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Attempts to Establish an Animal Model for HTLV- I infection and Development of Leukemia/Lymphoma in Rabbits

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Abstract HTLV- I positive rabbit lymphoblastoid cell lines (T2M-1 and T2M-2) were established by the coculture of a HTLV-I infected human T cell line MT-2 and lymphocytes from two rabbits (M-1 and M-2). Cells of T2M-1 and T2M-2 lines were inoculated autologously in these animals, and HTLV-I specific antibodies appeared 2 and 6 weeks post inoculation (p.i.), respectively. Immunoprecipitation showed that these anti-HTLV- I antibodies were mainly directed against viral glycopolypeptides, gp68 and gp46. HTLV-I specific antigens (ATLA) were detected on peripheral lymphocytes from both rabbits with short term cultivation method. Two ATLA positive cell lines, RM-2(2) and RM-2(6), were established from these cultured cells. Biochemical analysis of these cell lines showed that they had ATLA and proviral DNA of HTLV-I. Genomic DNA patterns with Southern blotting analysis represented distinct patterns from that of inoculated cell line. Twenty-four weeks p.i., ATLA was detected in peripheral lymphocytes from inoculated rabbits, but no animal developed leukemia/lymphoma. At this time, cell line could not be established by the cell culture.

Althogh we failed to develop leukemia/lymphoma in rabbits, this system is useful to study the mechanism of development of leukemia/lymphoma from HTLV- I carrier or smoldering state of ATL.

Key Words : HTLV- I infection, Animal model of ATL, Rabbit, Lymphoma/leukemia, ATLA

Introduction

Adult T-cell leukemia (ATL)[1] is a malignant hematological disorder, which has been etiologically linked to infection with human T-cell leukemia virus type I (HTLV - I)[2][3][4]. From sero-epidemiological studies, the three major transmission routes of HTLV- I are considered : 1) from parents to children (vertical or horizontal), 2) between spouses, and 3) blood transfusion. Mother-to-child transmission by orally given breast milk was thought to be the main route[5] as natural infection, but it is still unknown why some of these carriers develop lymphoma/leukemia after several decades of

latency.

HTLV-I antibodies were found in Japanese monkeys[6)7)8)] and African green monkeys[9)] and lymphocytes from sero-negative Japanese monkeys were also shown to be transformed by cocultivation with a HTLV-I producer cell line, MT-2[7)]. Yamamoto et al.[10)]showed that cynomolgus monkeys were experimentally infectable with HTLV-I. Also, HTLV-I can infect and transform T cells from rabbit and rat[11)12)].

In this study, we report our trial to establish animal model for the study of leukemogenesis caused by HTLV-I infection. HTLV-I-infected cell lines were established from lymphocytes of two rabbits by the coculture with MT-2[13)]. We inoculated these cells back to each rabbit autologously.

Materials and Methods

Rabbits

Two Japanese white rabbits (M-1 and M-2) purchased from a commercial breeder were used. These rabbits were free of HTLV-I and anti-ATLA antibody.

Cells

MT-2 cell line was used for cocultivation with rabbit lymphocytes. MT-2 is a HTLV-I producing CD4 positive T-cell line. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100IU/ml), and streptomycin (100 μg/ml). They were subcultured routinely every 5 days.

Rabbit lymphocytes were obtained from heparinized blood by disrupting erythrocytes with a Tris buffer (9 parts of 0.83% NH_4Cl, 1 part of Tris (hydroxymethyl) aminomethane, pH7.65) followed by washing with complete medium.

Establishment of HTLV-I positive rabbit cell lines

Cocultivation of normal lymphocytes with MT-2 cells was carried out as described previously[14)]. Briefly, freshly prepared rabbit lymphocytes (1 x 10^6 cells/ml) were mixed with an equal volume of irradiated MT-2 cells (1 x 10^6 cells/ml). Karyotype of established cell lines was studied in order to identify the origin.

In vivo inoculation of transformed cells

The established HTLV-I-infected cell lines T2M-1 and T2M-2, were inoculated back into rabbits M-1 and M-2, respectively. Each rabbit was inoculated intravenously with 2 ml (2 x 10^6 cells/ml) of cell suspension. Five weeks after initial infection, 10^7 cells of each transformed cell line were inoculated for booster.

After inoculation, blood samples were collected several times for hematological and serological examination.

