Lysis of Herpes Simplex Virus (HSV) Infected Targets

IV. HSV-Induced Change in the Effector Population

Robert L. Hendricks and Joel Sugar

Herpes simplex virus type 1 (HSV-1) stimulation of peripheral blood lymphocytes from patients (PBL-P) with both recurrent corneal and oral-facial HSV-1 lesions results in altered natural killer (NK) activity. Freshly isolated PBL-P exhibit high lysis of HSV-1 infected allogeneic fibroblasts but low lysis of uninfected fibroblasts. After stimulation with HSV-1, PBL-P exhibit markedly increased lysis of uninfected fibroblasts such that the HSV-1 infected and uninfected fibroblasts are lysed with equal efficiency. The NK activity in freshly isolated PBL-P is mediated by effector cells that are phenotypically distinct from those responsible for the NK activity in HSV-1 stimulated PBL-P. In freshly isolated PBL-P most of the lysis of HSV-1 infected fibroblasts is mediated by lymphocytes expressing the phenotype OKM1+, OKT3+, and Leu-7+. After incubation with HSV-1, most of the lysis of HSV-1 infected and uninfected fibroblasts is mediated by lymphocytes that express the OKM1 antigen but lack detectable OKT3 or Leu-7 antigens. The change in function and phenotype appears to reflect in part the activation of Leu-7+, OKM1+, and OKT3- precursors of cytotoxic lymphocytes that are inactive in freshly isolated PBL-P. Invest Ophthalmol Vis Sci 26: 208-213, 1985

Natural killer (NK) cells are capable of preferentially lysing virus-infected fibroblasts as compared with uninfected fibroblasts.\(^1\) The human NK population is phenotypically heterogeneous,\(^2\) but it has not been possible to differentiate the NK cells that lyse virus infected fibroblasts from those that lyse uninfected fibroblasts.\(^3\) Bishop et al\(^6\) demonstrated that the increased susceptibility of HSV-1 infected fibroblasts to NK lysis required the expression of HSV-1 glycoproteins on the surface of the infected cells.

We have demonstrated that peripheral blood lymphocytes (PBL) from patients with recurrent corneal and oral-facial HSV-1 lesions (PBL-P) lose the capacity to distinguish HSV-1 infected fibroblasts from uninfected fibroblasts after in vitro stimulation with HSV-1.\(^7\) Freshly isolated PBL-P exhibited high lytic activity against HSV-1 infected fibroblasts but low lytic activity against uninfected fibroblasts. After incubation of PBL-P with HSV-1 for 48 hr, lysis of uninfected fibroblasts was greatly augmented, and the HSV-1 infected and uninfected fibroblasts were lysed with equal efficiency. The present study was designed to determine if the NK activity in freshly isolated PBL-P (directed primarily against HSV-infected fibroblasts) and the NK activity in HSV-1 stimulated PBL-P (directed primarily against uninfected fibroblasts) are mediated by phenotypically distinguishable subpopulations of NK cells.

Materials and Methods

Patients

Nine patients with both recurrent corneal (zero to one recurrences in the 2 years prior to testing) and oral-facial (one to five recurrences per year in the 2 years prior to testing) HSV-1 lesions participated in this study. All the patients were tested between recurrences. The mean age of the patients was 36, ranging from 24 to 55. Five of the patients were men and four were women. Informed consent was obtained from all patients and control donors prior to their inclusion in the study.

Effector Cells

PBL-P isolated from the peripheral blood of patients by carbonyl iron Ficoll-hypaque separation followed by adherence to plastic\(^4\) were, in all experiments, greater than 97% lymphocytes. Subpopulations of PBL-P were sensitized with monoclonal antibodies...
specific for different lymphocyte differentiation antigens (Table 1) and then lysed with rabbit complement (C'). PBL-P were incubated for 30 min on ice with the monoclonal antibodies in RPMI-1640 containing 0.5% gamma globulin-free calf serum (GG-free CS). The antibody concentrations used were determined to be in slight excess of that required for plateau killing.

Sensitized lymphocytes were lysed by treatment with C' (Pel-Freeze; Rogers, AR) at a final dilution of 1:3 for 45 min at 37°C. The cells were washed and subjected to a second cycle of C' treatment. The PBL-P were then washed twice and resuspended in assay medium (RPMI-1640 plus 10% GG-free CS, 10 mM HEPES buffer, and antibiotics). Cell counts were adjusted to reflect the viability of cells receiving C' only. In some experiments, cells lysed by antibody and C' treatment were depleted by Ficoll-hypaque flotation. The cells isolated at the Ficoll-medium interface were greater than 95% viable and less than 2% marker positive as assessed by immunofluorescent staining.

