BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF A CLONED LEU-3$^-$ CELL SURVIVING INFECTION WITH THE ACQUIRED IMMUNE DEFICIENCY SYNDROME RETROVIRUS

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A unique human retrovirus (RV)[1] has been consistently isolated from patients with the acquired immune deficiency syndrome (AIDS). Infection of human T lymphocytes or T lymphocyte lines with the AIDS RV can result in a variety of outcomes ranging from rapid cell death to the integration of functionally inert proviral DNAs (1–3). A typical AIDS RV infection is characterized by the appearance of multinucleated cells, a burst of reverse transcriptase (RT) activity, and profound cellular degeneration that extends over a 5–20-d period (1, 4). Two recent reports have described noncytotoxic effects of the AIDS RV on human lymphocytes. In one case (2), PHA-stimulated, IL-2-dependent cultures of helper-inducer human T cells exhibited the characteristic cytopathic effects of acute viral infection, but nonetheless, sufficient numbers of cells survived to continue to produce infectious virus during the 4 mo they were maintained in culture. In the other (3), we described Leu-3$^-$ non–virus-producing cells, derived from a Leu-3$^+$ T cell line, that survived infection with the AIDS RV, and which could be induced with 5-iodo-2′-deoxyuridine (IUdR) to express infectious virus (3).

In this report we have examined in greater detail IUdR induction of lymphocytes surviving acute viral infection. A cellular clone was isolated from a mass culture of survivor cells that contained a single copy of the AIDS RV provirus. The integrated proviral DNA was constitutively expressed but generated defective virus particles that failed to synthesize detectable reverse transcriptase. The

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[1] *Abbreviations used in this paper:* AIDS, acquired immune deficiency syndrome; CAT, chloramphenicol acetyl transferase; IUdR, 5-iodo-2′-deoxyuridine; LAV, lymphadenopathy-associated virus; LTR, long terminal repeat; PLPG, periodate/lysine/paraformaldehyde/glutaraldehyde; RT, reverse transcriptase; RV, retrovirus.

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characterization of the survivor cell clone is presented, and its potential role in IUdR-mediated induction is discussed.

Materials and Methods

Viral Infection, IUdR Induction, and Cell Cloning. ~2 × 10^6 A3.01 cells were infected with a 10^3 dilution of a lymphadenopathy-associated virus (LAV) stock as previously described (4). After adsorption for 1 h at 37°C, cells were washed and maintained (10^6 cells/ml) in RPMI 1640 medium supplemented with 10% FCS. When the RT activity of the Leu-3^- cultures that had survived infection became undetectable (7–10 d after the peak of RT), cells (10^6 cells/ml) were exposed to IUdR (100 μg/ml) for 24 h, and then cocultivated with Leu-3^+ A3.01 cells, as previously described (3). The induction of virus was heralded by a second wave of RT activity in the cocultured cells; when RT was no longer detectable, the survivor cells were subjected to a second cycle of IUdR induction and cocultivation. The RT^-, Leu-3^- cells surviving these treatments were cloned by limiting dilution in 96-well microtiter plates. 111 single cell clones were obtained, pooled into groups of 10, expanded to a total of 10^6 cells, exposed to IUdR for 24 h, cocultivated with Leu-3^+ A3.01 cells, and examined daily for expression of the AIDS RV by monitoring syncytia formation. A single clone (8E5) was obtained.

