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Dual Infection of Rabbits with Human T Cell Lymphotropic Virus Types I and II

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Attempts were made to generate a rabbit model of dual infection with human T lymphotropic virus (HTLV) types I and II. Four groups (A, B, C, and D) of three rabbits each were used. Group A was inoculated with the RW-1 cell line coinfected with HTLV-I and HTLV-II and group B was transfused from a dually infected rabbit. Polymerase chain reaction (PCR) using primers specific for the *pol* region of each virus detected both HTLV-I and HTLV-II in all group A and two group B rabbits, but HTLV-II only in the remaining group B rabbit. Groups C and D already infected with HTLV-I and HTLV-II, respectively, were inoculated with an HTLV-II- or HTLV-I-producing cell line. One group C rabbit became PCR-positive for both viruses but the other five resisted superinfection with the respective viruses. During prolonged observation, three of the six dually infected rabbits converted to single (HTLV-I or HTLV-II) infection. The *in vivo* dual infection was confirmed by *in vitro* establishment of a lymphoid cell line coinfected with HTLV-I and HTLV-II. It was also possible to establish coinfected lymphoid cell lines from HTLV-I-infected rabbits by coculture with lethally irradiated HTLV-II-producing cells and *vice versa*. The mechanism of viral elimination in dually infected rabbits, as well as that of protective immunity against superinfection, remains to be elucidated.

Key words: Dual infection — Rabbit — HTLV-I — HTLV-II

Human T cell lymphotropic virus (HTLV) types I and II share 60% sequence homology, antigenic cross-reactivity, and a common cellular receptor. HTLV-I is endemic in southwestern Japan, the Caribbean basin, and parts of Africa, while HTLV-II infection is prevalent among intravenous drug users and certain Amerindian populations.[1] HTLV-I is causally related to adult T cell leukemia (ATL), tropical spastic paraparesis/HTLV-I-associated myelopathy, and uveitis, whereas HTLV-II has been associated with sporadic cases of hematologic and neurologic disorders.[1] Some persons at high risk for HTLV infection were found to be dually or triply infected with HTLV-I, HTLV-II, and human immunodeficiency virus (HIV) type I.[2] Interactions between these retroviruses and their combined pathogenicity at the host level are poorly understood. Our *in vitro* studies showed that the same rabbit lymphocyte could be coinfected with HTLV-I and HTLV-II.[3] In the present study, rabbits were tested for susceptibility to dual infection with HTLV-I and HTLV-II by simultaneous or sequential virus exposure.

MATERIALS AND METHODS

Rabbits Japanese white rabbits weighing approximately 3 kg were purchased from Shimizu Laboratory Supplies (Kyoto) and were maintained in biosafety level 3 containment.

Virus Three virus-producing lymphoid cell lines were used: Ra-1 (male rabbit) infected with HTLV-I,[4] Si-IIA (male cynomolgus monkey) infected with HTLV-II,[5] and RW-1 (female rabbit) dually infected with HTLV-I and HTLV-II.[3]

Serologic tests Serum samples were tested by enzyme-linked immunosorbent assay (ELISA) for IgG antibodies against disrupted HTLV-I virions (Eisai, Tokyo) and positive results were confirmed by western blotting using HTLV 2.4 strips (Genelabs Diagnostics, Singapore). These strips incorporated HTLV-I virus lysate, recombinant *env* protein GD21 (rgp21) shared by HTLV-I and HTLV-II, HTLV-I-specific recombinant *env* protein MTA-1 (rgp46-I), and HTLV-II-specific recombinant *env* protein K55 (rgp46-II).

Virus isolation Twenty milliliters of blood was collected from rabbits experimentally infected with HTLV-I, HTLV-II, or both HTLV-I and HTLV-II, and peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Lympholyte-Rabbit (Cedarlane Laboratories, Hornby, Canada). PBMC were plated at a density of 1×10^6/ml in 35-mm Petri dishes containing 3 ml of RPMI 1640 medium supplemented with 20% fetal calf serum, 1 μg/ml of phytohemagglutinin, and antibiotics. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere and were fed twice weekly with medium containing 50 units/ml of recombinant human interleukin-2 (IL-2) (Ajinomoto, Tokyo) instead of phytohemagglutinin. For *in vitro* superinfection, 1×10^6/ml of PBMC from HTLV-I-infected rabbits were cocultured

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with an equal number of γ-irradiated (10,000 rad) HTLV-II-producing cells and *vice versa*.

