Brief Definitive Report

SERONEGATIVE VIRUS CARRIERS IN THE INFECTION OF RABBITS WITH HUMAN T LYMPHOTROPIC VIRUS TYPE I

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Human T lymphotropic virus type I (HTLV-I) (1) is a putative etiological agent of adult T cell leukemia (ATL) (2), which is endemic in southwestern Japan. Antibody against this virus is detected in the serum from most ATL patients, suggesting an etiological role of the virus in this disease (3). The same antibody is also detected in a high proportion (10–30%) of healthy inhabitants of the endemic area (4). Virus-infected cells can be recovered from the peripheral blood of these healthy seropositive individuals, indicating that they are carriers of the HTLV-I virus (5).

We recently succeeded in the induction of a fulminant ATL-like disease as well as a preleukemic stage of this disease in rabbits (6, 7). This animal model closely resembles human ATL in the development of leukocytosis associated with the appearance of abnormal lymphocytes and of leukemic infiltration in the major organs. The use of inbred rabbits in this model seems to facilitate studies on immunological aspects of the disease. We now report that neonatal infection of rabbits with HTLV-I could result in infection without antivirus antibody response, and we hypothesize that the same may happen in humans.

Materials and Methods

Rabbits. Rabbits of two inbred strains, B/J and Chbb:HM, and their F_1 hybrid were bred in our laboratory and used for the experiment. These strains were originally derived from The Jackson Laboratory (Bar Harbor, ME) and Dr. Karl Thomae GmbH (Biberach, FRG), respectively (6).

Cell Lines. HTLV-I-transformed cell lines were established either by coculture of uninfected peripheral blood lymphocytes (PBL) with the virus-producing human T cell line MT2, or by culture of PBL from HTLV-I-infected rabbits in RPMI 1640 medium supplemented with 10% FCS and 10% T cell growth factor (TCGF; Japan Immunoresearch Laboratories, Takasaki, Japan) (6). Two (B425 and H446) of six cell lines used for in vivo inoculation were obtained by the former method and the remaining four by the latter method. Establishment of cell lines from seronegative animals was carried out by culturing Ficoll Paque-isolated PBL or whole blood in RPMI 1640 medium supplemented with 10% FCS and 10% TCGF.

In Vivo Inoculation of Cell Lines. HTLV-I-transformed cells ($2–50 \times 10^6$) were injected subcutaneously or intraperitoneally into neonatal animals 3 d after birth. Injection into adult animals was carried out subcutaneously or intravenously with 2×10^6 cells. Cultured cells were washed twice with HBSS before inoculation.

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Assays for Detection of Anti-HTLV-I Antibody and HTLV-I Antigen. Antivirus antibody was assayed by indirect immunofluorescence (3) and by the agglutination test. In brief, HUT 102 cells were smeared on slide glasses, allowed to react with appropriately diluted serum, and then with FITC-labeled anti-rabbit Ig (goat). The smears were then examined under a fluorescence microscope. The agglutination test of virus antigen-coated particles was carried out with a Serodia ATLA kit (Fujirebio Co., Tokyo, Japan). The presence or absence of HTLV-I antigen in cells was determined by indirect immunofluorescence with mAb against HTLV-I core protein, p19.

Southern Blot Hybridization Analysis. High molecular weight DNA was digested with Eco RI, subjected to electrophoresis in 0.6% agarose gel, transferred to nitrocellulose membrane, and hybridized with a radiolabeled pMT2/65 probe. The membrane was washed in 0.5 × SSC with 0.1% SDS at 65°C.

Neonatal Inoculation of Shope Papilloma Virus (SPV). Glycerinated Shope papillomas were obtained from the Earl Johnson Farm (Rago, KS) and stored at −20°C until use. Virus suspension (10% tumor extract) was prepared as described previously (8) and 0.05 ml of the suspension was injected intradermally on the dorsal area.

Neutralization of SPV. A 10% SPV suspension was mixed with an equal volume of serum specimen and allowed to react at 37°C for 30 min. The mixture was examined for papilloma-inducing capacity by inoculation onto the skin of an adult rabbit by the scarification method, as described previously (8).

Results

Characteristics of cell lines used for in vivo inoculation are shown in Table I. All six cell lines were negative for surface Ig but positive for pan-T cell marker and for HTLV-I p19, indicating that all 6 lines are HTLV-I-infected T cells. All but one (B684) cell line were Ia^+, and B425 cell line alone required TCGF for its growth. Two (B405 and B684) of six cell lines, but not four others, could induce lethal leukemic infiltration in syngeneic newborn animals when inoculated intraperitoneally at a dose of 10^7 cells (data not shown).

