Peripheral blood T lymphocytes obtained from two patients with paroxysmal nocturnal hemoglobinuria (PNH) were immortalized with human T-lymphotropic virus type 1 (HTLV-1). These cells showed interleukin-2 (IL-2)-dependent cell growth in culture. Cell surface analysis showed that they had the phenotype of a helper/inducer T subset that was positive for CD2, CD3, and CD4, but negative for CD8, similar to adult T-cell leukemia cells induced by HTLV-1. These cell lines lacked glycosylphosphatidylinositol (GPI)-anchored proteins, CDw52, CD55 (decay-accelerating factor; DAF), and CD59 on the cell surface, whereas intracellular DAF protein was detected. These T-subset cell lines with a PNH phenotype did not synthesize GPI anchor, whereas a control cell line, similarly prepared from the T cells of a healthy volunteer, produced the anchor. The control cells expressed CDw52, DAF, and CD59 on the cell surface and showed the phenotype of a helper/inducer subset. Southern blot analysis confirmed the clonality of each cell line. These CD4+ T-cell lines with a PNH phenotype and a subset-matched control counterpart could be a useful model for PNH investigation. © 1994 by The American Society of Hematology.

P A R O X Y S M A L  N O C T U R N A L  h e m o g l o b i n u r i a (PNH), an acquired hemolytic disease in which there is a stem cell disorder of a clonal nature, is associated with complications such as venous thrombosis, bone marrow dysplasia, and leukemic conversion.1 The hemolytic features are caused by the enhanced susceptibility of affected cells to the lytic action of autologous complement.2 This abnormal sensitivity is predominantly caused by a deficiency in complement regulatory membrane glycoproteins, such as decay-accelerating factor (DAF, CD55)3,4 and CD59,5,6 which are localized on the cell membrane via a glycosylphosphatidylinositol (GPI) anchor.7,8 Recent studies have showed impaired synthesis of the GPI anchor.9,10 Although the biochemical interruption site in the synthesis of GPI anchor and the molecular basis of PNH in some cases have already been established,11-15 the steps required for the synthesis of GPI anchor16,17 indicate that several interruption sites and resultant PNH subclasses are possible.18

PNH is a rare disease associated with dysplastic hematopoiesis. The establishment of cultured PNH cell lines is essential for obtaining sufficient numbers of affected cells to perform PNH studies. PNH blood cells, including lymphocytes, share common membrane abnormalities caused by the clonal stem cell disorder.19,20 Therefore, lymphocytes may be useful for the establishment of cultured PNH cell lines because it is easy to isolate circulating lymphocytes and to induce both B- and T-cell lines, using Epstein-Barr virus21,22 and human T-lymphotropic virus type 1 (HTLV-1),23-25 respectively. HTLV-1, in particular, can be used to selectively immortalize the CD4+ T-cell subset.24,25 Moreover, the expression of GPI-anchored proteins in T lymphocytes of PNH and healthy individuals has been well characterized.26 Thus, the affected and control T-cell lines can be classified into subsets, in contrast to peripheral granulocytes and B lymphocytes. In the present study, we prepared PNH–T-cell lines of a helper/inducer subset, together with a subset-matched control counterpart.

M A T E R I A L S  A N D  M E T H O D S

Cells and culture conditions. K.T., an HTLV-1–producing adult T-cell leukemia (ATL) cell line27 (a gift from Dr N. Arima, Kagoshima University) was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS; SebaK, Aidenbach, Germany) and antibiotics (100 μg/mL kanamycin and 100 U/mL penicillin G) at 37°C in a humidified atmosphere of 5% CO2 in air. Peripheral mononuclear cells (PMNC) containing lymphocytes were prepared by Ficoll-Conray (Takeda Co, Osaka, Japan) density gradient centrifugation of heparinized blood obtained from healthy volunteers and from three patients with PNH, after obtaining their consent. The disease was diagnosed on the basis of the clinical features, the blood picture, sugar water test, Ham’s acidified serum test, and cell surface analysis of blood cells by cytofluorometry with antibodies against the GPI-anchored proteins DAF and CD59.

