REFERENCES AND NOTES

1. M. Barany, *J. Gen. Physiol.* **50**, 197 (1967); K. Schwartz *et al.*, *J. Mol. Cell. Cardiol.* **13**, 1071 (1981).
2. R. G. Whalen *et al.*, in *Muscle Development: Molecular and Cellular Control*, M. L. Pearson and H. F. Epstein, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), pp. 25–33.
3. H. Czosnek *et al.*, *EMBO J.* **1**, 1299 (1982); L. A. Leinwand *et al.*, *Science* **221**, 766 (1983).
4. B. Nadal-Ginard *et al.*, in *Muscle Development: Molecular and Cellular Control*, M. L. Pearson and H. F. Epstein, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), pp. 143–168; V. Mahdavi *et al.*, in *Molecular Biology of Muscle Development*, C. Emerson, D. A. Fischman, B. Nadal-Ginard, M. A. Q. Siddiqui, Eds. (Liss, New York, in press).
5. R. G. Whalen *et al.*, *Nature (London)* **292**, 805 (1981).
6. N. A. Rubinstein *et al.*, *J. Cell Biol.* **79**, 252 (1978); F. Jolez and F. A. Sreter, *Annu. Rev. Physiol.* **43**, 531 (1981).
7. S. Salmons and F. A. Sreter, *Nature (London)* **263**, 30 (1976); A.-M. Lompre *et al.*, *ibid.* **282**, 105 (1979).
8. Y. Yazaki and M. S. Raben, *Circ. Res.* **36**, 208 (1975); D. Ianuzzo *et al.*, *Nature (London)* **270**, 74 (1977); J. F. Y. Hoh, P. A. McGrath, H. T. Hale, *J. Mol. Cell. Cardiol.* **10**, 1053 (1978); W. Dillman, *Diabetes* **29**, 579 (1980); E. Morkin *et al.*, *Prog. Cardiovasc. Dis.* **25**, 435 (1983).
9. A.-M. Lompre, B. Nadal-Ginard, V. Mahdavi, *J. Biol. Chem.* **259**, 6437 (1984); V. Mahdavi, A. P. Chambers, B. Nadal-Ginard, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2626 (1984).
10. A. M. Shinha *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5847 (1982).
11. A. M. Shinha *et al.*, *J. Biol. Chem.* **259**, 6674 (1984).
12. The nomenclature used for the different MHC mRNA's, cDNA clones, and corresponding genes follows the most common usage in the literature reflecting the developmental stage, muscle, or fiber type where their expression is predominant, without implying that their expression is restricted to these tissues or developmental stage.
13. D. F. Wieczorek *et al.*, *J. Cell Biol.* **101**, 618 (1985).
14. M. Periasamy, D. F. Wieczorek, B. Nadal-Ginard, *J. Biol. Chem.* **259**, 13,573 (1984); M. Periasamy *et al.*, *ibid.* **260**, 15,856 (1985).
15. B. Gambke *et al.*, *FEBS Lett.* **156**, 335 (1983); G. S. Butler-Browne, D. Herlicoviez, R. G. Whalen, *ibid.* **166**, 71 (1984).
16. G. F. Gauthier and S. Lowey, *J. Cell Biol.* **74**, 760 (1977); G. F. Gauthier *et al.*, *ibid.* **92**, 471 (1982).
17. A. C. Nag and M. Cheng, *Biochem. J.* **221**, 21 (1984); ——, R. Zak, *Exp. Cell Res.* **158**, 53 (1985).
18. L. Nwoye *et al.*, *Am. J. Physiol.* **242**, R401 (1982); E. C. B. Hall-Craggs, M. M. Wines, S. R. Max, *Exp. Neurol.* **80**, 252 (1983).
19. R. G. Whalen *et al.*, *J. Cell Biol.* **101**, 603 (1985).
20. J. H. Oppenheimer and H. H. Samuels, Eds., *Molecular Basis of Thyroid Hormone Action* (Academic Press, New York, 1983).
21. R. M. Evans, N. C. Birnberg, M. G. Rosenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7659 (1982); B. M. Yaffe and H. H. Samuels, *J. Biol. Chem.* **259**, 6284 (1984); M. A. Shupnick *et al.*, *ibid.* **260**, 2900 (1985).
22. P. K. Umeda *et al.*, in *Molecular Biology of Muscle Development*, C. Emerson, D. A. Fischman, B. Nadal-Ginard, M. A. Q. Siddiqui, Eds. (Liss, New York, in press).
23. F. E. Stockdale, *Dev. Biol.* **21**, 462 (1970); J. L. Doering and D. A. Fischman, *ibid.* **36**, 225 (1974); R. Zak, *Circ. Res.* **34** (Suppl. 2), II (1974).
24. R. M. Medford, H. T. Nguyen, B. Nadal-Ginard, *J. Biol. Chem.* **258**, 11,063 (1983).