Nucleic Acid Hybridization. DNA and RNA were prepared from AIDS RV–infected cells or 8E5 cells, and analyzed for the presence of virus-related sequences by Southern or Northern blot hybridization using cloned long terminal repeat (LTR) or representative *gag-pol-env* probes as previously outlined (4–6). For in situ hybridization, cultured cells were sedimented onto polylysine-coated glass slides, fixed in periodate/lysine/paraformaldehyde/glutaraldehyde (PLPG), and pretreated with HCl and proteinase K to allow the labeled probe to enter the cells (7–9). Cells were prehybridized in 10 mM Tris pH 7.4, 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 50% formamide, 1× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA), and 200 μg/ml yeast tRNA at 45°C for 2 h and hybridized in this solution containing 10% dextran sulfate, 5 μM DTT and 10^6 cpm of ^{35}S-labeled AIDS RV RNA in 10-μl reaction mixtures. Subgenomic viral DNA fragments present in pB1 (6), pBenn6 (4), pB11 (6), and a recombinant plasmid (pRG-B), which contains a 1.35 kb Hind III fragment mapping between 8.25 and 9.6 kb on the proviral DNA, were subcloned into SP6/T7 vectors (Promega Biotec, Madison, WI) and the pooled DNAs were transcribed using ^{35}S-UTP (Amersham Corp., Arlington Heights, IL). The labeled RNAs were incubated with 40 μM $NaHCO_3$/60 μM Na_2CO_3, pH 10.2, before hybridization to facilitate their entry into cells.

Hybridization was performed at 45°C for 8 h. The samples were then washed twice in: 2× SSC at 22°C for 10 min; 2× SSC, 0.1% Triton X-100 at 60°C for 30 min; 2× SSC with RNase A (40 μg/ml) and RNase T_1 (10 U/ml) at 37°C for 30 min; and 2× SSC at 60°C for 10 min. All solutions, excluding those with RNase, contained 5 μM DTT and 1 μM EDTA. Autoradiography was performed for 1–2 d as previously described (8, 9).

To control for the specificity of in situ hybridization, probes synthesized in the sense orientation (same polarity as viral mRNA) were incubated with duplicate cell preparations; in addition, uninfected cells were hybridized with antisense probes (i.e., complementary to viral mRNA). Infected cell preparations were also treated with RNase before addition of the probe.

Immunoblotting. Total cell lysates prepared from AIDS RV–infected A3.01 or 8E5 cells (10^8) were electrophoresed through 3–27% gradient polyacrylamide gels (Integration Separation Sciences, Newton, MA) by the method of Laemmli (10) and transferred to nitrocellulose membranes (11). The filters were then treated with 5% nonfat dry milk in 10 mM Tris (pH 7.4) and 155 mM NaCl (TN) for 30 min, and further rinsed in TN buffer containing 0.3% Tween-20, 0.5% NP-40 (TN-TN), and 1% BSA. Pooled AIDS patients' sera, diluted (1:1,000) in TN-TN containing 3% BSA, were incubated with the nitro-cellulose filters overnight at room temperature. The membranes were then washed three times in TN-TN buffer, incubated for 2 h with ^{125}I–protein A (200,000 dpm/ml), washed, air dried, and exposed to x-ray film.

Electron Microscopy. A3.01 cells infected with the AIDS RV, or 8E5 cells, were centrifuged at 650 *g* in standard Beem capsules. The pellets were then gently washed in serum-free RPMI 1640 medium and fixed in situ for 1–2 h in 2.5% glutaraldehyde in the presence of 0.1 M sodium cacodylate buffer, pH 7.2. After three 5-min washes in 0.1 M sodium cacodylate buffer containing 4% sucrose, the pellets were treated with 1% osmium tetroxide for 1–2 h. They were then washed three times in 0.1 M sodium cacodylate buffer and dehydrated in 30–100% ethanol, followed by a final dehydration using propylene oxide. The samples were then embedded in epon-Araldite LX-112 (Ladd Research Industries, Inc. Burlington, VT), and thin sections were prepared. After sequential staining with lead acetate and uranyl acetate, the preparations were examined in a 100B electron microscope at 80 kV (JEOL USA, Peabody, MA).

Results

We have previously described (4) a continuous Leu-3$^+$ human T cell line (A3.01) that is >95% susceptible to infection with the AIDS RV. After infection, a majority of the A3.01 cells is killed; Leu-3$^-$ survivor cells emerge that can be induced with IUdR to synthesize infectious virions (3). At least two discrete non–virus-producing cells survive infection with the AIDS RV: most cells are spontaneous Leu-3$^-$ revertants that arise despite repeated subcloning of the uninfected parental Leu-3$^+$ A3.01 cell line, and contain no proviral DNA (4); others (0.1–1% of the survivor cells) harbor inducible virus (3).

