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Experimental Infection of Subpopulations of Human Peripheral Blood Leukocytes by Herpes Simplex Virus[1] (40185)

SUSAN PLAEGER-MARSHALL AND JERRY W. SMITH

Department of Microbiology, Louisiana State University Medical Center, New Orleans, Louisiana 70112

Evidence is increasing that infection with herpes simplex virus (HSV) may cause impairment of host immunologic function. O'Reilly *et al.* (1) have determined that transient deficiencies of specific leukocyte migration inhibitory factor and interferon occur in patients with herpetic vesicular eruptions. In addition, lymphocytes cultured from patients with recurrent disease have been found to be deficient in production of lymphokines (2, 3), as well as in the ability to destroy HSV-infected cells (4). Inhibition of mitogen-induced lymphocyte blastogenesis (5) and decreased monocyte chemotaxis also have been attributed to this virus (6).

Still unclear is whether the immune impairment associated with HSV infection is mediated indirectly or is the result of interaction of virus and immune effector cells, with subsequent cellular alteration and/or destruction. It has been known for some time, however, that HSV replicates in continuous lymphoblastoid cell lines and in mitogen-stimulated cultures of peripheral blood lymphocytes from adult donors (5, 7-11). Recent reports indicate that infection of blood cells may also occur *in vivo*, since HSV has been isolated from buffy coats obtained from patients with recurrent herpetic disease (12). Further, unstimulated human cord blood mononuclear cells support replication of HSV, suggesting that dissemination of virus by means of blood cells may be an important factor in the pathogenesis of neonatal infections (13). The purpose of the present study was to gain further insight into the interactions between blood cells and HSV by studying the ability of virus to replicate in defined subpopulations of leukocytes.

Materials and Methods. Virus. The KOS strain of HSV type 1 (HSV-1) was propagated in a continuous line (Vero) of African green monkey kidney cells as described previously (14). Virus preparations contained greater than 10^8 plaque-forming units (PFU) per ml when assayed on Vero monolayers (15).

Human lymphoblastoid cell lines and peripheral blood leukocytes. A cell line of B lymphocyte origin, IM-1, was obtained from Dr. Dean Mann, National Institutes of Health (via Dr. L. A. Wilson, L.S.U. Medical Center), and a line of T lymphocyte origin, MOLT-3, was obtained from Associated Biomedic Systems, Inc., Buffalo, NY. Lymphoblastoid cell lines were carried as stationary cultures in eight oz glass prescription bottles containing RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml), hereafter referred to as standard medium.

Leukocyte subpopulations were separated from heparinized venous blood (heparin, 20 units/ml) obtained from five healthy donors. The presence of serum antibody to HSV was determined by the ^{51}Cr-release test for cytolytic antibody to surface antigens of HSV-infected BHK-21 cells (14, 15). Results showed three of the subjects to be positive for antibody to HSV and two negative.

Mononuclear cells were obtained by centrifugation of heparinized blood on Ficoll-Hypaque gradients according to the method of Mansfield and Wallace (16). Monocytes were separated from the mononuclear cells banding at the interface by adherence to glass or plastic as described below. The purity of the isolated fraction (>95% monocytes) was determined by the uptake of neutral red and peroxidase staining (17). Nonadherent mononuclear cells were separated into T and B lymphocyte fractions by centrifugation of rosetted (T cell) and unrosetted (B cell) cells on Ficoll-Hypaque, as described by Joseph *et al.* (18). Rosettes consisted of lymphocytes bound to sheep red blood cells treated with 2-amino-ethylisothiouronium bromide (19).

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Direct staining for surface immunoglobulin on B cells with fluorescein isothiocynate (FITC) conjugated goat-anti-human IgG, heavy and light chains (Cappel Laboratories, Cochranville, PA), showed the purity of the B cell fractions to be greater than 90%, and T cells greater than 80%.