25. We thank D. Bois, K. Kubota, and R. Matsuoka for their help. S.I. thanks W. Grossman for his continuous support. Supported in part by grants from the National Institutes of Health, American Heart Association, and Muscular Dystrophy Association. S.I. is supported by the American Heart Association Massachusetts Affiliate. V.M. is an Established Investigator of the American Heart Association.

5 August 1985; accepted 29 October 1985

Induction of HTLV-III/LAV from a Nonvirus-Producing T-Cell Line: Implications for Latency

THOMAS FOLKS, DOUGLAS M. POWELL, MARILYN M. LIGHTFOOTE, STEVEN BENN, MALCOLM A. MARTIN, ANTHONY S. FAUCI

When the human T-cell line A3.01 is infected with HTLV-III/LAV, the virus associated with the acquired immune deficiency syndrome (AIDS), most of the cells are killed. However, a small number of cells that lack the Leu-3 surface marker survive. Under normal conditions these surviving cells do not produce virus, nor can they be infected by the virus, but they can be induced to produce virus by treatment with 5-iodo-2′-deoxyuridine. This response can be induced for as long as 3 months after the initial infection, suggesting that the cells may harbor a latent form of HTLV-III/LAV.

THE PRIMARY ETIOLOGIC AGENT OF the acquired immune deficiency syndrome (AIDS) has been identified as a human T-lymphotropic retrovirus (RV) that has been referred to as human T-lymphotropic virus type III (HTLV-III), lymphadenopathy-associated virus (LAV), or AIDS-associated virus (*1–8*). When phytohemagglutinin-stimulated human lymphocytes are infected with this virus, the cells undergo a characteristic cytopathic effect that includes syncytia formation and, ultimately, cell death (*1, 2*). In contrast, a continuous lymphocyte line, H9, which has been used for the large-scale production of HTLV-III/LAV exhibits only modest cytopathic effects and no detectable cell death as a consequence of virus infection. We have recently described a variant of the CEM T-cell line A3.01, which is more than 95 percent susceptible to infection and undergoes all of the cytopathic changes associated with HTLV-III/LAV, including cell death (*9*). During the late stages of productive infection of A3.01 cells, a small percentage of the cells survive. These surviving cells lack the Leu-3 surface marker, and, although they do not produce the virus spontaneously, they clearly contain proviral DNA since infectious virus can be induced by treating the cells with 5-iodo-2′-deoxyuridine (IUdR). These findings have important implications in our understanding of the chronicity or possible latency of HTLV-III/LAV infection in humans and may serve as a useful model for investigations of the noncytocidal effects of the virus on human T-cell function.

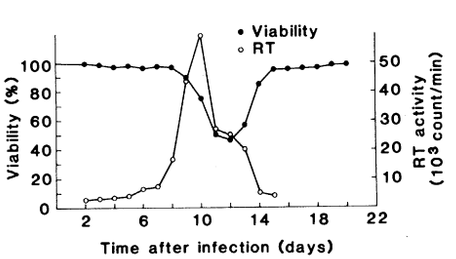

Fig. 1. Establishment of survivor NP cells. A3.01 cells (1×10^6) were infected with the LAV isolate (*1*) of HTLV-III/LAV as previously described (*9*). Cell viability was determined by trypan blue exclusion.

Cells of the A3.01 line grow as two discrete populations (*9*). One, representing ~95 percent of the culture, expresses the Leu-3 (CD4, or T4) surface marker as well as Leu-1, class I HLA, and the transferrin receptor. The second population, comprising <5 percent of the culture, is Leu-3$^-$. Subclones of A3.01 cells, although initially Leu-3$^+$, consistently give rise to cells that make up the 2 to 5 percent of the culture that are Leu-3$^-$. One of these Leu-3$^-$ cells was cloned by limiting dilution (A2.01) and did not revert to the Leu-3$^+$ phenotype during an 8-month observation period. The A3.01 and A2.01 cells were indistinguishable by morphology and growth characteristics.

