Leukocyte migration inhibitory factor in HSV infections

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The cell-mediated immune (CMI) response as measured by a direct assay of leukocyte migration inhibition factor (LMIF) was determined in a population of patients with recurrent herpes simplex virus (HSV) infections in the quiescent stage as well as in healthy volunteers. The migration of leukocytes incubated in the presence of HSV antigens was compared to that without viral antigens for the calculation of the migration index (MI). Eleven out of 41 control subjects (16.8%) had a MI below 0.8, indicating a positive CMI response. In contrast, all the herpes patients tested had a MI above 0.8, suggesting an impairment in the production of LMIF at this stage of their disease. This difference was statistically significant (t = 4.296; p < 0.0001) and was not dependent on the age of the population. This study indicates that individuals with recurrent HSV infections have impaired CMI response between attacks which may be associated with the stage of the disease.

Seroepidemiological studies on herpes simplex infections show that the primary infection with this virus is followed by the production of circulating neutralizing antibodies. They also show that a large portion of the population carries such antibodies, indicating a prior asymptomatic infection with this virus. In the case of ocular herpes simplex infections, secretory antibodies are present in patients with ocular dendritic keratitis as well as in healthy individuals. A study done by Centifanto et al. in herpes simplex ocular infections as specifically related to secretory IgA in rabbits shows that the secretory IgA antibodies present in the tears of animals topically immunized with herpes were capable of neutralizing the virus and indeed were protective against challenge with the same virus. However, it was found that topical immunization did not reduce the number of herpetic recurrences, although secretory IgA antibodies were readily produced, and the level of these antibodies was comparable in animals with and without recurrences. Within this study we made the observation that in the diseased eye which is subject to inflammation and transudation of immunoglobulins from the sera, an elevated amount of IgG antibodies did prevent the neutralization of the virus by the IgA antibodies present in tears. Therefore the relative amount of both IgA and IgG in the tears of the diseased eye is important for the effective neutralization of the virus.

These studies indicate the underlying cause for herpetic recurrences is other than a deficiency in the local and humoral antibody production. Because of the apparent lack of beneficial effects of antibodies, interest has developed in cell-mediated host defense mechanisms in an effort to find out whether there is a relationship between any of the several cell-mediated immunity parameters and herpes recurrences.

In a study on herpes stomatitis Wilton et al. reported that patients had a deficiency or...
decreased levels of macrophage migration inhibition factor as compared to the controls, and he postulated this impairment as the reason for the herpetic recurrences. The in vitro stimulation of lymphocytes by herpes simplex virus (HSV) antigens was found unimpaired, a finding that was confirmed by Russell in his study of lymphocyte proliferation in patients with frequent herpetic lesions.

Further studies of other parameters of cell-mediated immunity, such as lymphocyte cytotoxicity as measured by 51Cr released from infected cells and inhibition of leukocyte migration in patients with recurrent herpes labialis showed no defect in this cell-mediated immune (CMI) response. On the other hand, Steele et al. in a study of recurrent herpes labialis found an increased blastogenic index in HSV patients as opposed to controls and a decrease of cytotoxicity assay in these patients, showing a dissociation of these parameters. Thong et al., using the 51Cr cytotoxicity assay in herpes labialis patients, found a fluctuation in the specific immune release, that is to say, an increased immune response during the active stage of the disease and a decrease during the quiescent stage. This apparent impairment between attacks was postulated as the reason for the recurrent lesion.

The apparent diversity in these observations may be due to the different populations studied, the particular methods used, and the times of sampling in relation to infection. Therefore it is the object of this study to investigate one parameter of CMI function, specifically, leukocyte migration inhibitory factor (LMIF), in a population of patients with recurrent herpes infections and to compare them with age-, sex-, and race-matched controls who have been exposed to HSV but have no history of clinical infection.

Methods and materials

**Tissue culture and media.** Tube cultures of human embryonic kidney were obtained from Microbiological Associates, Rockville, Md. The medium used for the preparation of the antigen was RPMI-1640 supplemented with 2% fetal calf serum, 1% glutamine, sodium bicarbonate, and antibiotics.

