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In Vitro CD4$^+$ Lymphocyte Transformation and Infection in a Rabbit Model with a Molecular Clone of Human T-Cell Lymphotropic Virus Type 1

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We transfected human and rabbit peripheral blood mononuclear cells (PBMC) with the ACH molecular clone of human T-cell lymphotropic virus type 1 (HTLV-1) to study its in vitro and in vivo properties. PBMC transfected with ACH were shown to transfer infection to naive PBMC. ACH transformed rabbit PBMC, as indicated by interleukin-2-independent proliferation of a transfectant culture. This transformant culture was shown by flow cytometric analysis to be a CD4$^+$ CD25$^+$ T-lymphocyte population containing, as determined by Southern blot analysis, at least three integrated HTLV-1 proviral copies. HTLV-1 infection was produced in rabbits inoculated with ACH-transfected, irradiated PBMC. Inoculated rabbits seroconverted to positivity for antibodies against HTLV-1 and had steady or rising HTLV-1 enzyme-linked immunosorbent assay antibody titers. Western blot (immunoblot) analysis revealed sustained seroconversion of rabbits to positivity for antibodies against all major viral antigenic determinants. Infection of rabbits was further demonstrated by antigen capture assay of p24 in PBMC and lymph node cultures and PCR amplification of proviral sequences from PBMC. These data suggest that ACH, like wild-type HTLV-1, infects and transforms primary CD4$^+$ T lymphocytes and is infectious in vivo. This clone will facilitate investigations into the role of viral genes on biological properties of HTLV-1 in vitro and in vivo.

Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (42) and is closely linked with a degenerative neurological disorder known as HTLV-1-associated myelopathy/tropical spastic paraparesis (31), as well as with several other inflammatory disorders (26, 27, 29). Despite relatively high prevalence rates of the viral infection in regions where it is endemic, less than 5% of infected individuals develop disease after a latent period of months to years (38, 41).

The molecular and cellular mechanisms that control the latency and leukemogenic potential of the virus have been the subject of many recent investigations (12). However, molecular studies of HTLV-1 have been hampered by the lack of availability of infectious clones of the virus. HTLV-1 is highly cell associated and spreads in vitro principally through cell-to-cell contact (37). Cell-free virus is poorly infectious, and as a consequence, HTLV-1-infected or -transformed cells have traditionally been used in viral pathogenesis studies (10, 25, 32). These forms of the virus are difficult to genetically manipulate, and furthermore, the cellular inocula often contain defective viral variants which limit their usefulness in studies of virus replication (20, 30). Similar difficulties in other retroviral systems have been overcome by the use of infectious molecular clones (3, 8, 11, 23, 35). However, such clones of HTLV-1 have been difficult to produce. Three recent reports have described the construction of infectious HTLV-1 molecular clones. An in vitro study by Derse et al. demonstrated transfection of HeLa and 293 T-cell lines with a plasmid clone derived from the HTLV-1-infected T-cell line CS-1 (6). Transfected cells yielded infectious virus, as shown by cell-free infection of MOLT4 and CEM cell lines and human cord blood mononuclear cells (6). In another study, the K30 clone, derived from an HTLV-1-transformed rabbit T-cell line, was transfected into the herpesvirus-transformed RL-5 rabbit T-cell line, as well as into the human T-cell line CEMss and a rabbit fibroblast line (43). Transfected RL-5 cells were used to infect human and rabbit primary cells by coculture. We reported on the construction of a clone derived from the HTLV-1$_{CH}$ strain (19). Designated ACH, this clone contains 5′ and 3′ long terminal repeats derived from HTLV-1$_{ATK}$ and HTLV-1$_{EL}$, respectively. ACH was transfected into the COS7 and HOS cell lines, yielding viral antigen production and Tax protein activity. Furthermore, transfection of primary peripheral blood mononuclear cells (PBMC) by ACH resulted in continuous proliferation of PBMC and production of infectious virus.