Polymorphonuclear leukocytes (PMNL), which formed a pellet at the base of the initial Ficoll-Hypaque gradient, were treated with 0.83% NH_4Cl to remove contaminating erythrocytes. Cells were then washed twice by centrifugation and placed in 25 cm^2 plastic culture flasks. All isolated subpopulations were cultured in standard medium at 37° in a humidified incubator containing 5% CO_2.

Replication of virus. For infection of cell lines, lymphocytes and PMNL subpopulations, a cell pellet containing a designated number of cells was exposed to 1 ml of viral suspension at a multiplicity of infection (MOI) of either 1 or 10. After a 2-hr adsorption period in a 37° shaker water bath, cells were washed twice by centrifugation (150*g* for 10 min) in RPMI-1640 medium to remove unadsorbed virus, resuspended in 30 ml standard medium, and incubated at 37° in 25 cm^2 plastic flasks. One ml samples were removed at predetermined times and stored for future assay. Cells were frozen and thawed an additional cycle, and virus yield was determined by assay on Vero monolayers as described previously (14). Each leukocyte subpopulation contained at least 90% viable cells at the time of infection, as determined by trypan blue exclusion.

To convert lymphocytes to blast cells, 15 μg/ml of phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, MI) or 50 μg/ml of pokeweed mitogen (PWM; Sigma Chemical, St. Louis, MO) was used to supplement the standard medium. These concentrations had previously been shown to give optimum stimulation under test conditions. Pokeweed mitogen has been shown to stimulate B as well as T cells, and was therefore used to stimulate both lymphocyte subpopulations; PHA is a selective T cell mitogen (18, 20). Cells were infected immediately after separation on Ficoll-Hypaque, or after four days of culture in the presence of mitogen.

For infection of monocytes, which do not remain in suspension but adhere to plastic or glass (21), three different methods were used. The first method involved isolation of monocytes in 96-well microtiter plates (Falcon Plastics, Oxnard, CA). Cells were counted and dispensed at a concentration of 10^6 cells per well in 0.2 ml amounts of standard medium supplemented with 0.25 m*M* HEPES buffer to stabilize pH. Nonadherent lymphocytes were removed after 12–18 hr and fresh medium added. After 72 hr of additional incubation, medium was removed and virus was added to each well at an MOI of 1. After 2 hr of incubation at 37° in 5% CO_2, unadsorbed virus was removed from the wells by three cycles of washing with RPMI-1640. One tenth ml of standard medium with HEPES buffer was then added to each well. Sampling was done at designated times by scraping and combining the cells from each of eight wells. Suspensions were stored at −70° and assayed for infectious virus as described. The second method was identical to the above procedure with the exception that nonadherent cells were removed after 48 hr, instead of 12–18 hr, and infection proceeded immediately rather than after 72 hr of additional culture.

The third method was a modification of that described by Schultz *et al.* (22). Briefly, mononuclear cells (2×10^7) that had been separated on Ficoll-Hypaque were placed in 10 ml of standard medium in a 16-oz glass prescription bottle. After 2 hr of incubation at 37° in 5% CO_2, nonadherent cells were removed with two washes of medium, and adherent monocytes were gently scraped off the glass with a rubber policeman. Cells were suspended in 1 ml of virus in standard medium, and placed in a 50 ml plastic centrifuge tube (Falcon Plastics, Oxnard CA). After 2 hr at 4° with intermittent mixing, unadsorbed virus was removed by two washes in medium. Two $\times 10^5$ cells were added to individual wells of a microtiter plate and incubated and sampled as described above.

Fluorescent antibody experiments. For fluorescent antibody analysis, about 2×10^5 infected cells were applied to glass slides and spread onto a circular area about 2 cm in diameter. Slides were air-dried and fixed for 10 sec in cold acetone and then immersed in phosphate buffered saline (PBS, pH = 7.4) followed by distilled water. One drop of FITC conjugated antiserum to HSV-1 (Flow

Laboratories, Rockville, MD) at a 1:5 dilution was applied to dried slides, which were then incubated for 45 min at 37° in a humidified chamber, washed in distilled water, and air-dried. Cover-slips were added over a 9:1 solution of glycerol in PBS and the cells were examined for fluorescence using a Reichert Zetopan microscope (Vienna, Austria). Nonspecific fluorescence was minimized by absorbing conjugated antiserum with uninfected IM-1 and MOLT-3 cells (23).