The HTLV-III/LAV was the same as that used in our original report on the A3.01 cell line (*9*). The time course of a typical infection is shown in Fig. 1. Reverse transcriptase (RT) activity was initially detected in the supernatant fluid on day 8, peaked on day 10, and reverted to background levels by

T. Folks, D. M. Powell, M. M. Lightfoote, A. S. Fauci, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
S. Benn and M. A. Martin, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

day 15. A decrease in cell viability was evident on day 9, and by day 12 only 50 percent of the infected A3.01 cells were alive. Cell viability in the infected culture returned to normal levels (>95 percent) by day 15. By day 10, ~85 percent of cells were synthesizing viral structural proteins as monitored by indirect immunofluorescence with the use of pooled AIDS sera adsorbed on uninfected A3.01 cells. The expression of the CD4 molecule was detected by flow cytometry with the monoclonal antibody Leu-3. Only 5 percent of the infected A3.01 cells expressed the Leu-3 marker on day 10 (Fig. 1); by day 15 no detectable Leu-3$^+$ cells remained in the culture.

The Leu-3$^-$ cells surviving HTLV-III/LAV infection were indistinguishable from A3.01 and A2.01 cells in morphology and growth properties. Like their A3.01 parent, these Leu-3$^-$ survivors were Leu-1$^+$, class I HLA$^+$, and transferrin receptor–positive. The cells surviving infection were also evaluated for their ability to produce infectious virus. No budding retroviral particles were observed by electron microscopy and, as noted above, RT activity was at background levels by day 15. In addition, no infectious particles could be demonstrated by coculturing survivor cells with Leu-3$^+$ A3.01 cells; neither synctytia formation nor RT activity was detected during a 30-day coculture period.

To determine if HTLV-III/LAV genome was present in A3.01 cells that had survived infection, we treated cells with the halogenated pyrimidine IUdR, a well-known inducer of retroviruses (*10*). Survivor cells harvested 25, 45, and 90 days after infection with HTLV-III/LAV were exposed to IUdR (100 μg/ml) for 24 hours and then cocultured with Leu-3$^+$ A3.01 cells. Induced virus, if produced, could be detected by monitoring the RT activity in the supernatants of the cocultured cells. In general, there was a correlation between the time of peak RT activity and the time after exposure of the survivor cultures to IUdR. For example, as shown in Fig. 2, in which virus was induced in the three different survivor cell populations, the peak of RT activity occurred 7, 13, and 19 days after exposure of the 25-, 45-, and 90-day survivor cell cultures, respectively, to IUdR.

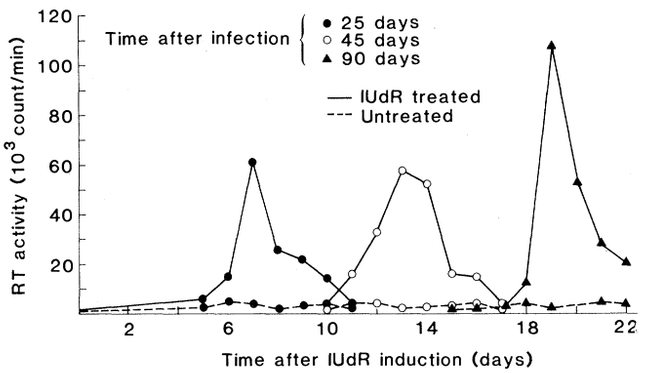

Fig. 2. Inducibility of HTLV-III/LAV after treatment with IUdR. Cells surviving HTLV-III/LAV infection (see Fig. 1) were maintained in culture for 25, 45, or 90 days. Aliquots (1×10^6) were treated with IUdR (100 μg/ml) for 24 hours, washed three times in medium, and cocultured with 1×10^6 A3.01 cells. Supernatants were tested daily for RT activity. Untreated survivor cells were also cocultured with A.301 cells and monitored for RT activity.

We have previously described the isolation of retroviruses from AIDS patients in New York, Alabama, and Zaire (*11*), and have demonstrated their similarity to LAV (*1*) and HTLV-III (*2, 3*). Seven of these isolates have been propagated in A3.01 cells and, in each instance, nonvirus-producing (NP) survivor cells were obtained. One culture of these NP cells derived from an infection with an HTLV-III/LAV isolate from New York could be induced to express infectious particles by induction with IUdR.

Since the A3.01 cells that survived HTLV-III/LAV infection were Leu-3$^-$, it was important to ascertain whether they were derived from the 2 to 5 percent of the Leu-3$^-$ cells present in the original culture or arose from Leu-3$^+$ cells that no longer expressed the CD4 antigen as a consequence of infection. Therefore, we used the A2.01 clone to determine whether these Leu-3$^-$ cells could be infected with HTLV-III/LAV. A2.01 cells were infected with dilutions (1×10^{-2}) of our HTLV-III/LAV virus stock shown to be infectious for A3.01 cells. The infected A2.01 cultures were maintained at a concentration of 2×10^6 cells per milliliter for 30 days at which time they were split into two aliquots. One aliquot was directly cocultured with A3.01 cells to assay the spontaneous release of HTLV-III/LAV. The second was treated with IUdR for 24 hours and then cocultured with A3.01 cells. In neither case was syncytia formation or RT activity detected (Table 1). NP cells were similarly refractory to viral infection. These results indicate that HTLV-III/LAV infection of the small population of Leu-3$^-$ cells present in the starting A3.01 culture is very unlikely to occur. The NP cells probably arise from Leu-3$^+$ A3.01 cells that survive infection.

