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Characterization and sensitivity to interleukin-2 and interferon-alpha of leukemic cells from a patient with large granular lymphocytic leukemia associated with chronic active Epstein-Barr virus infection.

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Abstract A patient presented with chronic large granular lymphocytic leukemia after 13 years of mosquito allergy, recurrent fever, hepato-splenomegaly, liver dysfunction and chronic active EBV infection. Surface marker analysis of the leukemic cells showed the presence of CD2, CD8, CD16 and CD56, and absence of CD3, CD57 and HLA-DR. Cell cycle analysis revealed a minimal growth fraction compatible with chronic lymphocytic leukemia, and Southern blots of the cells showed monoclonal integration of EBV. After 5 months of treatment, the patient died from acute transformation of the leukemia and severe liver dysfunction. Cells harvested during chronic phase were analyzed for sensitivity to interleukin-2 (IL-2) and interferon-alpha (IFNα) in vitro by means of surface phenotyping and cell cycle assay. IL-2 induced remarkable growth of the cells, whereas IFNα did not confer a growth advantage. Since IFNα was expected to have no growth induction effect on the leukemia cells, it was administered to the patient to treat the chronic active EBV infection. There is always a risk of conferring a growth advantage upon cancer cells when growth factors or cytokines are administered in vivo as shown in this report.

Introduction

Chronic active Epstein-Barr virus infection (CAEBV) is a group of disorders related to some granular lymphocyte-proliferative disorders (GLPD), virus-associated haemophagocytic syndrome (VAHS), and malignant histiocytosis [1-10]. Moreover, some T- and B-lymphomas, as well as Hodgkin's disease are thought to be involved in EBV infection [11-14]. GLPD are divided into two groups, CD3+ T-cell GLPD (T-GLPD) and CD3-/CD16+ natural killer GLPD (NK-GLPD), based on the surface phenotype of the leukemia cells. The clinical course of most GLPD is comparable to that of chronic lymphocytic leukemia (CLL), although it is likely to include an aggressive form of leukemia associated with high potency of proliferation. Despite several studies of aggressive GLPD, an efficient treatment for this disease remains known. Combination chemotherapy such as CHOP is usually not effective. Other trials, such as corticosteroids, cyclosporin A, and interferon are also reportedly ineffective. Thus, other means are required with which to treat this malignant disease.

A young female patient presented with progressive NK-GLPD. The cytokines, interleukin-2 and interferon-alpha, which are reportedly effective in treating CAEBV related disorders, were the candidates for treating this patient. Leukemic cells were harvested from the patient's blood and cultured in vitro to study their sensitivity to the cytokines in terms of cell growth activation. This procedure helped to predict the efficacy and safety of clinical trials.

