationship was observed between the lymphocyte responses of the patients and time elapsed since the previous herpetic lesion if either *C. albicans* (r = -0.37, n = 6) or PHA (r = -0.56, n = 6) was used as the stimulant. However, fewer patients were tested than with HSV.

**Effect of substituted sera.** Lymphocytes of six seronegative control subjects were cultured in the presence of serum from seropositive subjects. A total of 11 seropositive sera was used; 3 were from control subjects, 5 were from patients who had developed a recurrent herpetic lesion within the previous 7 days (acute phase), and 3 were from patients whose previous lesion had developed more than 14 days previously (nonacute phase). The SI to HSV of lymphocytes from seronegative controls was not increased in the presence of seropositive as compared to seronegative (autologous) serum (Table 1).

Lymphocytes of eight seropositive subjects were cultured in the presence of serum from seronegative controls. The seropositive subjects were three acute-phase patients, four nonacute-phase patients, and one control. The SI in response to HSV was not significantly decreased in the presence of seronegative as compared with autologous serum (Table 1).

Lymphocytes of 11 acute-phase patients were cultured in the presence of nonacute-phase sera, which had been collected previously. The nonacute-phase sera were autologous in four cases and homologous in six cases, but since no differences were seen between them, the results were combined. The SI in response to HSV in the presence of autologous fresh acute-phase serum was 17.6 ± 3.5, and in the presence of nonacute-phase sera it was 15.6 ± 4.3. The decrease was not significant at the 5% level.

Lymphocytes of six nonacute-phase patients were cultured in the presence of three autologous acute-phase sera, two autologous and homologous acute-phase sera, or one homologous acute-phase serum. The mean counts per minute of unstimulated lymphocytes was slightly depressed by acute-phase serum, whereas those of HSV- and *C. albicans*-stimulated lymphocytes were slightly increased, when compared with cultures in the presence of autologous nonacute-phase serum (Table 2). None of these changes approached the 5% level of significance. How-
ever, when SIs to HSV were calculated, an increase was seen in the presence of acute-phase serum ($P < 0.05$), and a corresponding increase was also found with $C. albicans$ ($P < 0.01$; Table 2).

**Macrophage migration inhibition.** (i) **HSV.** Supernatants of HSV-stimulated lymphocytes of seropositive and seronegative controls showed macrophage migration indexes that did not differ significantly from each other. Observations were made in eight patients, both when a recurrent herpetic lesion was present and after its resolution (Fig. 5). The mean time between the observations was 25 days. An increase in migration inhibition was observed in six cases; the maximum change in migration index was $-77$. A decrease in migration inhibition was observed in two cases; the maximum change in migration index was +8. The mean increase in migration inhibition between the two observations was significant at the 5% level. At the time of the second observation the mean migration index was $59 \pm 7$, which differed significantly from that of the seropositive controls (mean = $87 \pm 6$, $P < 0.01$).

(ii) **PPD.** No difference was seen between the migration inhibition response to PPD of lymphocytes from patients with a herpetic lesion of up to 7 days' duration (migration index = $83 \pm 9$), patients in whom more than 14 days had elapsed ($90 \pm 11$), or control subjects ($94 \pm 5.5$).

(iii) **C. albicans.** No difference was seen between the migration inhibition response to $C. albicans$ of lymphocytes from patients with a herpetic lesion of up to 7 days' duration (migration index = $99 \pm 13$) or patients in whom more than 14 days had elapsed ($103 \pm 13.5$). Control subjects showed rather greater migration inhibition (mean migration index = $70 \pm 8.5$), but the difference did not reach the 5% level of significance.

**DISCUSSION**

Primary herpetic infections result in the development of HSV antibody (3) and the maintenance of the virus in the local sensory ganglion in a latent form (26). A minority of infected individuals later suffer recurrent infections, despite the presence of circulating antibody, and the present study investigated the role of cell-mediated immunity in the pathogenesis of recurrences. The highest mean SI to HSV was seen in lymphocytes from patients with herpetic lesions of less than 3 days' duration, and the

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**TABLE 1. Effect of substituted seropositive or seronegative sera on the response to HSV of lymphocytes from seronegative or seropositive donors**

<table>
<thead>
<tr>
<th>Lymphocyte donor</th>
<th>Serum donor</th>
<th>SI ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative</td>
<td>Seronegative</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Seropositive</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Seropositive</td>
<td>Seronegative</td>
<td>14.7 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>Seropositive</td>
<td>12.3 ± 3.0</td>
</tr>
</tbody>
</table>

* Ratio of counts per minute in HSV-stimulated cultures to unstimulated cultures containing the same serum. Mean ± standard error.

**TABLE 2. Effect of substituted sera from acute- or nonacute-phase patients on responses of lymphocytes from nonacute-phase patients**

<table>
<thead>
<tr>
<th>Serum donor</th>
<th>cpm</th>
<th></th>
<th>SI ±</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>HSV stimulated</td>
<td>C. albicans stimulated</td>
</tr>
<tr>
<td>Nonacute phase</td>
<td>2,312 ± 662b</td>
<td>13,825 ± 4,187</td>
<td>11,266 ± 3,216</td>
</tr>
<tr>
<td>Acute phase</td>
<td>1,531 ± 207</td>
<td>14,667 ± 2,406</td>
<td>16,811 ± 3,250</td>
</tr>
</tbody>
</table>

* Ratio of counts per minute in antigen stimulated to unstimulated cultures containing the same serum.

b Mean ± standard error.

c Increase in stimulation index in presence of acute-phase sera significant at $P < 0.05$ level.

d Increase in stimulation index in presence of acute-phase sera significant at $P < 0.01$ level.
response declined after resolution of the lesion (Fig. 1). A similar fall in response was found by Rosenberg et al. (21) but not by other workers (17, 25). Patients with a lesion showed a higher response to HSV if only a short time had elapsed since the previous lesion, but the correlation reached the 5% level of significance only when patients were excluded if their previous lesion had occurred more than 90 days earlier. This might indicate that the SI in response to HSV declines linearly with time for about 90 days and then remains constant at a low level. The increased response at the time of a lesion was evidently associated with a period of susceptibility to a recurrence, and not with resistance to infection: the SI was probably increased by antigenic stimulation from virus replicating in the lesion.

