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Inability of Simian Virus 40 To Establish Productive Infection of Lymphoblastic Cell Lines

Sophie Shaikh, Christine Skoczylas, Richard Longnecker, and Kathleen Rundell*

Department of Microbiology-Immunology, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, Illinois 60611

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Lymphoblastic cell lines were infected with simian virus 40 (SV40) and then monitored for evidence of a productive infection. No evidence of early gene expression was found 2 days following infection, as determined by assaying viral mRNAs and early antigens. Furthermore, only small amounts of virus could be detected by plaque assay 2 days after infection, and levels slowly declined until they were undetectable after a few weeks in culture. Thus, human lymphocytes are not readily infectible with SV40 and do not provide a simple model for studying interactions of SV40 with a human cell type.

Recent reports on the association between the DNA tumor virus simian virus 40 (SV40) and human tumors have been quite provocative and have stimulated much discussion about the transmission and prevalence of this virus in the human population. Tumor-associated SV40-like sequences were first described for rare childhood brain tumors (1, 14) and shortly thereafter for mesothelioma (4). Since these early studies, SV40 has been reported in various human malignancies, as reviewed recently (9, 10, 19).

With one exception, it has been difficult to clearly define the host-virus relationships between SV40 and the cells that gave rise to these various tumors, in part because the cell types involved are so highly differentiated. The exception has been some very interesting studies done with primary mesothelial cells in culture (2). These studies have shown that SV40 DNA is maintained episomally in human mesothelial cells, without evidence of ongoing cytopathic effects (CPE), thus defining a novel host cell interaction not previously reported for SV40. Furthermore, mesothelial cells can be transformed in vitro when both large-T (LT) and small-t (ST) antigens are present in the absence of other transforming agents (2, 23).

Most recently, SV40 sequences were found in a sizeable frequency of DNAs from patients with non-Hodgkin's lymphoma (20, 21). Shortly thereafter, it was reported that SV40 could be found in some lymphoblastoid cell lines (LCLs) and that it could infect and persist in previously SV40-negative LCLs (8). This report had a firm historical basis because SV40 had been known to cause B-cell lymphomas in experimental animals for some time (5, 7) and several laboratories had reported the detection of SV40 in human blood samples (6, 15–17, 22).

Although the description of infection of lympoid cells in vitro was novel, few details of the infectious process were investigated, and it was simply stated that SV40 could be detected by cocultivation with permissive monkey kidney cells for as long as 6 months after infection in vitro. This report raised the possibility that it might be possible to study SV40-lymphocyte interactions in cell culture. To explore this possibility, we first asked whether we could detect SV40 DNA in LCLs currently in use for ongoing studies of Epstein-Barr virus (EBV). These lines are LCL1, LCL2, LCL3, and WT65. We also included in these studies two Burkitt's lymphoma cell lines, Daudi (EBV+) and BJAB (EBV−). Cellular DNA was prepared from each of these immortalized cell lines by using a Qiagen DNeasy kit and tested by PCR analysis for SV40 sequences. PCR analyses were modeled on standard protocols used by others to detect SV40 in mesothelioma and brain tumor DNAs. Three DNA primer sets (RA1/RA2, RA3/RA4, and SV.for3/SV.rev) were defined in other studies (12, 13) and were used in these studies. The RA primers recognize the SV40 regulatory region, while the SV set amplifies highly conserved sequences of the LT antigen. Analyses were performed using 45 PCR cycles with the annealing and extension temperatures outlined in earlier studies (12, 13).

Although the data are not shown here, all six of the cell lines were negative for SV40 DNA as detected by ethidium bromide staining. The DNA quality appeared to be good, and all six cell lines gave successful amplifications of a low-copy-number human gene coding for amyloid precursor protein (11). In reconstruction experiments, we were able to detect 10^{-8} μg of SV40 viral DNA when it was used to "spike" reaction mixtures that contained 1 μg of cellular DNA. This amount of SV40 DNA represents about 2,000 molecules based on Avagadro's number and a molecular weight for viral DNA of 3.6×10^6. We recovered 1 μg of cellular DNA from 2×10^5 cells; thus, we were able to detect 2,000 molecules of SV40 in DNA from 2×10^5 cells. In other words, we should have been able to detect SV40 in the LCLs if only 1 cell in 1,000 retained viral sequences.

Because the LCLs did not contain detectable SV40 DNA, we used them in infections with wild-type (WT) SV40 or an ST-negative virus, dl-888 (3). Cells were collected and then resuspended in media at 10^6 per ml, and then 10^7 PFU of SV40 in 0.2 ml was added for 1 h at 37. Cells were then collected by centrifugation, washed once with phosphate-buffered saline (PBS), resuspended at 2×10^5 cells per ml, and returned to the

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-5917. Fax: (312) 503-1339. E-mail: krundell@northwestern.edu.

