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Effect of Accessory Cells on Stimulation of Murine T-Cell Leukemia with Antibodies to the CD3/T Cell Antigen Receptor Complex

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Stimulation of EL4 and RL♂1 leukemia cells *in vitro* with immobilized anti-CD3ε monoclonal antibody (mAb) (145-2C11) or anti-TCRβ mAb (H57-597) in the absence of accessory cells induced interleukin-2 (IL-2) production, and caused growth inhibition. The growth inhibition was, however, transient and the tumors started to grow again within 5 days in immobilizing plates treated with antibodies at concentrations of 2.5–100 μg/ml. Addition of mitomycin C-treated accessory cells to the culture inhibited IL-2 production and resulted in augmented and persistent growth inhibition. No recovery of tumor growth was observed. Furthermore, DNA from EL4 and RL♂1 leukemia cells stimulated with anti-CD3/TCR mAbs was fragmented even in the absence of accessory cells, but fragmentation was much greater in the presence of accessory cells. Marginal and high expression of the *bcl*-2 gene were observed in EL4 and RL♂1, respectively, indicating that apoptosis of these leukemias mediated by signalling through the CD3/TCR complex has no direct relationship with expression of the *bcl*-2 gene.

Key words: Murine T-cell leukemia — CD3/TCR stimulation — Accessory cell

Antigen receptors on most peripheral T-lymphocytes are composed of a dimer of α and β polypeptides that is associated with the CD3 molecular complex.[1] Antibodies to the CD3/T-cell antigen receptor (TCR) complex stimulate mature T-cells to produce interleukin-2 (IL-2) and proliferate in the presence of accessory cells, but not in their absence.[2,3] Activation of immature thymocytes with these antibodies in the presence of accessory cells, on the other hand, results in death by nucleosomal fragmentation of DNA.[4] These findings have been discussed in relation to the mechanisms by which autoreactive T-cell clones are negatively selected during T-cell differentiation in the thymus.[5]

Activation of T-cell hybridomas by antibodies to the CD3/TCR complex causes IL-2 production even in the absence of accessory cells, but inhibits growth.[6,7] This growth inhibition was shown to result from apoptosis, in which disintegration of DNA was typically observed.[8] In this study, we investigated the effect of accessory cells on activation of T-cell leukemias through the CD3/TCR complex. We showed that, in the absence of accessory cells, immobilized antibodies to the CD3/TCR complex stimulated murine T-cell leukemias EL4 and RL♂1 to produce IL-2 and transiently inhibited their growth. Addition of accessory cells to the culture inhibited IL-2 production, and caused greatly augmented and persistent growth inhibition.

MATERIALS AND METHODS

Mice BALB/c, C57BL/6 (B6) and (BALB/c × C57-BL/6)F_1 (CB6F_1) mice were purchased from Japan SLC (Shizuoka). These mice were housed in the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Tumors and cell lines EL4 is a chemically induced leukemia of C57BL origin.[9] RL♂1 is a radiation-induced leukemia of BALB/c origin.[10] RV1, RV2 and RVC are radiation leukemia virus (RadLV)-induced leukemias of BALB/c origin.[11] P815 is a mastocytoma of DBA/2 origin.[12] A20-2J is a B-cell lymphoma of BALB/c origin.[13] WEHI164 is a fibrosarcoma of BALB/c origin.[14] UV♂1 is a fibrosarcoma of CB6F_1 origin.[15] NS-1 is a myeloma of BALB/c origin.[16] FBL-3 is a Friend virus-induced erythroleukemia of B6 origin.[17] 38-B-9 is a pre-B cell line of BALB/c origin.[18] YAC-1 is a Moloney leukemia virus-induced leukemia of A/Sn origin.[19] These tumors were maintained in ascites form in their strain of origin and in tissue cultures.

Antibodies Anti-CD3ε monoclonal antibody (mAb), a hamster antibody of the IgG immunoglobulin class, produced by hybridoma 145-2C11,[20] was provided by Dr. J. A. Bluestone, University of Chicago, Chicago, IL. Anti-TCRβ mAb, a hamster antibody of the IgG class, produced by hybridoma H57-597,[21] was provided by Dr. R. Kubo, National Jewish Center, Denver, CO. Anti-TCRδ

