Archives of Virology 49, 165—174 (1975)

Infection of Human and Rhesus Lymphoblastoid Cells with *Herpesvirus Macaca*

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With 3 Figures

Accepted August 5, 1975

Summary

The interaction between *Herpesvirus macaca* and lymphoblastoid cells grown in continuous culture, or purified from rhesus blood, was studied as a model for latent or chronic herpesvirus infection of leukocytes. Continuous lymphoblastoid cell lines infected *in vitro* were PA3, a human cell line with bone-marrow derived (B)-lymphocyte characteristics; LM/DM, a rhesus cell line with B-lymphocyte characteristics; and MOLT-4, a human cell line with thymus-derived (T)-lymphocyte characteristics.

A distinct pattern of interaction was found for each lymphoblastoid cell line. A small but stable fraction of cells continued to express virus-specific antigens for more than three weeks following infection of the B-type lymphoblastoid cell lines of rhesus and human origin. *Herpesvirus macaca* replicated only in PA3 cells. Viral replication did not occur and viral antigens were not detected in either peripheral lymphocytes or MOLT-4 cells. The observations suggest that *Herpesvirus macaca* interacts in distinct patterns with different lymphoid cell lines *in vitro*, and that within a given culture, subpopulations of lymphoid cells are present at a given time which respond to the virus differently.

Introduction

Interest in persistent viral infections has intensified as latent viruses have been increasingly implicated in carcinogenesis. The ability of herpseviruses to persist in the tissues of animals despite the presence of circulating antibodies directed against them is characteristic of the group. Lymphocytes appear to be target cells for infection by several primate herpesviruses. Thus, Epstein-Barr virus (EBV) infects bone marrow-derived (B) lymphocytes of man (17, 26), and EBV antigens can be detected for years after infection in the lymphocytes of

individuals who have EBV antibody (5). Human cytomegalovirus (CMV) may be recovered from leukocytes of infected patients (11). Lymphocytes are the target cells for *Herpesvirus saimiri* (HVS) infection of the saguinus, aotus, and saimiri species of New World primates (1, 8, 33) and also for infection by *Herpesvirus ateles* (21).

Herpes simplex virus (HSV) has been found to replicate *in vitro* in lymphocytes only after they have been stimulated with a mitogen like phytohemagglutinin (PHA) (24). Peripheral lymphocytes permit replication of EBV *in vitro*; the infected lymphocytes undergo blastoid transformation (14, 27). Continuous lymphoblastoid cell lines (LCLs) composed of cells with unlimited growth potential have been demonstrated to support the replication of many viruses including EBV (7, 16) and HVS (29). An advantage of LCLs in experimental study is that they may be propagated indefinitely, permitting long term observation and manipulation of the system.

Herpesvirus macaca (HVM), first designated the "leukocyte-associated herpesvirus", or LAHV (12), was originally isolated from the leukocytes of juvenile rhesus monkeys by cocultivation with a suitable indicator fibroblast line. Virus was recovered from leukocytes for months following experimental or natural infection despite the presence of circulating neutralizing and complement-fixing antibodies in the host animal (12, 3). Antigenically distinct from all other known primate herpesviruses, HVM is the only herpesvirus of Old World primates yet described which is recoverable from host peripheral leukocytes (2). Although HVM has not been shown to cause detectable illness in naturally or experimentally infected host animals, the oncogenic potential of this virus has not been completely explored. Like HVS, EBV, and CMV, HVM is capable of long term persistence in peripheral leukocytes, and could provide a useful model for the investigation of herpesvirus-host relationships.

Materials and Methods

Cell Cultures

Peripheral lymphocytes were obtained from *Macaca mulatta* raised from infancy in isolation cages (Litton Bionetics, Rockville, MD) and free of HVM neutralizing antibody. Blood samples were collected in preservative-free heparin (Hynson, Westcott, and Dunning, Baltimore, MD); lymphocytes were purified by the Ficoll-Hypaque technique (4). The lymphocytes were washed and resuspended in RPMI 1640 media containing neomycin 80 μg/ml, glutamine 2 mM, and 10 per cent fetal calf serum (RPMI 1640 + 10 per cent FCS). After overnight incubation at 37° C in 5 per cent CO_2, nonadherent cells were decanted for experimentation.

