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Establishment of a Lymphocyte Clone
Expressing Granzyme B and Perforin mRNAs Immortalized with *Herpesvirus saimiri*

Osamu Horie and Ryukichi Ryo

Minor histocompatibility antigens play a crucial role in inducing graft-versus-host disease (GVHD) after human leukocyte antigen (HLA)-identical allogeneic bone marrow transplantation. To identify unknown antigens, we need cytotoxic T lymphocyte (CTL) recognized by specific antigens. In this study *Herpesvirus saimiri* (HVS) subgroup C488 was used to establish a stable CTL clone. First, normal mononuclear cells were infected with HVS in owl monkey kidney (OMK) supernatant and cultured in RPMI 1640 medium supplemented with 10% FBS and 20% T cell growth factor (TCGF). The most proliferative cells were then subjected to five limiting dilutions. After this, the presence of the CD3⁺CD4⁻CD8⁺ T lymphocyte clone was observed. Interestingly, CD4⁺ T cells soon disappeared in the presence of TCGF after being infected with HVS. To confirm whether our lymphocyte clone was cytotoxic, the presence of mRNAs for cytotoxic granule granzyme B and perforin was examined using RT-PCR, and both mRNAs were identified. Our results thus demonstrate that the established clone might be a CD8⁺ cytotoxic T lymphocyte clone and that HVS was a useful tool for the immortalization of CTL. The disappearance of CD4⁺ T lymphocytes in our system suggests that some inhibitory factor may be present in TCGF.

**Key Words:** *Herpesvirus saimiri*, Cytotoxic T lymphocyte, GVHD, Granzyme B.

**Introduction**

Recently allogeneic bone marrow transplantation (BMT) is generally performed with an HLA-identical donor. Nevertheless, occurrence of GVHD is observed in most cases after BMT. It is speculated that GVHD is induced by attacks from incompatible minor histocompatibility antigens. In order to identify a target antigen in allogeneic HLA-identical GVHD, we need a stable cytotoxic T lymphocyte (CTL) clone established from the recipient with GVHD. The *herpesvirus saimiri* (HVS) subgroup C488 has been found to immortalize T cells and γδ T cell. In this study we attempted to establish a CTL clone using HVS and observed the characteristics of the cloned cells.
Materials and Methods

Preparation of T cell growth factor (TCGF)

Mononuclear cells from the buffy coat prepared from packed red cells of 10 healthy individuals were isolated with the Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) method and precultured in 100 ml of RPMI 1640 (Gibco BRL, NY, USA) medium supplemented with 1% human serum in a 5% CO₂ incubator at 37°C for 20 minutes. After inversion of the culture flask, the cells were cultured for a further 20 minutes. The lymphocytes suspended in 500 ml RPMI 1640 at a concentration of 1 to 2 × 10⁶ cells/ml were incubated with 0.2% PHA-P (Becton Dickinson, MD, USA), 0.1% indomethacin (Sigma, MO, USA) (1 ml/l) and 1% human serum for 48 hours. TCGF was extracted from the culture supernatant.

Separation of mononuclear cells from peripheral blood

Buffy coats were isolated from normal heparinized peripheral blood and mononuclear cells were separated from this buffy coat with a Ficoll-Paque solution. The isolated mononuclear cells were then cultured in RPMI 1640 medium supplemented with 10% FBS and 20% TCGF in a 5% CO₂ incubator at 37°C.

Infection of mononuclear cells with HVS

HVS in owl monkey kidney (OMK) supernatant was added to the cultured mononuclear cells from healthy donors, which were then cultured together for 30 minutes.⁷,⁸

Cloning of viable T lymphocytes

The feeder cells were prepared from X-ray irradiated lymphocytes from five normal individuals. T lymphocytes infected with HVS were seeded at a concentration of 1 cell/well in flat-bottom microtiter wells (Asahi Techno Glass, Chiba, Japan) containing 200 μl of culture medium supplemented with feeder cells. The clones were prepared by means of five limiting dilutions.

Histochemical analysis of the hematopoietic cells

Morphological characteristics of cells stained with May-Grünwald Giemsa were observed under a microscope.