Case report

The patient (a 16-year old girl) had experienced recurrent fever, hepato-splenomegaly and liver dysfunction triggered by a mosquito allergy accompanied with skin ulceration and leg edema since 3 years of age. Her parents were not consanguinous. In October, 1992, she suffered from a high grade fever and developed a sensory disturbance in lower extremities. She was then admitted to our hospital. Moderate edema was present in the face and legs, ulcer scars were evident in legs, and red papules were diffusely observed over the four extremities. Remarkable hepato-splenomegaly (liver was palpable 3 finger breadths below the costal margin and spleen, 5 f.b. below the navel) and no lymphoadenopathy were revealed by computed-tomography. She showed tenderness, dysesthesia and paresthesia in the legs, as well as other signs of peripheral neuropathy and cerebrospinal involvement [15]. Laboratory examination showed mild leukocytopenia with granular lymphocytosis (Figure 1-A) and thrombocytopenia (WBC 3,200 /μl, LGL 37 %, Plt 79 $x10^3$/μl), liver dysfunction (GOT 575 IU/l, GPT 246 IU/l, LDH 2,029 IU/l, Alp 3,650 IU/l, γ-GTP 291 IU/l, TB 1.2 mg/dl (dB 0.9 mg/dl)), a mild abnormality of coagulation (PT 14.9 sec, 100.0 %, aPTT 46.3 sec (control: 31.6 sec), Fbg 154.6 mg/dl, FDP 3.24 μg/ml, D-dimer 1.22 μg/ml), and high serum ferritin (302 ng/ml). The ultramicroscopic structure of LGL in the blood showed multivesicular granules in the cytoplasm. Three-color surface marker analysis of LGL showed the presence of CD2, CD8, CD16, CD56, CD122, and the absence of CD3, CD4, CD57, HLA-DR, CD25, TCRα/β, and TCRδ1. On the other hand, the cells stained for cytoplasmic CD3 in situ using anti-Leu4 monoclonal antibody and APAAP method. The bone marrow was hypercellular, and the number of monocytes and macrophages was high. EBV infection was serologically tested as follows. EB-VCA-IgG, x 5,120 (normal 10 - 160); EB-VCA-IgM, lower than (LT) x 10 (LT 10); EB-VCA-IgA, x 160 (LT 10); EB-EA-DR-IgG, x 5,120 (LT 10); EB-EA-DR-IgA, x 160 (LT 10) and EB-EBNA, x 40 (10 - 320). Pleocytosis and increased protein levels in the cerebrospinal fluid were found. Peripheral blood lymphocytes (LGL, higher than 80 %) were Southern blotted to detect terminal repeats of the EBV genome and monoclonal integration was found (Figure 3). The procedure to analyze the integrated EBV was described elsewhere [16]. JH and TCR were also examined, and JH, TCRβ, TCRγ as well as TCRδ were not rearranged. On the other hand, TCRβ mRNA varied in size on Northern blots (data not shown). Cell cycle analysis of leukemia cells in the chronic phase showed a low percentage of the growth fraction compatible with CLL (Figure 2-A). She was diagnosed as having a granular lymphocyte proliferative disorder (GLPD) accompanied with chronic active EBV infection and virus associated hemophagocytic syndrome (VAHS). She was administered with 1,000 mg of daily intra-venous Acyclovir from October, 1992, and 3 million units of native interferon alpha i.m. was added 3 times each week from January, 1993. A bolus injection of methyl-prednisolone (mPSL) to treat interstitial pneumonitis and respiratory failure caused by the IFN therapy, improved the liver dysfunction and GLPD associated peripheral neuropathy. A second trial of bolus-mPSL therapy combined with intravenous injections of vincristine and etoposide was attempted to treat the GLPD, and there was another tentative improvement in the disease. In March, 1993, liver dysfunction rapidly progressed (GOT 3,260 IU/l, GPT 2,226 IU/l, LDH higher than 9,999 IU/l, Alp 796 IU/l, TB 21.5 mg/dl) and leukocytosis (WBC 15,200 /μl, LGL 89 %) was accompanied by morphological acute transformation of GL (Figure 1-B), high grade fever, respiratory failure, thrombocytopenia (22 $x10^3$/μl), anemia (Hb 7.2 g/dl), and a remarkable ferritin level (16,000 ng/ml), followed by death. The surface markers of the blasts in the acute and chronic phases were the same. On the other hand, cell cycle analysis showed a higher ratio (%) of the growth fraction compatible with ALL (Figure 2-B, C). Postmortem examination showed LGL infiltration and expansion of macrophages in the bone marrow, liver, spleen and lungs. The in situ phenotypes of the LGL cells that infiltrated the liver showed the presence of CD45RO, CD43, CD57, and absence of CD45R, CD30, L26 using monoclonal antibodies and peroxidase. The EBV genome was detected in the liver, spleen, lungs, and heart by means of Southern hybridization. In situ hybridization detected EBER-1 RNA in the liver and spleen using EBER-1 probe labeled with digoxigenin. Immunological activities and cytokine levels in circulating blood taken from the patient during the acute phase of leukemia were measured.

Soluble IL-2 receptors (EIA) and macrophage colony-stimulating factor (ELISA) were increased in plasma at 1,480 pmol/l (normal range: 28.6 - 75.9) and 3,731 U/ml (756 $\pm$ 147), respectively, indicating the activation of the monocyte-macrophage system. Other cytokine levels in plasma were as following: IL-1 beta (EIA), lower than (LT) 15.6 pg/ml (normal: LT 15.6); IL-4 (EIA), LT 31.3 pg/ml (LT 31.3); IL-6 (EIA), LT 10.0 pg/ml (LT 10.0); TNF alpha (EIA), LT 7.0 pg/ml (LT 7.0) and granulocyte-macrophage colony-stimulating factor (EIA), LT 2 pg/ml (LT 5). Interferon (IFN) levels (RIA) were: IFN alpha (plasma), LT 10 IU/ml (LT 10) and IFN gamma (serum), 27.9 U/ml (LT 0.4). The immunological activities of ADCC, NK, basal LAK, and in vitro activated LAK measured by means of radioactive chromium release were 68 (normal: 41 - 72), 69 (18 - 40), 34 (LT 10) and 85 % (20 - 80), respectively.

