Selection of Rabbit CD4-CD8- T Cell Receptor-γ/δ Cells by In Vitro Transformation with Human T Lymphotropic Virus-I

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Summary

In vitro transformation of rabbit peripheral blood mononuclear cells (PBMC) with human T lymphotropic virus-I (HTLV)-infected human or rabbit cells resulted in CD4-CD8- cell lines, some of which caused acute leukemia when injected into rabbits. Structural analyses of the proviruses from cell lines with diverse pathogenic effects provided no clear correlation with lethality. The rabbit lines were provisionally designated T cells because they express interleukin 2R (IL-2R) and CD5 and lack surface immunoglobulin, but none express functional T cell receptor (TCR) α or β transcripts. A more detailed characterization of the HTLV-I-infected cells was required to determine cell lineage and its potential influence on pathogenic consequences. Probes for rabbit TCRγ and δ genes were derived and used to detect γ and δ TCR RNA transcripts, identifying the in vitro transformed lines as γ/δ T cells. CD4+ and CD8+ lines were derived from PBMC of HTLV-I-infected rabbits and CD4+ TCR-α/β HTLV-I lines were derived from rabbit thymus, eliminating the possibility that the HTLV-I isolates used here transform only CD4-CD8- TCR-γ/δ cells. The percentage of γ/δ cells in rabbit PBMC is relatively high (23% in adult rabbits); this with diminution of CD4+ and CD8+ cells in IL-2-supplemented PBMC or thymocyte cultures may account for selection of rabbit HTLV-I-infected γ/δ T cell lines in vitro. The availability of well-characterized T cell lines with diverse in vivo effects in the rabbit HTLV-I disease model allows evaluation of roles played by cell type in HTLV-I-mediated disease.

Although the majority of individuals infected with human T lymphotropic virus-I (HTLV-I)1 remain asymptomatic (1), HTLV-I is the etiological agent for at least two human diseases: adult T cell leukemia/lymphoma (ATL/L) (2, 3) and HTLV-I-associated myelopathy/tropical spastic paraparesis (4). The factors controlling pathogenesis of HTLV-I infection remain obscure; manifestation of different diseases does not correlate with the minor sequence variations among different strains of this highly conserved virus (5-7). Likewise, the influence of infected cell type on the outcome of infection is not known; most transformed cell lines derived from HTLV-I-infected individuals are CD4+ T cells (8, 9), although HTLV-I-transformed CD8+ T cells (10, 11), B cells (12-14), and dendritic cells (15) have also been reported. Development of relevant animal models for HTLV-I infection may provide insight into factors contributing to the varied pattern of HTLV-I pathogenesis.

1 Abbreviations used in this paper: ATL/L, adult T cell leukemia/lymphoma; HTLV-I, human T lymphotropic virus-I; Rb, rabbit.
This report documents that in vitro HTLV-I-transformed rabbit cell lines, including those causing lethal leukemia, are of the CD4+CD8+ TCR-γ/δ phenotype. Derivation of HTLV-I-infected CD4+CD8−α/β T cell lines from rabbit thymus and in vivo derivation of CD4+CD8+ and CD4−CD8−α/β T cell lines indicate that HTLV-I also infects rabbit cell types analogous to those normally obtained from HTLV-I-infected humans. The presence of a high percentage of γ/δ T cells in the peripheral blood of adult rabbits and selection by conditions of cell culture contribute toward preferential in vitro transformation of γ/δ T cells by HTLV-I. The development of reagents and characterization of cell lines will allow assessment of the roles played by TCR-α/β and γ/δ T cells in HTLV-I infection and pathogenesis.

Materials and Methods

HTLV-I Infection. All primary cells and tissues used in this study were obtained from New Zealand White rabbits (Hazeltone Research Products, Inc., Denver, PA) housed at Spring Valley Laboratories (Woodbine, MD). PBMC were isolated from EDTA-treated whole blood by centrifuging at 750 g for 20 min. Theuffy coat was removed and the residual red blood cells were lysed with ACK lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4 [B&K/Scott Lab., Fiskville, RI]). Thymocytes were obtained from thymus by mechanical dissociation. Infection of PBMC or thymocytes was carried out by coculture of 5 × 10^6 cells with 10^6 human MT-2 cells for 3 mo.