**Preparation of antigen.** Tube cultures of human embryonic kidney were infected with 500 pfu of HSV type 1 (Shealy strain) and allowed to incubate at 37°C for 24 hr. At that time the cells were scraped off with a rubber policeman, and all the cultures were pooled in one batch to insure reproducibility. The pooled cell suspension was frozen and thawed twice prior to sonication for 2 min in the cold. The disrupted cells were spun down at 900 x g for 30 min to sediment large cell debris. The resultant supernatant, which contained live virus, was subjected to ultraviolet irradiation for the inactivation of live virus. This preparation was tested for infectivity and dispersed in small vials and kept frozen at −70°C. The antigen was tested for its antigenicity in lymphocyte blastogenesis to see if it would elicit a T cell response. The control antigen was uninfected cultures processed in the same manner.

**Migration inhibition assay.** A modification of the method of Clausen for LMIF was employed in this study. Heparinized blood in 12 ml amounts was thoroughly mixed with 3 ml of 6% dextran in saline (Travenol Labs., Inc., Deerfield, Ill.) and allowed to separate for 1 hr at 37°C. The leukocyte-rich layer was then removed and centrifuged at 900 x g for 5 min in the cold. The disrupted cells were spun down at 900 x g for 30 min to sediment large cell debris. The resultant supernatant, which contained live virus, was subjected to ultraviolet irradiation for the inactivation of live virus. This preparation was tested for infectivity and dispersed in small vials and kept frozen at −70°C. The antigen was tested for its antigenicity in lymphocyte blastogenesis to see if it would elicit a T cell response. The control antigen was uninfected cultures processed in the same manner.

For the neutralization test, the medium used was Basal Medium Eagle supplemented with 2% fetal calf serum, 1% glutamine, sodium bicarbonate, and antibiotics.

A total of six wells with antigen and four wells with control antigen were done per patient. The fans were fixed by the addition of 1 ml of crystal violet in formaldehyde for 20 min. After this period, the dye was gently aspirated, and the fans
were washed with phosphate-buffered saline in a similar manner to remove excess dye. This procedure has the advantage of providing a permanent record of the migration area.

The fans were measured by projecting them in an Image Analyzer (Bausch & Lomb, Rochester, N. Y.). Five readings per well were obtained, and the migration index (MI) was determined according to the following formula:

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MI = \frac{\text{mean area of migration in HSV antigen cultures}}{\text{mean area of migration in control antigen cultures}}
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In this test an MI of less than 0.8 was considered positive and a demonstration of a CMI response.

**Neutralization tests.** Twofold dilutions of serum (1:4 through 1:64) in a 0.5 ml volume were mixed with an equal volume of virus (10^3 pfu/ml) and incubated at 37° C for 30 min. The virus control consisted of the same virus dilution plus 0.5 ml of medium, incubated for the same amount of time. At the end of the incubation period, 0.2 ml of each dilution was inoculated into triplicate tube cultures of human embryonic kidney, followed by the addition of 1 ml of complete medium. The cultures were read after 48 hr of incubation, and the titer was that dilution which reduced the cytopathogenic effects by at least 50% as compared to the virus control. This method for determining HSV-neutralizing antibodies with a cutoff dilution of 1:64 was chosen to ascertain whether CMI responses were associated with either low or high neutralizing antibody titers, rather than a precise measurement of humoral immunity.

**Study population.** The study population consisted of a total of 31 herpes patients and 41 control subjects. The patient population, obtained from the Eye Clinic, Shands Teaching Hospital, Gainesville, Fla., had a well-documented history of recurrent ocular or labial herpes infections and ranged in age from 12 to 84 years. This allowed for the study of individuals in several decades of life. All the patients were in the quiescent, or resting, stage of disease, at least 1 mo or more after a disease episode.

The control population consisted of healthy individuals with no history of ocular herpes infections, herpes labialis, or herpes genitalis, who were matched by age, race, and sex to the patient population.

Student's t test was used routinely to evaluate the differences between the groups. A probability (p) value of 0.01 was considered significant.

Results

The production of LMIF in patients as well as control subjects is presented in Fig. 1. The overall study showed that 11 out of 41 (26.8%) of the control individuals had a vigorous response to HSV antigens with an MI that ranged from 0.58 to 1.35 (mean, 0.89). In contrast, within the HSV population, the range was from 0.8 to 1.76, which in reality reflects an enhancement of leukocyte migration. Only one individual (3.2%) had an MI of 0.8, and the mean MI for the group was 1.06. These findings strongly indicate a deficiency in the production of LMIF in the population with history of recurrent herpes disease. It is
important to note that these measurements were done in the resting, or quiescent, stage of the disease, and none of the patients had a recent recurrence either prior to or after these data.