Results. Replication of HSV in lymphoblastoid cell lines. Lymphoblastoid cells of both T (MOLT-3) and B (IM-1) cell origin were infected with the KOS stain of HSV-1. Results are shown in Fig. 1. At an MOI of 1, both lines gave evidence of viral reproduction, although MOLT-3 cells appeared to have a greater capacity to replicate virus than did IM-1. Both lines showed maximum virus titers at 48 hr after infection. Control thermal inactivation cultures consisted of lymphoblastoid cells that had been frozen and thawed after viral adsorption. Infectious virus titers dropped markedly in these control cultures, indicating that input virus was inactivated quickly under culture conditions. At the end of the 96-hr culture period, less than 25% of the cells remained viable as determined by trypan blue exclusion.

Replication of HSV in peripheral blood subpopulations. Subpopulations of peripheral blood leukocytes from each of five subjects were infected with HSV and examined for virus yield. Results for all subjects were essentially the same. Representative experiments are shown in Fig. 2–4.

Initial experiments were concerned with the ability of mononuclear cells (lymphocytes and monocytes) to support replication of virus (Fig. 2). Cells infected after 72 hr of culture in the presence of PHA showed a log increase in virus titer, whereas cells cultured similarly but without mitogen showed a decrease in titer much like control cultures.

Because the overall patterns of viral replication observed might actually have masked individual responses of the several types of leukocytes, the various subpopulations were separated and examined for the ability to support viral replication. Results are shown in Fig. 3. No increase in viral yield was seen after mixture of PMNL with HSV. The decay of infectious virus was similar to that in control cultures of leukocytes frozen and thawed after addition of virus. In most experiments, the decay was more rapid than that observed in controls. PMNL, however, did not often survive *in vitro* for more than 48 hr, a phe-

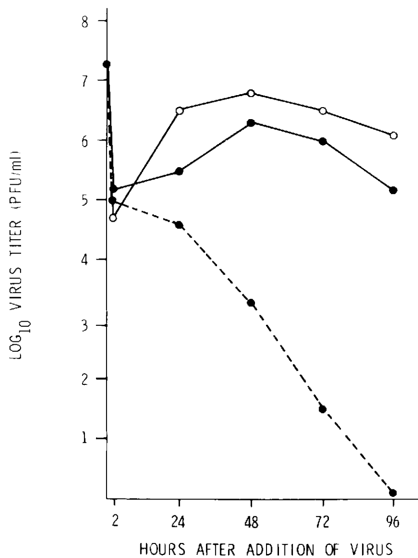

FIG. 1. Replication of HSV-1 in lymphoblastoid cell lines. IM-1 cells, ●——●; MOLT-3 cells, ○——○; frozen and thawed IM-1 control cells ●– – –●.

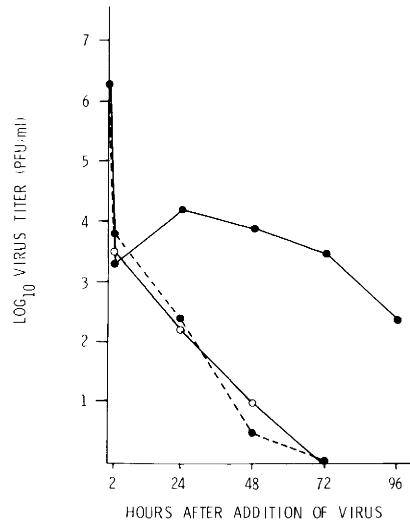

FIG. 2. Replication of HSV-1 in human peripheral blood mononuclear cells separated by centrifugation on Ficoll-Hypaque. Cells were infected after 72 hr of culture in the presence or absence of mitogen. PHA added, ●——●; no PHA, ○——○; frozen and thawed control cells, ●– – –●.

