NOTES

Infection and Persistence of Rhesus Monkey Rhadinovirus in Immortalized B-Cell Lines

John P. Bilello, Sabine M. Lang, Fred Wang, Jon C. Aster, and Ronald C. Desrosiers

New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts 01772, and Channing Laboratory and Pathology Department, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Received 7 July 2005/Accepted 18 January 2006

Rhesus monkey rhadinovirus (RRV) is a gamma-2 herpesvirus (a rhadinovirus) that is a close relative of human herpesvirus 8 (HHV-8; the Kaposis sarcoma-associated herpesvirus) (1, 6, 20). The vast majority of the open reading frames present in HHV-8 have at least one corresponding homolog in RRV, and there is a close sequence similarity in corresponding genes. Unlike HHV-8, RRV can be grown lytically and to high titers in permissive monolayer cells (6). The CD20+ B lymphocyte is the primary cell type harboring RRV in the peripheral blood of persistently infected rhesus monkeys (2, 13). This situation parallels HHV-8 in humans, where the B lymphocyte has also been found to be the principal cell type that harbors the virus during persistent infection (11, 14, 17). Here we describe a rhesus monkey B-cell culture system for the study of RRV persistence and latency.

Growth of RRV-infected B cells. The gamma-2 herpesviruses not only persist in lymphoid cells but often immortalize or otherwise alter the growth properties of these lymphoid target cells (4, 9, 21–23). To examine if RRV infection alters the growth of rhesus monkey B cells, we selected three rhesus monkey B-cell lines, 211-98, 260-98, and 309-98, for RRV infection. These three rhesus monkey B-cell lines have been described previously (18); they were established by immortalization with the Epstein-Barr virus (EBV)-related virus of rhesus monkeys (rEBV; also called rhesus lymphoepithelial virus [LCV]) (15, 18). The three monkeys used as the sources of cells for these immortalizations were rEBV negative. RRV strain 26-95 was harvested from the supernatant of infected rhesus fibroblasts at the time of complete cell lysis to use for B-cell infections. One million cells from each B-cell line were incubated for 1 h with RRV 26-95 in a volume of 0.5 ml, and control cells were incubated in 0.5 ml of Dulbecco modified Eagle medium (DMEM) in parallel. Cells were diluted with DMEM containing 10% or 2% fetal bovine serum (FBS) to 0.1 × 10^6 cells per ml, and live-cell counts were determined by trypan blue exclusion for each culture up to day 15 postinfection (Fig. 1, top). On day 7, cultures supplemented with 10% FBS were arbitrarily diluted 1:6 and those supplemented with 2% FBS were arbitrarily diluted 1:3. No differences in growth properties were detected in the RRV-infected versus the uninfected B-cell lines maintained in the presence of 10% FBS. At the 2% serum concentration, the RRV-infected 260-98 cell line displayed a slightly reduced growth rate compared to that
of its uninfected counterpart (Fig. 1B, top), but this effect was marginal for 211-98 and 309-98 (Fig. 1A and C, top). All cell lines were maintained for an additional 8 weeks post RRV infection and then analyzed again for their growth properties. Cultures were diluted with DMEM containing 10% or 2% FBS to 0.1 × 10^6 cells per ml, and live-cell counts were determined for each culture up to day 15 postinfection. On day 7 (arrows), cultures were arbitrarily diluted 1:6 for cultures supplemented with 10% FBS and 1:3 for cultures supplemented with 2% FBS. (Bottom) Following 4 weeks of culture postinfection, the same cell lines were diluted again to a density of 0.1 × 10^6 cells per ml of medium supplemented with 10% or 2% FBS and growth properties were analyzed as outlined above.

Although RRV has been associated with lymphomas in the setting of simian immunodeficiency virus-induced immunodeficiency at one primate center (24), we were not able to detect any alterations in the growth potential of the B cells in our present study, even at reduced serum concentrations. It is, of course, possible that any growth-altering properties of RRV were overshadowed by the potent rhEBV cell growth transformation.