Cell lines used were PA3, derived from EBV-transformed human peripheral lymphocytes (26); LM/DM, derived from peripheral leukocytes of a rhesus monkey with myelogenous leukemia (6); and MOLT-4, derived from peripheral lymphocytes of a human with acute lymphoblastic leukemia (22). LM/DM and MOLT-4 lack EBV genome (18, 32). PA3 contains the EBV genome but lacks demonstrable EBV viral capsid antigen (VCA) (26). PA3 and LM/DM have B-lymphocyte characteristics (32), whereas MOLT-4 has thymus-derived (T) lymphocyte characteristics (22).

WI-38 cells were used as the indicator cell line for HVM. Monolayers were maintained in Eagle's Basal Essential Medium containing 80 μg/ml, glutamine 2 mM, and 2 per cent fetal calf serum (EMEM + 2 per cent FCS).

Virus

The 551 strain of HVM was used exclusively in these studies. Virus pools were prepared by infecting WI-38 cells with HVM and harvesting when 50 per cent of the cells in the monolayer demonstrated cytopathic effect (CPE). Infected cells and supernatant were subjected to three cycles of freezing and thawing. The viral suspension was clarified by centrifugation at 2000 r.p.m. for 10 minutes, then passed through a 0.45 μ millipore filter, and stored at —70° C. Virus titrations were performed by determining the $TCID_{50}$ in WI-38 cultures by a microtiter method (12) using the Reed-Muench calculation (30).

Infection of LCLs and Rhesus Lymphocytes

Lymphocytes and LCLs were washed with RPMI 1640, then resuspended in 1 ml virus suspension at a multiplicity of infection of 1. After a 2 hour adsorption period at 37° C, cells were washed 5 times and resuspended in RPMI 1640 + 10 per cent FCS at a concentration of 1×10^6 cells/ml. Aliquots of the final wash supernate were assayed for infectious virus. Duplicate infected cultures of each LCL, or peripheral lymphocytes, were maintained in 75 cm^2 plastic flasks (Falcon Plastics, Oxnard, CA) at 37° C, in 5 per cent CO_2. In some experiments, peripheral lymphocyte cultures were infected and subsequently maintained in the presence of PHA (Wellcome Laboratories, Beckenham, England) 2 μg/ml. Control cultures received the same inoculum volume of virus free media, and were maintained under identical conditions. To maintain each culture under the same conditions, cell concentrations were adjusted at 24—48 hour intervals to 1×10^6 cells/ml.

At 24—72 hour intervals, aliquots of infected and control cultures were taken for total and viable cell counts, extracellular and cell-associated virus titrations, cocultivation assay on WI-38 indicator monolayers, and for HVM specific immunofluorescence.

Extracellular virus was assayed by inoculating WI-38 monolayers with serial dilutions of supernatants from lymphocyte cultures which were first passed through 0.45 μ millipore filters. Cell-associated virus was assayed by inoculating WI-38 monolayers with serial dilutions of 0.45 μ millipore-filtered lysates prepared by freezing and thawing of washed lymphocytes. Serial dilutions of viable, 5× washed lymphoid cells were cocultivated with WI-38 monolayers overnight. Nonadherent cells were then removed, and fresh EMEM + 2 per cent FCS added. All cultures were observed for CPE for 8 weeks before being discarded as negative.

Immunofluorescence

An indirect immunofluorescence test for the presence of intracellular HVM specific antigens was developed using hyperimmune rabbit anti-HVM serum which was demonstrated to be free of cross-reacting antibodies for rhesus VCM or other primate herpesviruses, including EBV, by immunofluorescence. A fluorescein isothyocionate-conjugated caprine antirabbit globulin (Meloy Laboratories, Springfield, VA) was used. Cells to be examined were resuspended in a phosphate-saline buffer pH 7.4, cytocentrifuged onto glass microscope slides, air dried, then fixed for 16 hours in cold acetone.