Analysis of surface antigens of cells

Expression of surface molecules such as CD3, CD4, CD8 and CD19 on the cells was analyzed by immunohistochemical staining and by direct immunofluorescence using a flow cytometer (FACS Calibour; Becton Dickinson). The avidin-biotin-peroxidase complex method (Vectastain ABC-AP kit; Vector, CA, USA) was used for immunohistochemical staining. Cells were stained with anti CD3, CD4 and CD8 monoclonal antibodies (Zymed, CA, USA) using the avidin-biotin-peroxidase complex. Fluorescein-isothiocyanate (FITC) conjugated CD3 mAbs, FITC conjugated CD4 mAbs, phycoerythrin (PE) conjugated CD8 mAbs and PE conjugated CD19 mAbs (Coulter Immunology, FL, USA) were used for flow cytometric analysis.
RT-PCR analysis for mRNA expression of granzyme B and perforin

Total RNA was extracted from cells with ISOGEN (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from this total RNA with reverse transcriptase from Bacillus caldotenax (TaKaRa Biomedicals, Shiga, Japan). This cDNA was then amplified for 27 cycles with the primers 5'-CGGGATCCATGCAACCAATCTGCTT-3'(sense) and 5'-CCGAATTCATCTAGTAGCGTCATGGTTTT-3'(antisense) and using a Thermal Cycler (Perkin Elmer, NJ, USA) to observe the expression of granzyme B mRNA. The amplified products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide to determine their molecular weight. For identification of the presence of perforin mRNA, primers 5'-CGGAGATTCAATGGCAGCCCTCCTGCTGCCT-3'(sense) and 5'-CCCTCGACGTACCACACGGCCCCT-3' (antisense) were used.

Results

Various hematopoietic cells including CD3⁺ T lymphocytes, CD19⁺ B lymphocytes, monocytes and neutrophils were found in the mononuclear cells separated from peripheral blood (data not shown). Ten days after being infected with HVS, most of the proliferating cells consisted of CD3⁺ cells (Fig. 1). Interestingly, CD4⁺ T cells soon disappeared in the presence of TCGF. CD8⁺ cells accounted for 96% of the cells (Fig. 2B). Limiting dilutions were repeated 5 times for the most proliferative cells, after which the CD3⁺ CD4⁻CD8⁺CD19⁻ T lymphocyte cell line was established (Fig. 2). The lymphocyte clones obtained from five other individuals had similar characteristics. Expression of grB mRNA and perforin mRNA, which is specific for CTL, was examined in the cloned cells. Unfortunately, we could not obtain data concerning the expression of grB and perforin mRNAs in the cells after 2, 3, and 4 limiting dilutions. Fig 3 shows that the stable CD8⁺ T lymphocyte clone expressed mRNAs for grB and perforin.

Figure 1. Characteristics of cultured hematopoietic cells. The mononuclear cells were stained 5 days after being infected with HVS. M·G staining shows that most of the cells are lymphocytes and that other hematopoietic cells had disappeared (A). ABC staining with anti CD3 antibody. The cells were positive for CD3 antigen (B).
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in, thus indicating that the clone may be derived from CTL.

Discussion

In vitro culture of CTL with IL-2, IL-12, TCGF and some other cytokines has been tried by many investigators. It has been generally accepted that various cytokines do not have the capability to maintain T cell clones. However, it seems possible to establish a stable CTL clone by repeated antigenic stimulation. This method is

Figure 2. Characteristics of the cloned cells. The HVS-infected T cells were subjected to five limiting dilutions. ABC staining with anti CD4 antibody shows that the cells were negative for CD4 antigen (A). ABC staining with anti CD8 antibody shows that the cells were positive for CD8 antigen (B). CD4 and CD8 expression in the cells was immunohistochemically confirmed (C).

Figure 3. mRNAs for grB and perforin in the cloned T cells. Expression of mRNA for grB and perforin was examined using RT-PCR. The arrow indicates perforin cDNA (A). The arrow indicates granzyme B cDNA (B).
not suitable for the establishment of a clone from patients with GVHD after HLA-identical allogeneic BMT, since the antigeneic target has not been identified. In this study HVS was used to obtain a stable clone. After being infected with HVS, CD3+ T lymphocytes were found to proliferate. Interestingly, CD4+ T cells soon disappeared in the presence of TCGF, while CD8+ cells were found to continue to proliferate. The reason why CD4+ T cells disappeared remains unclear, but it seems possible that some inhibitory factors were present in our TCGF. However, further investigation is required to clarify the mechanism of inhibition of CD4+ T lymphocyte growth. Limiting dilution resulted in the establishment of the CD8+ T lymphocytes clone, which has been maintained for nearly 6 months. It is known that CTL plays an important role in inducing the apoptosis of target cells through Fas/Fas ligand, perforin/granzyme, and TNFα. Thus, the presence of grB and perforin mRNAs indicate that T cell is cytotoxic. When we therefore observed the expression of mRNAs of grB and perforin, we found that both mRNAs were strongly expressed in our established clone. Our study indicates that HVS is a useful tool for the immortalization of cytotoxic T cells, and that some factor which inhibits the growth of CD4+ T lymphocyte may be present in our TCGF.

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References

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