Cell surface and viral antigens Cells were stained by indirect membrane immunofluorescence using monoclonal antibodies (mAbs) to rabbit CD4, CD5, CD8, CD25, and major histocompatibility complex (MHC) class II antigens, as described previously.[3] HTLV antigens were examined by fixed-cell indirect immunofluorescence with reference sera from ATL patients and HTLV-II carriers, mAbs to HTLV-I p19 and p24 purchased from Chemicon International (Temecula, CA), and HTLV-I gp46-specific mAb (B1E191N43).[6]

Polymerase chain reaction (PCR) Genomic DNA, extracted from rabbit PBMC and rabbit cell lines, was examined for HTLV sequences by PCR, using oligonucleotide primer pairs SK54/55 for HTLV-I *pol* and SK58/59 for HTLV-II *pol*.[2] DNA (1 μg) was subjected to 40 cycles of denaturation (94°C for 1 min) followed by annealing (55°C for 2 min) and extension (72°C for 1.5 min). Amplification was performed using a thermostable DNA polymerase in an automated DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). DNA controls were included with each analysis. The amplified products were electrophoresed on 6% polyacrylamide gels, transferred to nylon membranes, and hybridized with ^{32}P-end-labeled probes SK56 and SK60. Autoradiographic detection of amplimers of 119 and 103 bp was considered positive for the HTLV-I and HTLV-II *pol* genes, respectively.

RESULTS

Simultaneous transmission of HTLV-I and HTLV-II All three male rabbits in group A (1 to 3) inoculated intravenously with 1×10^7 RW-1 cells seroconverted to HTLV after 2 weeks. Three months later, three group B female rabbits (4 to 6) were transfused with 5 ml of blood from rabbit 3. Seroconversion to HTLV occurred in all three recipient rabbits after 4 to 6 weeks. Gene amplification by PCR, conducted 6 months after inoculation of group A rabbits with RW-1 cells, revealed the presence of both HTLV-I and HTLV-II sequences in all group A rabbits (Table I). On repeat analyses at 10 and 13 months, loss of HTLV-I infection was observed in one (rabbit 2) of the three rabbits (Fig. 1). In group B, rabbits 5 and 6 were found to be dually infected with HTLV-I and HTLV-II and rabbit 4 singly infected with HTLV-II at 2.5 months post-transfusion (Table I). When re-examined at 6 and 9.5 months, HTLV-II sequences were no longer detectable in rabbits 5 and 6 (Fig. 1).

Fifteen months after inoculation with RW-1 cells, PBMC from rabbit 1 cultured in the presence of IL-2 gave rise to an IL-2-independent lymphoid cell line after 2 months (Fig. 2A). This cell line, designated W1, grew in suspension with clumps of cells and consisted of prim-

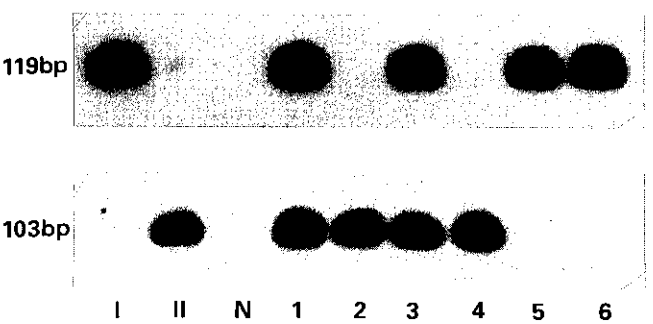

Fig. 1. PCR analysis on group A and B rabbits using a set of primers and probes specific for the *pol* region of each of HTLV-I and HTLV-II. Rabbits 1 and 3 show *pol* sequences of both viruses, while rabbits 2 and 4 show HTLV-II *pol* sequences only, and rabbits 5 and 6 show HTLV-I *pol* sequences only. I and II denote an HTLV-I- and HTLV-II-infected control rabbit, respectively, and N, a normal control rabbit. These data correspond to the final PCR analysis shown in Table I.