It was previously suggested that recurrent herpetic infections could result from inability of the patients' lymphocytes to release migration inhibitory factor after stimulation by HSV (29). In the present study, supernatants of HSV-stimulated lymphocytes from seropositive controls and of patients with a herpetic lesion did inhibit the migration of guinea pig peritoneal macrophages, but this response increased significantly during recovery from infection (Fig. 5). Sequential observations were made in only eight subjects, and the results therefore require confirmation in a larger series. Nevertheless, the association between an increase in the response and recovery from infection is consistent with this mechanism being protective against infection, unlike the SI, which fell simultaneously.

The mechanism by which release of migration inhibitory factor by HSV-stimulated lymphocytes could be protective against recurrent HSV infection is uncertain. However, as well as inhibition of migration, soluble mediators of cell-mediated immunity potentiate the bactericidal (16) and viricidal functions of macrophages, and in animals macrophages are essential in protection against HSV infection (11). The soluble mediators of cell-mediated immunity are usually released simultaneously (18), and a lack of migration inhibitory factor might be associated with a lack of other mediators. Although interferon production is within the normal range, the lower the concentration, the shorter the time before the next herpetic lesion (9, 17). However, production of chemotactic factor and lymphotoxin seems to be normal (21). A cyclical pattern in the release of a soluble mediator of cell-mediated immunity is supported by O'Reilly and Lopez (16a) and may account for the negative results in those investigations in which the tests were carried out in the absence of lesions (23).

An alternative interpretation of the cyclical change in the migration inhibition response is that viral infection depresses some lymphocyte functions. However, immunosuppression by viruses in vivo results in loss of lymphocyte responses to unrelated antigens and mitogens (5, 10, 28), which was not detected in the present study when PPD, C. albicans, or PHA was used as the control. It would seem possible that an insufficient migration inhibition response is therefore a predisposing cause rather than a result of infection, although a lesion may only develop when this coincides with local factors, allowing derepression of the viral genome (15) and spontaneous shedding of viral particles (4).

The possibility that the response of lymphocytes to HSV might be modulated by serum factors was not supported by the results of this investigation. Lymphocytes from patients prone to RHL responded equally well to HSV by thymidine incorporation whether cultured in the presence of autologous or homologous seronegative serum. The increased lymphocyte response from a donor with a lesion was maintained in the presence of serum from a donor without a lesion. The lymphocyte response was not increased when the cells from a donor without a lesion were cultured in the presence of serum from a donor with lesions. However, the latter sera caused a slight decrease in the [3H]-thymidine uptake of unstimulated lymphocytes and a slight increase in the uptake in response to HSV or C. albicans, as compared with that in the presence of autologous fresh serum. As a result, the SIs to both antigens were raised, and the increase reached the 5% level of significance in the case of C. albicans. However, since the change in SI was seen with two unrelated antigens, and was largely due to a fall in background uptake of thymidine, it was considered to be immunologically nonspecific. These results suggest that the varying responses of lymphocytes from patients prone to RHL are entirely cell mediated and are not related to serum factors. Although a slight increase in the response of lymphocytes in the presence of seronegative as compared with seropositive serum was recently reported (9), this was not statistically significant.

Hyperimmune serum can inhibit the responses of rabbit spleen lymphocytes to cell-bound, but not cell-free, HSV (6, 19, 20), although in humans it is probable that the antibody titer is insufficient to cause lymphocyte suppression. The immunoglobulin class of the antibody may also be relevant, since inhibition of the response to rubella of rabbit lymphocytes by serum is due to immunoglobulin M antibody alone (14). The lack of any influence of different sera on the lymphocyte responses to HSV seen in the present study is consistent with both the
lack of immunoglobulin M antibody to HSV in serum of patients with RHL (8) and the constant titer of whole serum antibody to HSV, regardless of the presence or absence of a lesion; this was seen in the present and in past investigations (4, 13, 17).

A very significant correlation was observed between lymphocyte responses to HSV-1 and HSV-2 (Fig. 4), suggesting that lymphocytes are stimulated by an antigen common to both HSV types. This is in contrast to the finding that rabbits immunized with one HSV type have lymphocytes sensitized to both types but a stronger response to the immunizing type (12, 22). Infants with neonatal HSV-2 infection develop a stronger lymphocyte response to HSV-2 than to HSV-1 (24). The adult patients in the present study were prone to RHL, which is usually due to HSV-1, but it cannot be excluded that some had had an HSV-2 infection in the past.

The sequential observations during the present study have shown that cyclic changes occur in the specific cell-mediated immune response to HSV related to the presence of a herpetic lesion. It is suggested that a periodic defect in the macrophage migration inhibition response to the virus allows the infection to develop, and that the increased lymphoproliferative response then results from antigenic stimulation by the virus replicating in the lesion. Recent observations suggest that both the macrophage inhibition and lymphoproliferative responses in this system are functions of the T lymphocyte (23a).

ACKNOWLEDGMENTS

The technical assistance of J. Blackburn and J. Walton is gratefully acknowledged. This work was carried out under a grant from the Cancer Research Campaign.

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