bcl-2 proto-oncogene encodes an inner mitochondrial membrane protein that blocks programmed cell death (apoptosis). There is now increasing evidence that regulation of bcl-2 expression is a determinant of life or death in normal lymphocytes. We have recently described that activated (CD45RO+) CD4+ and CD8+ T cells in acute infectious mononucleosis (IM) undergo apoptotic cell death on culturing, indicating an activation-driven cell death of mature T cells. In this work, we examine bcl-2 expression by activated T cells in acute IM using a flow-cytometric analysis with an anti-bcl-2 monoclonal antibody (MoAb). It was consistently observed that most T cells from acute IM patients displayed only much less bcl-2, while normal T cells expressed bcl-2 relatively strongly. Multicolor analysis showed that bcl-2–lacking T cells in acute IM were restricted to the CD45RO+ (activated) populations of CD4+, as well as CD8+ T cells. In contrast, the relatively intense levels of bcl-2 were expressed in both CD45RO+ and CD45RO– T-cell populations from normal subjects. This marked difference in bcl-2 expression of CD45RO+ T cells between acute IM and normal controls was also confirmed by Western blot analysis. Activated (CD45RO+) T cells with low bcl-2 expression, but not bcl-2–expressing CD45RO– T cells, in acute IM patients were found to die easily when cultured without added growth factors. However, in normal individuals, both CD45RO+ and CD45RO– T cells were relatively stable on culturing. These findings suggest that lack of bcl-2 expression by activated (CD45RO+) T cells in acute IM might be associated with their susceptibility to programmed cell death.

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MATERIALS AND METHODS

Cell preparation and cell cultures. Twelve patients, ranging from 5 to 15 years of age, who had characteristic clinical, hematologic, and serologic findings of acute EBV-induced IM, were entered onto this study as described. Healthy adult volunteers and age-matched children served as normal controls. Blood samples were collected after parental informed consent was taken. Thymic tissues were obtained from children undergoing cardiac surgery. Viable mononuclear cells from heparinized blood and the thymus were separated by Ficoll-Hypaque density gradient centrifugation. Event setting T cells were isolated by the rosette formation with 2-amino-
ethylisothiouronium bromide-treated sheep erythrocytes, followed by Ficoll-Hypaque gradient centrifugation. The cells were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics (culture medium), and cultured in the culture medium alone in 24-well culture plates (Corning Glass Works, Corning, NY) at a cell density of 5 x 10^4/mL at 37°C in 5% CO₂ and 95% air. The cultured T cells were collected after a 48-hour incubation, and viable cells in harvested cells were evaluated for the relationship between bel-2 and CD45RO expression as below.

**Antibodies.** The Dako bel-2, 124 MoAb (IgG1) against human bel-2 (Dako Japan, Kyoto, Japan) has been generated by immunizing with synthetic peptide sequence comprising amino acids 41 to 54 of bel-2 protein. KNT3 (IgG1) MoAb was used as the irrelevant control antibody. KNT3 was produced against recombinant IL-2 in this laboratory, and was found not to be reactive with either natural IL-2 or leukocytes. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG1 antibody (Zymed Laboratories, San Francisco, CA) was used as the second antibody for bel-2 staining. Phycoerythrin (PE)-conjugated antibodies for two-color immunofluorescence included OKT3 (anti-CD3; Ortho Diagnostic Systems, Tokyo, Japan) and UCHLI (anti-CD45RO; Dako Japan) MoAb. For three-color immunofluorescence analysis, Per-CP-conjugated anti-CD3 (anti-Leu-3a) and anti-CD8 (anti-Leu-2a) MoAb were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA).