Table I. Detection of HTLV-I and HTLV-II *pol* Gene Sequences by PCR in Simultaneously Infected Rabbits

PCR done after (months)	Virus	Group A inoculated with RW-1[a]			PCR done after (months)	Virus	Group B transfused from coinfected rabbit 3		
		1	2	3			4	5	6
6	HTLV-I	+	+	+	2.5	HTLV-I	−	+	+
	HTLV-II	+	+	+		HTLV-II	+	+	+
10	HTLV-I	+	−	+	6	HTLV-I	−	+	+
	HTLV-II	+	+	+		HTLV-II	+	−	−
13	HTLV-I	+	−	+	9.5	HTLV-I	−	+	+
	HTLV-II	+	+	+		HTLV-II	+	−	−

a) RW-1 is a rabbit cell line dually infected with HTLV-I and HTLV-II.[3]

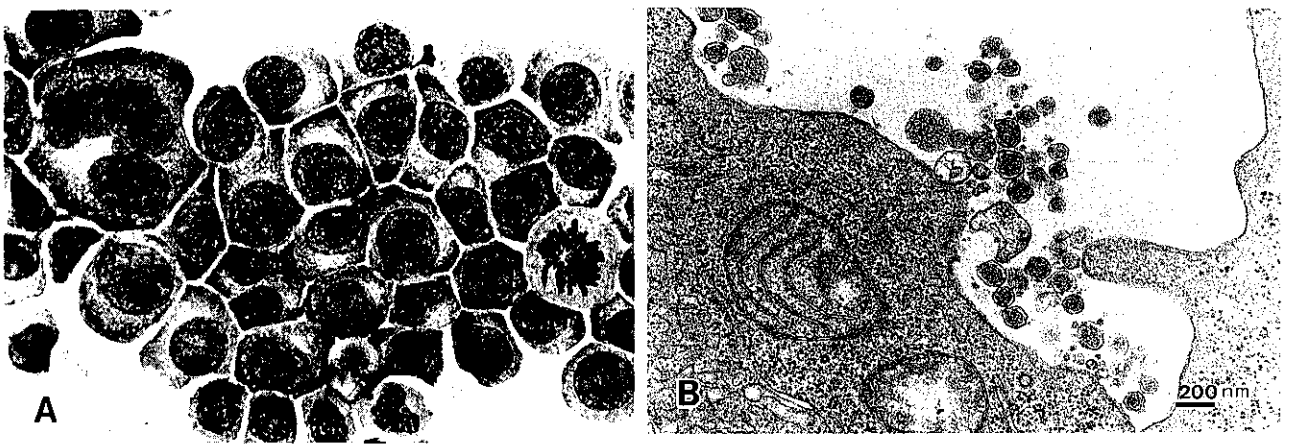

Fig. 2. The W1 cell line established from rabbit 1 coinfected with HTLV-I and HTLV-II. A, Light micrograph showing primitive lymphoid cells with multinucleate giant cells (May-Grünwald-Giemsa, ×630). B, Electron micrograph showing type C virus particles in the intercellular spaces (uranyl acetate and lead citrate, ×26,700).

Table II. Detection of HTLV-I and HTLV-II *pol* Gene Sequences by PCR in Sequentially Infected Rabbits

PCR done after (weeks)	Virus	Group C inoculated with HTLV-I-infected blood followed by Si-IIA[a]			PCR done after (weeks)	Virus	Group D inoculated with Si-IIA followed by Ra-1[b]		
		7	8	9			10	11	12
10	HTLV-I	+	+	+	10	HTLV-I	−	−	−
	HTLV-II	−	−	+		HTLV-II	+	+	+
15	HTLV-I	+	+	+	15	HTLV-I	−	−	−
	HTLV-II	−	−	+		HTLV-II	+	+	+
25	HTLV-I	+	+	+	25	HTLV-I	−	−	−
	HTLV-II	−	−	+		HTLV-II	+	+	+

a) Si-IIA is an HTLV-II-infected cynomolgus monkey cell line.[5]
b) Ra-1 is an HTLV-I-infected rabbit cell line.[4]

itive lymphoid cells with multinucleate giant cells (Fig. 2A). W1 cells had a normal male rabbit karyotype, and expressed rabbit CD4, CD5, CD25, and MHC class II antigens, but lacked CD8 antigens. Immunofluorescence staining demonstrated that most of the W1 cells were reactive with HTLV-I p19 and p24 mAbs and HTLV-I-specific B1E191N43, as well as sera from ATL patients and HTLV-II carriers. Electron microscopy of these cells revealed many type C virus particles thought to represent HTLV-I and HTLV-II (Fig. 2B). PCR analysis verified the presence of both HTLV-I and HTLV-II proviral DNA in this cell line at 6 and 12 months after the beginning of culture.