As shown in Table II, none of 3 B/J cell lines (B405, B425, and B684) induced antibody response upon neonatal inoculation into B/J rabbits, irrespective of the route of injection and the number of cells inoculated, whereas all Chbb:HM cell lines (H446, H676, H681) did induce a response in Chbb:HM rabbits. B/J cells, however, were able to induce antibody response in adult B/J rabbits, and Chbb:HM cells induced a response in a newborn B/J rabbit. Similarly, it was a Chbb:HM cell

TABLE I

Characteristics of Transformed Cell Lines

	Cell lines					
Cell marker	B405	B425	B684	H446	H676	H681
Rabbit strain*	B	B	B	H	H	H
sIg	−‡	−	−	−	−	−
Pan T marker (mAb L11/135)	+	+	+	+	+	+
Ia (mAb 2C4)	+	+	−	+	+	+
HTLV-I p19	+	+	+	+	+	+
Karyotype	44,XY	44,XY	44,XX	44,XY	ND	ND
TCGF requirement	No	Yes	No	No	No	No

* B: B/J, H: Chbb:HM

‡ Determined by indirect immunofluorescence. −, Negative; +, positive. ND, not determined.

TABLE II

Anti-HTLV-I Titer in Rabbits Inoculated with HTLV-I-transformed Cells

Rabbit		Inoculation of transformed cells				
Strain	No.	Age*	Cell	× 10^6	Route	Antibody titer‡
B/J	618	N	B425	2	s.c.	– (B425, –§)
	721	N	B425	10	i.p.	– (B405, 1:40)
	722	N	B425	50	i.p.	– (B684, 1:20)
	761	N	B684	5	i.p.	– (B405, 1:80)
	779	N	B405	5	i.p.	– (B684, 1:10)
	790¶	N	B405	5	i.p.	–§
	802¶	N	B405	5	i.p.	–§
	780	N	H446	5	i.p.	1:40
Chbb:HM	776	N	H681	10	i.p.	1:40
	777	N	H676	10	i.p.	1:80
	778	N	H446	10	i.p.	1:40
B/J	612	5 wk	B425	2	s.c.	1:10 (B425, 1:320)
	613	5 wk	B425	2	s.c.	1:10 (B425, 1:320)
	630	5 wk	B425	2	i.v.	1:10
	619	10 wk	B425	2	s.c.	1:10
	620	13 wk	B425	2	s.c.	1:10
F_1	634	N	B425	2	s.c.	– (B425, –§)
	637	N	B425	2	s.c.	– (B425, –§)
	649	N	B425	2	s.c.	– (H446, 1:160)
	650	N	B425	2	s.c.	– (H446, 1:80)
	647	N	H446	2	s.c.	1:160
	648	N	H446	2	s.c.	1:640

* Age at which cells were injected. N, neonatal; wk, weeks.

‡ Determined 5 wk after inoculation by indirect immunofluorescence. –, <1:10. Shown in parentheses are cells reinoculated i.v. at a dose of 2 × 10^6 and antibody titer after reinoculation.

§ Remained seronegative for more than 15 wk after inoculation.

¶ Simultaneously given HTLV-I and SPV. Papilloma and anti-SPV antibody were induced and persisted.

line, but not a B/J cell line, that induced antibody response in F_1 hybrids upon neonatal inoculation. Absence of antivirus antibody was confirmed also by the agglutination test using virus antigen-coated particles. Reinoculation of the same quantity of the same cell line into two F_1 hybrids (Nos. 634 and 637) 10 wk after the primary inoculation could not induce antibody, whereas reinoculation of the same quantity of Chbb:HM cells into two F_1 (Nos. 649 and 650) did induce antibody, apparently abrogating unresponsiveness induced by the primary inoculation of B/J cells. Unresponsiveness in B/J rabbits was abrogated by reinoculation of two TCGF-independent B/J cell lines (B405 and B684), but not of TCGF-dependent cell line (B425), at the age of 10 or 30 wk. Two B/J rabbits (Nos. 790 and 802) simultaneously given HTLV-I transformed cells and SPV were seronegative for anti-HTLV-I, but positive for anti-SPV; HTLV-I infection did not impair antibody production against SPV. When sera of other seronegative animals were examined by ELISA, sera of four of nine animals contained antibody against BSA, which could have been induced by the antigen in the inoculum despite washing before inoculation (data not

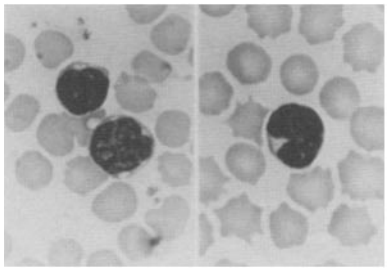

FIGURE 1. Atypical lymphoid cells with convoluted and lobulated nuclei in the peripheral blood of seronegative rabbit No. 618.

shown). These results indicate that immunological tolerance against HTLV-I antigens could be induced in B/J and F_1 rabbits neonatally inoculated with B/J cells and that such tolerance could be abrogated by inoculation of Chbb:HM or of B/J cells.