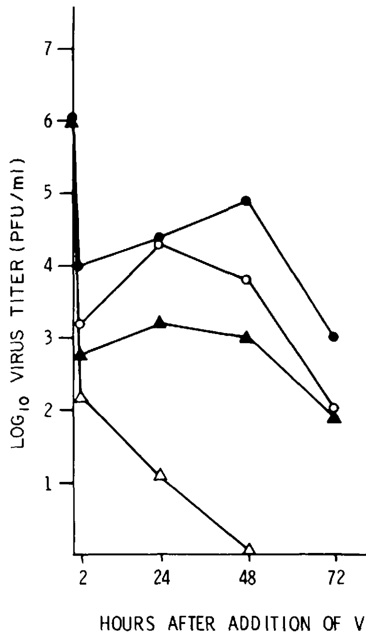

FIG. 3. Replication of HSV-1 in human peripheral blood leukocyte subpopulations. Lymphocytes were stimulated with pokeweed mitogen prior to infection. T lymphocytes, ○——○; B lymphocytes, ▲——▲; PMNL, △——△; monocytes, ●——● (cultured for 72 hr following isolation and prior to infection).

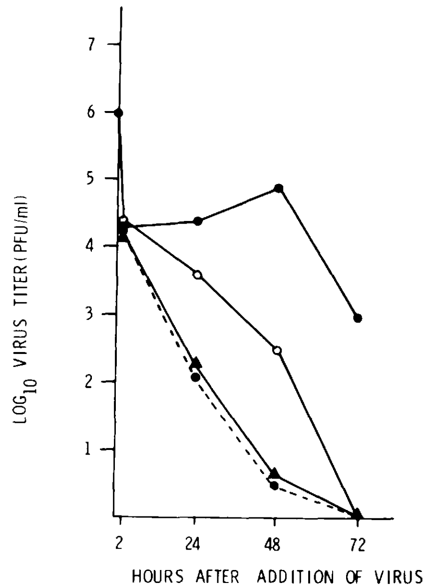

FIG. 4. Replication of HSV-1 in human peripheral blood monocytes. Comparison of three methods for culturing and infecting cells. ●——●, mononuclear cells cultured for 12–18 hr; adherent cells cultured an additional 72 hr before infection; ○——○, mononuclear cells cultured for 48 hr, adherent cells infected immediately; ▲——▲, mononuclear cells cultured for 2 hr, adherent cells removed and infected in suspension; ●- - -●, frozen and thawed control monocytes.

nomenon also noted by others (18). T and B lymphocytes transformed with pokeweed mitogen prior to infection both showed increases in virus yielded. The T cell fraction showed a rise in titer of about one log, whereas B cells showed less of an increase; rapid decrease of infectious virus was observed in unstimulated cultures.

Infection of monocytes cultured for 72 hr after removal of nonadherent cells also resulted in increased virus yield (Fig. 3). However, because experimental techniques and results of viral infection of animal monocytes (or macrophages) have been somewhat varied, two additional procedures were attempted. In these, length of time of the cells in culture and method of infection were examined. Results are shown in Fig. 4. The ability of monocytes to replicate virus was seen to be a function of the procedure used. Only cells cultured for 72 hr before infection, and infected while adherent to culture dishes, showed a significant increase in virus yield.

Fluorescent antibody experiments. Experiments were done to determine the extent of infection of the several leukocyte subpopulations by staining for fluorescent antibody to virus-specific proteins (data not shown). A pattern of strong fluorescence typical of HSV infection was observed in cells that showed a rise in virus titer (lymphoblastoid cells, monocytes, stimulated T and B cells). Uninfected control cells, however, and cells that showed a decrease in virus yield (PMNL, unstimulated T and B cells) did not react to any extent with labeled antibody. Based on counts of 200 cells each, fluorescent cultures of monocytes and stimulated lymphocytes contained less than 50% positive cells, whereas cultures of lymphoblastoid cells (IM-1, MOLT-3) contained greater than 75% positive cells. Infection was also confirmed by electron microscopic examination of thin sections of cell pellets (data not shown).