TABLE 1. Virus recovery from infected LCLs

Cells	Virus recovered at day 2 (PFU)[a]
LCLs	
LCL1	1.3×10^4
LCL2	7.0×10^4
LCL3	0.9×10^4
WT65	2.3×10^4
Burkitt's lines	
Daudi (EBV+)	1.3×10^4
BJAB (EBV−)	0.3×10^4

[a] Titers were determined using frozen and thawed cell lysates, and the values reported reflect the total amount of virus recovered after an initial infection of 10^6 cells with 10^7 PFU of WT SV40. To search for virus in cell lysates from P4, 0.1-ml portions of the undiluted lysate were used, representing 10 to 20% of the total lysate available. No virus was recovered from P4. Results from a representative experiment are shown.

incubator to continue growing. Portions of these infected cells were maintained in culture over a period of several weeks, maintaining cell density between 10^5 and 10^6 cells per ml. In general, this involved a weekly 1:10 subculture. Each subculture was considered a passage, and a passage number (e.g., P1 for passage 1) was assigned.

At 48 h postinfection, each flask contained about 2×10^6 cells. New flasks were seeded for the P1 culture, and then the remaining cells were collected by centrifugation to separate the media from the cells. The media were saved for later plaque assays, and the cells were resuspended in 1.5 ml of PBS. A portion (0.5 ml) of the cell suspension was frozen and thawed three times to prepare lysates for titering. Cells in the remaining 1.0-ml portion were sonicated in protein lysis buffer (18) for Western blotting for viral early proteins.

Plaque assays performed on frozen and thawed cells showed that extremely low levels of virus were detected (Table 1). These levels were so low that residual virus remaining after the initial infection period and washing of the cells could account for the presence of virus. For example, when 10^7 PFU of virus was used to initiate the infection, <1% of this amount of virus could be recovered 2 days later in any of the cell lines used. It is also worth noting that it is not likely that the presence of EBV in cells does not limit SV40 replication, because the