**Flow-cytometric identification of bel-2-bearing cells.** The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 20 minutes at room temperature, and permeabilized in 0.1% Triton X-100 in Tris-buffered saline (pH 7.4) with 0.1% bovine serum albumin for 30 minutes. After three washes in PBS with 3% FBS and 0.1% sodium azide (washing buffer), these fixed, permeabilized cells were incubated with anti-bel-2 or irrelevant control antibodies for 20 minutes on ice, and washed twice in a washing buffer. Anti-bel-2 antibody was used at a 4 µg/mL on the basis of results of preliminary titration experiments. Anti-bel-2-treated cells were treated with FITC-conjugated anti-mouse IgG1 antibody at a 1:1,000 dilution for 20 minutes on ice, washed twice in a washing buffer, and incu-bated for 10 minutes with 10% mouse serum to block unbinding sites of the second antibody. Next, PE- or Per-CP-conjugated corresponding antibodies alone or together with each other were added to anti-bel-2-treated cells at optimal concentrations for 20 minutes on ice. Preliminary experiments indicate that the fixation and permeabilization has no deleterious effect on following flow-cytometric analysis of surface antigens using fluorochrome-conjugated antibodies, except for anti-CD3, resulting in reasonable staining of corresponding surface antigens. For CD3, the cells were incubated with PE-conjugated anti-CD3 antibody before fixation and permeabilization, which allow the detection of immature T cells containing intracytoplasmic CD3 antigen, especially in the thymus.

**Single-, two-, or three-color immunofluorescence analyses** were performed as previously described. The stained cells were analyzed on a Cytoron Absolute flow-cytometer (Ortho Diagnostic Systems) in which a single 488-nm argon laser was used to excite FITC (green), PE (orange), and Per-CP (red). Single- or two-color staining patterns were obtained for lymphocytes gated forward and 90° light scatter. For three-color analysis, data were obtained by electronic gating on red fluorescence and forward-angle light scatter to identify CD4⁺ or CD8⁺ T cells, and then analyzed for the other two colors, i.e., FITC for bel-2 and PE for CD3 or CD45RO.

**Western blot analysis.** For Western blot analysis of different cellular bel-2 expression, various cell populations were isolated by an electronic sorting with the use of an Epics-C flow-cytometer ( Coulter Electronics, Hialeah, FL) as described elsewhere. The cells were lysed in 10 µL/10⁶ cells of the lysis solution (1% Triton X-100, 150 mMol/L NaCl, 10 mMol/L Tris-HCl, pH 7.6, 5 mMol/L EDTA, and 2 mMol/L phenylmethylsulfonyl fluoride) for 30 minutes on ice. The cells were centrifuged for 10 minutes at 15,000g to remove nuclei, and the supernatants was diluted in Laemmli’s sample buffer and boiled for 2 minutes. Samples were electrophoresed in the sodium dodecyl sulfate (SDS)-polyacrylamide 10% to 20% gradient gel (Daichi Pure Chemicals, Tokyo, Japan), and electroblotted onto nitrocellulose filters using a HORIBA BLOT apparatus (ATTO, Tokyo, Japan). Blots were blocked in 5% skimmed milk in PBS for 1 hour, reacted with anti-bel-2 antibody diluted at 4 µg/mL in PBS-0.1% Tween 20 (PBS-T) for 1 hour, and further incubated with horseradish peroxidase-conjugated sheep anti-mouse Ig antibody (Amersham International, Amersham, UK) at a 1:1,500 dilution in PBS-T for 60 minutes. Immunoblots were developed by using an ECL Western blotting detection system (Amersham International).

**RESULTS**

We used a flow-cytometric analysis using anti-bel-2 MoAb to examine comparatively bel-2 expression on peripheral T cells from acute IM patients and normal subjects, because this method might allow for the simultaneous identification of intracytoplasmic bel-2 protein and surface phenotypes expressed in individual cells. As shown in Fig 1, a flow-cytometric analysis indicated that the majority of peripheral blood lymphocytes (PBL) in healthy donors were relatively strongly stainable with anti-bel-2 antibody, whereas only a few thymocytes were reactive with the antibody. Two-color staining indicated that both T and non-T cells in PBL expressed bel-2 intensely, and bel-2 expression was found on bright CD3⁺ thymocytes but not on others, consistent with the results of previous immunohistologic studies. Of importance, we consistently observed a marked difference between PBL from acute IM patients and healthy age-matched children with respect to bel-2 expression. In marked contrast to normal subjects, a large proportion of PBL in acute IM patients were not reactive with anti-bel-2 antibody, while only a few cells were positive for bel-2. Two-color analysis indicated that the majority of T cells in acute IM patients displayed much less bel-2 than normal T cells.