Target Cells

HSV-1 infected and uninfected HEp-2 cells were used as targets. Procedures for propagating the HSV-1 (KOS strain) and infecting and 51Cr-labelling the target cells have been described in detail elsewhere.9 Greater than 90% of the HSV-1 infected HEp-2 targets expressed HSV-1 glycoproteins as assessed by immunofluorescent staining with fluorescein-conjugated antiserum to HSV-1 (M. A. Bioproducts; Walkersville, MD).

Cytotoxicity Assay

Our Cr-release cytotoxicity assay has been described in detail.9 Titrated numbers of effector cells and 10^3 Cr-51 labelled fibroblasts at effector to target (E/T) ratios of 100, 50, 25, and 10 were mixed and incubated for 4 hr at 37°C in a humid 5% CO2-95% air atmosphere. Controls included 10^3 targets in assay medium (spontaneous chromium release [Cr Rel]) or in 1% nonidet P-40 (NP40, Particle Data Laboratories; Elmhurst, IL) in water (maximum Cr Rel). The radioactivity in 100 µl of supernatant fluid was determined with a Beckman 7000 liquid scintillation counter using Ready-Solv HP cocktail (Beckman Instruments, Inc.; Lincolnwood, IL). The percentage 51Cr-release (% Cr Rel) was determined using the formula:

\[
\% \text{ Cr Rel} = \frac{\text{experimental Cr Rel} - \text{spontaneous Cr Rel}}{\text{maximum Cr Rel} - \text{spontaneous Cr Rel}} \times 100
\]

Table 1. Monoclonal antibodies used in lymphocyte subpopulation depletion studies

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Vendor</th>
<th>Concentration (µg/10^6 lymphocytes)</th>
<th>Lymphocyte subpopulation identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Leu-7</td>
<td>Becton Dickinson</td>
<td>0.300</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>OKM1</td>
<td>Ortho Diagnostics</td>
<td>0.100</td>
<td>Natural killer cells, K-cells and null cells</td>
</tr>
<tr>
<td>OKT3</td>
<td>Ortho Diagnostics</td>
<td>0.125</td>
<td>T-lymphocytes</td>
</tr>
</tbody>
</table>

The spontaneous release for most experiments was less than 10% of the maximum release and never exceeded 20% of the maximum release. The data are reported as lytic units (LU) per 10^6 lymphocytes. The LU, defined as the number of effector cells required for 20% Cr Rel from 10^3 target cells, was calculated according to the equation: y = A(1 - e^-kx) where y = 20% Cr Rel, A = the maximum cell mediated lysis, k = a constant equal to the negative slope of the target cell survival curve, obtained by plotting ln(A - y) versus x, and x = E/T ratio producing 20% Cr Rel. A nonlinear least-squares fitting program (NOLLIN, courtesy of Dr. Hugh Pross, Queens University; Kingston, Ontario, Canada) was used to fit the data to this equation. The use of this equation for calculating lytic units has been described in detail10 and shown to be more accurate than extrapolating from the linear portion of the exponential dose response curve.11

In Vitro Stimulation of Cytotoxic Cells

Preliminary experiments determined the concentrations of HSV-1 and lymphocytes yielding optimum levels of lytic activity after 48 hr of incubation. These experiments established the culture conditions used in all the experiments described in this manuscript. Five million PBL-P in 1 ml of assay medium were dispensed into each well (16 mm diameter) of a 24-well tissue culture plate (Costar 3524). Each well received 1 ml of assay medium containing 10^6 PFUs of HSV-1 or 1 ml of assay medium alone. The plates were then incubated for 48 hr in a humid atmosphere of 5% CO2-95% air with constant rocking (approximately 6 cycles per min). After incubation, the PBL-P were removed from each well, washed twice, and counted. Incubation with HSV-1 did not result in decreased viability, as assessed by trypan blue dye exclusion.