Optimally, a useful molecular clone of HTLV-1 will behave in a fashion similar to that of wild-type virus. HTLV-1, in the form of infected cell lines, infects and transforms primary T lymphocytes in vitro upon cocultivation. To determine the in vitro infectivity and transforming properties of ACH, we transfected this clone into primary cells. Mitogen-stimulated PBMC from four rabbits (R46 through R49) and one human donor were transfected by electroporation as previously described (2) with 7.5 μg of the ACH plasmid. Transfectants were maintained as previously described (28). A p24 antigen capture assay (Coulter Corp., Hialeah, Fla.) was used daily to monitor viral antigen production. Production of viral antigen was detectable in one rabbit transfectant culture supernatant 2 days posttransfection (R49 PBMC) and by 6 days in all rabbit

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PBMC cultures (concentration range, 19.4 to 109.5 pg/ml). Viral antigen production was transient in two of the rabbit PBMC cultures (R46 PBMC and R48 PBMC), falling to undetectable levels by 2 weeks posttransfection (concentration, <15 pg/ml). Cultures derived from R47, R49, and human PBMC continued to produce detectable viral antigen at 2 weeks posttransfection (concentration range, 36.3 to 257.8 pg/ml) and were subsequently used in rabbit inoculation procedures. Lethally irradiated ACH-transfected human PBMC were shown to produce infectious virus, as evidenced by coculture with naive, activated human PBMC in two separate trials. Cocultures continued to produce high levels of p24 (>250 pg/ml) at 2 weeks postirradiation. However, viral p24 antigen was no longer detectable at 2 weeks in cultures of irradiated cells alone. After continuously proliferating and producing viral p24 antigen for 4 weeks, R49 transfectants were cryopreserved for in vivo inoculations. This in vitro production of viral protein and passage of infection to naive PBMC are similar to the results of experiments utilizing HTLV-1-infected cell lines (32).

To evaluate the ability of ACH to transform rabbit lymphocytes, the cryopreserved, ACH-transfected R49 cells were thawed and maintained in culture with and without supplementation with recombinant interleukin-2 (rIL-2). The cells supplemented with rIL-2 continuously proliferated for 24 weeks. After 6 weeks of no growth in culture, the cells deprived of exogenous rIL-2 began proliferating and remained rIL-2 independent. To determine the proviral copy number, we examined this culture, designated R49ACH, by Southern blot analysis using previously described methods (4, 16, 39). High-molecular-weight DNA was digested with *Eco*RI or *Pst*I and probed with a ^{32}P-labeled random-primer (Ambion, Austin, Tex.)-generated probe of the pMT-2 full-length HTLV-1 clone (kindly provided by F. Wong-Staal, University of California, San Diego, La Jolla, Calif.). The hybridization signal was analyzed with a PhosphorImager 445 and ImageQuaNT software (Molecular Dynamics, Sunnyvale, Calif.). *Eco*RI does not cut within the viral genome and thus yields a unique restriction fragment for each viral integration. Results indicated clonal integration of at least three proviral copies (Fig. 1). *Pst*I cuts at four sites within the viral genome. The four *Pst*I restriction sites present in the parent plasmid were conserved in R49ACH, as revealed by the presence of internal fragments of 1.2, 1.6, and 2.6 kb (Fig. 1). The other bands seen in the *Pst*I digestion of R49ACH correspond to the six polymorphic terminal fragments expected from three integrated proviral copies. R49ACH was tested by flow cytometry for cell surface CD receptor expression. Cells were directly labeled with fluorescein isothiocyanate-conjugated monoclonal antibodies against rabbit CD4, CD5, and CD25 receptors (Spring Valley Laboratories, Woodbine, Md.). Fluorescence intensity was measured with a Coulter Elite cytometer, and data analysis was performed with the Immuno-4 analysis program (Coulter Corp.). R49ACH was typical of HTLV-1-transformed cells, with 99.6% of cells positive for CD4 expression, 66.3% positive for CD5 expression, and 99.9% positive for CD25 expression. No functional anti-rabbit CD8 reagents were available, and therefore we cannot rule out the possibility that R49ACH coexpresses CD4 and CD8, as recently reported for HTLV-1 (36). Like wild-type HTLV-1, ACH appears to transform lymphocytes and to cause continuous cellular proliferation in the absence of stimulation with exogenous IL-2. Two other molecular clones have been used to infect primary human or rabbit lymphocytes (6, 43). Derse et al. infected human cord blood lymphocytes with cell-free virus obtained from transfected 293 cells but did not observe proliferation for more than 4 to 6 weeks. However, no attempt to directly transfect primary cells was made (6).