mAb, a hamster antibody of the IgG class, produced by hybridoma 3A10,[22] was provided by Dr. S. Itohara, M.I.T., Cambridge, MA. Anti-L3T4 (CD4) mAb, a rat antibody of the IgG2b class, produced by hybridoma GK1.5,[23] was provided by Dr. F. Fitch, University of Chicago, Chicago, IL. Anti-Lyt-2.2 (CD8) mAb and anti-Thy-1.2 mAb, both mouse antibodies, were described previously.[24] Anti-Thy-1 mAb, a rat antibody of the IgG2c class, produced by hybridoma G7[25] was provided by Dr. N. Shinohara (Mitsubishi Kasei Institute of Life Science, Tokyo). Anti-Pgp-1 (CD44) mAb, a mouse antibody of the IgG3 class, was produced by hybridoma 5-50. Anti-Ly-5 (CD45) mAb, a rat antibody of the IgG2c class, produced by hybridoma M1-9.3HL, was obtained from Bethesda Research Laboratories (Bethesda, MD).

Accessory cells $CB6F_1$ spleen cells (5×10^7 in 0.2 ml) were treated with anti-Thy-1.2 mAb (ascites diluted 1:10 with MEM) for 30 min on ice. Preselected rabbit complement (0.75 ml) was then added and the cells were incubated for 45 min at 37°C. Residual cells were washed and then incubated for 2 h in plastic tissue culture flasks (Corning 28630, Corning, NJ) in a volume of 5 ml in an atmosphere of 5% CO_2 in air at 37°C. The nonadherent cells were collected and used as accessory cells. Accessory cells (3×10^6/well) were used after their treatment with mitomycin C (MMC) at a concentration of 50 μg/ml for 30 min at 37°C. In some experiments, peritoneal exudate cells (PEC) (1.5×10^6/well) and tumor cells (1×10^6/well) were used as accessory cells after treatment with MMC.

Paraformaldehyde (PFA) fixation Accessory cells were fixed with 0.5% PFA in phosphate-buffered saline (PBS) for 30 min at 4°C. The cells were washed 3 times with RPMI1640 medium before use.

Assay of *in vitro* leukemia growth Leukemia cells (1×10^4) were cultured with immobilized mAb in flat-bottomed 96-well plastic plates with or without accessory cells. For immobilization of mAbs, purified mAbs diluted with PBS were added to each well at a concentration of 2.5 μg/ml. In some experiments, 0.1 and 1.0 μl of mAb ascites (in 100 μl PBS) were added to the wells. The plates were incubated for 3 h at 37°C and then washed three times with PBS before use. After culture for several days, ^{3}H-thymidine (0.1 μCi) was added and incubation was continued for 16 h. The cells were then harvested and their incorporation of ^{3}H-thymidine was determined.

IL-2 assay The culture supernatant from leukemia cells was added to IL-2-dependent NRB cells (5×10^3) in 96-well plastic plates. The cells were cultured for 8 h and then pulsed with ^{3}H-thymidine for 16 h. After culture, the cells were harvested and the incorporation of radioactivity was determined.

DNA fragmentation The cell pellets were lysed in 5 m*M* Tris buffer (pH 7.4) containing 1 m*M* EDTA and 0.5% Triton X-100 for 20 min on ice. The preparation was then centrifuged at 2,700*g* for 20 min, and low-molecular-weight DNA in the supernatant was precipitated with ethanol. Electrophoresis was carried out in 1.5% agarose gels in 40 m*M* Tris-acetate buffer (pH 8.0) containing 1 m*M* EDTA. Gels were stained with ethidium bromide.

Northern blot analysis Total cellular RNA was extracted by the acid phenol method with some modification. RNA samples (30 μg) were separated on 1% agarose-formaldehyde gels and transferred to a Gene Screen plus membrane (DuPont, USA). Hybridization with [α-^{32}P]-dCTP-labeled murine *bcl*-2 cDNA probe was performed in 50% formamide, 1 *M* NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA at 42°C for 16 h. The filter was then washed twice for 15 min in 2×SSC and once for 10 min in 2×SSC, 1% SDS at 65°C, and autoradiographed.

RESULTS

Production of IL-2 by leukemia cells on stimulation with anti-CD3ε mAb and anti-TCRβ mAb We investigated TCR expression on murine leukemia lines by cytofluorometric analysis with anti-CD3ε mAb and anti-TCRβ mAb and found that of 9 leukemias tested, a chemically induced leukemia EL4 (C57BL), a radiation induced leukemia RL♂1 (BALB/c), and two RadLV-induced leukemias, RV1 (BALB/c) and RVC (BALB/c), gave positive reactions with both mAbs. Of these four leukemias, EL4 and RL♂1, but not RV1 or RVC, secreted IL-2 on stimulation with immobilized anti-CD3ε mAb and anti-TCRβ mAb (Fig. 1).