Materials and methods

DNA/RNA staining reagents and monoclonal antibodies.

We purchased 7-amino-actinomycin-D (7AAD) [17, 18] from the Sigma Chemical Company, St. Louis, MO. Pyronin Y (PY) [19] was purchased from Polysciences, Warrington, PA. DNA/RNA dye was dissolved in PBS at 20 times the final concentration, and 50 µl of dye was added to 1 x 10^6 cells in 1 ml.

FITC-conjugated anti-Leu-5b (CD2), Leu-4 (CD3), Leu-2a (CD8), control IgG1, PE-conjugated anti-Leu-4 (CD3), Leu-19 (CD56), control IgG1 and Per/CP-conjugated anti-Leu-2a (CD8) and control IgG1 were purchased from Becton Dickinson Immunocytometry Systems, Mountain View, CA.

Cells and culture conditions.

Heparinized peripheral blood and bone marrow cells were obtained from the patient. Mononuclear cells were isolated by means of Lymphoprep (1.077, Nycomed Pharma AS, Oslo, Norway) density centrifugation. Light density mononuclear cells were washed with RPMI-1640 (Gibco Laboratories, Grand Island, NY) medium containing 100 U/ml penicillin G sodium (PCG; Biowhittaker, Walkersville, MD), 100 µg/ml streptomycin sulfate (SM; Biowhittaker) and 10% heat-inactivated fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan), resuspended in the same medium, then cultured with 500 JRU/ml of human recombinant interleukin-2 (Shionogi Pharmaceuticals, Osaka, Japan) or 100 U/ml of human native interferon alpha (Sumitomo Pharmaceuticals, Osaka, Japan) for 2 or 4 days at 37^{o}C in 5% CO_2 in humidified air.

Flow cytometry

For surface marker analysis, 1 x 10^6 cells were stained simultaneously with FITC-, PE- and Per/CP-conjugated monoclonal antibodies, washed twice with PBS then analyzed by means of flow cytometry.

Cell kinetics were analyzed using 7AAD/PY as described [20, 21]. In brief, up to 1 x 10^6 cells were stained with FITC-conjugated monoclonal antibody, then washed twice with IFA buffer (10 mM/l Hepes buffer in 0.15 M NaCl supplemented with 0.1% NaN_3 and 4% FBS). Pelleted cells were suspended in 1 ml of nucleic acid staining solution (NASS; 0.15 M NaCl in 0.1 M phosphate-citrate buffer supplemented with 5 mM sodium EDTA (Sigma) and 0.5% bovine serum albumin (Sigma; BSA, fraction V), pH 6.0) containing 0.004% saponin (from quillaja bark, Sigma). Thereafter, 50 µl of 400 µM 7AAD (final 20 µM) was added and the cells were incubated at RT for 30 min. After an incubation with the DNA dye, cells were cooled on ice for at least 5 min, then 50 µl of 100 µM PY (final 5 µM) was added. The cell suspension was incubated for an additional 10 min on ice, then analyzed.

Cell fluorescence was analyzed with a FACScan^{TM} Flow Cytometer (Becton-Dickinson, Mountain View, CA). The cells were excited with a single, 488 nm argon laser, then simultaneous green (FL1; 525 nm), orange (FL2; 570 nm), and red (FL3; >650 nm) fluorescence emission was analyzed. The thousand events were collected. The data were analyzed using Lysis II^{TM} and CellFit^{TM} software (Becton-Dickinson).

Results

Surface phenotype changes in culture

Peripheral blood mononuclear cells were harvested from the patient during chronic phase of the disease, December 9, 1992, and cultivated in the presence and absence of IL-2 or IFNα. The phenotypic changes of CD2, CD3, CD8 and CD56 in the culture are shown in Figure 4. The pathological cells (red dots in Figure 4)

cultivated in the absence of IL-2 and IFNα expressed CD3 antigen on days 2 and 4, although to a lesser extent than T-cells in the same culture. At the same time, the level of CD8 expression on the pathological cells increased comparable to that of CD8 positive T-cells at days 2 and 4.

The cells cultivated in the presence of IFNα showed the same change on the surface compared with the control culture. On the other hand, IL-2 induced different changes compared with the control culture. CD2 and CD56 expression on the pathological cells increased on days 2 and 4, and the level of CD2 expression was higher than that of CD8 positive T-cells.