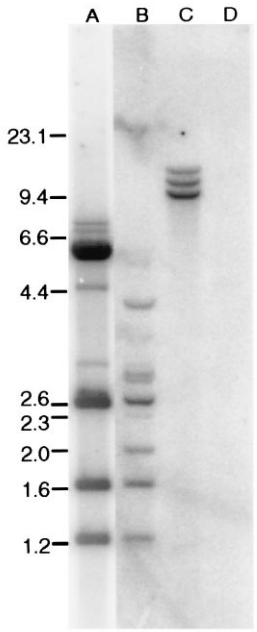

FIG. 1. Detection of HTLV-1 provirus in R49ACH cells by Southern blot analysis. Molecular size markers are on the left in kilobases. Lane A, DNA from plasmid clone ACH digested with *Pst*I; lane B, DNA from R49ACH digested with *Pst*I; lane C, DNA from R49ACH digested with *Eco*RI; lane D, DNA from an HTLV-1-negative cell line (Jurkat) digested with *Eco*RI. No provirus was detected in DNA from Jurkat cells. At least three proviral copies were detected in R49ACH when DNA was digested with *Eco*RI, and three internal HTLV-1 fragments generated by *Pst*I digestion (1.2, 1.6, and 2.6 kb) showed identity between the ACH plasmid and R49ACH DNA. The minor bands seen in lane A represent residual, incompletely digested plasmid fragments.

Zhao et al. demonstrated only transient p24 production in transfected primary rabbit and human cells but were able to produce cell lines from PBMC cocultured with transfected, herpesvirus ateles-transformed cells (43). However, the phenotype, HTLV-1 status, and herpesvirus status of these cell lines are unclear. Our R49ACH transformed cell population was $CD4^+$ and $CD25^+$ and contained at least three clonally integrated copies of the HTLV-1 genome, suggesting that R49ACH is an outgrowth of an HTLV-1-infected $CD4^+$ T-cell population expressing high levels of IL-2Rα, typical of HTLV-1-transformed cells from adult T-cell leukemia/lymphoma patients (15, 38). To determine the clonality of R49ACH, we evaluated T-cell receptor rearrangement by Southern blot analysis as described above. *Eco*RI-digested genomic DNA was hybridized with a rabbit $C_T\beta$ probe PCR amplified from the pRTB92 clone (kindly provided by Thomas J. Kindt, National Institute of Allergy and Infectious Diseases). The results did not reveal monoclonal T-cell receptor rearrangement (data not shown), suggesting that R49ACH consists of an oligoclonal population of ACH-infected $CD4^+$ lymphocytes, as previously described for HTLV-1-infected T lymphocytes (18).

HTLV-1 persistently infects rabbits after injection of infected cell lines (1, 5, 22). Although the infection is generally asymptomatic, rabbits represent an excellent model for studying mechanisms of HTLV-1 infectivity and expression. Furthermore, rabbits have much potential as a model for testing HTLV-1 vaccines (21). To determine if ACH could produce HTLV-1 infection in rabbits, we intravenously inoculated 12-week-old New Zealand White rabbits (1.0×10^7 cells; via lateral ear vein) with lethally irradiated (7,500 rads), ACH-transfected cells. Initially, four rabbits were inoculated with

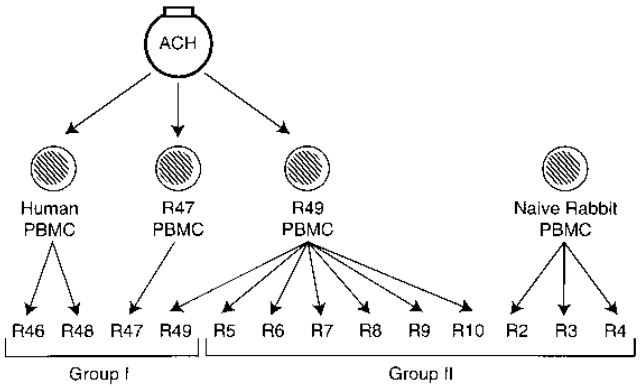

FIG. 2. Diagram of in vivo inoculations. Rabbits designated by numbers at the bottom received ACH-transfected PBMC or naive PBMC from rabbit or human sources as indicated by arrows. For details, see the text.