RRV production from persistently infected rhesus B-cell lines. Following RRV infection, B-cell lines 211-98, 260-98, and 309-98, as well as the uninfected parental cell lines, were continuously maintained in culture for 4 months. At this time point, clarified supernatants from RRV-infected B-cell lines were analyzed for RRV production and supernatants from parental cell cultures were used in parallel as negative controls. As additional controls, RRV titers in supernatant from lytically infected rhesus fibroblast cells and uninfected parental cells were determined in parallel. Fifty percent tissue culture infectious dose (TCID_{50}) endpoint titers were determined in 48-well plates in duplicate by serial 10-fold dilution on a rhesus fibroblast line until complete cell lysis by 2 weeks (Table 1). All RRV-infected B-cell lines produced infectious RRV at titers of 10^3 to 10^4 per ml, indicating that the rhesus B cells were persistently infected with RRV. These titers were lower by a

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TCID_{50}/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>211-98</td>
<td>Negative</td>
</tr>
<tr>
<td>211-98 with RRV</td>
<td>10^4</td>
</tr>
<tr>
<td>260-98</td>
<td>10^4</td>
</tr>
<tr>
<td>260-98 with RRV</td>
<td>Negative</td>
</tr>
<tr>
<td>309-98</td>
<td>10^3</td>
</tr>
<tr>
<td>309-98 with RRV</td>
<td>10^3</td>
</tr>
<tr>
<td>Rf 388-93</td>
<td>10^7</td>
</tr>
<tr>
<td>Rf 388-93 with RRV</td>
<td>10^7</td>
</tr>
</tbody>
</table>

Supernatants from the indicated cell lines, uninfected and RRV infected, were analyzed for the concentration of infectious RRV. Rf 388-93 is a rhesus monkey fibroblast cell line. TCID_{50} endpoint titers were determined in duplicate by serial 10-fold dilution on rhesus fibroblast line Rf 388-93 following complete cell lysis after 2 weeks.
factor of 1,000 to 10,000 than titers obtained from infected fibroblast cultures (typically, $10^7$ TCID$_{50}$/ml). Infectious RRV was detected in supernatant taken at earlier and later times as well. No overt cytopathic effects were observed in the RRV-infected B-cell lines. These data demonstrate the persistence of RRV in the B-cell lines and the continuous production of low levels of infectious RRV.

To further analyze the status of RRV persistence, we compared viral protein expression in persistently infected B cells with that in lytically infected fibroblasts. To generate cell lysates of the RRV-infected B cells and the parental cell lines, 10 million cells were lysed in 1 ml of RIPA buffer (phosphate-buffered saline containing 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) and clarified by centrifugation (13,000 rpm for 15 min at 4°C). In addition, lysates were prepared from rhesus fibroblast cells at 81 h post RRV infection and from uninfected control fibroblasts. Protein concentrations were measured by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). For the B-cell lines, 20–$\mu$g samples of whole-cell lysates were analyzed by Western blotting and compared to 10–$\mu$g of fibroblast-derived lysates. Viral proteins were detected with a polyclonal rabbit serum raised against purified Triton X-100-denatured RRV particles (Fig. 2A). While RRV-infected fibroblast cells displayed a complex pattern of RRV polypeptide expression, only one or two of the bands were detected in the RRV-infected B cells. These polypeptides exhibited mobilities corresponding to approximately 13 and 10 kDa and were specific for RRV since they were absent in the parental B-cell lines and in uninfected fibroblasts. Two bands with the same mobility were detected in column-purified RRV virion preparations with the rabbit anti-RRV serum but not with normal rabbit control serum (Fig. 2B, right and left sides respectively). Mass spectrometry was performed on the excised material of 13 kDa (p13), and a total of nine peptides were identified. Eight peptides matched the gene product of open reading frame 52 (Orf52), and one peptide matched Orf47 of RRV. Orf52 of RRV is a homologue of BLRF2 of EBV, which constitutes one component of the highly immunogenic viral capsid antigen complex (8, 10). All sera from RRV-infected rhesus monkeys that were tested showed good reactivity to p13 by Western blotting (data not shown).

**RRV genomes in rhesus B cells.** We showed previously that in situ hybridization with pooled rhEBV cosmid probes can reliably identify cells that are lytically or latently infected with rhEBV (12, 18). An analogous approach was used here to detect cells infected with RRV. To first address the question of what percentage of the cells were infected with RRV, fluorescence in situ hybridization (FISH) was performed with cosmid clones spanning the RRV genome. The RRV cosm id library was established by subcloning genomic RRV DNA into mod-
TABLE 2. Status of RRV infection in rhEBV-immortalized rhesus B cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Latent RRV, percent</th>
<th>Productive RRV, percent</th>
<th>Latent rhEBV, percent</th>
<th>Productive rhEBV, percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>211-98 with RRV</td>
<td>40.4</td>
<td>0.2</td>
<td>59.2</td>
<td>0.2</td>
</tr>
<tr>
<td>260-98 with RRV</td>
<td>4.8</td>
<td>0.6</td>
<td>94.6</td>
<td>0</td>
</tr>
<tr>
<td>309-98 with RRV</td>
<td>26.6</td>
<td>1.2</td>
<td>71.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* A total of 500 cells of each cell line were evaluated by two-color FISH for RRV and rhEBV latent persistence or productive replication. Only cells that were positive for rhEBV were scored for RRV. Values represent percent cells with the indicated staining pattern. A small percentage of cells (less than 10%) did not show a signal for rhEBV; it is likely that these represent false negatives. None of the cells were rhEBV negative and RRV positive.