Three-color immunofluorescence analyses were further performed to investigate the relationship between bel-2 expression and CD45RO surface phenotypes in each T-cell population. As shown in Fig 2, an appreciable expression of bel-2 was demonstrable in both CD45RO⁺ and CD45RO⁻ populations of T cells from normal subjects. However, it should be noted that a defined fraction of CD45RO⁺ T cells in normal subjects expressed low levels of bel-2, implying the presence of a small number of recently activated T cells in the blood. As expected, T cells lacking bel-2 in acute IM patients resided largely in CD45RO⁺ populations of both CD4⁺ and CD8⁺ T cells. CD45RO⁻ T cells in acute IM, a minor T-cell population in the blood of patients, actually expressed bel-2.

Western blot analysis was performed for various cell populations to examine whether the flow-cytometric evaluation might reflect the real expression of bel-2 protein in each cell. On Western blot analysis using anti-bel-2 antibody, the
Fig 1. Cellular expression of bcl-2 evaluated by a flow-cytometric analysis using anti-bcl-2 antibody. Thymocytes, PBL from a healthy child, and PBL from a 6-year-old patient with acute IM were incubated with PE-conjugated anti-CD3 antibody, and further stained for bcl-2. Stained cells were analyzed on a Cytoron absolute flow cytometer. (A) Histogram patterns for bcl-2 and (B) two-color immunofluorescence profiles of bcl-2 and CD3 were obtained from lymphocytes gated by forward and 90° light scatters. Dashed lines show the controls containing irrelevant antibodies. For each quadrant, the percentage of total cells is given.

Normal IM band of the same size (~26 Kd) was detected in the lysates of all cell samples as previously described23 (Fig 3). This was present at high levels in PBL from normal controls. Although a band from the whole thymocytes was faint, isolation of bright CD3+ thymocytes resulted in an enhancement of a band of bcl-2 protein. Supporting the above flow-cytometric assessment, Western blot analysis indicated that CD45RO+ T cells from acute IM patients lacked bcl-2 protein, while normal CD45RO+ T cells possessed bcl-2 intensely. The expression of bcl-2 was equally demonstrated in CD45RO− T cell populations from acute IM subjects, as well as normal subjects.

Finally, we examined changes in bcl-2 and CD45RO expression on T cells from acute IM subjects after an incubation without any added growth factors. It has been shown that apoptotic dead cells are distinguishable from viable cells by the measurement of forward and side light scatters on a flow cytometer.33 Confirming previous findings,24-25 a flow-cytometric light scatter analysis demonstrated that the majority of acute IM T cells abruptly died when cultured for 2 days (Fig 4A). These dead cells showed morphologic changes characteristic of apoptosis as evaluated by May-Grünewald-Giemsa staining as described25 (data not shown).

On the other hand, most T cells from normal donors remained viable on culture. Concomitant with the death of acute IM T cells, a simple culture led to a marked reduction of CD45RO+ T cells, with low bcl-2 expression seen in these patients, resulting in the enrichment of CD45RO− cells that expressed bcl-2 relatively intensely (Fig 4B). Expression patterns of bcl-2 on normal T cells, whether CD45RO+ or CD45RO−, were largely unaffected during the same period of incubation. These results seemed to support the notion that low bcl-2 expression on activated (CD45RO+) T cells in acute IM patients might be associated with their susceptibility to apoptosis in vitro.

**DISCUSSION**

Elimination of excess and unnecessary lymphoid cells activated by antigenic stimuli via a programmed cell death mechanism is a physiologic event that essentially occurs in normal immune responses.34 A spontaneous ex vivo apoptotic cell death of activated CD4+ and CD8+ T cells during acute IM may provide an example of activation-mediated depletion of peripheral mature T cells.25 We proposed that apoptosis of most activated T cells in acute IM might play a beneficial role for termination of exaggerated T-cell im-
mune responses against EBV-infected B cells. Unlike activated (CD45RO+ T cells in acute IM, memory T-cell populations with the same phenotype in normal individuals hardly undergo apoptosis in culture. The Fas/APO-1 antigen has been recently cloned as a putative cell-membrane molecule that mediates apoptosis.35,36 The MoAb against this surface structure causes apoptotic cell changes in some positive cell lines.35,38 In a previous study, we examined the different expression of Fas antigen on CD45RO+ T cells in acute IM and normal subjects as a possible cellular situation involved in a discrepant susceptibility to apoptosis between both cells.35 Nevertheless, CD45RO+ T-cell populations from IM and normal subjects coexpressed Fas antigen similarly. It is likely that CD45RO+ T cells in acute IM have been programmed for apoptotic cell death, whereas normal CD45RO+ T cells are circulating as relatively stable memory cells in the peripheral pool. In fact, normal CD45RO+ T cells are resistant to cytolytic action of anti-Fas MoAb, even though they express Fas antigen.39