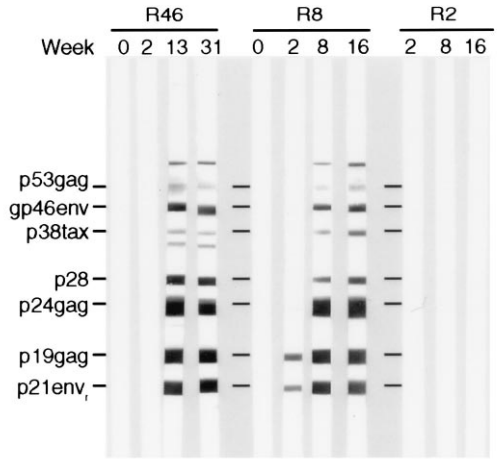

FIG. 3. Representative serial Western blots for ACH-inoculated rabbits (R46 and R8) and a negative control rabbit (R2). No seroreactivity against HTLV-1 antigens was noted for plasma from the negative control rabbit or from ACH-inoculated rabbits at preinoculation (week 0). Plasma from ACH-inoculated rabbits was not reactive (R46) or was mildly reactive (R8) to Env (p21) and Gag (p19 and p24) at week 2, whereas at all later time points plasma was strongly reactive against all major viral antigenic determinants.

either ACH-transfected human PBMC (rabbits R46 and R48) or ACH-transfected autologous rabbit PBMC (rabbits R47 and R49). These four rabbits were designated group I (Fig. 2). Approximately 4 months later, cryopreserved ACH-transfected R49 rabbit PBMC (R49ACH) were thawed, expanded in culture, and used to inoculate a group of six rabbits (R5 through R10). Concurrently, three rabbits (R2 through R4) were inoculated with allogeneic naive PBMC from a healthy rabbit donor. The six R49ACH-inoculated rabbits and the three negative controls were designated group II (Fig. 2).

To determine the presence of antibodies in and degree of seroreactivity of rabbits inoculated with ACH, plasma from the rabbits was tested for titers of anti-HTLV-1 antibodies with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cambridge Biotech, Worcester, Mass.) which was adapted for rabbit plasma by the use of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:400 dilution) (Sigma, St. Louis, Mo.). Twofold dilutions of plasma from 1:100 to 1:6,400 were tested, and titers were expressed as the reciprocal of the highest plasma dilution to yield a positive reaction. Plasma was collected from group I rabbits at 0, 2, 4, 8, 13, and 31 weeks postinoculation (p.i.) and from group II rabbits at 0, 2, 4, 8, 12, and 16 weeks p.i. and stored at −20°C until tested. Except for R46, which seroconverted at 8 weeks p.i., all ACH-inoculated rabbits developed antibody reactivity by 4 weeks p.i. and remained persistently seroreactive, with steady or rising antibody titers (100 to 6,400), through the remainder of the study, as evidenced by HTLV-1 antibody ELISA. In contrast, no anti-HTLV-1 seroreactivity could be detected at any time point among any of the control rabbits (Table 1).

Reactivity to specific viral antigenic determinants was detected with a commercial HTLV-1 Western blot (immunoblot) kit (Cambridge Biotech) adapted for rabbit plasma by use of avidin-conjugated goat anti-rabbit immunoglobulin G (1:3,000 dilution) (Vector, Burlingame, Calif.). Plasma showing reactivity to capsid (p24) and envelope (p21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity (14). Western blot analysis was performed on plasma obtained on weeks 0, 2, 12, and 31 p.i. (group I) or 0, 2, 8, and 16 p.i. (group II). Eight of ten ACH-inoculated rabbits were initially seroreactive to HTLV-1 p19 matrix and recombinant p21 transmembrane envelope at 2 weeks p.i. (Fig. 3). Furthermore, all ACH-inoculated rabbits were strongly seroreactive to all major viral antigenic determinants, including p21 envelope, p19 matrix, p24 capsid, p38 Tax, and gp46 surface glycoproteins, at all remaining time points tested. None of the naive PBMC-inoculated control rabbits showed any evidence of seroreactivity to HTLV-1 by Western blot assay (Fig. 3).

To test ACH-inoculated rabbits for viral infection, PBMC were isolated by Ficoll-Hypaque separation from rabbit whole

TABLE 1. Anti-HTLV-1 antibody ELISA titers in plasma from rabbits inoculated with ACH-transfected or naive PBMC

Wk p.i.	Antibody titer[a] for indicated rabbit												
	Group II (naive)			Group II (R49ACH transfected)						Group I (ACH transfected)			
	R2	R3	R4	R5	R6	R7	R8	R9	R10	R46	R47	R48	R49
0	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
2	<100	<100	<100	<100	400	<100	<100	100	800	<100	<100	<100	200
4	<100	<100	<100	400	800	200	400	400	3,200	<100	200	100	1,600
8	<100	<100	<100	400	1,600	400	400	3,200	3,200	800	400	200	3,200
12	<100	<100	<100	200	1,600	800	400	3,200	1,600	ND[b]	ND	ND	ND
13	ND	ND	ND	ND	ND	ND	ND	ND	ND	1,600	400	400	3,200
16	<100	<100	<100	800	3,200	800	400	6,400	1,600	ND	ND	ND	ND
31	ND	ND	ND	ND	ND	ND	ND	ND	ND	1,600	400	800	1,600

[a] Expressed as the reciprocal of the highest plasma dilution to yield a positive result.
[b] ND, not determined.