Cell cycle analysis of cells in culture

The kinetics of the cultivated cells was also analyzed using 7AAD/PY (Figure 5). The cells before culture showed a minimal growth fraction (G1, 98.9 %; S, 1.0 %; G2+M, 0.1 %), that mainly consisted of CD2+/CD3-/CD8 dim+ NK cells. When cultivated in the absence of IL-2 and IFNα, the growth fraction decreased on days 2 and 4 (G1, 99.5 %; S, 0.4 %; G2+M, 0.1 % in day 2, and G1, 99.4 %; S, 0.5 %; G2+M, 0.1 % in day 4). The growth fraction of the cells cultivated with IFNα decreased on day 2 (G1, 99.3 %; S, 0.5 %; G2+M, 0.2 %). In contrast, the cells started to proliferate and progress to early S-phase on day 4 (G1, 98.6 %; S, 1.2 %; G2+M, 0.1 %). The growing cells in the culture at day 4 were mainly CD2+/CD3-/CD8+ NK cells (compare with the phenotype analysis in Figure 4).

On the other hand, when cultivated in the presence of IL-2, NK cells remarkably proliferated on days 2 and 4. The novel growing fraction of NK cells progressed into the early S phase on day 2 (G1, 86.9 %; S, 12.3 %; G2+M, 0.8 %), reaching maximal growth on day 4 (G1, 53.3 %; S, 40.3 %; G2+M, 6.4 %). The growing cells in the culture with IL-2 were mainly CD2+/CD3-/CD8 dim+ NK cells. Cells growing in the presence of IFNα contained a relatively small amount of RNA in S phase, while the S fraction of the cells activated by IL-2 showed relatively a high level of RNA in the RNA/DNA dot plots in Figure 6. Further cell cycle analysis of IL-2 activated pathological cells is shown in Figure 6. In conclusion, IFNα did not induce a remarkable growth of the pathological NK cells, whereas, IL-2 induced maximal growth of the cells in culture.

Discussion

Chronic active EBV infection (CAEBV), EBV related virus associated hemophagocytic syndrome (EB-VAHS) and granular lymphocyte-proliferative disorders (GLPD) are common in Japan. The reason is not known. Some cases reportedly have a history of mosquito-allergy in childhood and other prospective followup studies of children with mosquito-allergy have shown that they developed EB-VAHS [22, 23]. The patient described here also had history of mosquito-allergy 13 years before the onset of GLPD.

The pathological cells in this patient were CD2+/CD3-/CD8+/CD16+/CD56+/CD57- non-T natural killer cells. Thus, the clonality of the cells could not be demonstrated by means of biological studies of the T-cell receptor gene. However, the terminal repeat region of the integrated EBV genome revealed the clonality. The cells expressed TCRβ mRNA before, as well as CD3 during culture in the presence and absence of IL-2 or IFNα. Therefore the cells may be intermediate between natural killer and killer T-cell.

Several authors have performed in vitro studies or attempted clinical trials to treat CAEBV, EB-VAHS, or GLPD. For example, acyclovir or ganciclovir has been used to treat EBV infection [24-27], splenectomy and/or interleukin-2 (IL-2) has been applied to CAEBV [28, 29], and interferon-alpha (IFNα) has been used against EBV-associated B-cell lymphoproliferative disorders [30]. Among these, we selected interferon-alpha (IFNα) and interleukin-2 as candidates with which to treat this patient. Both IFNα and IL-2, might stimulate the proliferation of clonal NK cells. We therefore studied the in vitro activity of these two factors upon clonal NK cells of this patient. IL-2 induced maximal growth of the cells, while IFNα did not induce remarkable proliferation in vitro. IL-2 might therefore activate clonal proliferation of the cells in vivo, so we regarded IFNα as a potentially safe means of treating this patient. IFNα administration to treat the chronic active EBV infection did not cause expansion of the leukemia cells in the patient.

New biological activities such as growth factors and cytokines are discovered year after year. Some of these factors have been used to treat hemopoietic disorders such aplastic anemia [31,

32], malignant lymphomas [33, 34], and acute leukemias [35, 36]. Despite these trends in modern clinical applications, there is always a risk of activating the growth advantage of cancer cells with these factors in vivo. Hence, it will be safe to assume that such factors activate the growth advantage in vivo until a treatment strategy is established. First of all, the sensitivity of cancer cells to factors should be determined ex vivo using individual clinical sample to predict the efficacy and at least, the safety of administering patients with biologically active materials.