Increasing evidence that bcl-2 may serve as a repressor of
lymphoid cell death led us to investigate bcl-2 expression by activated (CD45RO+) T cells during acute IM. We used a flow-cytometric analysis to examine differential expression of bcl-2 protein by CD45RO+ and CD45RO- T-cell populations in acute IM patients and normal controls. The validity of flow-cytometric evaluation was confirmed by Western blot analysis. The flow-cytometric analysis showed that an appreciable expression of bcl-2 was detected in the vast majority of normal PBL. The most important finding was that a large population of PBL in acute IM patients expressed less bcl-2 as compared with normal cells. Furthermore, three-color analysis showed that the cells with low bcl-2 expression in these patients resided largely in CD45RO+ (activated) populations of CD4+, as well as CD8+ T cells. In healthy donors, CD45RO+ (memory) T cells expressed the same levels of bcl-2 as naive (CD45RO-) ones.

The physiological role of bcl-2 for the survival of peripheral lymphoid cells has been delineated for the secondary follicles. Recirculating B cells in the mantle zone possess an abundant amount of bcl-2. Expression of bcl-2 protein is essentially absent from germinal center B cells, which are presumably activated with antigens and are susceptible to apoptosis in vitro. A subset of germinal center B cells are rescued from the cell death pathway through some survival selection steps to become long-lived memory B cells with bcl-2 expression. From the present results, it is feasible to suppose that activated CD45RO+ T cells expanded in acute IM may be a prototype of peripheral T cells corresponding to germinal center B cells. Considering the role of bcl-2 in determining the fate of activated T cells, it seems important to determine how bcl-2 expression is regulated in mature T cells after activation with antigenic stimuli, eg, viral infection. Although expression of abundant bcl-2 protein in freshly isolated PBL has been demonstrated by immunohistological studies, another study showed that expression of bcl-2 mRNA is somewhat low in unstimulated PBL, but can be markedly augmented by mitogenic stimulation. Reed et al recently reported that resting PBL contained detectable amounts of bcl-2 protein that was not augmented following stimulation with a combination of mitogens and IL-2. In preliminary experiments, we found that naive (CD45RO-) and memory (CD45RO+) T cells from normal donors did not show significant changes in bcl-2 following activation with anti-CD3 antibody. However, these anti-CD3-stimulated T cells exhibited markedly diminished levels of bcl-2 protein when further cultured free of IL-2, but their bcl-2 expression could be sustained by the presence of IL-2 in culture, suggesting a role for IL-2 in the regulation of bcl-2 expression in activated T cells. However, the mechanism by which bcl-2 expression is regulated in activated T cells awaits further investigation.

Current research interests are directed to a relevance of abnormal induction of programmed cell death to pathogenesis of acquired immune deficiency syndrome. In resemblance to acute IM, it has been observed that CD8+, as well as CD4+, T cells from asymptomatic human immunodeficiency virus (HIV)-infected individuals trended to die as a result of apoptosis on culturing. We had an opportunity to examine bcl-2 expression by T cells from two asymptomatic hemophiliacs chronically infected with HIV. It was found
that CD45RO+ populations of both CD4+ and CD8+ T cells from these HIV-infected patients expressed markedly lower levels of bcl-2 than healthy controls (unpublished observations). Taken together, the assessment of cellular bcl-2 expression may be important for evaluation of the fates of lymphoid or hematopoietic cell populations in viral infections and various other disease conditions, such as autoimmune diseases, immunodeficiency, or malignancies, although other genes (ced-9 or Elb) have recently been linked with cell survival.46,47

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Absence of bcl-2 expression by activated CD45RO+ T lymphocytes in acute infectious mononucleosis supporting their susceptibility to programmed cell death

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