T-cell immortalization with HTLV-1. T lymphocytes in the PMNC were selectively infected and immortalized with HTLV-1 by coculture with the virus-producing K.T cells in RPMI medium supplemented with 20% FCS and 10 ng/mL of recombinant interleukin-2 (IL-2; Takeda Co, Osaka, Japan), as described previously.26,27 Briefly, K.T cells (1072/mL well) in 12-well culture plates (Linbro; Flow Laboratories, McLean, VA) were irradiated with 100 Gy to inhibit cell proliferation and were then used as a feeder layer, which simply provided HTLV-1 in the medium. Freshly isolated PMNC (104 in 2 mL) were added to each well, one day after the irradiation. The concentration of the plated cells was then 105 PMNC/104 K.T/4 mL/well. This medium contained 0.5% (wt/wt) phytohemagglutinin (PHA HA15; Wellcome, London, UK) to stimulate lymphocyte growth. Half of the volume of the coculture medium was replaced with an equal volume of fresh medium two or three times a week. One week after the coculture plating, cells were transferred to expand in 10 mL of the medium without the

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lectin, being placed in 50-ml culture flasks (Nunc; Nunc, Roskilde, Denmark). Thereafter, the cells were subcultured two times a week.

Cell cloning by limiting dilution. After 3 months of culture, the cells were confirmed to be growing continuously, but they still required IL-2. We then attempted cell cloning by plating diluted cells, at a concentration of 0.1 to 1.0 cells/well, in ten 96-well U-bottomed tissue culture plates (Cell Wells; Corning Glass Works, Corning, NY) without feeder cells. An aliquot of the surviving and proliferating cells was analyzed by cytofluorometry to identify T cells with a cell surface deficiency in GPI-anchored proteins. Aliquots were also subjected to Southern blot analysis.

Cytofluorometry. The cell surface expression of membrane proteins was analyzed by cytofluorometry, as described previously, with monoclonal antibodies (MoAb) against CD2 (a pan-T marker), CD3 (T-cell receptor), CD4 (a marker for helper/inducer T subset), CD8 (a marker for suppressor/killer T subset), CD19 (a marker of B cells) (all of these lymphocyte markers were purchased from Becton Dickinson, Mountain View, CA), and with MoAb against GPI-anchored proteins, i.e., DAF (Wako, Osaka, Japan), CD59 (provided by Dr M. Tomita, Showa University, Tokyo), and CDw52 (Nippon-Wellcome, Osaka, Japan). Briefly, cells were treated at 4°C for 30 minutes with each of the mouse MoAb against cell surface proteins or standard DAF. The plates were then washed and treated with fluorescein-conjugated goat-antimouse IgG (Zymed, South San Francisco, CA), and finally analyzed with a flow cytometer analyzer (FACScan, Becton Dickinson Laboratories, Mountain View, CA).

Detection of DAF protein. Cell proteins were solubilized by freezing, thawing, and homogenization in a buffer consisting of 50 mmol/L TRIS/HCl (pH 7.4), 1 mmol/L EDTA, and 20 ng/mL leupeptin. The homogenate was centrifuged at 100,000 g for 30 minutes at 4°C, and the supernatant was used as the cytosol fraction. The supernatant was used as the cytosol fraction. The supernatant was used as the cytosol fraction.

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Southern blot analysis. High molecular weight DNA was extracted from the cells by proteinase K digestion, followed by phenolchloroform extraction. DNA was digested with a restriction enzyme (EcoRI), electrophoresed in 0.7% agarose gel, denatured, neutralized, and blotted onto a nylon filter. The membrane filter was then hybridized to nick-translated DNA probes (a viral cDNA, pX, prepared from pMT-2, and a T-cell receptor J3 gene; respectively provided by Drs R.C. Gallo at the National Cancer Institute, Bethesda, MD, and M. Matsuoka, Kumamoto University, Japan), and analyzed by autoradiography.

Karyotype analysis. Cytogenetic studies of the cultured cells were performed, using conventional trypsin-Giemsa chromosome-banding techniques. Karyotypes were analyzed according to the criteria of the international system for human cytogenetic nomenclature.