Cells were examined for EBV VCA by indirect immunofluorescence using human sera which was demonstrated to be positive or negative for EBV antibodies, and a fluorescein isothiocyonate-conjugated caprine anti-human (Meloy Laboratories, Springfield, VA). An average of 1000 cells from each of two preparations were examined for each sample.

Iododeoxyuridine (IUdR) Treatment

Aliquots of infected and uninfected lymphoblastoid cells were resuspended in RPMI 1640 + 10 per cent FCS containing 60 μg/ml IUdR. After 72 hours of incubation, the cells were washed 3 times in fresh media and resuspended at 1×10^6 cells/ml in RPMI 1640 + 10 per cent FCS without IUdR.

Results

Counts of viable and nonviable cells were made daily for each infected and uninfected culture. Doubling time for all LCL cultures under experimental conditions was approximately 4 days, and no significant differences in growth rate between infected and uninfected cultures were found. The observed interactions between HVM and 3 LCLs are summarized in Figures 1—3. Following the extensive initial wash procedure, virus recovered from culture medium was reduced from 10^6 $TCID_{50}$ to less than $10^{1.5}$ $TCID_{50}$/ml. The further drop in titer to zero during the first 24 hours in culture probably represents thermal inactivation of residual virus. The rate of inactivation of cell free HVM at 37° C under the experimental conditions was determined and is presented as the superimposed dashed line in Figure 1 b. Thus, any extracellular virus found in culture supernatants after 24 hours post-infection most likely represents virus released from infected cells.

A distinct pattern of interaction was found for each LCL. A small but stable fraction of cells continued to express virus-specific antigens for more than 3 weeks following infection of the B-type LCLs of rhesus and human origin. No virus-specific antigen was detected in MOLT-4, the T-type LCL of human origin.

The most common immunofluorescent pattern of infected cells was a diffuse but intense staining suggesting both nuclear and cytoplasmic viral antigens, although in some cells a distinct, speckled, intranuclear pattern was discerned. Uninfected WI-38 cells were not stained by this technique, and control (non-immune) rabbit sera or conjugates did not stain infected cells. The HVM-specific fluorescence could be readily discerned from the nonspecific, diffuse staining of occasional dead cells.

HVM appeared to replicate only in PA3, the human B-type LCL (Fig. 1). Extracellular virus titers increased reproducibly after 24 hours post infection, reaching a maximum on the fourth day. It is unlikely that permissive secondary infection of PA3 cells occurred, since no extracellular infectious virus was found by the seventh day post-infection or thereafter. The largest percentage (2.4 per cent, or 1 in 42 cells) of PA3 cells producing HVM antigen was also found on the fourth day, and a maximum of cells capable of transferring infectious virus (1 in 10^5 cells) by cocultivation was found at this time. A small but detectable fraction (1 in 10^6 cells) of PA3 cells remained capable of transferring infectious virus by cocultivation at 8 days post-infection, at a time when infectious extracellular virus could no longer be detected. Beyond 8 days post-infection, however, PA3 cells were not found to transfer infectious virus by cocultivation. Thus a small but persistent fraction of PA3 cells were abortively infected by HVM, in which viral antigen continued to be expressed for at least 3 weeks following infection; the fraction of PA3 cells capable of supporting replication of infectious virus appeared to be smaller and short-lived.

Although barely detectable infectious virus persisted in the supernatants of LM/DM cultures for 3 days post-infection, no increase in the titer of extracellular virus occurred. It is unlikely, therefore, that replication of HVM took place in LM/DM cells (Fig. 2). A small (0.5 per cent) but persistent fraction of the rhesus LCL cells, however, produced virus-specific antigen beginning at 24 hours post-infection and continuing for at least 3 weeks thereafter. Cells capable of transferring infectious

virus by cocultivation were detected 24 hours post-infection; the proportion increased to a maximum of 1 cell in 2×10^4 on the fourth day post-infection. By 6 days post-infection and thereafter, no LM/DM cells transferred infectious virus. Unlike PA3, then, LM/DM rhesus LCL cells did not appear to permit replication of HVM, but a proportion of cells were transiently capable of transferring infectious virus to permissive cells by cocultivation. As with PA3, a small persistent fraction of LM/DM cells, abortively infected with HVM, produced virus-specific antigen.