There are several means of monitoring growth such as tritium-thymidine uptake, bromodeoxyuridine incorporation [37, 38, 17], propidium iodide and acridine orange staining [39 - 43]. However, 7AAD/PY based on the cellular DNA/RNA content is one of the simplest and most accurate means of calculating distinct populations of G0, G1a, G1b, S, and G2+M [20, 21]. This procedure can also be applied to analyze heterogeneous clinical samples in combination with FITC-conjugated antibodies while preserving light scatter and cell morphology. Moreover, this method is also applicable to the simultaneous three color analysis of two surface phenotypes and DNA quantitation using FITC- and PE-conjugated monoclonal antibodies and 7AAD [44].

In conclusion, IL-2 is expected to expand NK leukemia mass, while interferon-alpha is safely administered in vivo without activation of the growth of NK leukemia cells.

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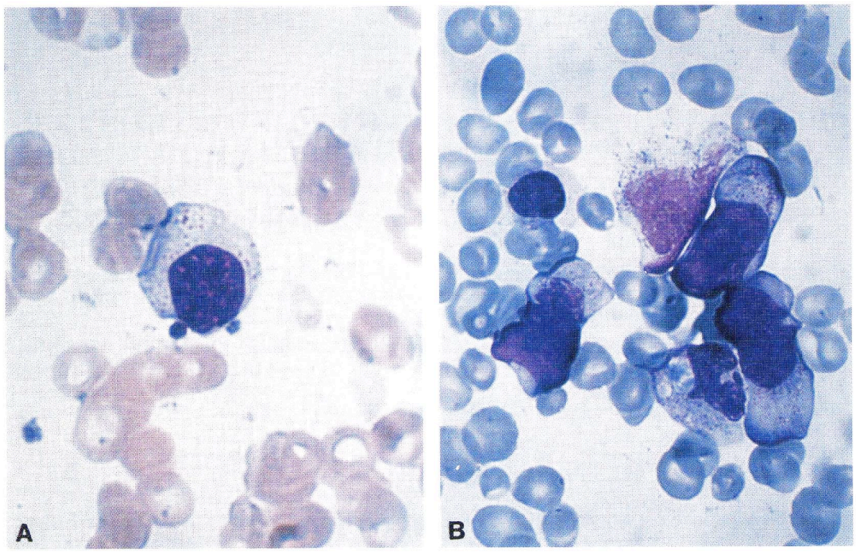

Figure 1. Morphology of leukemic large granular lymphocytes (May-Giemsa stain). (A) The granular lymphocytes in the blood upon admission were mature. (B) The cells in the blood at acute transformation were immature blasts. (original magnification x 1,000).

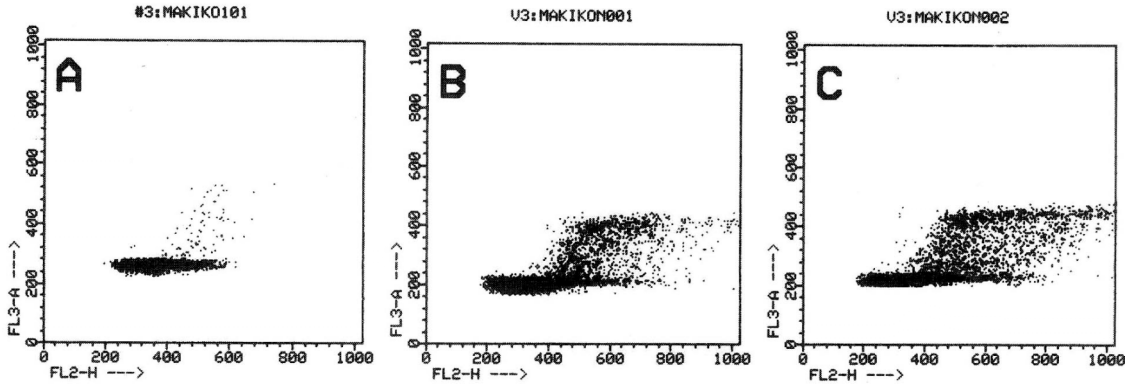

Figure 2. Cell cycle analysis of leukemic LGL using 7AAD/PY. LGL in the blood in chronic phase showed a minimal growth fraction compatible with CLL (panel A, S = 0.7%). On the other hand, the percentage of the growth fraction in the cells after blastic transformation in the blood (panel B, S = 6.5%) and bone marrow (panel C, S = 8.9%) was high, being compatible with ALL. X-axis, RNA; Y-axis DNA.