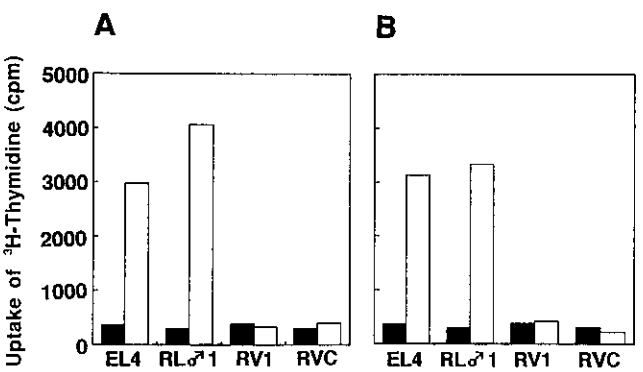

Fig. 1. IL-2 production by murine T-cell leukemias on stimulation with immobilized anti-CD3ε mAb (A) or anti-TCRβ mAb (B). Leukemia cells (1×10^4) were cultured with (open columns) or without (closed columns) mAb for 24 h. Then the supernatants were collected and added to the cultures of 5×10^3 IL-2 dependent NRB cells. ^{3}H-Thymidine uptake was determined after 24 h incubation.

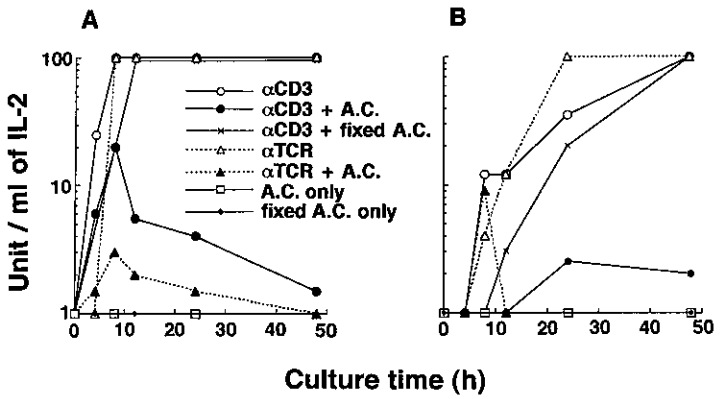

Fig. 2. EL4 (A) and RL♂1 (B) leukemia cells were stimulated with anti-CD3ε mAb (αCD3) and anti-TCRβ mAb (αTCR) in the absence or presence of PFA-fixed (fixed) or unfixed, MMC-treated splenic accessory cells (A.C.). Leukemia cells (1×10^4) were cultured for the indicated periods and then the amount of secreted IL-2 in the supernatant was determined using NRB cells as described in the legend to Fig. 1.

Effect of accessory cells on secretion of IL-2 from leukemia cells stimulated with anti-CD3ε mAb and anti-TCRβ mAb The effect of splenic accessory cells on secretion of IL-2 from EL4 and RL♂1 stimulated with immobilized anti-CD3ε mAb and anti-TCRβ mAb was studied kinetically. MMC-treated spleen cells that were depleted of Thy-1^+ cells and adherent cells were used as accessory cells. In the absence of splenic accessory cells, IL-2 secreted from these leukemia cells increased to a plateau (Fig. 2). On the other hand, in the presence of splenic accessory cells, secretion of IL-2 reached a peak after 8–24 h and then decreased. Treatment of splenic accessory cells with PFA abrogated their inhibitory effect.

Effect of splenic accessory cells on leukemia cell growth stimulated with anti-CD3ε mAb and anti-TCRβ mAb The effect of splenic accessory cells on growth of leukemia cells stimulated with immobilized anti-CD3ε mAb and anti-TCRβ mAb was then investigated. As shown in Fig. 3, growth inhibition of EL4 and RL♂1, but not RV1 or RVC was observed on day 5 of culture. No strain restriction of the source of splenic accessory cells for inhibition was observed (Fig. 4). Splenic accessory cells had no growth-inhibitory effect on leukemia cells treated with mAbs other than anti-CD3ε mAb and anti-TCRβ mAb; namely anti-TCRδ mAb, anti-Thy-1 mAb (G7), anti-Thy-1.2 mAb, anti-L3T4 (CD4) mAb, anti-Lyt-2.2 (CD8) mAb, anti-Pgp-1 (CD44) mAb and anti-Ly-5 (CD45) mAb.