Neither permissive nor abortive infection of the T-type LCL, MOLT-4, occurred (Fig. 3). These cells of human origin nevertheless appear to have surface receptors for HVM. Cells capable of transferring HVM by cocultivation persisted for 4 days following infection. Barely detectable extracellular virus also persisted for 4 days (and no longer) following infection.

To determine whether or not infectious virus might be present within infected cells without release into culture medium, aliquots of cells from each culture were taken, washed thoroughly, and subjected to 3 cycles of freezing and thawing. Previous experience in our laboratory indicated that this procedure did not significantly reduce the infectivity of HVM suspensions. No infectious virus was obtained from any LCL culture in this manner. An electron microscopic study of

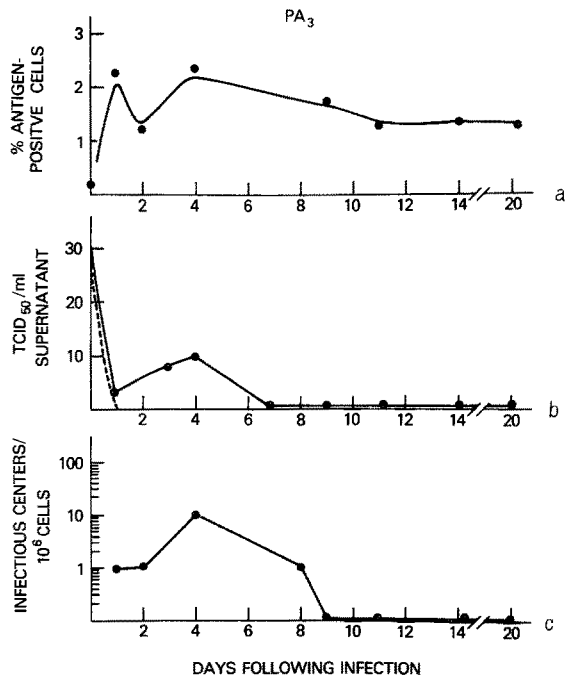

Fig. 1. Interaction of HVM with PA3 Cells

a) Percentage of cells with HVM-specific antigens. Standard error for each point is ± 0.01 per cent

b) Extracellular virus titration. The dashed line indicates the determined thermal inactivation for HVM at 37° C

c) Proportion of cells transferring infectious virus to WI-38 monolayer after cocultivation

infected LCL cells has thus far failed to reveal evidence of intracellular herpesvirus or subparticles. Since the number of LCL cells in any culture capable of producing virus or viral antigens was so low, however, it would not be surprising if such cells had been missed.

Since it had been previously shown (13, 15) that treatment of LCLs with inhibitors of DNA synthesis could induce synthesis of EBV antigens and/or virus in LCLs containing EBV genome, a similar procedure was attempted with LCLs which had been infected with HVM. By 4 weeks post-infection, none of the three LCLs produced infectious virus either spontaneously of following cocultivation with the WI-38 permissive cell line. PA3 and LM/DM cultures, however, continued to have a small proportion of cells producing HVM-associated antigens, indicating retention of at least part of the viral genome within these cells. IUdR treatment of these cultures, however, did not result in production of infectious virus nor did it alter the fraction of cells producing HVM antigen in any of the LCLs. Cell division owas inhibited as excepted.

Peripheral lymphocytes obtained from seronegative monkeys or from normal human donors showed no evidence of infection following inoculation with HVM. This may be analogous to HSV, which requires activation of lymphocytes before such cells will permit viral replication (24). However, we were unable to demonstrate replication of HVM in peripheral lymphocytes activated by PHA.