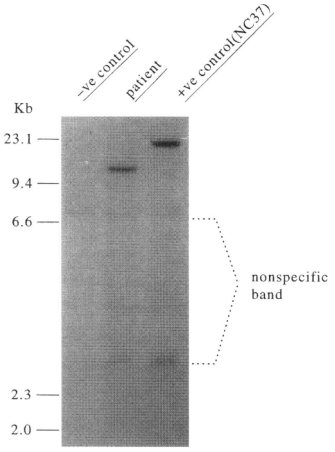

Figure 3. Monoclonal integration of EBV in lymphocytes. Peripheral blood lymphocytes (LGL over 80%) were separated and the EBV genome was detected by Southern blotting with an EBV terminal repeat probe. -ve control, negative control; +ve control, positive control of the EBV integrated cell line (NC37).

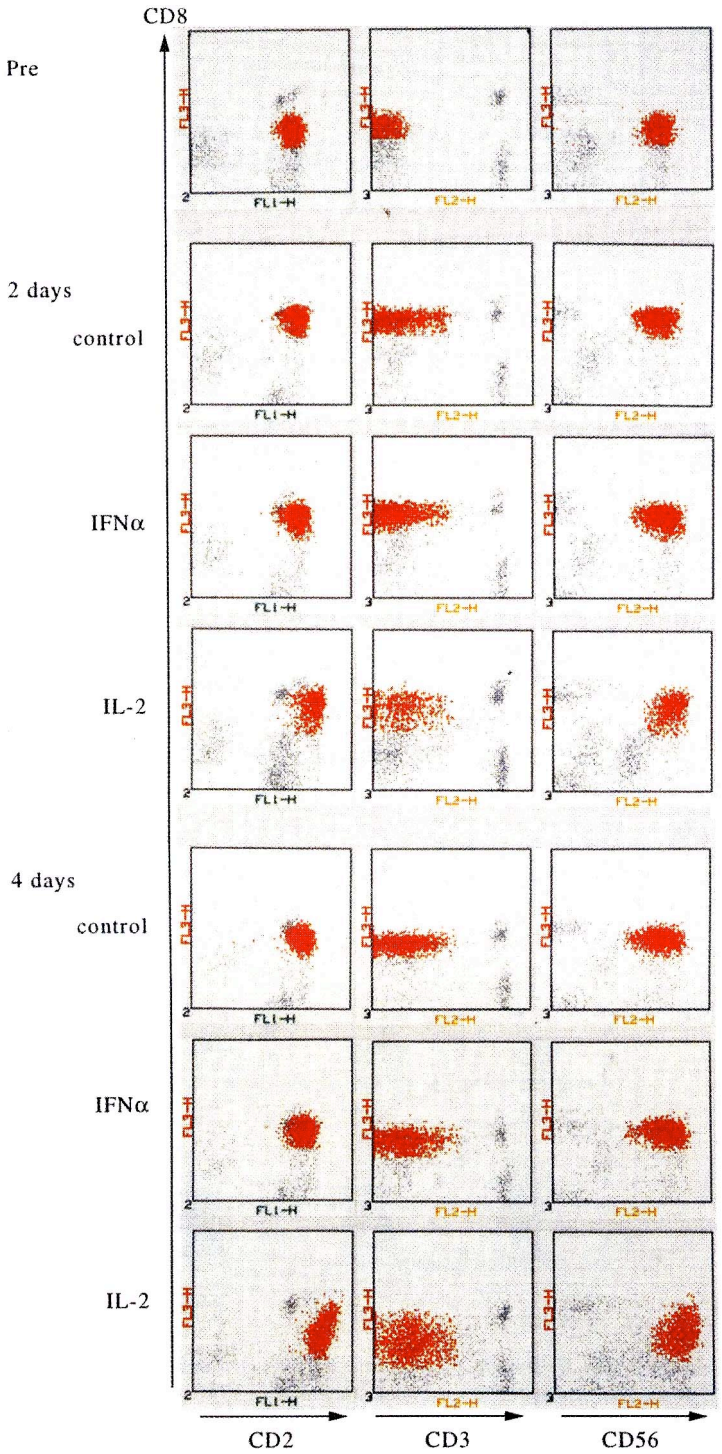

Figure 4. Surface phenotype of cultivated cells. Peripheral blood mononuclear cells were caltured for 2 or 4 days in the presence or absence of interferon-alpha or interleukin-2. CD2+/CD3-/CD8+/CD56+ pathological cells expressed CD3 in the culture either in the presence or absence of IFN-α or IL-2. Red dots, pathological cells; gray dots, other cells; pre, before culture.