TABLE 2. Detection of viral p24 core antigen in PBMC culture supernatants from rabbits inoculated with ACH-transfected or naive PBMC[a]

Wk p.i.	No. of rabbits positive for p24/no. monitored (p24 concn)[b]	
	Naive	ACH transfected
0	0/3 (<15)	0/10 (<15)
2	0/3 (<15)	10/10 (252 ± 113)
4	0/3 (<15)	10/10 (409 ± 263)
6	0/3 (<15)	10/10 (248 ± 97)
8	0/3 (<15)	10/10 (411 ± 256)
13	ND[c]	4/4 (149 ± 103)

[a] PBMC cultures were established from rabbits at time points indicated, and culture supernatants were tested for p24 by ELISA on day 3 of culture.
[b] ELISA sensitivity, ≥15 pg/ml. p24 concentrations are in picograms per milliliter and are presented as means ± standard deviations.
[c] ND, not determined.

blood at 0, 2, 4, 6, 8, and 13 (group I) or 2, 4, 6, and 8 (group II) weeks p.i. and cultured. Culture supernatants were tested for HTLV-1 p24 after 72 h of culture by antigen capture ELISA (Coulter Corp.). Viral antigen was detected for all ACH-inoculated rabbits at all time points tested. Control rabbit PBMC cultures were negative for viral p24 antigen (Table 2). Mesenteric lymph nodes were obtained from rabbits R46, R47, and R2 through R8 at necropsy. Rabbits were perfused with sodium phosphate buffer prior to lymph node harvest to minimize blood contamination. Viral p24 antigen was detected in lymph node culture supernatants from all ACH-inoculated group II rabbits at 16 weeks p.i. (R5 through R8). No viral antigen was detected in lymph node cultures from group I rabbits (R46 and R47) at 31 weeks p.i. or from group II negative control rabbits (R2 through R4) at 16 weeks p.i.

PCR was used to detect HTLV-1 provirus in PBMC from ACH-inoculated rabbits. PBMC were collected from group I rabbits on weeks 2, 8, and 31 p.i. and from group II rabbits on weeks 2, 8, and 16 p.i. Genomic DNA was extracted from PBMC by a spin column procedure (QIAamp; Qiagen, Chatsworth, Calif.) and amplified as previously described (5) with a Gag gene-specific primer pair (SG 296 and SG 166) (9). HTLV-1 Gag gene-specific sequences were amplified from all ACH-inoculated rabbits at all time points tested (Fig. 4). Specificity was confirmed by slot blot hybridization with SG 242 (Gibco-BRL), a Gag gene-specific oligonucleotide probe ^{32}P end labeled with T4 kinase (Boehringer Mannheim) (9). Amplified sequences, as analyzed by agarose gel electrophoresis, had molecular sizes compatible with the predicted size of 272 bp.

Since ex vivo PBMC from HTLV-1-infected humans undergo spontaneous proliferation in the absence of exogenous stimuli (17, 24, 33, 34), we tested whether this would occur in PBMC derived from ACH-inoculated rabbits. Spontaneous proliferation of PBMC from HTLV-1-infected rabbits was assessed by determining [^{3}H]thymidine incorporation as previously described (21). No significant difference in mean counts per minute of [^{3}H]thymidine-spiked PBMC between ACH-inoculated rabbits and controls was detected (data not shown). No clinical, hematologic, or pathologic alterations among the inoculated or control rabbits were detected during the course of the 31-week study (data not shown).