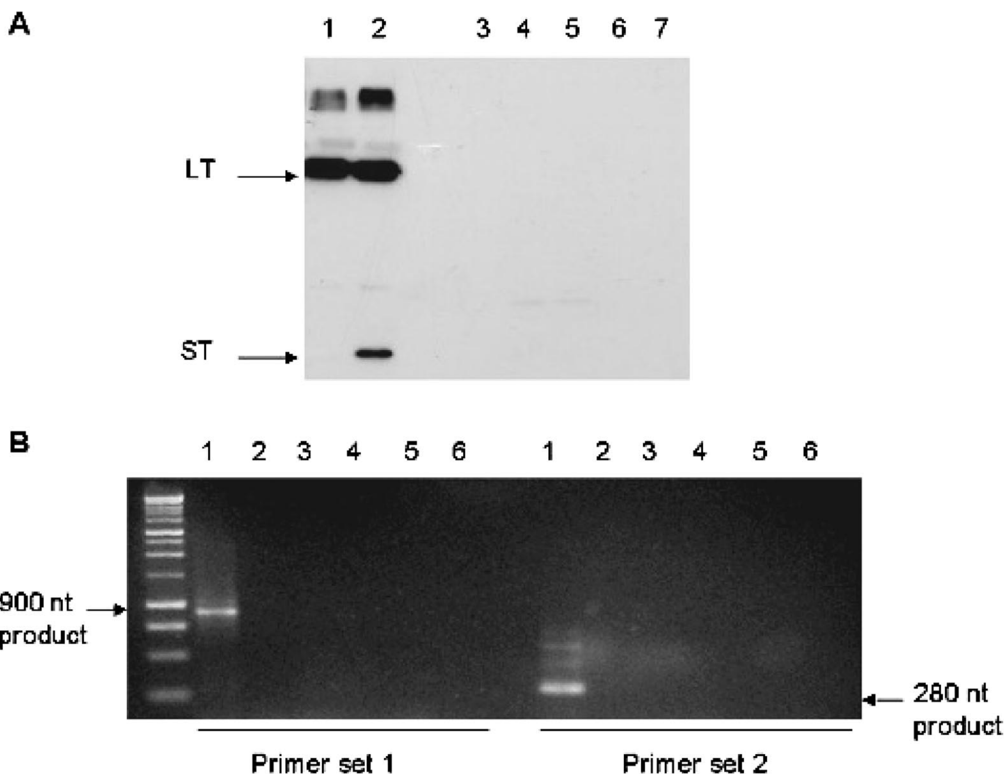

FIG. 1. Protein and mRNA products of SV40-infected cells. (A) LCLs were infected with WT SV40, and then extracts were prepared at 48 h postinfection. Twenty micrograms of each extract was analyzed on a sodium dodecyl sulfate–10% polyacrylamide gel, transferred to an Immobilon membrane, and then probed with monoclonal antibody Pab419 and the ECL enhanced chemiluminescence detection system with a secondary antimouse antibody. As a positive control, 4 μg of SV40-infected CV1 cell extracts was used. The lanes shown are extracts from CV1 cells infected with dl-888 (lane 1), CV1 cells infected with WT SV40 (lane 2), dl-888-infected LCL1 (lane 3), WT-infected LCL1 (lane 4), WT-infected LCL2 (lane 5), WT-infected LCL3 (lane 6), and WT-infected WT65 (lane 7). (B) Total RNA extracted from infected LCLs was analyzed with slight modifications of the Promega Access RT-PCR system. (55°C annealing temperature, 72°C extension, 45 cycles). Two primer sets were used (set 1, SV40 nt 5198 to 5178 and 3971 to 3950; set 2, nt 5198 to 5178 and 4541 to 4568). The expected products derived from splice LT mRNAs were 900 and 280 nt, respectively, and were detected from RNA prepared from human fibroblasts transfected with pw2t, a plasmid that contains the SV40 early region (lane 1). Other lanes show amplifications with RNA prepared from WT-infected LCL1 (lane 2), dl-888-infected LCL1 (lane 3), uninfected LCL1 (lane 4), WT-infected LCL3 (lane 5), and WT-infected WT65 (lane 6). No DNA contamination was detected even when RNAs were not treated with RQ1 DNase (lane 6). Also, 1,200- or 630-nt products that would be amplified from genomic SV40 DNA were not observed in any lanes.

EBV− cell line BJAB also shows only low levels of SV40 after 2 days of infection. In contrast to the behavior of LCL and Burkitt's cell lines, increased amounts of SV40 were recovered from infected monkey kidney (CV1) cells at 48 h postinfection (data not shown).

Amounts of recovered virus declined progressively as infected cells were subcultured postinfection. Plaque assays were performed on lysates made at weekly intervals after the initial infection. As shown in Table 1, by P4 no plaques were detected at all in any of the LCL or the EBV+ and EBV− Burkitt's cell lines.

When P3 or P4 LCL cells were added to permissive CV1 cells, CPE was occasionally observed after 1 to 2 weeks. This suggests that rare virus particles are present for several weeks after the initial infection, but because only a single infectious particle can result in cytopathicity in CV1 cells, the amounts of virus present may be too low to detect by plaque assays in which only an aliquot of the total culture is tested at one time. While this agrees with the earlier report, which stated that cocultivation showed the presence of SV40 in infected LCL cultures, no CPE was observed by P7 or P8. Thus, these findings do not support the claim that virus could be detected for prolonged periods after infection of LCLs, and the amounts of virus found in plaque assays are so low that it is difficult to rule out the persistence of the residual inoculum.

LCLs become immortalized because of the presence of EBV. Although it is not known how EBV would influence expression of SV40 in lymphocytes, all experiments were performed with the EBV− cell line BJAB, and the results were identical. That is, extremely low levels of virus were detected by plaque assay after a few weeks in culture, and no virus remained by P8.

When SV40 infects nonpermissive cells such as hamster or mouse cells, late proteins are not expressed in these cells and packaged virus is not found. In these infections, however, viral LT or ST antigens can be detected in lysates prepared even a day after infection. In contrast, neither LT nor ST was ever detected in infected LCLs either 48 h postinfection (Fig. 1A) or at subsequent passages (not shown). To look for very low levels of virus gene expression in these cells, RNA was prepared from infected cells and then analyzed by reverse transcription-PCR (RT-PCR) using the Promega Access RT-PCR system. As a positive control, small amounts of RNA from cells transfected with plasmids that contained the SV40 early region were used. The primers used for these analyses came from the two different exons of LT, so that processed mRNAs could easily be detected from the much larger genomic products.

Primer set 1 yields a 1,248-nucleotide (nt) product from either genomic DNA or unprocessed mRNA and a 1,186-nt product from an ST message. The intron removed from an LT mRNA is 350 nt, and amplification of this message yields a 900-nt product. The 900-nt product was readily detected from positive control RNA templates, as shown in Fig. 1B. Similarly, primer set 2 yielded a product of 280 nt. No products were ever detected when RNA prepared from infected LCLs was used as a template in these reactions.

Taken together, it seems highly unlikely than any productive infection, even very-low-level infection, is occurring in LCLs. The amounts of virus detected by cocultivation several weeks after infection are so low that it is impossible to rule out residual inoculum. More importantly, no virus can be detected by plaque assay, and we could find no evidence of processed viral messages—a readout that would provide the best evidence of a meaningful infection. We undertook these experiments in the hope that infections of lymphocytes would provide a model for further understanding infection of defined human subtypes by SV40, especially cells that might be significant in human malignancy. Unfortunately, it does not appear that a potential interaction with SV40 and human lymphocytes can easily be modeled in cell culture.

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