Analysis of T- and B- cell markers

Cell lines were examined for T-cell marker (Leu 1) and B-cell marker (surface, cytoplasmic immunoglobulin) by indirect immunofluorescence using their respective monoclonal antibodies[14)]. As a T-cell marker, the rosette formation with neuraminidase-treated sheep red blood cells and rabbit erythrocytes was also tested (E-receptor test)

Southern blotting analysis of HTLV-I provirus DNA

Chromosomal DNA was digested with restriction endonuclease EcoRI and was separated on 0.7% agarose gel electrophoresis. Southern blotting experiment was performed as described previously[15)]. Sst-1 fragment of HTLV-I clone# 42, which contains gag, pXs and U_3RU_5 portion of HTLV-I[15)], was used to detect HTLV-I provirus sequence in each cell line.

Detection of serum antibodies against HTLV-I antigen (anti-ATLA antibodies)

Indirect immunofluorescence (I.F.)

Sera were titrated for anti-ATLA antibodies by I.F. using MT-1 cells as targets. For second antibodies, fluorescence-conjugated anti-rabbit IgG was used in a similar manner as described[3)].

Immunoprecipitation and SDS-PAGE

Complete procedures are described[9)16)17)]. Briefly, 8 x 10^6 cells were labeled for 16 hrs in 10 ml of medium containing 50 μCi/ml of L-^{3}H-leucine (146.5 Ci/m mol) (NEN, Tokyo). Incorporation efficiency of radioactive precursors was increased by the use of dialysed FCS in which the concentration of unlabeled leucine was reduced, and then 1 x 10^6 labeled cells were washed twice by pelleting in PBS. The cells were treated for 20 min at 4℃ with 0.5 ml lysis buffer (10 mM Tris-HCl, pH8.0, 0.14 M NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoried, 0.5% NP40). The lysates were cleared by centrifugation at 14,000g for 10 min at 4℃.

Each serum collected from inoculated rabbits was incubated for 15 hrs at 4℃ with cell lysate. Immunoprecipitates were bound to 10 μl of protein A-sepharose (Pharmacia, Tokyo) for 1 hr and were washed four times, twice with 1 ml of high-salt washing buffer (20 mM Tris-HCl pH7.6, 0.5M NaCl, 1 mM EDTA, 0.5% NP40, 1% desoxycholate) and twice with 1 ml of low-salt washing buffer (10 mM Tris-HCl pH7.6, 10 mM NaCl).

The washed immunoprecipitates were extracted for 1 min at 100℃ with 15 μl of sample buffer[7] supplemented with 6 M urea, and then 25 μl of each sample was applied to 8-16% SDS-PAGE[18].

The gels were processed for fluorography, then dried in order to locate radioactive bands on sensitized X-ray film (X-O-mat AR, Kodak, Tokyo)

Detection of HTLV-I antigens (ATLA)

To detect the HTLV-I antigen positive lymphocytes, 10 ml of peripheral blood was collected from rabbits at various intervals after inoculation. Mononuclear lymphocytes were separated by disrupting erythrocytes. Cells were cultivated in RPMI 1640 medium supplemented with 20% FCS and 25% crude T-cell growth factor (TCGF). Five days after cultivation, expression of HTLV-I antigen was examined by immunofluorescence with murine monoclonal antibody to p19[19], a HTLV-1 component.

Results

Establishment of HTLV-I infected rabbit cell lines by coculture with MT-2 cells

After 2 to 3 weeks of culture, scattered foci of cell aggregates were formed. These cell aggregates gradually increased in size and number, and the first subculture was made 6 weeks after coculture with or without the use of TCGF. Two cell lines originated from rabbit M-1 and M-2 were designated T2M-1 and T2M-2, respectively. These cell lines have now been maintained in continuous culture. For the establishment of cell line, T2M-1 required TCGF, while T2M-2 did not. Interestingly, T2M-1 cell line turned to be TCGF independent after 6 months of culture. Both cell lines grew in suspension forming clumps.

Both cell lines lacked the surface and cytoplasmic immunoglobulin and E-receptors.

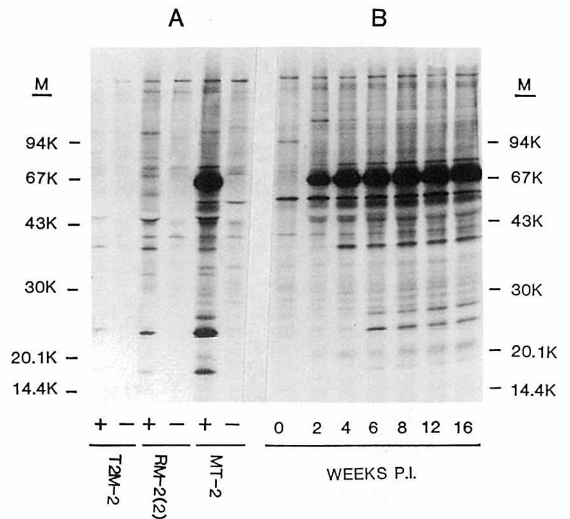

Fig.1A Immunoprecipitation of HTLV-1 specific polypeptides extracted from T2M-2, RM-2(2) and MT-2 cells. Cells were labeled with ^{3}H-leucine. Cell extracts were immunoprecipitated with serum from an antibody positive ATL patient (+), and serum from an antibody negative individual (−). Marker polypeptides (M) were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and 2-lactoalbumin (14,000).