Results

We have observed previously7 that PBL-P freshly isolated from the peripheral blood of patients with
corneal and oral-facial herpetic recurrences lyse acutely HSV-1 infected allogeneic fibroblasts more efficiently than uninfected fibroblasts of the same cell line. The data in Figures 1 and 2 are consistent with these findings. The difference in lytic activity of freshly isolated PBL-P treated with C' only against HSV-1 infected (T = 0, Figs. 1B, 2B) versus uninfected (T = 0, Figs. 1A, 2A) targets was significant at $P < 0.001$ as assessed by a paired $t$-test. In six separate experiments PBL-P were isolated and depleted of one or more lymphocyte subpopulation. Freshly isolated PBL-P from three patients were treated with anti-Leu-7 and C', while PBL-P from the other three patients were treated separately with OKM1 and OKT3 and C'. The treated cells then were assayed immediately for lytic activity against uninfected and HSV-1 infected fibroblasts (T = 0, Figs. 1, 2). The percent reduction of lytic activity listed below represents the mean and standard error of the mean obtained with PBL from three patients. PBL-P lysis of the uninfected fibroblasts was reduced by 93% ± 2% with anti-Leu-7 + C' (Fig. 1A), 79% ± 4% with
OKM1 + C (Fig. 2A), and 64% ± 2% with OKT3 and C (Fig. 2A) compared with PBL-P treated with C' only. PBL-P lysis of HSV-1 infected fibroblasts was reduced by 92% ± 2% with anti-Leu-7 + C' (Fig. 1B), 89% ± 5% with OKM1 + C' (Fig. 2B), and 70% ± 4% with OKT3 + C' (Fig. 2B) compared with PBL-P treated with C' only. The lytic activities of untreated PBL-P and PBL-P treated with antibody only were not significantly different from that of C' treated PBL-P and are not shown. Thus, in freshly isolated PBL-P, most of the lytic activity against HSV-1 infected and uninfected fibroblasts was mediated by lymphocytes expressing the phenotype Leu-7+, OKM1+, and OKT3+ in agreement with our previous findings.8

PBL-P from the same patients were stimulated for 48 hr with HSV-1 and then treated with anti-Leu-7, OKM1, or OKT3 and C' immediately prior to assay for lytic activity against uninfected and HSV-1 infected fibroblasts (T = 48, Figs. 1, 2). Lysis of uninfected fibroblasts by HSV-1 stimulated PBL-P was reduced by a mean of 35% ± 2% with anti-Leu-7 + C' (Fig. 1A), 80% ± 5% with OKM1 + C' (Fig. 2A), and 7% ± 3% with OKT3 + C' (Fig. 2A) compared with lysis by HSV-1 stimulated PBL-P treated with C' only. Lysis of HSV-1 infected fibroblasts by HSV-1 stimulated PBL-P was reduced by a mean of 17% ± 6% with anti-Leu-7 + C' (Fig. 1B), 84% ± 5% with OKM1 + C' (Fig. 2B), and 4% ± 4% with OKT3 + C' (Fig. 2B) compared with lysis by HSV-1 stimulated PBL-P treated with C' only. Thus, incubation with HSV-1 resulted in a change in the phenotype of most of the effector cells that lyse uninfected and HSV-1 infected fibroblasts from Leu-7+, OKM1+, OKT3+ in freshly isolated PBL-P to Leu-7−, OKM1−, OKT3− in HSV-1 stimulated PBL-P. It should be noted that this change in phenotype was associated with a loss of the capacity of PBL-P to preferentially lyse HSV-1 infected fibroblasts. Figure 1 shows that HSV-1 stimulated PBL-P lysed the uninfected fibroblasts (T = 48, Fig. 1A) as effectively as the HSV-1 infected targets (T = 48, Fig. 1B).

It could not be determined from these experiments if the change in phenotype of the lytic effector cells during incubation with HSV-1 reflected a different population of effector cells or the loss, through antigen modulation, of the Leu-7 and OKT3 antigens from the surface of the same effector cells. Therefore, lymphocytes expressing the Leu-7, OKM1, and OKT3 surface antigens were depleted individually from freshly isolated PBL-P by antibody and C' treatment followed by removal of dead cells on Ficoll. The viable lymphocytes were then tested immediately (T = 0) or after incubation for 48 hr with HSV-1 (T = 48) for lytic activity against uninfected or HSV-1 infected targets.

PBL-P that were depleted of Leu-7 antigen-bearing lymphocytes (Leu-7− PBL-P) and OKM1 antigen-bearing lymphocytes (OKM1− PBL-P) exhibited significantly (P < 0.001) reduced lytic activity against HSV-1 infected (Fig. 3B) targets when tested at T = 0. HSV-1 stimulation resulted in a significant (P < 0.001) increase in the lytic activity of the Leu-7− PBL-P against HSV-1 infected and uninfected targets but did not affect the lytic activity of the OKM1− PBL-P when tested at T = 48.