Atypical lymphoid cells with convoluted or lobulated nuclei, which are characteristic of ATL, were found in the peripheral blood of both seronegative and seropositive animals at 0.5–5% of total leukocytes (Fig. 1). Leukocyte count in the peripheral blood was raised up to 1–2 × $10^4/mm^3$ and was similar between seronegative and seropositive animals. Peripheral blood lymphocytes from the seronegative 10-wk-old animals were cultured in vitro in the presence of TCGF for 3 mo and examined for the expression of viral core antigen p19 by indirect immunofluorescence. Although the viral antigen was not detected in cells from any of 11 animals at the start of culture, >80% of cells became positive at 4 wk of culture in four animals that were inoculated with B405 and B684 cells; no positive cells were detected in cultured cells from seven other animals that were inoculated with B425 cells. Five cell lines were established from these animals: three TCGF-dependent cell lines from B425-inoculated animals, and two TCGF-independent cell lines from B405- and B684-inoculated animals. Southern blot hybridization analysis of these cell lines revealed integration of proviral genomes in one TCGF-dependent and both of two TCGF-independent cell lines (Fig. 2). Thus, a rabbit neonatally infected with HTLV-I may become a seronegative virus carrier under certain conditions. No pathogenic effects of immune unresponsiveness were observed in these animals.

Discussion

It is generally accepted that immune tolerance is not easily induced to viral antigens (9–11), but the absence of serum antibody in the present experiment is very probably due to immune tolerance to HTLV-I antigens. First, the absence of cir-

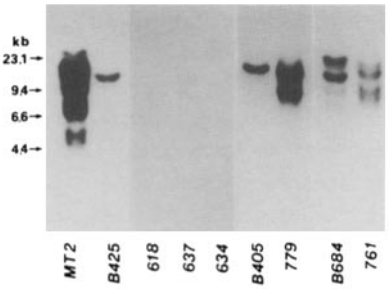

FIGURE 2. Integration patterns of HTLV-I proviral genome in inoculum cells and cell lines derived from seronegative animals. High molecular weight DNA was digested with Eco RI and hybridized with a radiolabeled pMT2/65 probe. MT2, HTLV-I-producing human T-cell line; B405, B425, and B684, inoculum cells; 618, 634, and 637, cell lines derived from animals given B425; 779 and 761, cell lines derived from animals given B405 and B684, respectively. Analyzed cells were at the 13th week of culture (for 618, 634, and 637) and at the 4th week (for 761 and 779).

culating antibody does not seem to be due to the formation of virus-antibody immune complexes that are eliminated from the circulation, because virus antigen-positive cells were not detected in fresh PBL from any animals. HTLV-I-infected cells usually do not express detectable virus antigens in vivo (12), and no viral transcripts are found in fresh leukemic cells (13). Second, the absence of antivirus antibody does not seem to be ascribed to general immune deficiency induced by HTLV-I infection, because antibody production against SPV and BSA was not impaired in seronegative animals. Third, the absence of antibody response in one but not in another strain of rabbits favors the possibility that such an absence of response is due to immune tolerance; such a strain difference has been repeatedly demonstrated in the induction of unresponsiveness and is thought to be related to differences in macrophage function (14). The present findings imply that absence of antibody should not be considered proof of the absence of virus, at least in rabbits. Whether or not similar tolerance is induced in humans is an important issue that needs investigation. The presence of seronegative virus carriers among inhabitants of endemic areas is suggested by the findings that transfusion of antivirus antibody-free blood can result in the seroconversion of 3 out of 600 recipients (15). Serological methods presently in use to detect virus carriers may have to be replaced by another method to detect virus genome or antigens themselves, if some human virus carriers can also be seronegative.

Summary

Six HTLV-I-transformed T cell lines were prepared from PBL of three rabbits each of B/J and Chbb:HM strains, and were inoculated into newborn rabbits of these two strains, and of their F_1 hybrid. None of three B/J cell lines induced anti-HTLV-I antibody response in newborn B/J rabbits, whereas all three Chbb:HM cell lines did induce a response in newborn Chbb:HM rabbits. These B/J cell lines however could induce antibody response in adult B/J as well as newborn Chbb:HM rabbits, and a Chbb:HM cell line could induce a response in a newborn B/J rabbit. Similar unresponsiveness was observed in (B/J × Chbb:HM)F_1 hybrids neonatally inoculated with B/J cells. Unresponsiveness was abrogated by reinoculation of some but not other cell lines. Viral antigen-positive cell lines harboring HTLV-I provirus genomes were established from such seronegative B/J and F_1 rabbits. Simultaneous inoculation of HTLV-I-transformed cells and SPV resulted in the induction of papilloma and antibody against SPV, but not antibody against HTLV-I. The present findings thus reveal that neonatal infection of HTLV-I could result in immunological tolerance to the virus antigens, thereby leading to a persistent infection without antibody induction.

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