Role of T lymphocytes in cellular immune responses during herpes simplex virus infection in humans

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ABSTRACT Lymphocyte blast transformation and interferon production in mononuclear cell cultures prepared on Ficoll-Hypaque gradients from individuals with herpes simplex virus-1 infection were enhanced by a disease recurrence. Responses to both herpes simplex virus-2 and phytohemagglutinin were unaltered. Transformation to herpes simplex virus-1 antigen was adversely affected by depleting either thymus-derived (T) lymphocytes or bone marrow-derived (B) lymphocytes together with monocytes from cultures. The transformation response was reconstructed when the selectively depleted lymphocyte populations were recombined. X-irradiation of either T or B lymphocytes and monocytes showed that T lymphocytes incorporated [3H]thymidine with the aid of a radioresistant non-rosetting cell, probably a monocyte. Depletion of B lymphocytes and monocytes, but not of T lymphocytes, resulted in reduction in interferon production. Irradiated B lymphocytes and monocytes failed to produce significant quantities of interferon, suggesting that a radiosensitive B cell was a major interferon source.

There is evidence for the importance of cellular immunity in limiting herpesvirus infections. Adoptive transfer of spleen cells from mice immunized with herpes simplex virus (HSV) protected syngeneic irradiated mice against lethal challenge with the virus (1, 2). Treatment of mice with anti-lymphoid sera (3-5) potentiates HSV infection, pointing to a role for sensitized lymphoid cells in host defense.

In humans, cellular immunity to HSV has been studied by using different in vitro variables, most commonly, lymphocyte transformation. The significance of lymphocyte transformation as a correlate of susceptibility to recurrent HSV infection is not established. Defects in transformation to HSV antigen associated with acute disease have been detected by some investigators (6), but not by others (7-11). Defects in lymphokine production in vitro, for example, in migration inhibiting factor and interferon production have been reported during acute HSV infection (7, 12). In cardiac transplant recipients, low responses of lymphocyte interferon production and transformation to HSV antigen are associated with both severe and prolonged HSV infection (13).

It is important to measure responses of thymus-derived (T) lymphocytes to specific antigens in cellular immune reactions. There are observations that viral antigens stimulate the incorporation of [3H]thymidine in B as well as T lymphocytes (14-16). This indicates that transformation may not correlate only with T cell function. The production of lymphokines is also complex. Various immunocytes yield soluble factors such as migration inhibiting factor (17), mitogenic factor (18), lympho toxin (19), and interferon (20, 21), raising doubts about the validity of measuring lymphokines as indicators of T cell function.

The goals of this study were to determine (i) whether either lymphocyte transformation or interferon production was altered by recurrent HSV infection in humans and (ii) if the origin of these responses reflected the activity of T cells.

MATERIALS AND METHODS

Viruses and Antigens. HSV (strain PH) and HSV-2 (strain MS) from Lavelle Hanna, University of California School of Medicine, San Francisco, CA, were grown in HeLa cell cultures with Eagle's minimal essential medium and 1% newborn calf serum. Cell cultures in 32-oz (0.95-l) prescription bottles were infected with a multiplicity of 0.01 plaque-forming units/cell and incubated at 32° in 30 ml of medium for 24-48 hr. Virus infected cells were shaken from the surface with glass beads, rapidly frozen and thawed 2 times, and sonicated for 45 sec in a Branson model 185 D oscillator at a setting of four. After centrifugation at 600 × g, the supernatant was heat inactivated for 2 hr at 56° and then stored at −70°. The titer by plaque-forming unit assay before heat inactivation was 10⁷/ml for HSV-1 and 10⁶/ml for HSV-2. After inactivation, no plaques were detected when 0.1 ml was inoculated into each of 10 replicate cultures. Control antigen was prepared from uninfected HeLa cell cultures.

Mitogens. Lyophilized phytohemagglutinin-P (PHA) (Difco, Detroit, MI) was reconstituted in 5 ml of phosphate buffered saline and diluted 1:30. Pokeweed mitogen (PWM) (Gibco, Grand Island, NY) was reconstituted in 10 ml of the buffered saline and diluted 1:60.

Mononuclear Cell Cultures. The microculture assay for lymphocyte transformation and interferon production from cells separated on Ficoll/Hypaque (FH) gradients has been described (22). T lymphocytes were purified on nylon fiber columns (23). The eluate was centrifuged on a FH gradient. T lymphocytes so prepared gave at least 60% rosette formation with papain-treated sheep erythrocytes (24), and at least 95% were killed by anti-human T lymphocyte sera (25).