Detection of GPI anchor. Glycolipids, including the GPI anchor, were labeled in vivo with [3H]-mannose, as described previously. Briefly, cells were preincubated in a glucose-free medium and then radiolabeled with 50 μCi/mL of [3H]-mannose. Radiolabeled glycolipids were extracted with organic solvents, separated by thin-layer chromatography (TLC), and analyzed by autoradiography. The GPI anchor in the cell lipids was identified by labeling with [3H]-sugars (mannose, glucosamine, inositol, and ethanolamine), constructing the core structure of the anchor, and by both TLC and structural characterization with the enzymes GPI-specific phosphodiesterase D, phosphodiesterase, and mannosidase. Assays for cellular proliferation. Cells were plated at a concentration of 5 × 10^6/200 μLwell of a 96-well culture plate, in quadruplicate, and incubated for 6 or 24 hours in the culture medium containing 20% FCS and IL-2 at the designated concentrations (0.0, 2, or 10 ng/mL). For the last 4 hours of each incubation, the cells in each well were metabolically labeled with 1 μCi of [3H]-thymidine (Amersham Life Science, Tokyo, Japan). Cells were then washed with PBS, precipitated in 5% trichloroacetic acid, filtered onto a glass fiber filter membrane (Cambridge Technology, Inc, Watertown, MA). The radioactivity on the membrane was measured by a liquid scintillation counter.

RESULTS

Immortalized cells. In the PMNC cultures from the three patients with PNH and the healthy volunteers, the cells from two of the PNH patients and a healthy control proliferated continuously. After cell cloning by limiting dilution, these cells were designated KM-1 and YT-1, for the PNH cell lines, and TM-1 for the control cell line. These cultured cells still required IL-2 (Fig 1). Another PNH cell line, KM-1, showed a similar response to IL-2 (data not shown). The doubling times of KM-1, YT-1, and TM-1 were, respectively, 20, 21, and 18 hours. All of the IL-2–dependent cell lines have been maintained for more than 20 months.

Cell surface and intracellular expression of membrane proteins. Figure 2 shows the cytofluorometrical analysis of cell surface proteins. The PNH cell lines had the phenotype of a helper/inducer subset of T cells, that is, they were positive for CD2 and CD4, but negative for CD8, or CD19, while they were deficient in the GPI-anchored proteins DAF, CD59, and CDw52. The control T-cell line had the phenotype of a helper/inducer subset of T cells, that is, they were positive for CD2 and CD4, but negative for CD8, or CD19, while they were deficient in the GPI-anchored proteins DAF, CD59, and CDw52.
IL-2-DEPENDENT PNH-T CELL LINES

2-CD2  CD4  CD8  DAF  CD59  CDw52

Fig 2. Cytofluorometric analysis of cell surface expression of lymphocyte markers (CD2, CD4, and CD8) and GPI-anchored proteins (DAF, CD59, and CDw52) in established PNH cell lines (YT-1 and KM-1) and a control cell line (TM-1). (-----), background staining with a control antibody; and (----), staining with specific antibodies.

Fig 3. Integration sites of HTLV-1 proviral DNA (A) and configurations of T-cell receptor Jβ2 genes (B). Arrow indicates a 4.0-kb band detected by the Jβ2 gene probe in EcoR1 digests of germline DNA.

Table 1. Intracellular Expression of DAF Determined by ELISA

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>TM-1</th>
<th>YT-1</th>
<th>KM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate membrane</td>
<td>20.2</td>
<td>1.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Cytosol</td>
<td>84.6</td>
<td>68.5</td>
<td>66.0</td>
</tr>
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</table>

Values show DAF protein (ng/10 mg of cell protein). Each value given is the mean of triplicate assays.

Mitogenic response to IL-2. Both of the cell lines showed IL-2-dependent growth, and PNH cells were particularly highly dependent (Fig 5). Moreover, the mitogenesis of control cells was faster than that of PNH cells.

DISCUSSION

HTLV-1 selectively infects and then immortalizes the helper/inducer T-subset.24,25 Similarly to ATL cells,39 we found here that all of the established cell lines immortalized with HTLV-1 had the phenotype of a helper/inducer T-subset and the monoclonal integration of the HTLV-1 genome into their cellular DNA. Although the cells synthesized DAF protein intracellularly, the PNH-T-cell lines were deficient in the GPI-anchored proteins DAF, CD59, and CDw52 on the cell surface; control T cells were positive for all of the GPI-anchored proteins.

As compared with CD3 of T cells in the PMNC, CD3 expression in control and PNH cell lines was variably decreased (data not shown), as is often observed in HTLV-I-transformed and ATL cells.24,25,36,37 The intracellular localization of DAF was determined by ELISA (Table 1). PNH cell lines produced DAF protein, but to a lesser extent than control cells, especially in the particulate membrane fraction.