Northern blot Analyses. Northern blot analyses were used to determine expression patterns of RbCD4, RbCD8, RbTcR-α, RbTcR-β, RbTcR-γ, RbIL-2Rα, and RbMHC class I transcripts by rabbit cell lines. cDNA probes for RbCD4 were radiolabeled with [32P]dCTP (Amersham Corp., Arlington Heights, IL) by random hexamer priming (37, 38) and used as a template for synthesis of complementary DNA probes specific for rabbit TCR-γ (RbTcR-γ), TCR-δ (RbTcR-δ), CD8α (RbCD8α), and IL-2Rα (RbIL-2Rα). Reported nucleotide sequences of the homologous molecules from different species were aligned using the MacVector DNA analysis program (IBI-A Kodak Co., New Haven, CT). Conserved regions were identified and degenerate oligonucleotide primers were synthesized by an oligonucleotide synthesizer (Applied Biosystems Inc.) based on these sequences. An exception was IL-2Rα, for which the forward and reverse human primers were derived from Clontech Laboratory (Palo Alto, CA). Rabbit cDNA probes were radiolabeled with [32P]dCTP (Amersham Corp., Arlington Heights, IL) by random hexamer priming (37, 38) and used as a template for synthesis of complementary DNA probes specific for rabbit TCR-α, RbCD4, RbCD8, and RbMHC class I transcripts by rabbit cell lines. First-strand cDNA was reverse transcribed from 1 µg of total RNA using the oligo dT primer and subsequently reverse transcriptase-PCR amplified (Perkin Elmer Cetus, Emeryville, CA) using gene-specific primers.

The PCR products were separated by electrophoresis and the appropriate size fragments were excised, purified by GeneClean (BIO 101, Inc., Vista, CA), and ligated to the pCR™II vector (Invitrogen, San Diego, CA). Cloned cDNA was sequenced by the Sanger method (32) using Sequenase Ver.2 (United States Biochemical Corp., Cleveland, OH). The gene fragments obtained by this technique were identified by alignment of sequences to the analogous genes from other species and by cell and tissue distribution patterns of RNA transcripts hybridizing to them. Percent nucleotide identity was determined by MicroGenie sequence software (Beckman Instruments, Inc., Palo Alto, CA).

Results and Discussion

In Vitro Derivation of HTLV-I-infected Cell Lines. HTLV-I-transformed cell lines were derived from rabbit PBMC by coculture with human or rabbit HTLV-I-infected cell lines. The cell lines RH/K30, RH/66A, RH/66E, and RH/66F were derived by coculture with irradiated human MT-2 cells (26). RH/K34 and RH/K32 were obtained using the in vivo transformed rabbit cell line RHT-16 (27) as virus source. First-strand cDNA was reverse transcribed from 1 µg of total RNA using the oligo dT primer and subsequently reverse transcriptase-PCR amplified (Perkin Elmer Cetus, Emeryville, CA) using gene-specific primers.

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I-transformed lines become IL-2 independent and require no further growth factor supplement. All the transformed lines are pleomorphic and tend to form cell clumps, whether they are adherent or grow in suspension.

All derived lines are HTLV-I infected as indicated by various criteria. Since there are no internal EcoRI sites within the HTLV-I proviral genome, each site of HTLV-I integration generates a unique EcoRI restriction fragment that is revealed by hybridization with the HTLV-I probe (Fig. 1 A). The autoradiogram indicates that certain cell lines possess more than one HTLV-I copy (or, alternatively, the lines are oligo-clonal). RH/K34, RH/66F, and RHT-16 have a single integrated provirus. No signal was detected with the HTLV-I probe in the lane containing EcoRI-digested DNA from the Herpesvirus atelels—transformed rabbit α/β T cell line, RL-5 (39). Nucleotide sequence determination of cloned EcoRI fragments containing provirus from RH/K30 and RH/K34 cell lines revealed that each of these cell lines possesses a full-length HTLV-I genome (23). Reaction of cell lines with a fluorescent mAb directed against HTLV-I p19 matrix protein revealed the presence of this protein on all lines. These data, along with the ability of the lines to infect other rabbit PBMC, indicate that all are productively infected with HTLV-I.