Bone marrow-derived (B) lymphocyte-enriched populations were obtained by incubating 5 × 10⁶ mononuclear cells per ml with 0.5% papain-treated sheep erythrocytes in 50% fetal calf serum for 15 min at room temperature. The mixture was centrifuged at 200 × g for 5 min and held for 1.5 hr at room temperature. After gently resuspending the cell pellet and centrifuging at 400 × g for 30 min on a FH gradient, the nonrosetting cells at the interface of the FH and medium had residual rosette-forming cells of less than 5% and, for most experiments, less than 1%. The nonrosetting cells, 10-70% had surface immunoglobulins and 10-40% were monocytes.

For monocyte depletion, leukocytes from dextran-sedimented, heparinized blood were centrifuged at 200 × g, resuspended in 30 ml McCoy's 5A medium, and incubated for

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Abbreviations: T, thymus-derived; B, bone marrow-derived; HSV, herpes simplex virus; PHA, phytohemagglutinin P; PWM, pokewe wood mitogen; FH, Ficoll-Hypaque.
20 min at 37° with 25 mg iron carbonyl powder (GAF Corporation, New York, NY). Cells with ingested iron carbonyl particles were removed with a magnetic bar. The remaining cells, when centrifuged on FH gradients had less than 1% monocytes. For some experiments, after monocyte removal, cells were subsequently depleted of T lymphocytes.

T lymphocytes were quantitated by mixing 1 × 10^6 cells in 0.1 ml with 0.1 ml of 0.5% papain-treated sheep erythrocytes, centrifuging for 5 min at 200 × g, and incubating the mixture at room temperature for 1.5 hr. By using a hemacytometer, mononuclear cells with three or more attached sheep erythrocytes were scored as positive for rosette formation. B lymphocytes were quantitated by using direct immunofluorescent staining of surface immunoglobulins (25). Monocytes were identified by cytoplasmic esterase staining (26).

Study Population. All donors were healthy adults who were diagnosed at the time of lesion recurrence by either isolation of virus or detection of cells with HSV-1 antigen by immunofluorescence with monospecific rabbit anti-HSV-1 sera.

RESULTS

Effect of Recurrent Infection on Lymphocyte Responses to HSV Antigen. Fig. 1 shows the kinetics of (A) lymphocyte transformation and (B) interferon production by cells from individuals at different times after HSV-1 recurrence. Statistically significant differences (P < .005) in mean transformation indices were detected by comparing the following groups: (i) those with no detectable antibody (no complement fixation at 1:4 dilution) with all groups with history of infection and (ii) those studied within the first 12 weeks after infection with those studied more than 12 weeks after disease. Interferon production was highest in the group studied within 0 to 7 days of lesion appearance as compared to all other groups (P < .005).

When cells from 16 donors were stimulated with HSV-2 antigen at 2 days and 4 weeks after HSV-1 recurrence, the mean transformation indices were 11 ± 2 and 15 ± 3, respectively (data not shown). PHA stimulation produced transformation indices of 40 ± 8 in the acute disease and 50 ± 9 for convalescent individuals. Interferon responses to HSV-2 antigen were 1.3 ± 0.3 and 1.2 ± 0.2 log_10 units.PHA-stimulated cultures gave 1.2 ± 0.3 log_10 units of interferon in both the acute and convalescent stages of HSV-1 disease. Thus, HSV-1 infections stimulated antigen-specific increases in both lymphocyte transformation and interferon that were maximum at the time of lesion recurrence.

Effect of Selective Depletion of Either T Lymphocytes or B Lymphocytes and Monocytes on Responses to HSV. Comparisons of (A) [3H]thymidine incorporation and (B) interferon production in cell cultures selectively depleted of either B cells and monocytes or T lymphocytes are shown in Fig. 2. Depletion of either of these categories of mononuclear cells produced decreases in lymphocyte transformation. In 1 of 10 experiments in which a reduction of [3H]thymidine incorporation was not observed with B cell and monocyte depletion, 10% cells with surface immunoglobulins remained in the depleted mixture. Depletion of B cells and monocytes, but not T lymphocytes, resulted in reduction of interferon production; in seven of nine experiments, interferon titers were diminished by 0.5 log or greater. In two experiments not resulting in a decreased response, there were approximately 5% residual cells with surface immunoglobulin. When T cells were depleted, in six of seven experiments interferon titers were either unaffected (four experiments) or increased (two experiments). In one experiment, depletion of T lymphocytes produced a reduction in interferon titers.