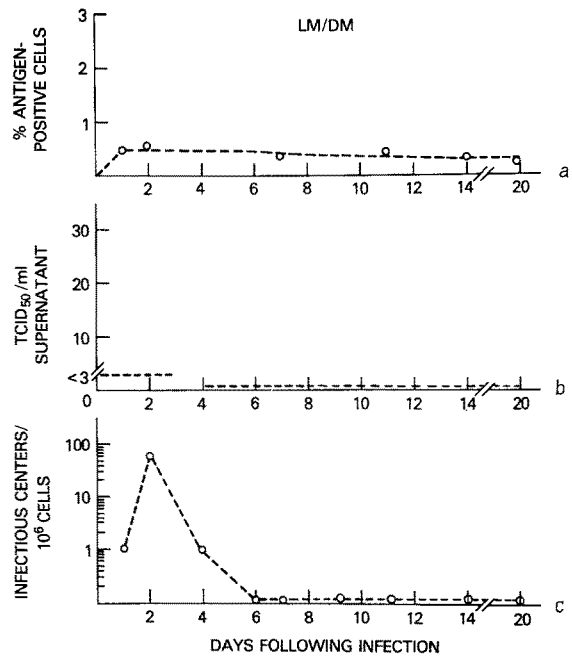

Fig. 2. Interaction of HVM with LM/DM Cells
a) Percentage of cells with HVM-specific antigens. Standard error for each point is ± 0.01 per cent
b) Extracellular virus titration
c) Proportion of cells transferring infectious virus to WI-38 monolayer after cocultivation

Although PA3 contained EBV genome, it did not spontaneously produce EBV VCA antigens. To determine whether HVM superinfection might induce synthesis of EBV-specific antigen, we examined PA3 cells for EBV antigen by immunofluorescence and found none before or after exposure to HVM. As expected, LM/DM and MOLT-4 cells, which lack the EBV genome, were negative for EBV-specific immunofluorescence before or following exposure to HVM.

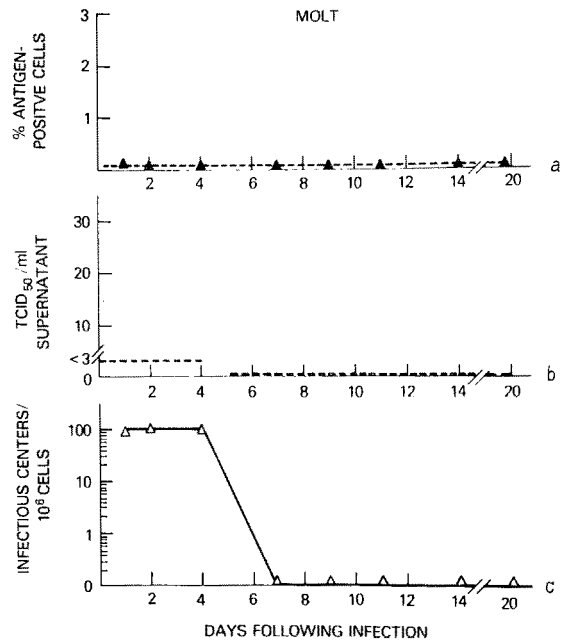

Fig. 3. Interaction of HVM with MOLT-4 Cells
a) Percentage of cells with HVM specific antigens. Standard error for each point is 0.01 per cent
b) Extracellular virus titration
c) Proportion of cells transferring infectious virus to WI-38 monolayer after co-cultivation

Discussion

Only a small fraction of cells in a given B-type LCL sustained permissive or abortive infection, and despite evidence that intracellular viral genome persisted for at least there weeks in these cultures, the fraction of antigen positive cells remained small and did not increase. This phenomenon has been noted in other *in vitro* sytems of primate herpesvirus infected lymphoid cells. Thus, in PHA activated leukocyte cultures exposed to HSV for ten days, virus-specific antigen was demonstrated in only 1 per cent of cells (24). The P3J LCL infected with HSV supported viral replication, while the EB3 LCL sustained abortive infection in which a persistent fraction of 0.2—3.0 per cent of cells contained HSV antigen for at least 20 days following exposure to the virus (10). Infection of EBV genome-containing LCLs with HSV did not induce the synthesis of intracellular EBV

antigens nor infectious EBV particles (10), consistent with our observations with HVM.