Isolation of a Survivor Cell Clone Containing AIDS RV DNA. Definitive biologic and biochemical evaluation of IUdR-mediated virus induction clearly requires the cloning of the few cells containing the AIDS RV provirus that survive acute infection. Although the assay system used to detect induced viral particles is quite sensitive (3), it is not readily applicable to a cell cloning project in which the desired cell occurs at a frequency of $<10^{-2}$. In a typical assay (3), survivor cells are treated with IUdR, and because they are Leu-3$^-$ and therefore not susceptible to infection with the AIDS RV, must be cocultured with Leu-3$^+$ A3.01 cells, which adsorb and efficiently replicate the virus. Induced virus can be monitored by assaying for RT activity 5–10 d after induction. In view of the logistic demands attending the screening of several hundred survivor cell clones for the few that harbor inducible virus, several modifications were incorporated into the assay and selection system.

A3.01 cells were infected with the LAV strain of the AIDS RV at a multiplicity of infection that resulted in a peak of RT activity on day 10. 7 d later, when RT activity was no longer detectable, $\sim 2 \times 10^6$ of the Leu-3$^-$ survivor cells were treated with IUdR and cocultured with susceptible A3.01 to induce the AIDS RV. The cells surviving the initial induction and coculture procedure were subjected to a second cycle of IUdR treatment and A3.01 cocultivation in an attempt to increase the proportion of inducible cells. Surviving cells, after this second induction and cocultivation procedure, became RT$^-$ on day 8 after IUdR treatment, and were then cloned by limiting dilution. 111 clones derived from the surviving mass culture were pooled in groups of 10, treated with IUdR, and cocultivated with A3.01 cells. Virus induction was monitored daily by screening the cocultures for syncytia formation. We elected to follow the appearance of syncytia by light microscopy rather than RT assays, since the former was logistically more suitable for the rapid identification of the rare cell harboring AIDS RV proviral DNA.

A single clone (8E5) was isolated that produced syncytia in cocultured Leu-3$^+$ A3.01 cells after exposure to IUdR. When 8E5 cells were expanded and characterized in greater detail, an unanticipated result was obtained; despite repeated treatments with IUdR, no RT activity could be detected in the cocultures of 8E5 and the Leu-3$^+$ A3.01 cells that were maintained for >30 d. This was puzzling in view of the readily demonstrable syncytia that appeared 24 h after IUdR treatment of the 8E5 cells.

Detection of Viral RNA, DNA, and Protein in 8E5 Cells. Since it was unclear which, if any, AIDS RV gene products were being expressed in induced 8E5 cells, we used in situ hybridization to monitor the IUdR effect. As shown in Fig. 1*A*, virtually all of the IUdR-treated 8E5 cells synthesized viral RNA. Of interest was the fact that 8E5 cells, growing in the absence of IUdR, also expressed high levels of AIDS RV RNA (Fig. 1*B*). The in situ hybridization results shown in Fig. 1 also indicated that cloned 8E5 cells contained amounts of viral mRNA comparable to present those in a cell infected with virus (Fig. 1*C*). To more fully evaluate the RNA present in the 8E5 clone, polyadenylated RNA was isolated and examined by Northern blot hybridization using an LTR probe as previously described (5). Fig. 2*B* shows that, similar to A3.01 cells, which actively produce virus, uninduced 8E5 cells also contain substantial amounts of 9.1 (genomic/*gag-pol*), 5.0, 4.3 (*env*), and 1.8–2.0 kb viral RNAs. Although this Northern analysis suggested that the expression of the 5.5-kb species of viral RNA might be reduced in 8E5 cells, S1 nuclease protection experiments (S. Venkatesan, data not shown) indicated that RNAs processed at the putative splice acceptor for 5.5 kb viral RNA were readily identified.

The number and state of proviral DNA copies of 8E5 cells were analyzed by Southern blot hybridization of Eco RI–digested cellular DNA. Since the parental LAV provirus contains two internal Eco RI restriction sites defining a 1.1 kb viral DNA segment, the presence of only three reactive cleavage products (Fig. 2*A*) is consistent with the clonality of the 8E5 line and the existence of a single integrated copy of viral DNA.