The inhibitory effect of splenic accessory cells on growth of leukemia cells stimulated with immobilized anti-CD3ε mAb and anti-TCRβ mAb was then studied kinetically. In the absence of splenic accessory cells, growth was inhibited transiently in immobilizing plates treated with antibodies at concentrations of 2.5–100 μg/ml (Fig. 5). The inhibition of growth of EL4 cells persisted for 3 days and that of RL♂1 for 1–2 days. On the other hand, in the presence of splenic accessory cells, growth of these cells was inhibited permanently, and no

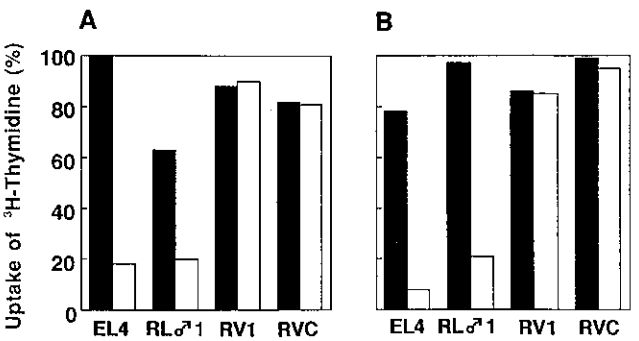

Fig. 3. Leukemia cells were cultured with immobilized anti-CD3ε mAb (A) or immobilized anti-TCRβ mAb (B) in the absence (solid columns) or presence (open columns) of MMC-treated, splenic accessory cells. Proliferation of leukemia cells was determined on day 5 by measuring ^{3}H-thymidine uptake and expressed as a percent of that in control cultures (leukemia cells alone).

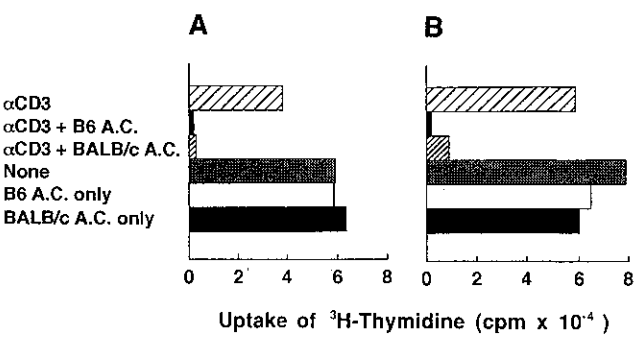

Fig. 4. Growth of EL4 (A) and RL♂1 (B) leukemia cells stimulated with anti-CD3ε mAb (αCD3) in the presence of syngeneic or allogeneic, MMC-treated splenic accessory cells (A.C.). Proliferation of leukemia cells was determined on day 5 by measuring ^{3}H-thymidine uptake.

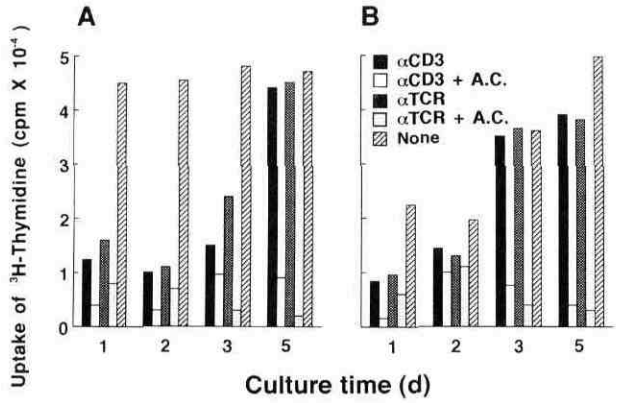

Fig. 5. Kinetics of growth inhibition of EL4 (A) and RL♂1 (B) leukemia cells stimulated with anti-CD3ε mAb (αCD3) and anti-TCRβ mAb (αTCR) in the absence or presence of MMC-treated splenic accessory cells (A.C.). Proliferation of leukemia cells was determined by measuring ^{3}H-thymidine uptake.