Sequential transmission of HTLV-I and HTLV-II Two male and one female rabbits in group C (7 to 9) were first transfused with 5 ml of blood from an HTLV-I-infected rabbit and were challenged with 1×10^7 HTLV-II-infected Si-IIA cells 13 months later. When analyzed by PCR at 10, 15 and 25 weeks post-challenge, dual infection with HTLV-I and HTLV-II was found in one (rabbit 9) of them and rabbits 7 and 8 showed only HTLV-I infection (Table II, Fig. 3). Conversely, three female rabbits in group D (10 to 12) were first inoculated intravenously with 1×10^7 HTLV-II-infected Si-IIA cells and were challenged with 1×10^7 HTLV-I-infected Ra-1 cells 14 weeks later. PCR analysis, performed at 10, 15 and 25 weeks post-challenge, repeatedly demonstrated only HTLV-II infection in all of them (Table II, Fig. 3). At 26 weeks post-challenge, these three rabbits were transfused with 5 ml of blood from an HTLV-I-infected

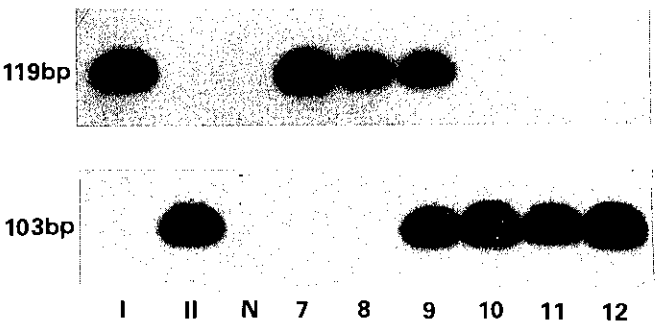

Fig. 3. PCR analysis on group C and D rabbits using a set of primers and probes specific for the *pol* region of each of HTLV-I and HTLV-II. Rabbit 9 shows *pol* sequences of both viruses, whereas rabbits 7 and 8 show HTLV-I *pol* sequences only, and rabbits 10, 11, and 12 show HTLV-II *pol* sequences only. These data correspond to the final PCR analysis shown in Table II.

rabbit but they all remained PCR-negative for HTLV-I after 11 weeks. At this point, three normal rabbits were inoculated with 1×10^7 Ra-1 cells as controls and they all became infected with HTLV-I as indicated by seroconversion and PCR-positivity for HTLV-I after 10 weeks.

Four weeks prior to superinfection *in vivo*, PBMC from three group C rabbits were cocultured with lethally irradiated Si-IIA cells. Lymphoid cell lines, designated W7, W8 and W9, were established from each of the group C rabbits after one month. Two of these cell lines (W7 and W9) became IL-2-independent one month later. The three cell lines expressed an activated helper T-cell phenotype positive for CD4, CD5, CD25, and MHC class II antigens and possessed a normal rabbit karyotype. All three cell lines expressed viral antigens as detected by immunofluorescence, and type C virus particles were detected by electron microscopy. In addition, HTLV-I and HTLV-II proviral DNA were demonstrated in each cell line by PCR 2 and 6 months after the start of coculture. On the other hand, six months after rechallenge with HTLV-I-infected blood, PBMC from rabbit 12 in group D were cocultured with lethally irradiated Ra-1 cells. This led to the establishment of a female lymphoid cell line (W12) shown to be dually infected with HTLV-I and HTLV-II by PCR after 2 months.

In addition, we analyzed DNA extracted from the thymus, mesenteric lymph node, and spleen of rabbits 5 and 9 more than two years after the start of the experiment. The thymus and spleen from rabbit 5 were PCR-positive for HTLV-I only and all three organs from rabbit 9 contained both HTLV-I and HTLV-II. These results were concordant with the PCR data on PBMC from these two rabbits.

DISCUSSION

In the present study, we have demonstrated that rabbits can be dually infected with HTLV-I and HTLV-II, when inoculated with a dually infected cell line or transfused with blood from a dually infected rabbit. In contrast, superinfection of HTLV-I-infected rabbits with HTLV-II and *vice versa* resulted in coinfection with HTLV-I and HTLV-II in only one of six rabbits. Dual infection in these animals was demonstrated by PCR using *pol* primers specific for each virus and was confirmed by *in vitro* establishment of a dually infected lymphoid cell line from one rabbit. These findings provide an experimental basis for the reported occurrence of HTLV-I and HTLV-II coinfection among intravenous drug users.[2] Presumably, HTLV-I and HTLV-II will each infect different lymphocyte populations in rabbits and humans. However, possible coinfection of the same lymphocyte *in vivo* cannot be excluded, since rabbit lymphocytes were shown to be dually infected at the single cell level, when they were exposed simultaneously to HTLV-I and HTLV-II *in vitro*.[3] Nevertheless, lymphoid cell lines already infected with HTLV-I could not be superinfected with HTLV-II and *vice versa*, apparently due to viral receptor interference.[3]