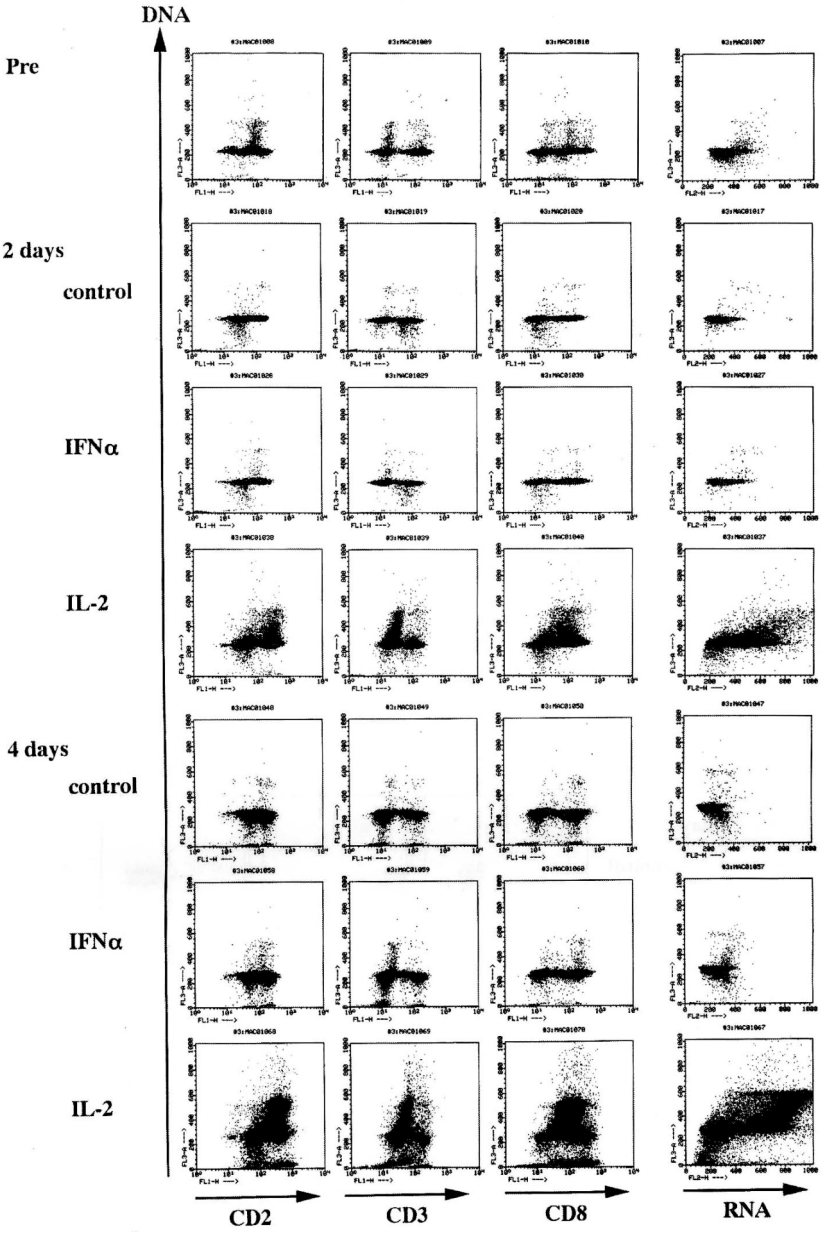

Figure 5. Cell cycle analysis of cultured cells. Peripheral blood mononuclear cells were incubated for 2 or 4 days in the presence or absence of interferon-alpha or interleukin-2. The cells were then harvested, serially stained with FITC-conjugated monoclonal antibodies, 7AAD and PY, then analyzed. The cycling cells before culture (top 4 dot plots) were CD2+/CD3-/CD8 dim+ pathological cells, and the level of S-phase was 1.0 %. Cells cultured without lymphokines contained very few in S-phase. Cells incubated with IFN-α entered early to middle S-phase at 4 days. The activated cells were CD2+/CD3 dim+/CD8+, and the S-population was 1.2 %. On the other hand, cells cultured with IL-2 entered into early S-phase in 2 days, and showed maximal growth in 4 days, and the activated cells were CD2+/CD3 dim+/CD8+ as well. The S-population was 12.3 % in 2 days, and 40.3 % in 4 days. A detailed cell cycle analysis is shown in Figure 8.