The results presented thus far clearly indicate that the one copy of the LAV provirus in 8E5 cells is constitutively expressed as viral RNA, but they provide no explanation for the absence of RT activity after IUdR treatment. As a first step to address this question, the viral proteins present in 8E5 cells were analyzed by immunoblotting using pooled AIDS sera, as described in Materials and Methods. A side-by-side comparison with the immunoreactive peptides present in virus-producing A3.01 cells (Fig. 2*C*) revealed the presence of virus-encoded gp120, p55, p41, and p25 proteins and the absence of the prominent 64 kD and fainter 34 kD viral polypeptides in 8E5 cells. The immunoblot of the 8E5 clone also suggested the presence of a reactive 50 kD band, perhaps representing a truncated form of the 64 kD polypeptide identified in productively infected cells. A similar immunoblot was obtained when a lysate, prepared from 8E5 cells treated with IUdR, was examined (data not shown).

Characterization of Virus Particles Synthesized in 8E5 Cells. Although the analysis of the cell lysates indicated that most of the major AIDS RV structural proteins were synthesized in 8E5 cells, we did not know whether they were assembled into virus particles. When 8E5 cells were examined with an electron

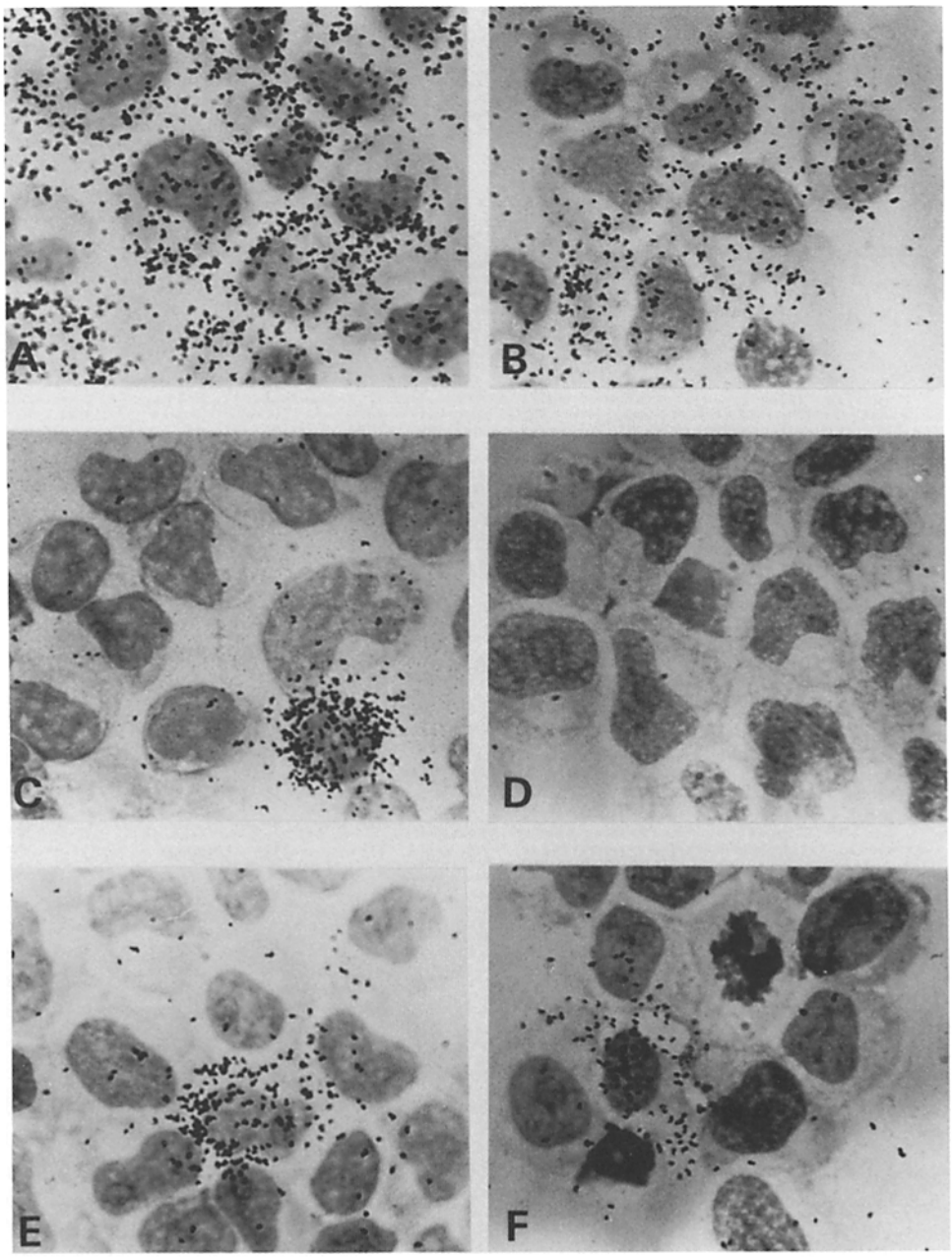

FIGURE 1. In situ hybridization of human T lymphocyte lines synthesizing AIDS RV RNA. Photomicrographs of: 8E5 cells after (*A*) and before (*B*) treatment with IUdR; A3.01 cells 3 d after infection (*C*) with the AIDS RV; uninfected A3.01 cells (*D*); IUdR-inducible cells before (*E*) or after (*F*) incubation with IUdR. Original magnification, × 400.

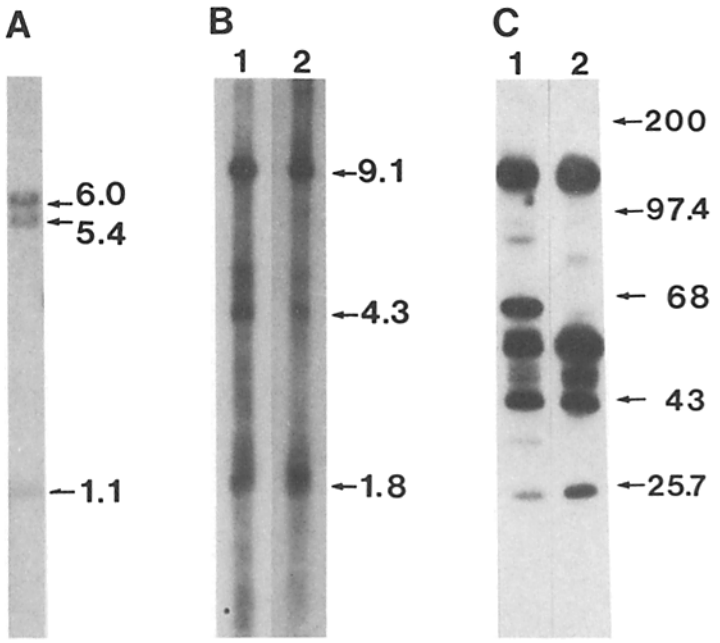

FIGURE 2. Characterization of AIDS RV DNA, RNA, and proteins in 8E5 cells (*A*). 5.0 μg of 8E5 DNA were digested with Eco RI and analyzed by Southern blot hybridization using the pBenn6 (4) probe. (*B*) Polyadenylated RNA was prepared from virus-infected A3.01 (lane *1*) or 8E5 (lane *2*) cells and subjected to Northern analysis using a viral LTR probe as previously described (5). (*C*) Immunoblots of infected A3.01 (lane *1*) or 8E5 (lane *2*) cell lysates carried out as described in Materials and Methods.

microscope, innumerable RV-like structures were seen budding from their surfaces (Fig. 3). These particles could be readily distinguished from infectious virions produced in A3.01 cells because they lacked the condensed rod-shaped nucleoid characteristic of the AIDS RV.