Statistical comparison between the two groups showed this difference to be very significant ($t = 4.2, p < 0.0001$). When these observations were examined as related to age, the strong difference is real and not dependent on the age of the population (Fig. 2).

Lymphoproliferative responses to HSV antigens were measured at random in a small number of both control and patient populations, and there was no correlation between LMIF and a blastogenic response; that is, lymphocyte stimulation by HSV antigens was found in subjects who failed to produce LMIF, indicating a dissociation of these two parameters of CMI (Centifanto et al., unpublished results).

Neutralizing antibody titers in both the control and patient populations were independent of the production of LMIF. In the control population, positive CMI response was found in individuals with low as well as high antibody titers. Three of the positive-responding controls had antibody titers of less than 1:8; four individuals had titers of 1:16 to 1:32, and the remaining had titers.
In the patients, who in most cases exhibited high antibody titers (≥ 1:64), the production of LMIF was nil. These results point to the independence of both types of immunity.

Discussion

Studies on the pathogenesis of recurrent herpes infections in animals revealed that during the quiescent stage of the disease the virus resides in latent form in the trigeminal ganglia.\textsuperscript{15, 16} Since these animals have high titers of neutralizing antibodies to HSV, it has been postulated that either the virus is inaccessible to these antibodies or it is in a form that precludes effective neutralization, and thus this may be the reason why neutralizing antibodies do not appear to have a beneficial effect.

It is not clear how or why the latent virus, which is harbored in the neurons,\textsuperscript{17} is reactivated to an infectious form leading to the appearance of lesions, but studies on herpes infections on compromised hosts (immunosuppressed)\textsuperscript{18, 19} indicate a role for cell-mediated immunity in the resistance or susceptibility to this virus.

In this study we have shown that patients with recurrent herpes keratitis had a deficiency in the production of LMIF and that this difference is not age or sex dependent.

The CMI response of these patients during the quiescent stage of the disease is significantly different (p < 0.0001) from that found in the control population. In this sense we are in agreement with Wilton et al.\textsuperscript{8} and Thong et al.,\textsuperscript{13} since both found a decreased CMI response in patients with herpes simplex recurrences. However, our results are not entirely in accordance with the findings of O'Reilly et al.\textsuperscript{20} In their studies, the production of LMIF was more pronounced during the convalescent stage of the disease, although LMIF was also found in patients during the period of active lesions. A strict comparison cannot be made between the two studies because we have designed an MI of 0.8 or less as a positive CMI response. In addition, their patient population consisted of individuals with more frequent episodes of infection, whereas ours were without infection for 1 month or more.

It is dangerous to ascribe a particular deficiency in CMI response as the cause for recurrent herpes simplex infections. Such conclusion should be reached only after an extensive longitudinal study of patients in all stages of the disease.

There are many lymphocyte-mediated functions which are independent of each other, and the apparent decrease in one need not be an absolute deficiency but merely the production of another lymphokine with an opposite effect, such as migration-enhancement factor.\textsuperscript{21} If herpes simplex recurrent infections are related to fluctuations in the CMI status of the host, this has to be viewed relative to the state of the latent virus in the host, since the immune response of the host can be altered by the presence of infectious virus or antigens during intermittent shedding, which in turn serves as a source of continuous stimulation.

Neutralizing antibodies to HSV were found in both the patient and control populations, and the titer of these antibodies did not have any relation to the level of LMIF response within either patients or healthy subjects. It has been our observation that as a group, the patients with recurrences have a higher titer of HSV-neutralizing antibodies than their asymptomatic countergroup, perhaps due to frequent re-exposure to the virus. Although antiviral IgG has been implicated as the humoral factor that controls the reactivation of latent virus from the ganglia of mice,\textsuperscript{22} the fact that IgG-HSV complexes have been found in herpes simplex infections\textsuperscript{7, 23} suggests that HSV survives in the presence of these circulating antibodies, circumventing this host defense mechanism.

This study of LMIF clearly shows a decrease in this lymphocyte-mediated function at a quiescent stage. Translating this information into a clinically useful approach to halt recurrences may be difficult, however, since previous studies\textsuperscript{24} with staphylococcal lysate and BCG have been ineffective.
REFERENCES