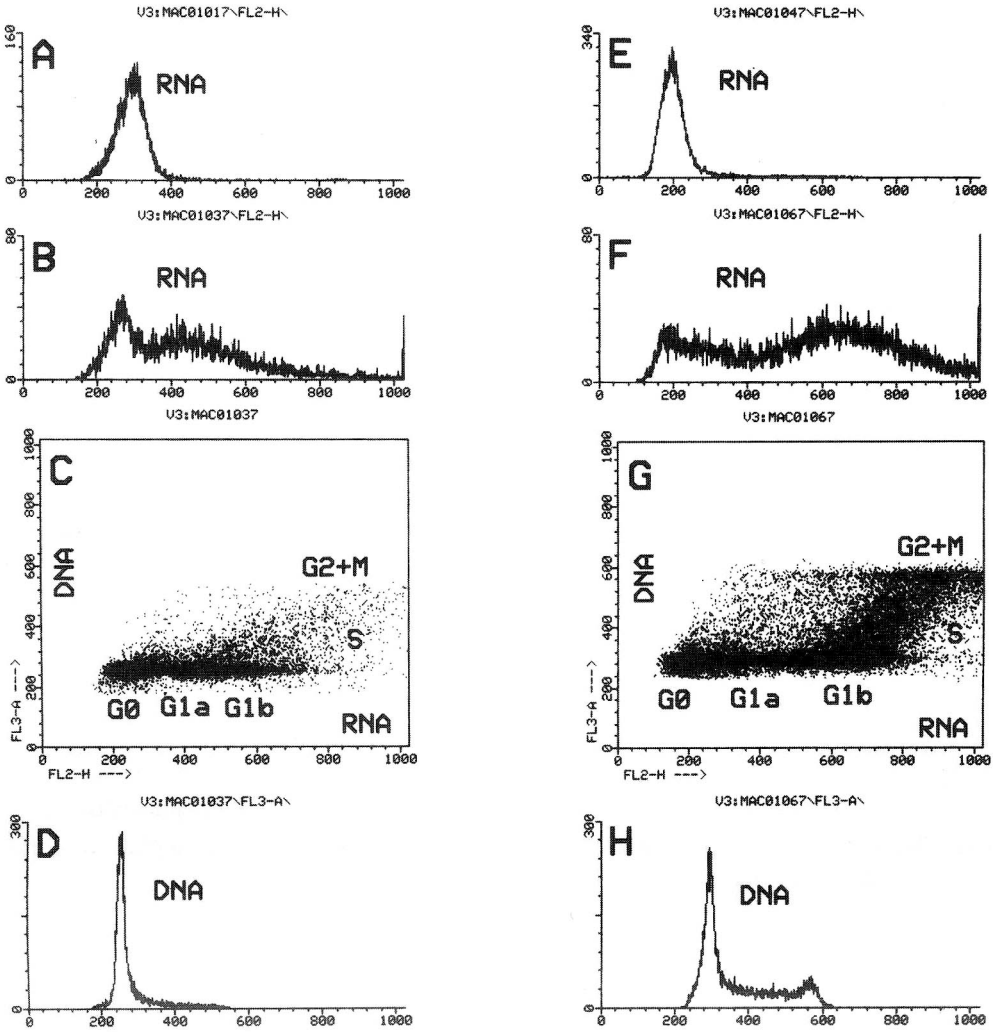

Figure 6. Further analysis of IL-2 activated cells in the culture. Panels A, B, C, D, 2 days of culture; E, F, G, H, 4 days of culture. Panels A, E, control; panels B, C, D, F, G, H, with IL-2. Panels A, B, E, F, RNA histograms; panels C, G, RNA/DNA dot plots; and panels D, H, DNA histograms. Cell cycle analysis data in panel C was as follows: G0 = 34.4 %, G1a = 29.4 %, G1b = 23.1 %, S = 12.3 %, G2+M = 0.8 %. Cell cycle analysis data in the panel G was as follows: G0 = 16.5 %, G1a = 18.4 %, G1b = 18.4 %, S = 40.3 %, G2+M = 6.4 %.