**Surface Markers of HTLV-I Cell Lines.** The transformed cell lines were reacted with a panel of mAbs specific for rabbit lymphoid cell markers (Table 1) and subjected to FACS analyses. None of the six PBMC-derived, in vitro transformed cell lines express rabbit CD4 or CD8, regardless of the viral source used to transform them. Analysis of peripheral blood lymphocytes from 20 normal rabbits yielded average values of 26.4% for the CD4+ subpopulation and 9.5% for the CD8+ subpopulation. This indicates that CD4+ and CD8+ cells are present in PBMC as potential targets for HTLV-I infection. Exceptions to the double negativity include the CD8+ line, RHT-16, and the CD4+ line, RHT-K29; both were derived from PBMC of HTLV-I-infected rabbits.

With respect to the other cell surface molecules, all cell lines express low to moderate levels of CD5, but none express surface Ig. All lines showed binding of FITC-conjugated recombinant human IL-2. This indicates high level expression of IL-2R, which is typical of HTLV-I-infected human cells (8, 9). While most lines express rabbit MHC class II antigens to varying degrees, RH/66A, RH/66E, and RH/66F, all of which were derived from the same animal, express neither RLA-DQ nor -DR. Even though an average of 23.5% of normal rabbit peripheral blood lymphocytes react with CyM1, a mAb directed against human TCR-γ (40), none of the cell lines reacted. However, the absence of surface TCR-γ does not preclude the possibility that the lines are of the γ/δ lineage, as HTLV-I infection has been reported to downregulate or prevent surface expression of both the TCR-α/β and γ/δ complexes (41, 42).

**Selection of CD4+ Cell Lines.** Because in vitro HTLV-I infections consistently resulted in transformation of rabbit CD4+CD8− cells, the question was raised whether the in vitro transformation protocol is capable of producing CD4+ HTLV-I transformants. It is known that rabbit CD4 differs in structure from human CD4 (33), but this should not influence HTLV-I infectivity as it is unlikely that either CD4 or CD8 serves as receptor for HTLV-I. Human chromosome mapping data localize the HTLV-I receptor to human chromosome 17 (43), whereas genes for human CD4 and CD8 are on chromosomes 12 (44) and 2 (45), respectively. To determine whether rabbit CD4+ cells could be infected in vitro, thymocytes rich in CD4+ and CD8+ cells (Fig. 2 A) from two young rabbits were cocultured with irradiated MT-2 or RH/66A cells. After 1 wk, the thymocyte cultures were selected for rabbit CD4+ cells by magnetic bead separation using anti-rabbit CD4 mAb. This procedure yielded HTLV-I-transformed CD4+ cell lines from each thymocyte donor: RH/Thy-2 from the MT-2 infection (Fig. 2 B) and RH/Thy66A from infections with RH/66A. Southern blot analyses confirmed that both cell lines harbor HTLV-I provirus.
Table 1. Cell Surface Markers of Rabbit HTLV-I Cell Lines Compared with PBMC from Normal Rabbits

| HTLV-I-transformed rabbit cell lines | CD4 | CD8 | CD5 | MHC II | TCR-γ |
|------------------------------------|-----|-----|-----|--------|-------|
| **Peripheral blood derived**        |     |     |     |        |       |
| Viral source: MT-2                  |     |     |     |        |       |
| RH/K30                             | −   | −   | +   | ±      | −     |
| RH/66A                             | −   | −   | ±   | −      | −     |
| RH/66E                             | −   | −   | ±   | −      | −     |
| RH/66F                             | −   | −   | +   | −      | −     |
| RHT-16*                            | −   | +   | +   | +      | −     |
| RH/K29†                            | +   | −   | +   | −      | −     |
| Viral source: RHT-16                |     |     |     |        |       |
| RH/K32                             | −   | −   | +   | +      | −     |
| RH/K34                             | −   | −   | ±   | +      | −     |
| **Thymus derived**                  |     |     |     |        |       |
| Viral source: MT-2                  |     |     |     |        |       |
| RH/Thy2                            | +   | −   | +   | +      | −     |
| Viral source: RH/66A                |     |     |     |        |       |
| RH/Thy66A                          | +   | −   | +   | −      | −     |
| Normal adult rabbit PBMC (n = 20)  | 26.4% | 9.5% | ND  | ND     | 23.5% |
| (SD 7.4)                           | (SD 5.8) |   |   | (SD 5.8) |   |