In a number of *in vitro* systems, including the HVM-LCL interaction, the fraction of cells producing virus or viral antigens may remain reproducibly small and persistent during long term culture. Thus, a maximum of 6 per cent of cells in thirty LCLs established from patients with infectious mononucleosis and containing EBV genome expressed EBV specific antigens (19, 23); the majority of these had less than 3 per cent antigen positive cells (5), and many had a persistent fraction of as little as 0.1 per cent with EBV specific immunofluorescence. Similarly, less than 1 cell in 10^6 in a LCL derived from an HVS infected owl monkey produced infectious virus; no herpes-like particles were seen by electron microscopy (9). Only 0.1—2.0 per cent of cells in a LCL established by *in vitro* infection of lymphocytes with HVS formed infectious centers when cocultivated with permissive cells, and no more than 2 per cent of cells produced intracellular HVS specific antigens (9, 28). Only 0.5 per cent of cells in the MLC-1 LCL derived from an HVS infected marmoset had electron microscopic evidence of herpesvirus infection, although 9 per cent of the LCL cells yielded HVS-like virus when cocultivated with permissive cells.

It may be that only a small fraction of LCL cells are susceptible to HVM infection at a given time. A smaller fraction of PA3 LCL cells appeared to permit replication of HVM at a given time during the first four days post infection, than produced viral antigen. Since permissive herpesvirus infections are lytic (31), the few cells permitting viral replication may have died out after a single replicative cycle (apparently 2—4 days) without significantly altering the growth rate of the whole culture. Replication of HSV in (PHA stimulated) lymphocytes is dependent on the multiplicity of infection (24). By analogy, since only a small proportion of PA3 cells appear to be susceptible to permissive HVM infection at a given time, the low effective secondary multiplicity of infection may have been insufficient to permit persistence of lytic infection.

In the case of LM/DM, in addition to a small fraction of cells sustaining abortive infection with HVM, there is a transient increase in the subpopulation of infected cells capable of transferring infectious virus by cocultivation with permissive cells. It is possible that this represents replication of virus which may be activated, then transmitted by cell-cell contact. This subpopulation of cells may represent a second form of abortive infection in which cocultivation may result in transfer of virus, but the infected LCL cells are at a selective disadvantage and eventually are outgrown by the uninfected cells.

We found no evidence of viral replication or abortive infection in MOLT-4 cells. T-LCLs generally do not contain EBV genome and are not capable of supporting EBV infection (17). Since LCLs derived from HVS-infected lymphocytes have T-cell characteristics (28), the significance of T-cell resistance to HVM infection is uncertain.

Our observations suggest that HVM interacts in distinct patterns with different LCLs, and that within a LCL, subpopulations of lymphoid cells are present at a given time which respond to HVM infection differently. The type of infection may be dependent not only upon characteristics of the host cell, but also upon the particular metabolic state, or phase of the cell cycle, within a given culture.

Recently, *Herpesvirus ateles* and *saimiri* have been demonstrated to be capable of inducing neoplasms of lymphoid cells in New World primates (20, 21) and EBV has been associated with lymphoid neoplasia in man (7). The implications of demonstrated HVM infection of LCLs of human and rhesus derivation for human disease remain to be fully assessed. Interactions of herpesviruses and LCLs, however, may provide a useful model for the further elucidation of the mechanisms regulating the establishment and maintenance of herpesvirus latency in lymphoid cells.

Acknowledgments

The authors are grateful to Drs. Francis Ennis and Paul Parkman for helpful discussions and critical reading of the manuscript.

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Received May 30, 1975