Identification of Cellular Interactions for Optimal Lymphocyte Transformation and Interferon. When T lymphocytes were recombined with B cells and monocytes, restoration of the transformation response to HSV antigen occurred, as shown in a representative experiment in Fig. 3, lower. Maximal restoration was seen in the range of the cell proportions in the original unseparated mononuclear cells, shown by the hatched bar. With decreasing numbers of T lymphocytes, a decrease in the proliferative response to both PHA and PWM was observed; these responses are consistent with the T cell origin of PHA-stimulated transformation and T-dependent B cell transformation by PWM (27). For interferon (Fig. 3, upper), the amount detected in the supernatants increased proportionally with numbers of B cells and monocytes.

In most instances, recombination resulted in [3H]thymidine incorporation and interferon production similar to that observed in unseparated cells. However on occasion, the proliferative response of the recombined cells substantially exceeded that of the unseparated cells (Fig. 4, lower left). The response of the separated and recombined cells was never less than that of the unseparated cells. Background levels in unstimulated T lymphocyte cultures in individual experiments were as low as between 50 and 150 cpm and increased progressively with non-T mononuclear cells to 500–600 cpm, with a maximum of 2000 cpm in some experiments. When monocytes were depleted from B lymphocytes, the progressive increases in background stimulation were dramatically diminished. These observations may relate to the stimulation of autologous T lymphocytes by non-T mononuclear cells (28, 29).

Supernatant from cultures of either T-depleted cells or unseparated mononuclear cells that were stimulated with HSV
antigen, harvested 5 days later, and tested in concentrations ranging from undiluted to 1:100 did not help purified populations of T lymphocytes incorporate \(^{3}H\)thymidine after stimulation with HSV-1 antigen.

To identify the cell incorporating \(^{3}H\)thymidine in response to HSV antigen, we irradiated suspensions of either T cells or B cells with monocytes with 3000 rads (1 rad = 0.01 J/kg) in a cesium irradiator by using a dose of 750 rads per minute. Two representative experiments are shown in Fig. 4. When irradiated T cells were combined with B cells and monocytes, no incorporation of \(^{3}H\)thymidine (A and B, lower) was observed. However, when irradiated B cells and monocytes were added to viable T cells, the transformation was similar to (4B) or substantially exceeded (A) that observed in the control reconstituted mixtures of cells. For interferon (A and B, upper), mixtures of irradiated T cells with viable B cells and monocytes after stimulation with HSV antigen produced titers similar to those of reconstructed control cells. Irradiation of B cells and monocytes resulted in a substantial reduction in interferon production.

Identification of the Helper Cell for Lymphocyte Responses to HSV Antigen. In Fig. 5, a representative experiment is shown in which B lymphocyte-enriched cell suspensions were depleted of monocytes. B cells alone gave minimal restoration of \(^{3}H\)thymidine incorporation as compared to B cells with monocytes. Monocyte depletion also resulted in less interferon from the B cell-enriched population.

Characterization of Interferons Produced by Mononuclear Cell Populations. Supernatants from HSV antigen-stimulated cultures of T cells or B cells and monocytes diluted to contain approximately 100 units of interferon were incubated for 1 hr at room temperature with sufficient antisera to human leukocyte type I interferon to neutralize 100 units of antiviral activity. The antiviral activity in supernatants from both T cells and B cells with monocytes was reduced by at least 60% after incubation with antisera to type I interferon (30) indicating that the interferon was Type I rather than immune (Type II).

**DISCUSSION**

In humans with recurrent HSV infection, T lymphocytes respond *in vitro* to HSV antigen with increased DNA synthesis. The role of the T lymphocyte in transformation was supported by several findings. When either B or T lymphocyte-enriched populations were irradiated and recombined with the viable complemental cells, DNA synthesis was observed only when viable T lymphocytes were present. Also, after HSV recurrence, cells responding to antigen by proliferation are present in the circulation for prolonged periods of time, a characteristic of the long-lived sensitized T lymphocyte (31). Studies with murine splenic cells have shown that B lymphocytes respond to HSV stimulation with cellular DNA synthesis (32). It is entirely possible that the human peripheral blood lymphocytes used in our study have a different spectra of responses when compared...
to those of murine splenic lymphocytes. Additionally, infectious virus used for stimulation of the murine cells may initiate cellular responses distinct from those of the inactivated virus used in our studies in humans.