Table 1. Induction of A2.01, A3.01, and NP cell lines by treatment with IUdR. Cells (1×10^6) were treated with or without a 10^{-1} dilution of stock virus for 2 to 4 hours, washed, and cultured for 30 days. The cells were then incubated with or without IUdR (100 μg/ml) for 24 hours, washed, and cocultured where indicated with 1×10^6 A3.01 cells. The supernatant was monitored daily for RT activity (+, >15,000 cpm/15 μl; −, no activity).

Cell	Treatment		Cocultured with A3.01	Days in culture												
	Virus	IUdR		4	5	6	7	8	9	10	11	12	13	14	15	16
NP				−	−	−	−	−	−	−	−	−	−	−	−	−
NP			+	−	−	−	−	−	−	−	−	−	−	−	−	−
NP		+		−	−	−	−	−	−	−	−	−	−	−	−	−
NP		+	+	−	−	+	+	+	+	+	−	−	−	−	−	−
NP	+			−	−	−	−	−	−	−	−	−	−	−	−	−
NP*		+	+	−	−	−	−	−	+	+	+	+	−	−	−	−
A3.01	+			−	−	−	−	+	+	+	+	+	+	−	−	−
A3.01		+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
A2.01	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
A2.01	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
A2.01	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−

*Supernatant (100 μl) from an IUdR-treated NP cell was added to A3.01 cells.

Table 2. Frequency of IUdR-inducible NP cells in the surviving population. Log_{10} dilutions of NP cells were treated with 100 μg of IUdR per milliliter for 24 hours, washed, and cocultured with 1×10^6 susceptible A3.01 cells. Supernatants were monitored daily for RT activity (+, >15,000 cpm/15 μl; −, no activity).

Number of NP cells	Days in culture													
	8	9	10	11	12	13	14	15	16	17	18	19	20	21
10^6	−	+	+	+	−	−	−	−	−	−	−	−	−	−
10^5	−	−	−	+	+	+	−	−	−	−	−	−	−	−
10^4	−	−	−	−	−	+	+	+	−	−	−	−	−	−
10^3	−	−	−	−	−	−	−	+	+	+	−	−	−	−
10^2	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^1	−	−	−	−	−	−	−	−	−	−	−	−	−	−

From these data we concluded that the Leu-3$^-$, virus-negative cells arising as a result of HTLV-III/LAV infection of A3.01 cells consisted of at least two cell populations: cells that were originally Leu-3$^-$ (that is, derivatives of the 5 percent of A3.01 cells that were Leu-3$^-$ from the start) and survivor cells that had lost their Leu-3 marker as a result of infection with HTLV-III/LAV. This indeed appeared to be the case when attempts were made to clone cells that could be induced with IUdR to express virus. In an initial experiment, none of 40 randomly selected survivor cell clones, obtained by limiting dilution, released virus after exposure to IUdR. Our interpretation of this result was that most of the "survivor" cells were an outgrowth of Leu-3$^-$ cells that were present in the starting culture and that only a minority harbored inducible virus. As shown in Table 2, when log_{10} dilutions of NP cells were exposed to IUdR and then cocultured with susceptible A3.01 cells, no virus was induced when 100 or fewer cells were tested. The result suggests that virus-inducible NP cells comprise between 0.1 and 1 percent of the surviving cell population.

At least two general models of latency can be considered to explain the inducibility of HTLV-III/LAV by IUdR from cells surviving productive infection (Fig. 3). In both, DNA copies of the viral genome have been inserted into chromosomal DNA of infected cells in reactions catalyzed by *pol* region gene products. During cytocidal infection of human T cells by HTLV-III/LAV, the proviral DNA is constitutively expressed, resulting in the synthesis of the five major classes (*12*) of viral RNA (Fig. 3A). In contrast, cells surviving infection may harbor a copy of the provirus that has been rendered functionally inert (Fig. 3B). If we assume that a faithful copy of the viral genome has been integrated into the DNA of the infected cell, HTLV-III/LAV gene activity can be "down regulated" by any of the mechanisms illustrated in Fig. 3B [that is, hypermethylation (line 1), insertion of proviral DNA into a region of densely packed chromatin (line 2), and the binding of regulatory proteins that could repress the expression of viral messenger RNA's (line 3)]. The addition of IUdR might obviate any of these hypothetical blocks.