Group A and group B rabbits (1 to 6) that were infected simultaneously with HTLV-I and HTLV-II were serially analyzed three times by PCR for HTLV status. Unexpectedly, three rabbits (2, 5, and 6) converted from dual to single infection between the first and second PCR analyses: rabbit 2 lost HTLV-I and rabbits 5 and 6 eliminated HTLV-II during the 3.5- to 4-month period. The first PCR analysis on groups A and B was performed at 6 and 2.5 months post-infection, respectively. It may be argued that the transient viral gene amplification was due to the inoculated lymphocytes used for infection. However, it is unlikely that virus-infected rabbit cells persisted up to the time of the first PCR analysis in the allogeneic hosts. In fact, our passive immunization experiments suggested that virus-infected rabbit lymphocytes would be rejected within two weeks of inoculation and that HTLV-I or HTLV-II would no longer be detectable by PCR at 10 weeks, unless virus had been transmitted to host lymphocytes.[7,8] Thus, it is more likely that HTLV-I and HTLV-II were initially transmitted to host lymphocytes and that one of these viruses was selectively eliminated, probably by cytotoxic T lymphocyte-mediated immunity[9,10] and/or antibody-dependent cellular cytotoxicity, which is cross-reactive between HTLV-I and HTLV-II.[11] No such virus elimination has been documented in humans infected with HTLV-I or HTLV-II or both.

Sequential infection with HTLV-I and HTLV-II gave somewhat unexpected results. One of the three HTLV-I-

infected rabbits in group C became superinfected with HTLV-II. In group D, none of the three HTLV-II-infected rabbits became superinfected with HTLV-I and they were refractory to repeated superinfection with HTLV-I. In our preliminary study, two other HTLV-II-infected rabbits likewise proved refractory to subsequent infection by blood transfusion from an HTLV-I-infected rabbit (unpublished observation). This cannot be explained by humoral immunity *per se*, since there is no cross-reactivity of neutralizing antibodies between HTLV-I and HTLV-II.[8] Nor is the resistance to superinfection accounted for by viral receptor interference that is applicable only to virus-infected lymphocytes. Roughly 1% to 10% of rabbit PBMC are thought to be infected with HTLV-I *in vivo*.[12] In view of a 100% virus transmission rate to naive rabbits by inoculation of 1×10^7 HTLV-I- or HTLV-II-producing cells or 5 ml of blood from HTLV-I-infected rabbits,[7,8,13] the lack of superinfection in group C and group D rabbits can be best explained by cellular immunity cross-reactive between HTLV-I and HTLV-II. The present observation may be relevant to the recent report that human HIV-2 infection provides significant protection from subsequent HIV-1 infection in a cohort of commercial sex workers in Senegal.[14] This issue of HIV-2-mediated inhibition of HIV-1 has been extensively analyzed *in vitro* by Arya and Gallo.[15] Despite the *in vivo* resistance to superinfection, PBMC from two group C rabbits were susceptible to *in vitro* HTLV-II infection in that they were transformed into lymphoid cell lines dually infected with HTLV-I and HTLV-II. Similarly, PBMC from one group D rabbit were transformed, after *in vitro* infection with HTLV-I, into a lymphoid cell line carrying both viruses. All these findings point to complex interactions between HTLV-I and HTLV-II and suggest that cross-protective cellular immunity (cytotoxic T lymphocyte response and/or antibody-dependent cellular cytotoxicity) renders HTLV-I-infected rabbits resistant to subsequent infection with HTLV-II and *vice versa*. Elucidation of the mechanisms responsible for this phenomenon, as well as virus elimination in dually infected animals, will contribute to the development of new strategies such as the use of attenuated or recombinant live viruses for the prevention of these retroviral infections.