The monocyte-dependent T lymphocyte transformation with HSV antigen is consistent with those of other investigators who used nonspecific mitogens and other viral antigens (33-36) as well as with our earlier studies that showed enhanced transformation by T cells with monocyte-derived macrophages (23). Since supernatants from mononuclear cells stimulated with HSV antigen were ineffective in promoting T cell proliferation, the mechanism by which the monocyte enhances transformation probably requires cell-to-cell contact rather than soluble mediators (37, 38). Soluble antigens can be concentrated by monocytes to give a more potent stimulus to lymphocytes (39).

No obvious defect in T lymphocyte transformation was associated with recurrence of HSV infection. Instead, the disease episode increased the T lymphocyte response to HSV-1 antigen, as shown in other studies (7-10). The enhanced T cell response during recurrent disease may reflect the activity of cells in immunologically normal individuals which are more important in restricting the disease to a local site as suggested by Russell (40) rather than governing the frequency of recurrences.

Whether the T lymphocyte that is transformed has a direct effector function for limiting virus infection remains to be determined. Effector functions in some murine virus infections have been attributed to histocompatibility restricted cytotoxic T lymphocytes present in spleens (41, 42). However, in humans it has not been possible to demonstrate cytotoxic effector cells in the peripheral blood of individuals immunized with vaccinia (43) despite the presence of T lymphocytes that transform in response to vaccinia virus antigen (44). In model systems using HSV infected mice (45) and rabbits (46), cytotoxic T lympho-

**FIG. 4.** Effect of X-irradiation on the ability of selectively depleted cell populations to reconstruct transformation and interferon responses. Either B or T lymphocytes were irradiated at 3000 rads and mixed with the unirradiated complementary cell population. Reconstructed mixtures were T lymphocytes + B lymphocytes and monocytes (O), X-irradiated T lymphocytes + B lymphocytes and monocytes (●) and T lymphocytes + X-irradiated B lymphocytes and monocytes (◊). The transformation (Lower) and interferon (Upper) responses in cultures or peripheral blood mononuclear cells isolated on Ficoll gradients are shown by the hatched bar placed on the abscissa to show the cell composition of the unseparated cells. All cultures for [3H]thymidine incorporation and interferon were stimulated with either HSV antigen or control HeLa cell antigen. [3H]cpm shown represent those in HSV antigen-stimulated cultures with subtracted background cpm from HeLa cell antigen-stimulated cultures. Two representative experiments are shown in panels A and B.

**FIG. 5.** Lymphocyte interferon production (Upper) and transformation (Lower) in reconstructed mixtures before [T + (B + mono) (O)] and after removal of monocytes [T + (B - mono) (●)] by iron carbonyl ingestion.
cytes could be demonstrated only at local sites. These studies imply that the proliferating cells in the peripheral blood of humans may represent a precursor which could give rise to effector cells that are sequestered locally.

The reduction in interferon production after irradiation of B lymphocytes and monocytes indicates that viable B cells are a major source for interferon production. Most monocyte functions are radioresistant; therefore, this cell most likely contributes to interferon in a helper capacity. In addition, we have been unable to detect significant interferon production in cultures of glass adherent cells, mostly monocytes, after stimulation with HSV antigen (data not shown). Finally, the amount of interferon produced in supernatants was proportional to the increases in the number of non-T lymphocytes.

The B lymphocyte responds to HSV antigen with interferon production under at least two circumstances. During acute recurrent HSV-1 disease, interferon production is in part immune specific since the increased response occurs only after stimulation with HSV-1 antigen. In nonimmune individuals—i.e., those with no detectable T lymphocyte transformation and minimal production of interferon by T lymphocytes (22)—the interferon detected in mononuclear cultures from FH gradients is most likely of B cell origin. This finding suggests that the B cell may be particularly responsive to interferon induction even without immune sensitization, as is the case for Sendai virus-induced interferon from human B cells (47).

The physicochemical type and immune nature of interferons may relate to the cell of origin. Type II interferon from T lymphocytes with macrophages is immune specific and may be quantitatively related to HSV lesion frequency (23, 30). Type I interferon from T lymphocytes alone is also immune specific (22). These studies show that the B lymphocyte yields more type I interferon than T lymphocytes and its production is unrelated to the presence of sensitized T lymphocytes. Interferon is important in resolution of HSV infection (48, 49). Whether or not a single type is critical in host defense is a subject for future investigation.

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