PBL-P that were depleted of OKT3 antigen-bearing lymphocytes (OKT3− PBL-P) expressed comparable lytic activity to unseparated PBL-P against HSV-1 infected and uninfected targets when tested at T = 0. It should be noted that in these experiments the lymphocytes that were lysed with antibody and C' were removed. Thus, treatment with antibody specific for an antigen that is not expressed on the lytic effector cells will enrich for lytic effector cells and cause a corresponding increase in lytic activity expressed as LU/106 PBL-P. Anti-Leu-7 and OKM1 lysed only approximately 15% of the PBL so that enrichment was not a major factor with these antibodies. However, OKT3 lysed 70% of the PBL so that if OKT3 were not expressed on the lytic effector cells one would expect a threefold increase in lytic activity. The fact that the OKT3− PBL-P expressed the same lytic activity as unseparated PBL-P suggests that some (approximately 70%) of the effector cells were depleted. HSV-1 stimulation significantly augmented the lytic activity of OKT3− PBL-P against uninfected (Fig. 3A) and HSV-1 infected (Fig. 3B) targets when tested at T = 48. These data suggest that at least 50% of the lytic activity in HSV-1 stimulated PBL-P may be generated during incubation with HSV-1. This activity appears to be mediated by Leu-7−, OKM1−, OKT3− lymphocytes that were inactive when freshly isolated.

Discussion

We have observed that HSV-1 stimulation of PBL-P from patients with recurrent corneal and oral-facial HSV-1 lesions results in altered NK function. Freshly isolated PBL-P from these patients effectively lyse HSV-1 infected but not uninfected allogeneic fibroblasts. After stimulation with HSV-1, these PBL-P lyse the uninfected fibroblasts as effectively as the HSV-1 infected fibroblasts. We have now observed that the lytic activity in freshly isolated PBL-P that is directed primarily against targets expressing HSV-
glycoproteins is mediated by lymphocytes that express the Leu-7, OKM1, and OKT3 lymphocyte differentiation antigens. In contrast, the lytic activity in HSV-1 stimulated PBL-P that is directed primarily against uninfected targets is mediated by lymphocytes that express the OKM1 antigen but lack detectable Leu-7 or OKT3 antigens. These Leu-7\(^-\), OKM1\(^+\), OKT3\(^-\) cytotoxic cells in HSV-1 stimulated PBL-P appear to derive from inactive precursor cells of the same phenotype in the freshly isolated PBL-P. This was indicated by the fact that in three experiments, Leu-7\(^-\) PBL-P, when tested immediately after depletion, possessed a mean of only 12% of the lytic activity against HSV-1 infected targets of undepleted PBL-P. After incubation with HSV-1 for 48 hr these Leu-7\(^-\) PBL-P still lacked Leu-7 antigen detectable by immunofluorescent staining but possessed a mean of 60% of the lytic activity of unseparated PBL-P.

We could not determine from these experiments if the augmented lytic activity of the Leu-7\(^-\) PBL-P resulted from proliferation of a small number of Leu-7\(^-\) cytotoxic lymphocytes in the freshly isolated PBL-P, from activation of inactive Leu-7\(^-\) precursors of cytotoxic lymphocytes, or from increased lytic efficiency of the same number of effector cells. In the latter two cases, it is possible that a Leu-7\(^-\) suppressor cell responsible for maintaining the Leu-7\(^-\) effector cells in an inactive state or at a low level of activity in fresh PBL is eliminated by treatment with anti-Leu-7 + C. The Leu-7\(^-\) effector cells are then able to escape from suppression during in vitro incubation.

We are currently using a single-cell cytotoxicity assay to distinguish among these possibilities.

The fact that the cytotoxic cells that do not express the Leu-7 and OKT3 surface antigens lacked the capacity to preferentially lyse HSV-1 infected targets suggested that one or both of these antigens may be involved in binding of the effector cell to HSV-1 glycoproteins on the surface of the infected targets. However, treatment of freshly isolated PBL-P with anti-Leu-7 or OKT3 in the absence of C did not significantly affect their capacity to lyse the HSV-1 infected targets (data not shown). This observation would seem to militate against a receptor role for these antigens.

It is interesting to note that these Leu-7\(^-\), OKM1\(^+\), OKT3\(^-\) cytotoxic cells are not generated when PBL from control donors with no known HSV involvement are incubated with HSV-1 or when PBL from patients are incubated without HSV-1.\(^7\) This raises the possibility that HSV-1 virions at sites of virus shedding may have a different effect on PBL from patients who get recurrent HSV-1 lesions and PBL from individuals who do not get these lesions. HSV-1 stimulation of PBL from patients may cause augmentation of nonspecific cytotoxic activity resulting in lysis of uninfected cells and tissue necrosis at sites of HSV-1 shedding.

**Key words:** herpes simplex virus, natural killer cells, surface phenotype
Acknowledgments

The authors acknowledge the expert technical assistance of Barbara Grajewski and Christine Rolnik and thank Dr. Morton Goldberg for reviewing the manuscript.

References