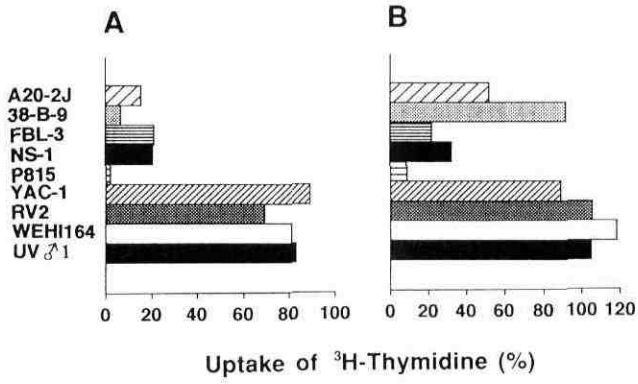

Fig. 7. Growth of EL4 (A) and RL♂1 (B) leukemia cells stimulated with anti-CD3ε mAb in the presence of various MMC-treated tumor cells as accessory cells. Proliferation of leukemia cells was determined on day 5 by measuring ^{3}H-thymidine uptake.

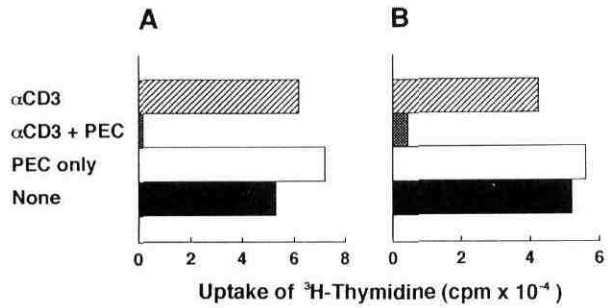

Fig. 6. Growth of EL4 (A) and RL♂1 (B) leukemia cells stimulated with anti-CD3ε mAb (αCD3) in the presence of MMC-treated, peritoneal exudate cells (PEC). Proliferation of leukemia cells was determined on day 5 by measuring ^{3}H-thymidine uptake.

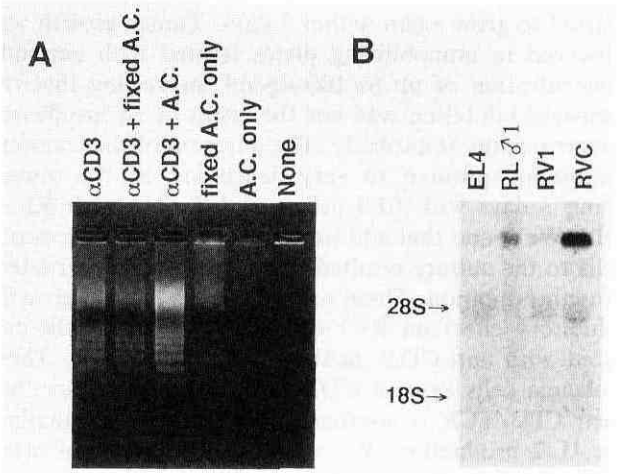

Fig. 8. A: DNA fragmentation. DNA from EL4 stimulated with anti-CD3ε mAb for 12 h in the absence or presence of PFA-fixed (fixed) or unfixed, MMC-treated splenic accessory cells was electrophoresed in agarose gel. B: Northern blot analysis of *bcl*-2 mRNA from leukemia cells.

recovery of tumor growth was observed on day 20 of culture (data not shown). Treatment of splenic accessory cells with PFA abrogated their inhibitory effect.

In the experiments described above, we used B-cell-enriched spleen cells as accessory cells. We observed that PEC had a similar growth-inhibitory effect (Fig. 6). We also examined whether various tumor lines including T and B-cell lines, fibroblast lines, and other myeloid cell lines showed similar growth-inhibitory effects. A20-2J, FBL-3, NS-1 and P815, but not YAC-1, RV2, WEHI164 or UV♂1, were found to inhibit growth of both EL4 and RL♂1 (Fig. 7). 38-B-9 inhibited growth of EL4, but not RL♂1. Treatment of these tumor cells with PFA abrogated their inhibitory effects.

DNA fragmentation of leukemia cells We investigated DNA fragmentation in EL4 and RL♂1 cells stimulated with anti-CD3/TCR mAbs with or without accessory cells. DNA fragmentation was observed on treatment with immobilized anti-CD3ε mAb and anti-TCRβ mAb, and was augmented by the addition of accessory cells (Fig. 8A). No augmentation was observed with the addition of PFA-fixed accessory cells. No DNA fragmentation was observed in the absence of anti-CD3ε mAb or anti-TCRβ mAb.

Northern blot analysis of *bcl*-2 The expression of *bcl*-2 mRNA in the leukemia cells was analyzed by Northern blotting. Transcripts of the *bcl*-2 gene were detected in RL♂1 and RVC, but not in EL4 and RV1 (Fig. 8B). Stimulation with anti-CD3ε mAb induced slight elevation of transcription of *bcl*-2 mRNA in EL4, RL♂1 and RVC, but not RV1 after 8–12 h culture.