Our data indicate that, like wild-type HTLV-1, ACH is capable of producing infection in rabbits. All rabbits inoculated with ACH-transfected primary cells became infected, as evidenced by their seroconversion to positivity for antibodies against all major viral proteins, the production of viral p24 antigen by PBMC and lymph node cultures, and the detection of virus in PBMC by PCR. While it may be argued that seroconversion in rabbits was in response to the initial large inoculum rather than infection, this possibility seems remote in light of the steady or rising antibody titers over the 16- or 31-week course of the experiments. Successful transfer of the infection was supported by detection of p24 antigen in PBMC and lymph node cultures and PCR amplification of viral sequences from PBMC. It is unlikely that these results are due to persistence or outgrowth of the foreign cellular inoculum since all cells were lethally irradiated prior to inoculation and, for 8 of 10 rabbits, the inoculum consisted of allogeneic or xenogeneic cells, which are unlikely to persist in an immunocompetent host. Finally, there were no hematological or postmortem changes in the rabbits that would have indicated an outgrowth of the introduced cell population.

A recent report by Zhao et al. provides preliminary data suggesting in vivo infectivity of a clone in two rabbits (43). Likewise, we show here that ACH was infectious in 10 of 10 inoculated rabbits. This consistency of infection makes ACH ideal for in vivo use. Furthermore, our use of primary cells as the inoculum for in vivo experiments should minimize the effect of unwanted variables on infectivity. Zhao et al. used a herpesvirus-transformed cell line as the inoculum in their in vivo experiments. It is unknown what effect the herpesvirus genome may have had on parameters of HTLV-1 infectivity such as virus production. There are precedents in other retroviral systems for upregulation of viral expression due to concomitant herpesvirus infection, as seen with human immunodeficiency virus and cytomegalovirus coinfection (7).

Viral mutants derived from HTLV-1 molecular clones can be used in studies similar to this one to investigate the role of viral genes in cellular transformation and proliferation. The use of HTLV-1 molecular clones in rabbits will facilitate the identification of viral gene products important for infectivity, latency, tissue distribution, and host immunologic response

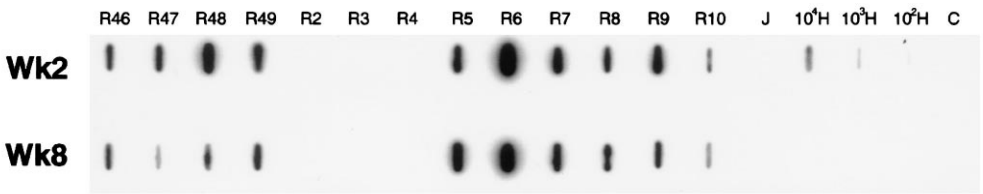

FIG. 4. Detection of HTLV-1 *gag* sequences in DNA isolated from PBMC of rabbits inoculated with ACH-transfected PBMC (R46 through R49 and R5 through R10) or naive PBMC (R2 through R4), performed at 2 and 8 weeks (WK) p.i. J, negative control for which DNA from an HTLV-1-negative T-cell line (Jurkat) was used; C, negative control for which water was used in lieu of template DNA; 10^4H, 10^3H, and 10^2H, positive controls for which DNA from 10-fold dilutions of an HTLV-1-positive T-cell line (HuT102) in constant numbers of Jurkat cells was used. No viral DNA was detected in PBMC from control rabbits, whereas viral DNA was detected in PBMC from all ACH-inoculated rabbits.

and has the potential to provide a system for the creation and testing of attenuated live virus vaccines. For instance, it has recently been shown that mutations in the R3 and G4 genes of the closely related bovine leukemia virus resulted in an attenuated phenotype in vivo, with decreases in viral loads and frequency of leukemia in sheep compared with those produced by wild-type virus (40). HTLV-1 *tof* and *rof* show some homology with R3 and G4 and may have similar effects.

Complete understanding of the pathogenesis of HTLV-1 infection will require the development of molecular clones capable of reproducing the natural infection. While capable of infecting a number of cell targets, the virus preferentially targets and transforms $CD4^+$ T lymphocytes (13, 15, 28, 35). Utilization of HTLV-1 molecular clones that demonstrate this ability has great biological significance. ACH appears to be ideally suited for such investigations since it mimics the behavior of wild-type HTLV-1. In this report we demonstrate that the HTLV-1 ACH molecular clone, following transfection in both human and rabbit PBMC, caused sustained virus replication and subsequently transformed rabbit $CD4^+$ lymphocytes. Importantly, lymphocytes infected with ACH produced infectious virus and transmitted HTLV-1 infection to rabbits. Further delineation of the in vivo tissue expression of ACH is the subject of ongoing studies. These studies will provide the necessary background for investigations designed to learn the effects of viral mutants derived from ACH on viral infectivity and tissue distribution, as well as to facilitate the development of live virus vaccines against HTLV-1 infection.

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