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REFERENCES

1) Blattner, W. A. and Gallo, R. C. Epidemiology of HTLV-I and HTLV-II infection. *In* "Adult T-Cell Leukaemia," ed. K. Takatsuki, pp. 45–90 (1994). Oxford University Press, Oxford.
2) Ehrlich, G. D., Glaser, J. B., La Vigne, K., Quan, D., Mildvan, D., Sninsky, J. J., Kwok, S., Papsidero, L. and Poiesz, B. J. Prevalence of human T-cell leukemia/lymphoma virus (HTLV) type II infection among high-risk individuals: type-specific identification of HTLVs by polymerase chain reaction. *Blood,* **74,** 1658–1664 (1989).
3) Kubota, T., Morishita, N., Tanaka, Y., Sawada, T., Miyagi, T., Ohtsuki, Y. and Miyoshi, I. Establishment of novel lymphoid cell lines dually infected with human T cell lymphotropic viruses types I and II. *J. Infect. Dis.,* **172,** 220–224 (1995).
4) Miyoshi, I., Yoshimoto, S., Taguchi, H., Kubonishi, I., Fujishita, M., Ohtsuki, Y., Shiraishi, Y. and Akagi, T. Transformation of rabbit lymphocytes with T-cell leukmia virus. *Gann,* **74,** 1–4 (1983).
5) Miyamoto, K., Tomita, N., Hayashi, K. and Akagi, T. Transformation of animal cells with human T-cell leukemia virus type II. *Jpn. J. Cancer Res.,* **81,** 720–722 (1990).
6) Tanaka, Y., Tanaka, R., Terada, E., Koyanagi, Y., Miyano-Kurosaki, N., Yamamoto, N., Baba, E., Nakamura, M. and Shida, H. Induction of antibody responses that neutralize human T-cell leukemia virus type I infection *in vitro* and *in vivo* by peptide immunization. *J. Virol.,* **68,** 6323–6331 (1994).
7) Tanaka, Y., Ishii, K., Sawada, T., Ohtsuki, Y., Hoshino, H., Yanagihara, R. and Miyoshi, I. Prophylaxis against a Melanesian variant of human T-lymphotropic virus type I (HTLV-I) in rabbits using HTLV-I immune globulin from asymptomatically infected Japanese carriers. *Blood,* **82,** 3664–3667 (1993).
8) Morishita, N., Ishii, K., Tanaka, Y., Sawada, T., Hoshino, H., Foung, S. K. H. and Miyoshi, I. Immunoglobulin prophylaxis against human T cell lymphotropic virus type II in rabbits. *J. Infect. Dis.,* **169,** 620–623 (1994).
9) Jacobson, S., Shida, H., McFarlin, D. E., Fauci, A. S. and Koenig, S. Circulating $CD8^+$ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature,* **348,** 245–248 (1990).
10) Kannagi, M., Harada, S., Maruyama, I., Inoko, H., Igarashi, H., Kuwashima, G., Sato, S., Morita, M., Kidokoro, M., Sugimoto, M., Funahashi, S., Osame, M. and Shida, H. Predominant recognition of human T-cell leukemia virus type I (HTLV-I) pX gene products by human $CD8^+$ cytotoxic T cells directed against HTLV-I-infected cells. *Int. Immunol.,* **3,** 761–767 (1991).
11) Zhang, X.-Q., Yang, L., Ho, D. D., Kuritzkes, D. R.,

Chen, I. S. Y., Ching, W. T. W., Chen, Y. M. A. and Schooley, R. T. Human T lymphotropic virus types I- and II-specific antibody-dependent cellular cytotoxicity: strain specificity and epitope mapping. *J. Infect. Dis.,* **165,** 805–812 (1992).

12) Franchini, G., Tartaglia, J., Markham, P., Benson, J., Fullen, J., Wills, M., Arp, J., Dekaban, G., Paoletti, E. and Gallo, R. C. Highly attenuated HTLV type I_{env} poxvirus vaccines induce protection against a cell-associated HTLV type I challenge in rabbits. *AIDS Res. Hum. Retroviruses,* **11,** 307–313 (1995).

13) Kataoka, R., Takehara, N., Iwahara, Y., Sawada, T., Ohtsuki, Y., Dawei, Y., Hoshino, H. and Miyoshi, I. Transmission of HTLV-I by blood transfusion and its prevention by passive immunization in rabbits. *Blood,* **76,** 1657–1661 (1990).

14) Travers, K., Mboup, S., Marlink, R., Guèye-Ndiaye, A., Siby, T., Thior, I., Traore, I., Dieng-Sarr, A., Sankalé, J.-L., Mullins, C., Ndoye, I., Hshieh, C.-C., Essex, M. and Kanki, P. Natural protection against HIV-1 infection provided by HIV-2. *Science,* **268,** 1612–1615 (1995).

15) Arya, S. K. and Gallo, R. C. Human immunodeficiency virus (HIV) type 2-mediated inhibition of HIV type 1: a new approach to gene therapy of HIV infection. *Proc. Natl. Acad. Sci. USA,* **93,** 4886–4891 (1996).