Fig.1B Detection of antibodies in sera collected from rabbit (M-2) inoculated with autologous HTLV-I positive lymphoid cells (T2M-2). MT-2 cells were labeled for 16 hrs with ^{3}H-leucine (50 μCi/ml) in unlabeled leucine free medium. Details of immunoprecipitattion and SDS-PAGE analysis are described in Materials and Methods. Numbers below each slot indicate weeks post inoculation (p.i.).

Chromosomal analysis showed that the karyotype of T2M-1 and T2M-2 cells was that of rabbit. More than 90% of cells of these cell lines were positive for ATLA. Immunoprecipitation, using a serum from an ATL patient, and SDS-PAGE analysis showed that

HTLV-I specific polypeptide patterns of both cell lines were very similar to each other, containing gp61, p53, p33, p22 and p19, but different from the pattern of HTLV-I -infected MT-2 cells that were shown to contain gp68 and p28 additionally. Representative results with T2M-2 cell line are shown in Fig.1A. Southern blotting analysis showed that this cell line contained 3 discrete bands of proviral DNA, 22.5 kbp, 15.5 kbp and 8. 8kbp. The proviral DNA integration sites were different from those of MT-2 cells (Fig. 2). More precise analysis of HTLV-I provirus DNA of T2M-2 cell line, using specific probes to each component of virus DNA, showed that at least 22.5 and 15.5 kbp provirus genomes were integrated (data not shown). In the experiments carried out in parallel, it was shown that neither irradiated MT-2 cells nor rabbit leukocytes cultured alone were alive more than two months.

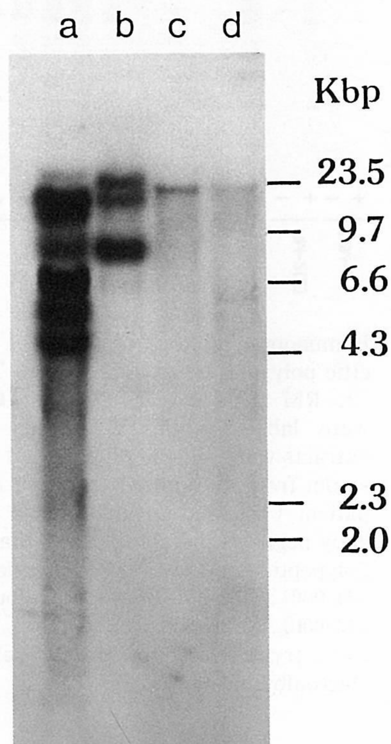

Fig. 2 Detection of HTVL-I provirus in chromosomal DNA of T2M-2, RM-2(2) and RM-2(6) cell lines. Five μg of DNA from MT-2(a), T2M-2(b), RM-2(2) (c) and RM-2(6)(d) was digested completely with restriction endonuclease, EcoRI. Fragments were separated by electrophoresis on 0.7% agarose gel. DNA was transferred from the gel to nylon membrane filter. To detect HTLV -I clone #42 Sst I fragment (4 x 10^8 cpm/ μg DNA). After hybridization, filter was exposed to Kodak X-O mat AR film at -70℃ for 2 days using intensifying screen (Dupont lightning-plus). Hind III -digested DNA was used as molecular size standard.

Inoculation of HTLV-I-transformed cell line autologously to rabbits

Rabbit M-1 and M-2 were inoculated with

TABLE I -APPEARANCE OF ANTI-HTLV ANTIBODIES IN RABBITS AFTER INOCULATION OF HTLV-I TRANSFORMED AUTOLOGOUS CELLS

Animal	Time after inoculation (Weeks)								
	0	2	4	6	8	12	16	20	24
#1	<10[a]	<10	<10	40	40	40	40	40	40
#2	<10	80	160	160	160	320	320	160	160

a. Titer of indirect immunofluorescence with MT-1 cells.

T2M-1 and T2M-2 cells, respectively, 3 months after establishment. At this time, the former was TCGF dependent, while the latter was not.