Discussion. Our results showed that HSV may replicate in T lymphocytes, B lymphocytes, and monocytes from human peripheral blood, but not in PMNL. In agreement with previous reports, replication of virus in lym-

phocytes was confined to mitogen-stimulated cultures (5, 7–11). This was demonstrated both by the production of infectious virus (plaque assay) and by detection of virus-induced cellular antigens by direct immunofluorescence.

Replication occurred in leukocytes obtained from both seropositive and seronegative individuals, suggesting that susceptibility to infection was independent of immune status to HSV. The similar results obtained from infection of lymphocyte subpopulations, however, do not eliminate the possibility that immune cells may have some effect upon viral replication. Since the blastogenic transformation necessary for infection was induced by non-specific mitogens, the ability of virus to replicate in specific memory cells cannot be fully evaluated. A slight decrease was noted in virus yields obtained from monocytes of seropositive donors as compared to seronegative donors (data not shown). The difference was small, however (approximately 1 log), and additional subjects will have to be tested to determine if this is a general phenomenon.

Some recent reports have indicated that HSV replicates only in stimulated T cell subpopulations (24, 25). We have shown that replication occurred in B cell fractions as well, suggesting that susceptibility to infection may not be restricted by cell type. Furthermore, we found that a similar production of infectious virus occurred in both T and B derived lymphoblastoid lines. Other viruses, including measles and dengue, have been examined in this respect. Results showed that the ability of virus to replicate in either or both subpopulations of peripheral blood lymphocytes correlated with ability to replicate in the corresponding lymphoblastoid cell line (18, 26).

Infection with HSV may result in T lymphocyte dysfunction (1–6). Our findings suggest that HSV may also interact with stimulated B lymphocytes, resulting in the production of infectious virus. Further study is therefore needed concerning the immunological consequences of interaction of HSV with these cells. Although T lymphocytes have been seen to be of prime importance in defense against herpetic infection, Rager-Zisman and Allison (27) recently demonstrated that antibody also is involved.

To our knowledge, HSV has not been previously studied with regard to its ability to replicate in human monocytes, and analyses in animal models have given conflicting results (28–30). Our findings suggest a possible reason for these variations. Using three experimental approaches, significant viral replication was demonstrated in only one, in which incubation of isolated monocytes was required for a period of time prior to infection. The absolute need for this incubation period remains unexplained but may be related to cell surface changes governing susceptibility to viral adsorption.

From our results, PMNL appear to be unable to support replication of HSV. The titer of infectious virus exposed to these cells dropped dramatically. Similar results have been reported for measles virus (18), suggesting that this may not be an isolated phenomenon. Loss of viral infectivity was often greater and more rapid than that seen in killed control cells, suggesting that virus not only did not replicate, but was inactivated. Although PMNL have at present no known specific role in defense against herpetic disease, this possibility, suggested by our observations, provides an interesting point for further investigation.

Our results show that HSV can, under appropriate conditions, infect most subpopulations of peripheral blood leukocytes. These data, combined with those obtained with other animal viruses (18, 26, 31), indicate that infection of peripheral blood leukocytes may be a common phenomenon associated with viruses that cause immune dysfunction.

Summary. Results from examination of peripheral blood leukocytes from five subjects, three with antibody to HSV and two without, showed that HSV type 1 may replicate in mitogen-stimulated T and B lymphocytes and in continuous lymphoblastoid cell lines derived from either B or T cells. Significant replication was also noted in monocytes from each subject but not in polymorphonuclear leukocyte subpopulations. Replication in monocytes was found to be dependent on certain conditions of culture and viral inoculation. Only adherent cells cultured for 72-hr prior to infection produced significant yields of virus.

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