The tissue culture medium from untreated or IUdR-treated 8E5 cells was also examined for the presence of infectious virus particles. Supernatants, concentrated from 10^8 8E5 cells, were incubated with 2×10^6 Leu-3^+ A3.01 cells, as previously described (4), in an attempt to amplify any released viral particles. In repeated experiments, the synthesis of infectious progeny virions, as monitored by RT assays, was invariably negative. Nonetheless, A3.01 cells exposed to concentrated 8E5 tissue culture supernatants did exhibit minimal syncytia formation. However, the development of syncytia could not be shown after the serial (and blind) passage of the 8E5 supernatant through A3.01 cells.

In Situ Hybridization of IUdR-inducible Cells. Since the characterization of the cloned 8E5 survivor cell indicated the presence of a single copy of proviral DNA that directed the synthesis of defective virus particles, we wished to determine how and when this cell arose. Accordingly, we used in situ hybridization to evaluate the IUdR-inducible cells that survived the initial infection with LAV. As shown in Fig. 1*E*, cells constitutively expressing AIDS RV mRNA could be identified in the mass culture of IUdR-inducible cells existing at a frequency of ≥1/1,000 at least two IUdR-induction/cocultivation cycles before the isolation

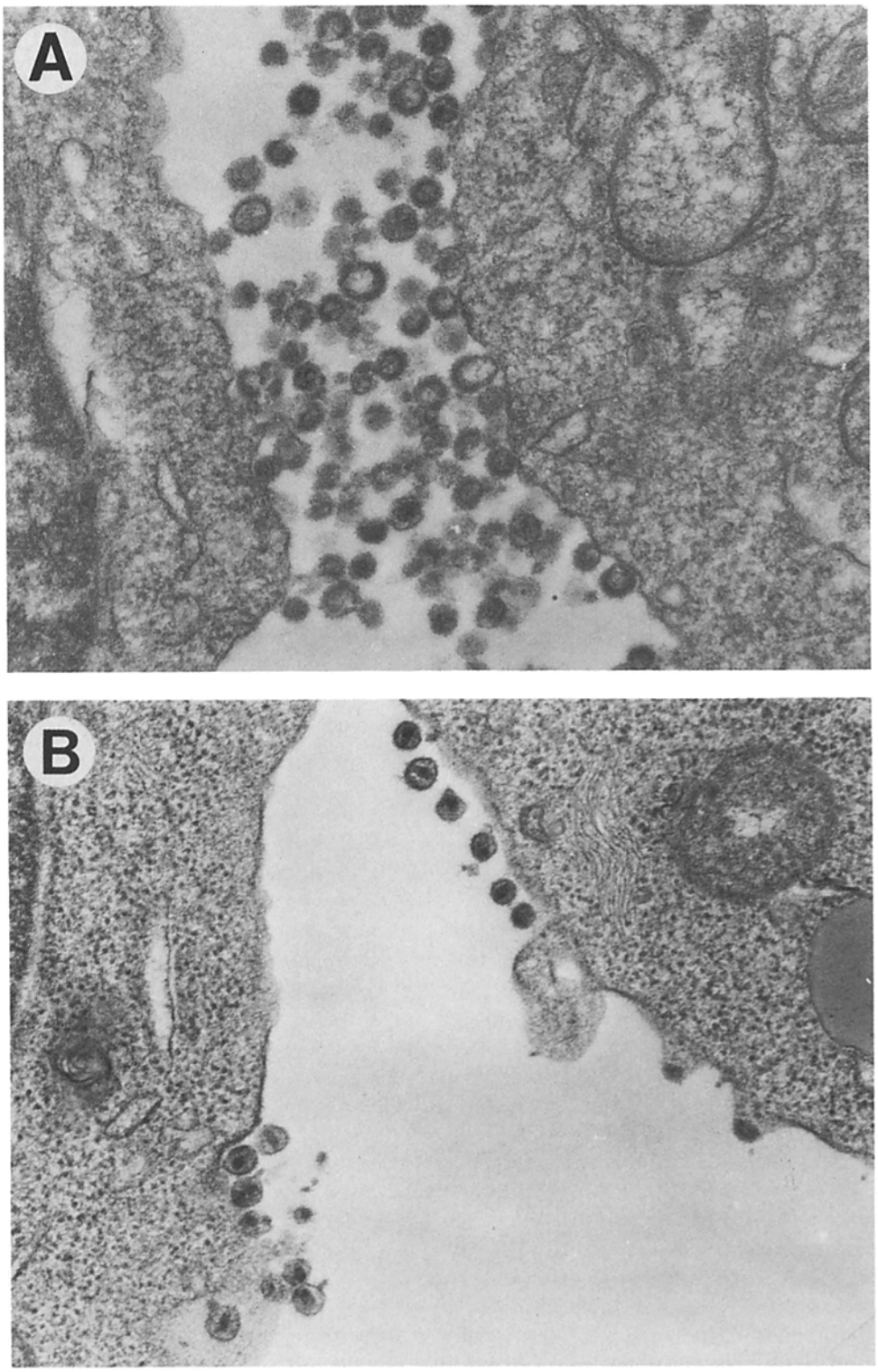

FIGURE 3. AIDS RV particles synthesized from 8E5 (*A*) or virus-infected A3.01 (*B*) cells. Original magnification, × 50,000.

of the 8E5 clone. In the presence of IUdR, the level of viral RNA synthesis in the survivor cells did not appreciably change (Fig. 1 *F*).

Discussion

The results presented here describe the isolation of a cellular clone (8E5) that harbors an integrated defective AIDS RV genome. The clone is derived from an IUdR-induced mass culture that has survived AIDS RV infection.

IUdR and other halogenated pyrimidines are known inducers of RVs (12–14). Although their precise mechanism of action is unknown, it has been assumed that these chemicals act on functionally inactive but structurally intact integrated copies of proviral DNA. It has been proposed (15), for example, that IUdR modulates transcription of integrated viral DNA, resulting in increased expression of dormant proviruses, and ultimately to the appearance of infectious retroviral particles. IUdR also has the potential for producing other effects in eukaryotic cells, such as unscheduled DNA replication, recombination, and mutagenesis.