These rabbits were examined clinically and hematologically after inoculation of autologous lymphocytes. Up to 24 weeks after inoculation, none of the inoculated rabbits developed either physical or hematological abnormalities.

Appearance of serum anti-ATLA antibodies after inoculation

Anti-ATLA antibodies were developed in both rabbits M-1 and M-2 after inoculation of T2M-1 and T2M-2 cells, respectively. I.F. test showed that M-2 rabbit started to devolop serum antibodies 2 weeks post inoculation and the titer of serum antibodies increased according to the time course. Rabbit M-1 showed a similar response to the inoculation of autologous cells, but with delayed and lower antibody production compared to M-2 rabbit (Table I).

Collected sera from M-2 rabbit were subjected for immunoprecipitation analysis using ^{3}H-leucine-labeled MT-2 cell extracts. As shown in Figure 1B, rabbit M-2 already developed antibodies against HTLV-I specific glycopolypeptides, gp68 and gp46, 2 weeks after inoculation. Reactivities of the sera to those polypeptides increased corresponding to the increase of I.F. titer. Apparently, gp68 was precipitated by the sera obtained at each time. Besides these polypeptides p76, p53, p36, p28, p24, p21 and p19 were visibly immunoprecipitated with sera collected at 6 weeks post inoculation and thereafter.

Appearance of ATLA positive lymphocytes after inoculation

To investigate whether HTLV-I was harbored in ATLA positive rabbit or not, peripheral leukocytes were obtained at 2,4,6,8,12,16, 20 and 24 weeks after inoculation. ATLA were examined with I.F. after in vitro cultivation.

As shown in Table II, peripheral leukocytes in M-1 and M-2 rabbits included ATLA positive lymphocytes 4 and 2 weeks after inoculation, respectively. Thereafter, peripheral lymphocytes positive for ATLA were not detected in those two rabbits by short term in vitro culture system (up to 24 weeks). But ATLA positive lymphocytes were detected in both rabbits 24 weeks after inoculation. Interestingly, continuous cell lines were developed from the peripheral lymphocytes from M-2 rabbit at 2 and 6 weeks after inoculation. Although less than 1% cells were ATLA positive after short term in vitro cultivation, positive cells increased apparently after long term culture. Both cell lines initially required TCGF, but the former began to grow independently. Continuous lymphoid cell lines obtained from peripheral lymphocytes of M-2 rabbit col-

TABLE II-APPEARANCE OF HTLV-I ANTIGENS (ATLA) IN LYMPHOCYTES OF VIRUS-INOCULATED RABBITS AFTER SHORT-TERM CULTURE[a]

Animal	Time after inoculation (Weeks)								
	0	2	4	6	8	12	16	20	24
#1	0	0	0.1-1[b]	0	0	0	0	0	0.1-1
#2	0	1-3 (>90)c	0	0 (0.5-1)	0	0	0	0	1-2

a. Frequencies of ATLA positive cells were determined at 5 days of cultivation by indirect immunofluorescence with a mouse monoclonal antibody GIN-14[19].

b. % of ATLA positive cells.

c. Number in parenthesis represents % of ATLA positive cells after establishing cell lines.

lected at 2 and 6 weeks after inoculation, were designated RM-2(2) and RM-2(6), respectively.

Comparison between T2M-2 cell line and cell lines (RM-2(2), RM-2(6)) established from lymphocytes of T2M-2 inoculated animal

It is of interest to inquire whether the established cell lines represent some difference in nature from inoculated T2M-2 cell line or not. For this purpose we studied the characteristics of RM-2(2) and RM-2(6) cell lines extensively. Both cell lines had no surface Igs and E-receptors same as their parental T2M-2 line. More than 90% of RM-2(2) cells were stained with anti-p19 mouse monoclonal antibody, while less than 1% in RM-2 (6) cells.

Immunoprecipitation study of RM-2(2) cells using anti-HTLV-I antibody positive human sera, revealed that RM-2(2) cells contained HTLV-I specific polypeptides indistinguishable from of inoculated cell line (T2M-2) (Fig.1A). These results showed no definitive answer for whether newly established cell lines were directly derived from inoculated cells or originated as a consequence of infection of HTLV-I to normal lymphocytes in vivo. However, further analysis with Southern blotting technique revealed that patterns of proviral DNA of RM-2(2) and RM-2(6) were different from that of T2M-2. As shown in Fig.2, both established cell lines, had a distinct band of 18. 5 kbp proviral DNA which was never observed in the parental cell line. Although both RM-2(2) and RM-2(6) cell lines contained the same size of proviral DNA, the latter showed a faint band. This may reflect the low population of ATLA positive cells in RM-2(6) cells.