DISCUSSION

It has been demonstrated that stimuli that are mitogenic in normal T cells can induce cell death in T cell hybridomas.[6,7] This activation-dependent cell death can be triggered by the presentation of appropriate antigens as well as by treatment with lectins and antibodies specific for the T cell antigen receptor complex. Our findings that immobilized anti-CD3ε mAb and anti-TCRβ mAb inhibited growth of EL4 and RL♂1 leukemia cells in the absence of accessory cells are consistent with these observations. The growth inhibitions of EL4 and RL♂1 leukemia cells were, however, transient and the tumor cells started to grow again within 5 days. Tumor growth was observed in immobilizing plates treated with antibody concentration of up to 100 μg/ml, suggesting that the transient inhibition was not the result of an insufficient concentration of antibody. The duration of the transient inhibition appeared to vary depending on the tumor, being 3 days with EL4 cells and 1–2 days with RL♂1 cells. We found that addition of MMC-treated accessory cells to the culture resulted in augmented and persistent growth inhibition. These accessory cells had no growth-inhibitory effect on RV1 and RVC leukemia cells cultured with anti-CD3ε mAb or anti-TCRβ mAb. These leukemia cells express CD3/TCR on their surface, but their CD3/TCR is nonfunctional in terms of signalling for IL-2 production. We also tested a variety of other mAbs, including mAb G7 (rat anti-mouse Thy-1), which induces proliferation of peripheral T-cells, for growth-inhibitory effects on these leukemia cells in the presence of accessory cells, but none of these other mAbs had any inhibitory effect. These findings suggest that a costimulatory signal derived from the accessory cells augmented signalling via CD3/TCR, resulting in persistent inhibition of leukemia growth.

Earlier studies showed that activation-dependent cell death was associated with IL-2 and interferon-γ production.[6,7] In this study, we found that addition of accessory cells inhibited IL-2 production by EL4 and RL♂1 leukemia cells stimulated with immobilized anti-CD3/TCR mAbs, and augmented activation-induced leukemia cell death. Thus, lymphokine production and activation-induced leukemia cell death are apparently functionally independent. This idea is supported by our finding that mAb G7 (anti-Thy-1) in a range of 0.1–10 μl/well activated EL4 and RL♂1 to produce IL-2, but caused no growth inhibition in the presence of accessory cells. Recently, the Thy-1 molecule was found to cause apoptosis in mature, but not immature T-cells or thymomas.[26] In mature T-cells, stimulation via Thy-1 in the absence of TCR expression failed to induce IL-2 production, but caused apoptosis.[26] Our observations were consistent with these findings.

DNA from EL4 and RL♂1 leukemia cells stimulated with anti-CD3ε mAb or anti-TCRβ mAb was fragmented even in the absence of accessory cells, but the fragmentation was much greater in the presence of the accessory cells. Thus, the costimulatory signal derived from accessory cells augmented the apoptotic signal due to anti-CD3/TCR mAbs. The *bcl*-2 gene has been shown to promote cell survival.[27–29] The EL4 and RL♂1 leukemias, that were sensitive to apoptosis via CD3/TCR-mediated signalling, express only a marginal amount and a high amount of *bcl*-2 mRNA, respectively. Thus apoptosis of these leukemias mediated by signalling through the CD3/TCR complex has no direct relationship with expression of the *bcl*-2 gene.

The present findings suggest that a stimulatory signal via the CD3/TCR molecular complex in the presence of a costimulatory signal might be effective therapeutically for T-cell leukemias that express functional TCR. We recently demonstrated that *in vivo* administration of a relatively high dose (50 μg/mouse) of anti-CD3ε mAb (145-2C11) inhibited RL♂1 leukemia growth in BALB/c *nu/nu* mice (submitted). On the other hand, it has been shown that *in vivo* administration of a low dose (2 μg/mouse) of anti-CD3ε mAb (145-2C11) to C3H mice prevented the growth of syngeneic fibrosarcoma 1591-Pro-4L.[30] Since this tumor does not express surface CD3/TCR molecules, the effect would be due to activation of recipient T-cells.[31]

The molecular basis for the costimulatory signal is not clearly understood. Recently, however, it has been shown that several adhesion molecules on the APC augment TCR-driven proliferative response. Further studies are necessary to identify the molecules responsible for persistent inhibition of leukemia growth.

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