We previously reported (3) that IUdR treatment of virus-free, Leu-3^- lymphocytes surviving infection with the AIDS RV induced infectious virus, as monitored by RT assays. The IUdR-induced progeny virions were indistinguishable from their LAV parent in terms of tropism for Leu-3^+ lymphocytes, the cytopathic effect that occurred during their replication in human T cells, and in their kinetics of infection. We had expected to clone an IUdR-inducible survivor cell that contained a wild-type provirus, and were therefore surprised when it became apparent that 8E5 cells harbored a defective copy of the viral DNA. It was unclear whether the 8E5 clone was an artifact of viral infection, IUdR treatment, the cloning procedure, or whether it was a biologically relevant participant in the induction of infectious virus.

In a previous examination of IUdR inducibility (3), we noted that no virus was detected when $\leq$100 virus-free survivor cells were evaluated; virus was obtained when $\geq$1,000 cells were exposed to the halogenated pyrimidine. This result implied that between 0.1 and 1% of the cells surviving the initial LAV infection could be induced to express virus. In situ hybridization analyses of the IUdR-inducible cells indicated that a similar fraction also contained a provirus that synthesized AIDS RV mRNA in the presence or absence of IUdR. Thus, the mass culture of cells surviving infection clearly harbors a subpopulation, indistinguishable from the 8E5 clone, that constitutively expresses viral RNA.

Assuming that a copy of proviral DNA, similar to that present in 8E5 cells, somehow participates in IUdR induction of virus, and is not an artifact of the cloning procedure or IUdR-mediated mutagenesis, a model could be proposed explaining the origin of the defective provirus and this form of viral latency. The single mutated copy of AIDS RV proviral DNA in 8E5 cells very likely represents a reverse transcript of a defective virion present in the original LAV inoculum. In this regard, we have encountered AIDS RV proviruses in preparations of infected cellular DNA that contain small deletions or inappropriate termination codons that would preclude their expression as infectious virus (our unpublished observations). Thus it would be necessary for an 8E5-like virus, presumably containing a mutation in the *pol* gene, to successfully synthesize and

then integrate a DNA copy of its genome into the chromosome of an infected human lymphocyte. This could be accomplished if the RT activity required during the initial phase of a hypothetical 8E5 viral infection could be supplied by: (*a*) simultaneous infection with a replication-competent AIDS RV, or (*b*) the pseudotyping of the 8E5 virus with wild-type RT, since RT is a virion structural protein.