In a second model of latency (Fig. 3C) we assume that survivor cells harbor defective copies of the HTLV-III/LAV provirus containing point mutations or microdeletions rather than intact, potentially infectious proviral DNA's. The effect of IUdR on these cells is to stimulate complementation or recombinational events between defective viral genomes, ultimately resulting in a productive infection.

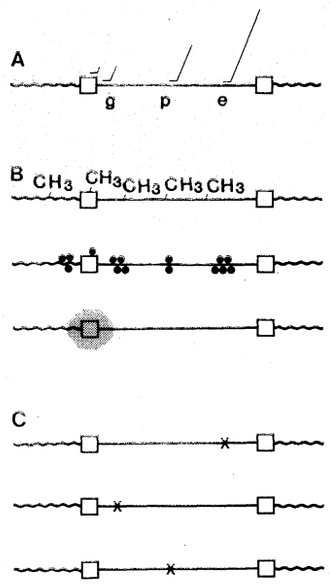

Fig. 3. Models of HTLV-III/LAV latency. (A) Integrated HTLV-III/LAV proviral DNA being expressed in cells productively infected with virus (g, *gag*; p, *pol*; e, *env*). (B) Transcriptional repression of intact proviral DNA by hypermethylation (line 1), chromatin conformation (line 2), or a DNA-binding protein (line 3). (C) Integrated HTLV-III/LAV proviruses containing mutations (X).

To investigate these hypotheses, we assayed survivor cells for the presence of proviral DNA and RNA transcripts by Southern and Northern blot hybridizations, respectively. No viral DNA or RNA was detected in mass cultures of survivor cells similar to those examined in Table 2. However, one survivor clone was selected from such a mass culture because it did cause syncytia formation after IUdR treatment and coculture with Leu-3$^+$ human lymphocytes. Preliminary characterization of this clone (8E5) indicates that high levels of viral RNA and protein are synthesized both before and after exposure to IUdR (*13*), but that no wild-type virus is produced.

Even after long-term culture (3 months) of one of the NP cell lines, infectious HTLV-III/LAV virus can be induced by IUdR. This phenomenon has potentially far-reaching clinical consequences, explaining how an individual who harbors the virus in a latent form may not, for significant periods of time, express viral proteins or infectious virus particles. Subsequently, the latent virus might be induced by any one of a variety of physicochemical stimuli such as irradiation, chemical exposure, stress, superimposed infections, or antigenic stimulation of latently infected T lymphocytes resulting in the production of infectious virus and the development of disease. Of note is the fact that Hoxie *et al.* (*14*) have recently reported persistent noncytopathic infection with human T4 cells by AIDS-associated virus in vitro in which infection also resulted in a loss of reactivity with the OKT4 monoclonal antibody (LEU-3). Taken together, these results provide evidence in AIDS for both productive and nonproductive latent noncytopathic infection of a small population of Leu-3$^+$ cells that lose the Leu-3 marker.

REFERENCES AND NOTES

1. F. Barré-Sinoussi *et al.*, *Science* **220**, 868 (1983).
2. M. Popovic, M. G. Sarngadharan, E. Read, R. Gallo, *ibid.* **224**, 497 (1984).
3. R. C. Gallo *et al.*, *ibid.*, p. 500.
4. D. Klatzmann *et al.*, *ibid.* **225**, 59 (1984).
5. A. G. Dalgleish *et al.*, *Nature (London)* **312**, 763 (1984).
6. D. Klatzmann *et al.*, *ibid.*, p. 767.
7. R. C. Gallo and F. Wong-Staal, *Blood* **60**, 545 (1982).
8. J. A. Levy, A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, *Science* **225**, 840 (1984).
9. T. Folks *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4539 (1985).
10. D. R. Lowy, W. P. Rowe, N. Teich, J. W. Hartley, *Science* **174**, 155 (1971).
11. S. Benn *et al.*, *ibid.* **230**, 949 (1985).
12. A. B. Rabson *et al.*, *ibid.* **229**, 1388 (1985).
13. T. Folks *et al.*, in preparation.
14. J. A. Hoxie, B. S. Haggarty, J. L. Rackowski, N. Pillsbury, J. A. Levy, *Science* **229**, 1400 (1985).

15. We thank A. Kinter and J. Justement for technical assistance, J. Sears for preparing art work, A. Rabson for critically reading the manuscript, and A. London for editorial assistance.

20 August 1985; accepted 13 November 1985