* RHT-16 was derived from PBMC of a rabbit infected by injection of irradiated MT-2 cells (27).
† RHT-K29 was derived from PBMC of a rabbit infected by injection of autologous PBMC transformed by HTLV-I (Sawasdikosol, S., unpublished results).

An aliquot of the thymocyte culture (not selected by the anti-CD4 mAb) was tested for CD4 and CD8 cells 6 mo after initiation of the culture and shown to contain only CD4−CD8− cells (Fig. 2 C). In a separate experiment, the kinetics of CD4 and CD8 cell depletion was monitored in thymocytes cocultured with MT-2 cells; the percentages of CD4 and CD8 cells increased during the first 2 wk of culture but then declined to undetectable levels within 3–4 wk (data not shown). Similar reductions of CD4+ and CD8+ cells were observed in normal PBMC cultures grown in IL-2-supplemented media, even without exposure to the virus. The effect of IL-2 alone on the selection of CD4+CD8− cells is difficult to assess since normal rabbit lymphocytes do not survive in its absence. This selection against CD4+ and CD8+ cells in culture provides a plausible explanation for the predominance of double negative cells in HTLV-I infection in vivo.

The dominance of CD4+CD8− transformed cells resulting from in vitro infection with HTLV-I is surprising since CD4+ is the predominant cell phenotype derived from HTLV-I-infected human patients (8, 9). However, since other infected cell types such as CD8+ T cells (10, 11), B cells (12–14), and γ/δ T cells (15) have also been described, it is possible that these results are specific to the rabbit model. The exact mechanism of this selection is not yet known, but it is clear that the virus has evolved to preferentially infect and expand certain cell subsets in infected rabbits.

Figure 2. FACS analyses of HTLV-I-transformed thymocytes during HTLV-I transformation. (A) Distribution of CD4+ and CD8+ cells in a 6-wk-old rabbit thymus. (B) RH/Thy-2, CD4+ HTLV-I-transformed thymocyte, was magnetic bead selected using anti-rabbit CD4 mAb 1 wk after the start of the HTLV-I coculture. No CD8+ cells were detected. (C) Total absence of both CD4 and CD8 cells in an unselected aliquot of the same thymocyte culture from which the CD4+ RH/Thy-2 was rescued, 6 mo after initiation. Isotype-matched mAb was used as the control (CTRL) for primary antibody.
(12–14), and dendritic cells (15) have been isolated from HTLV-I patients, the apparent CD4 selection may reflect mechanisms that enable infected CD4+ cells to predominate in vivo. It is not clear what other cell types are involved in human infection and/or what roles these cell types play in the diverse pathology associated with HTLV-I infection.

Information obtained thus far has not clearly assigned the lineage of the CD4−CD8− cell lines obtained by in vitro transformations of rabbit PBMC. It is possible that the CD4−CD8− cell lines were initially CD4+ or CD8+, but ceased to express these markers with time. This argument is weakened by the fact that the rabbit CD4+ and CD8+ cell lines have maintained high expression levels of CD4 and CD8 over a 1-yr period. Examination of TCR gene expression was required to ascertain the lineage of the rabbit HTLV-I cell lines. Since the majority of human γ/δ T cells are CD4−CD8−CD5+, it was postulated that the rabbit cells that have a similar surface phenotype are of the γ/δ lineage. To test this possibility, TCR gene expression patterns were determined by Northern blot analysis.