Clonality and karyotype. Figure 3A shows the integration of the HTLV-1 genome into the cellular DNA. Because HTLV-1 genome (10 kb) does not have an EcoR1 digestion site, a pX probe usually detects full-length viral DNA coupled with differential flanking regions of cellular DNA in the EcoR1-digested cellular DNA.25,31,32 DNA-fragments shorter than 10 kb may be detected in HTLV-1-transformed cells, partly because of defective HTLV-1 provirus.38 Cultured cells showed random integration and several copies of the HTLV-1 genome in their DNA, consistent with previous reports.25,32 To confirm their T-cell origin and clonality, configurations of T-cell receptor Jβ2 genes in the HTLV-1-transformed cells was examined (Fig 3B). Differential patterns of the gene rearrangement suggested that the cell lines originated from different T-cell clones. K'T, TM-1, and KM-1 respectively showed a rearranged and a nonrearranged germline fragment of Jβ2 genes, whereas YT-1 showed two rearranged Jβ2 genes.33 Karyotypes were: TM-1 control cell line, 91, XXXX, 6q−, +8, −11, −15; KM-1 PNH cell line, 46, XX; and YT-1 PNH cell line, 46, XY.

Synthesis of GPI anchor. The [3H]-mannose labeled GPI anchor was detected in control cells, but not in YT-1 PNH cells (Fig 4). This was consistent with previous reports of a synthetic defect of the anchor in PNH.10,11 The anchor was also labeled with other [3H]-compounds, and the other PNH cell line (KM-1) also showed a defect of the anchor (data not shown).
This indicated that the cultured cells had derived from the peripheral T cells of the PNH patients because they had been immortalized with HTLV-I produced by the K3T cells. However, the possibility that the cell lines were derived from the K3T cells (an ATL cell line) used as feeder cells was also ruled out. In the coculture of PMNC with irradiated K3T cells, there were no viable K3T cells after 100 Gy irradiation, as determined by the Trypan blue dye exclusion test. Moreover, in contrast to PNH cells, the K3T cells were positive for DAF and CD59 at the cell surface (data not shown). Even if K3T were fused with the PNH-T cells, the hybrid would not be cytofluorometrically negative for DAF and CD59 because the PNH phenotype is recessive. Furthermore, Southern blot analyses showed that the three cell lines were not identical to K3T cells. These findings thus indicate that the PNH and control cell lines were derived from T cells in the PMNC of PNH patients and a healthy volunteer, respectively.

Viral infection may affect the expression of DAF. However, HTLV-I infection in this present study did not alter the expression of surface proteins. Because DAF was not cytofluorometrically detected on the cell surface of PNH cells, the DAF detected by ELISA in the particulate fraction of PNH cells may have originated in the membrane of a microorganelle (microsome). However, in KM-1, we could not rule out the possibility of a slight expression of membrane DAF (Fig 2). This ELISA was previously established for DAF alone and DAF was measured as a representative of GPI-anchored proteins. Western blot analysis did not detect DAF proteins in the cell homogenates. Differences in the sensitivity of detection between ELISA and blot analysis may explain this. The lack of DAF on the cell surface, and the detection of DAF in cell proteins suggests impaired GPI anchoring of DAF. In addition, synthesis of the GPI anchor in the PNH–T cell lines was impaired (Fig 4).

We noted that PNH cells showed considerably slow mitogenesis as compared with subset-matched control cells. The molecular mechanism and pathophysiologic significance of this slow response are important because autocrine activation of T cells by IL-2 is a critical step in T-cell–mediated immune responses. To our knowledge, these are the first established T cell lines to have a well-characterized PNH phenotype and a strict control counterpart. In addition to granulocytes, B-cell lines, and a natural killer lymphocyte cell line, we believe that these established T-subset cell lines are useful for PNH studies, including the characterization of T-cell function in PNH and the investigation of PNH subclasses.

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Interleukin-2-dependent T-cell lines established from paroxysmal nocturnal hemoglobinuria patients

H Nakakuma, S Nagakura, K Horikawa, M Hidaka, T Kawaguchi, N Iwamoto, I Sanada, T Kagimoto and K Takatsuki