Discussion

Several decades after the HTLV-I infection to a child from infected mother, ATL develops either in a manner of leukemia or lymphoma at the low rate of about 1 out of several thousands. About a half year after infection by infected mothers milk[5], infants usually become positive for anti-ATLA antibody. HTLV-I was harbored polyclonally in various cell populations not only in T- but also B-and other cells for significantly long time. During this time some cells are considered to undergo a malignant change and they possibly escape from the pressure of host's immunological response and initiate to grow. After the preleukemic state, abnormal lymphocyte[5] with convoluted and lobulated nuclei or blastoid lymphocytes develops to leukemia/lymphoma. The mechanism, how leukemia/lymphoma develops, is still unknown. HTLV-I can infect and transform T-cells not only from human but also from monkey[3], rabbit[20], and rat[21]. Cynomolgus mondeys developed serum antibodies and viral antigens in peripheral lymphocytes in response to inoculation of HTLV-I transformed cells[10]. Several attempts have been made to establish an animal model of ATL by the inoculation of autologous or allogenic transformed cells thereafter, but no sign of ATL was observed. It was reported that adult T cell leukemia-like disease can be experimentaly induced through peritoneal inoculation of transformed cells to newborn rabbits[26][27]. However, the disease induced showed significant differences from ATL. For example, infiltration of leukemic cells and physiological symptoms appeared without the long latency which corresponds to carrier state of ATL patients. Moreover, induced leukemia was polyclonal origin, while leukemic cell in ATL patient is monoclonal.

We tried to establish an animal model in which leukemia/lymphoma develops after the immunologically suppressed carrier state. We thought that autologous inoculation of HTLV-I-infected cells may favor development of lymphoma/leukemia after establishment of the state positive for both anti-ATLA antibody and HTLV-I positive cells in blood just like a natural course of ATL development.

When rabbits were inoculated with their own lymphocytes after transformation with HTLV-I, the titer of ATLA antibodies began to increase as early as 2 weeks p.i.. These antibodies mainly directed against viral glycoproteins gp68 and gp46 in MT-2 cell

extract. Viral polypeptides p28, p24, p21 and p19 were detectable to some extent. This is similar to our previous data[7)15)] in which gp68 and gp46 were mainly immunoprecipitated with sera from ATL patients, healthy carriers or HTLV-I-infected monkeys. Accord ing to the increase of virus titer, other polypeptides could be detected.

HTLV-I antigens were detected on short term cultivated peripheral lymphocytes of inoculated rabbits. Detection of HTLV-I antigens was rather occasional but not constant in lymphocytes collected at various time. The reason for this occasional HTLV-I antigen detection is unclear at present. However, RM-2(6) established from peripheral lymphocytes collected at 6 weeks p.i. were apparently positive for antigens in spite that they were negative after short term cultivation. This result suggests that the number of affected cells varied after inoculation and occasionally escaped from antigen detection. Two cell lines were established from peripheral lymphocytes collected from 2 inoculated rabbits at 2 and 6 weeks p.i.. They were positive for HTLV-I specific polypeptides and provirus DNA. As far as immunoprecipitation of HTLV-I antigens, T2M-2 and two established cell lines were indistinguishable. Southern blotting analysis of provirus DNA, however, suggested that they were different from each other. Parental cell line, T2M-2, contained 22.5kbp, 15.5kbp and 8.8kbp DNA, while two of established cell lines had 18.5kbp. RM-2(6) seems to have 5.5 and 5.0kbp DNA, additionally. The amount of proviral DNA in RM-2(6) appears to be lower than that of RM-2(2). These data suggest that RM-2(2) and RM-2(6) cells were derived as the consequence of infection of lymphocytes with HTLV-I released from T2R-2 cells in vivo. However, we can not exclude the posibility that T2M-2 cell line had polyclonal cell populations in terms of proviral DNA integration site, and cell population might change while in vivo inoculation as well as in vitro culture.

We continued this experiment until 24 weeks p.i. but we could not observe any clinical symptoms suggesting ATL lymphoma/leukemia in the autologously inoculated animals. We could detect HTLV-I antigen positive lymphocytes, but failed to establish cell line for further experiments. Thinking about the time course of ATL, it is too early to conclude that the rabbits are susceptible to infection with HTLV-I but resistant to tumor development by this virus. It is thus neccessary to continue to follow up the fate of HTLV-I specific antigens and antibodies, and the development of malignancy in these animals. In any case the infection system with rabbits shown here may be a useful model system for HTLV-I studies, especially for the study of pharmacological intervention and vaccine development against HTLV-I.

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