**Probes for Rabbit TCR-γ, TCR-δ, CD8β, and IL-2Rα.** Complementary DNA probes specific for rabbit TCR-γ, TCR-δ, CD8β, and IL-2Rα were derived for test for the presence of TCR transcripts. Degenerate oligonucleotide primers with sequences based on reported homologues were used to amplify fragments from rabbit PBMC RNA. Probes cloned in this manner were compared with human and mouse homologues and percent nucleotide identity for each was determined (Table 2). In every case, the rabbit gene is more closely related to the human than to the mouse homologue. Attempts to derive a rabbit CD8α probe using this approach have been unsuccessful and a human CD8α probe did not hybridize with RNA from the cell lines.

**Expression of TCR Genes.** The rabbit gene fragments derived here (RbCD8β, RbTCR-γ, RbTCR-δ, and RbIL-2Rα), along with probes for rabbit CD4 (33), TCR-α (34), TCR-β (35), and class I MHC genes (36), were used to determine mRNA expression patterns by the rabbit cell lines (Fig. 3). The CD4−CD8− status, determined by FACS® analyses, was verified by Northern blots probed with rabbit CD4 and CD8β; only the lines selected from thymus with anti-CD4 express the CD4 transcript, and only the in vivo transformed RHT-16 cell line expresses CD8β (Fig. 3). The absence of both transcripts in other lines was verified by reverse transcriptase–PCR amplification using the CD4 and CD8β gene-specific primers; no amplification was detected in lines shown to be negative by Northern blot analysis.

TCR gene expression was detected by the use of rabbit TCR-α, TCR-β, TCR-γ, and TCR-δ probes (Fig. 3). Analyses revealed that all CD4−CD8− cell lines express TCR-γ and TCR-δ transcripts comparable in size to functional human transcripts. RNA from the RH/66E, RH/66F, and RHT-16 cell lines contain bands detected with a RbTCR-α probe. A strong single band corresponding to the functional transcript was detected in the RHT-16 RNA sample, whereas weak signals corresponding to both functional and truncated sterile transcripts were observed in RH/66E and RH/66F. Hybridizing sequences were also detected in these three cell lines with the RbTCR-β probe. RHT-16 expresses both functional and sterile TCR-β transcripts; bands corresponding only to the TCR-β sterile transcript are detected in RH/66E and RH/66F. The absence of a functional TCR-β transcript precludes expression of a functional TCR-α/β complex in RH/66E and RH/66F, despite expression of a full-length TCR-α transcript. These expression patterns indicate that only RHT-16 is capable of forming a functional TCR of the α/β type and suggest that the other PBMC-derived lines express γ/δ TCR.

**Analysis of Rabbit T Cells by Scanning Electron Microscopy.** Scanning electron microscopy further established the similarity

| Gene   | Forward primers used for amplification | Reverse primers used for amplification | Fragment length (bp) | NT sequence identity to prototype |
|--------|----------------------------------------|----------------------------------------|----------------------|-----------------------------------|
| TCR-γ  | 5′ CCCAA GCCC/TA CTATG/T TTT/CCT TCCTT C 3′* | 5′ CCAGC TG/AAAC/T TTCAT GTATG TG 3′ | 195                   | 82.3%–human                       |
|        | 5′ CCAGC TG/AAAC/T TTCAT GTATG TG 3′       |                                         |                      | 80.3%–mouse                       |
| TCR-δ  | 5′ CCATC T/C GCTT T/CG/ATC ATGAA AAATG G 3′ | 5′ GCAGT CAAGA GAAAA TTGAT/C GGC 3′    | 419                   | 80.6%–human                       |
|        | 5′ GCATC TACTG GCTGA GAGAG CGCCA G 3′      |                                         |                      | 77.7%–mouse                       |
| CD8β   | 5′ GCATC TACTG GCTGA GAGAG CGCCA G 3′      | 5′ GGCCC TTCTG GGTCT CTGG 3′            | 345                   | 80.0%–human                       |
|        | 5′ GCATC TACTG GCTGA GAGAG CGCCA G 3′      |                                         |                      | 62.8%–mouse                       |
| IL-2Rα | 5′ GAATT TACCAT TTTGG TGGT GGGCA 3′        | 5′ TCTTC TACTC TTTCT CTGTCT TCGG 3′    | 399                   | 77.4%–human                       |
|        | 5′ GAATT TACCAT TTTGG TGGT GGGCA 3′        |                                         |                      | 70.9%–mouse                       |

* Nucleotide positions written with slash (/) indicate introduction of degeneracy to maximize identity to reported homologues.