At present, we have no explanation of IUdR induction in the AIDS RV system. Clearly, the 8E5 clone cannot be induced to yield infectious virus after exposure to IUdR. However, it is tempting to speculate that cells harboring a single copy or multiple defective copies of viral DNA exist within the survivor population. The mass culture of survivor cells might therefore contain a spectrum of mutated proviral DNAs, no one of which could encode infectious virus. However, when exposed to an external stimulus such as IUdR, individual members of the survivor cell population might be induced to express larger amounts of AIDS RV RNA which, following complementation and/or recombination, could give rise to infectious virus. To test such a possibility, we are presently isolating additional independent clones from the mass culture that have survived an initial cycle of infection.

Studies are also in progress to map the defect in the 8E5 provirus. The results of immunoblotting and Northern hybridization analyses indicate that the LTR, *gag*, and *env* regions of the proviral DNA are functionally intact. The integrity of the *trans*-activating segments (16) of the integrated 8E5 provirus was shown by transfecting 8E5 cells and a Leu-3$^-$ spontaneous revertant of the parental A3.01 line (designated A2.01 [3]) with a chloramphenicol acetyl transferase (CAT) construction under the control of an AIDS RV LTR. Striking enhancement of CAT activity was detected in 8E5 (A. Adachi and M. A. Martin, unpublished data), but not in A2.01 cells, indicating that the *trans*-activating regions of the integrated 8E5 provirus were being actively expressed. The absence of the immunoreactive p64 and p34 viral proteins, coupled with the appearance of defective virus particles unable to synthesize RT, suggest a defect in the *pol* gene. The location of mutation(s) within the 8E5 proviral DNA is currently being evaluated by marker rescue and nucleotide sequencing procedures.

Although 8E5 cells cannot be directly used to study IUdR induction, they might be used in cocultures with other clones of survivor cells, also containing defective proviruses, as a model system. 8E5 cells, nonetheless, express most of the AIDS RV–encoded proteins and thereby represent a potentially useful reagent. For example, the development of syncytia attending the cocultivation of 8E5 and parental A3.01 cells suggests that biologically active viral envelope proteins are synthesized. These viral proteins could be used to develop diagnostic reagents, therefore obviating potential biohazards attending the isolation of analogous proteins from cells producing infectious virus. Since it contains a single copy of viral DNA, 8E5 cells may also prove to be extremely valuable in delineating the immune response to virus-encoded proteins. For instance, the use of 8E5 cells as targets for MHC-compatible cytotoxic lymphocytes could provide important new insights regarding the mechanisms of immune-mediated elimination of virus-infected cells.

The presence of the 8E5 proviral DNA and the synthesis of all viral proteins except p64 have no effect on cell viability. Thus, if a virus-encoded protein, rather than the complex process of virus replication, is responsible for viral cytopathic effects and cell death, such a protein is not synthesized in 8E5 cells and could certainly be identified during detailed evaluations of the cloned line.

Summary

Leu-3$^-$ cells that survive infection with the acquired immune deficiency syndrome (AIDS) retrovirus can be induced with IUdR to express infectious virus (1). A cellular clone (8E5), isolated by limiting dilution of a mass culture of survivor cells, was found to contain a single, integrated provirus that was constitutively expressed. Although IUdR treatment of 8E5 cells failed to induce infectious virus, cocultivation with Leu-3$^+$ cells generated the characteristic syncytia associated with acute AIDS retrovirus infection. The single integrated copy of proviral DNA directs the synthesis of all major viral structural proteins except p64, as monitored by immunoblotting. The relationship of the 8E5 clone to viral latency and persistence is discussed.

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