...
previously shown that lytic replication of rhEBV in epithelial cells is associated with a pattern of intense diffuse nuclear staining that is identical to the pattern observed in the small subset of rhEBV- and RRV-infected B cells that we describe here. Thus, a punctate staining pattern has been shown in previous publications to correspond to individual viral genomes in latent infection, the copy number of latent viral genomes can be variable, and the intense diffuse nuclear staining pattern is associated with productive replicative infection. Our analysis does not, however, specify the nature or level of repressed expression and latent infection with RRV in these cell lines.

**Time course and dose dependence of B-cell infection.** LCL211-98 cells were infected at increasing multiplicities of infection (MOIs) with a replication-competent, recombinant RRV expressing green fluorescent protein (GFP), RRV-GFP. RRV-GFP was generated and its titer was determined in rhesus monkey fibroblasts as described by Bilello et al. (3). At day 4 postinfection, triplicate cultures of LCL211-98 cells, either uninfected or infected with increasing numbers of PFU of RRV-GFP per cell, were analyzed by fluorescence-activated cell sorting (FACS) to determine the percentage of cells that were GFP positive at each MOI. As displayed in Fig. 5A, the percentage of GFP-positive cells was MOI dependent, ranging from approximately 0.2% to 47% GFP positive at day 4 postinfection, with MOIs of 0.009 and 0.6 PFU/cell, respectively. To determine if RRV was actively replicating and spreading within the cultures, LCL211-98 cultures were infected at a low MOI with 0.037 PFU of RRV-GFP per cell. Each day postinfection, cultures were examined for GFP positivity by FACS analysis until day 17. The percentage of GFP-positive cells increased steadily over time after infection with RRV-GFP to a maximum of approximately 47% at day 17 postinfection, when the experiment was concluded. Decreases in cell number (flow cytometry) and viability (trypan blue exclusion) were noted after 14 days postinfection (data not shown). Thus, RRV-GFP was able to replicate and spread slowly through the B-cell culture. The maximal number of GFP-positive cells (47% in Fig. 5) was similar to the number of RRV-positive cells obtained by FISH in separate experiments (41% in Table 2).

Bilello et al. (3) have recently described a neutralization assay in which sera from RRV-positive monkeys, but not RRV-negative monkeys, were able to block infection of rhesus fibroblasts by RRV-GFP. To examine the capacity of sera from rhesus monkeys naturally infected with RRV to neutralize infection of B cells by RRV-GFP, RRV-GFP (0.3 PFU/cell) was incubated with either medium alone or heat-inactivated sera from RRV-negative and RRV-positive rhesus monkeys at a dilution of 1:20 or 1:100. Following this 3-h incubation, LCL211-98 cells were inoculated with the virus-serum mixture. At day 4 postinfection, the percentage of GFP-positive cells was determined by FACS analysis. Sera from RRV-negative monkeys 288-94 and 232-03 did not neutralize RRV-GFP infection of LCL211-98 cells (Fig. 6). However, sera from rhesus monkeys 541-03, 140-83, and 488-03, which were naturally infected with RRV, neutralized RRV-GFP infection by 99%, 77%, and 63%, respectively, at a 1:20 dilution (Fig. 6). Sera from naturally infected monkeys 288-03 and 526-91 did not show neutralizing activity in this B-cell assay. The sera from these two monkeys also had the weakest neutralizing activity for RRV infection of rhesus fibroblasts (3). In fact, there was a strong correlation of the rank order of neutralizing activity on the two cell types. Of the five RRV-positive sera compared, 541-03 had the highest neutralizing activity on both cell types.

In summary, we have described here a rhesus monkey B-cell culture system that will allow the study of RRV’s ability to infect and persist in rhesus monkey B cells. Persistence of RRV and Kaposi sarcoma-associated herpesvirus in human...
B-cell lines has recently been described (5, 7). One line of investigation that will be made possible by the availability of this system is the extent to which different RRV glycoproteins and different cellular receptors may be used for infection of B cells versus fully permissive fibroblasts. It will now also be possible to investigate the ordered regulation of gene expression in the context of rhesus monkey B cells.

This work was supported by Public Health Service grants 1P01DE14388, 1RO1AI63928, RR00168, and ST32AI0724522.

We thank Charles Lee for assistance with the FISH studies.

REFERENCES