1 These sequence data are available from EMBL/GenBank/DDBJ under accession numbers: TCR-γ L22290; TCR-δ L22291; CD8β L22293; and IL-2Rα L22292.
Figure 3. Northern blot analyses of RNA from rabbit HTLV-I cell lines. Total cell RNA was isolated from lines derived from PBMC (left lanes) or thymus (right lanes) and probed for expression of genes associated with lymphocyte function. Probes for CD8β, TCR-γ and -δ, and IL-2Rα are described in Table 1. Probes for CD4 (33), TCR-α (34), TCR-β (35), and class I MHC genes (36) were described previously.

of rabbit α/β and γ/δ T cell lines to human T cell lines and gave further confidence that the rabbit CD4-CD8- lines are of the γ/δ type. Examination of three representative rabbit lines, RH/K34, RH/Thy-2, and RHT-16, revealed close correlation in cell morphology to human γ/δ T cells, while both the RHT-16 (Fig. 4 B) and RH/Thy-2 (Fig. 4 C) are similar in morphology to human α/β T cells (47). Most RH/K34 cells were ~15 μm long; however, it was possible to find adherent cells >20 μm in length between the widest points of the cytoplasmic extensions. The bipolar fusiform is the most common shape in the rabbit γ/δ culture, but large circumscribed discoid cells similar to other cultured human γ/δ T cells were also observed. By contrast, the CD4+CD8- and CD4-CD8+ α/β rabbit T cells were monomorphic, 8-12-μm-diam spherical cells similar in morphology to human α/β T cells (47).

Role of Infected Cell Type in HTLV-I Disease. Interest in the characterization of these rabbit HTLV-I cell lines is intensified by the fact that two of them, RH/K34 and RH/K32, cause acute leukemia-like disease and death when injected into outbred rabbits (22, 23). Other lines, including RH/K30 and RHT-16, given in equal or higher doses, cause infection but produce no acute disease. Even though primary structural differences exist between the RH/K30 and RH/K34 viruses, sequence analyses yielded no definitive answer about the role of HTLV-I variation in pathogenesis; not all cell lines possessing the HTLV-I sequence found in the lethal lines have the ability to cause lethal leukemia (23). For example, the CD8-TCR-α/β line, RHT-16, which has an integrated viral genome identical in sequence to RH/K34 and RH/K32, does not mediate lethal disease. The present data further suggest that lineage of the infected cell is not the sole factor in the development of leukemia-like disease, since both RH/K34, which causes lethal disease, and RH/K30, which does not,
are of the same CD4⁻CD8⁻ γ/δ phenotype. Although it may be argued that the presence of a given viral sequence in a cell of the appropriate type will provide whatever is necessary to cause fulminate disease, more extensive data will be required to substantiate this. The contribution of other factors, such as the immune response to the virus or to virus-infected cells, must be explored to determine why only certain HTLV-I cell lines are lethal.

Reports concerning the effects of HTLV-I infection on human γ/δ T cells are beginning to emerge. In vitro susceptibility of human γ/δ T cells to HTLV-I infection and the subsequent diminution of the cells' cytotoxic effects (42) implicate HTLV-I γ/δ T cell infection as a potential pathogenic factor. Detection of clonal TCR γ chain gene rearrangements along with monoclonal HTLV-I provirus in cells isolated from a skin lesion of an ATL/L patient (48) suggest γ/δ T cell involvement in skin infiltration characteristic of ATL/L. Lastly, an HTLV-I-infected child with primary immunodeficiency was reported to have increased CD4⁻CD8⁻ as well as CD4⁻CD8⁺ circulating γ/δ T cells (49). These data concerning the role of γ/δ T cells in HTLV-I pathology provide an impetus for further research. The rabbit model for HTLV-I disease, which is improved substantially by the cell lines and reagents developed in this study, can be exploited for studies of the puzzling variability seen in HTLV-I pathogenesis. The results of these studies will have significant impact on our understanding of the varied consequences of human HTLV-I infection.

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