A Bispecific Antibody Enhances Cytokine-Induced Killer-Mediated Cytolysis of Autologous Acute Myeloid Leukemia Cells

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An anti-CD3 Fab′ × anti-CD13 Fab′ bispecific antibody (BsAb) was generated. This BsAb reacted with both CD3+ T cells and CD13+ acute myeloid leukemia (AML) cells. We investigated whether cytokine-stimulated peripheral blood mononuclear cells (PBMC) could lyse patient AML cells after addition of the BsAb. When interleukin-2 (IL-2)–stimulated PBMC were assayed for their cytotoxicity against ^{51}Cr-labeled allogeneic and autologous CD13+ AML cells, their activity was markedly enhanced by the addition of the BsAb. PBMC stimulated with IL-2 plus anti-CD3 monoclonal antibody (MoAb) showed higher proliferative ability and higher cytotoxicity if this was expressed as lytic units per culture. IL-7–stimulated PBMC also exhibited enhanced cytotoxicity against CD13+ AML cells after addition of the BsAb. Ultrastructurally, CD13+ AML cells incubated with IL-2 plus anti-CD3 MoAb-stimulated PBMC and the BsAb showed apoptotic morphologic changes. A colony assay for AML blast progenitors showed that the colony formation of CD13+ AML cells was inhibited by the addition of autologous IL-2 plus anti-CD3 MoAb-stimulated PBMC, and that this inhibition was further enhanced by the addition of the BsAb. A colony assay for normal bone marrow progenitor cells showed that the addition of autologous IL-2 plus anti-CD3 MoAb-stimulated PBMC and the BsAb inhibited the formation of granulocyte-macrophage colonies and mixed-cell colonies. However, the degree of inhibition was smaller than that for the AML blast colonies. Taken together, these findings suggest that this BsAb may be useful for ex vivo purging of CD13+ AML cells in autologous bone marrow transplantation.

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$\mathbf{I}$N PATIENTS with hematologic malignancies it is difficult to induce and expand cytotoxic T lymphocytes (CTL) specific to host leukemia or lymphoma cells.[1,2] Recent findings that interleukin-2 (IL-2)–induced lymphokine-activated killer (LAK) cells are able to lyse freshly isolated patient tumor cells have made it feasible to use these LAK cells for cancer immunotherapy.[3,4] However, in vitro studies have shown that large numbers of LAK effector cells are required to produce target tumor cell lysis, and the level of cytotoxicity actually achieved tends to be low.[5-7]

Bispecific antibodies (BsAb) that have dual specificity for the T-cell antigen receptor/CD3 complex and for a surface antigen expressed by the target cells have been generated.[8-15] In both in vitro studies and in vivo animal experiments, such BsAb have been shown efficiently to enhance CTL reactivity against tumor cells by the probable mechanisms of bridging CTL and target tumor cells and triggering the lytic process. The cytolysis of target cells is totally independent of the original specificity of the TCR of CTL and is not major histocompatibility complex-restricted.

Regarding BsAb that are reactive with human hematologic malignancies, we have recently generated an anti-CD3 Fab′ × anti-CD10 Fab′ BsAb that reacts with CD3+ CTL and CD10+ acute lymphoblastic leukemia (ALL) cells.[11] This BsAb was shown to efficiently enhance cytolysis of IL-2–stimulated peripheral blood mononuclear cells (PBMC) against patient CD10+ ALL cells in vitro. However, in that study we did not address conditions for generating potent effector cells or the effects of BsAb on leukemic or normal bone marrow colony forming cells. Therefore, in this study we examined these phenomena, using a newly generated anti-CD3 Fab′ × anti-CD13 Fab′ BsAb that reacts with CD3+ CTL and patient CD13+ acute myeloid leukemia (AML) cells.

MATERIALS AND METHODS

Characteristics of patients and leukemic cells. Twelve patients were studied (Table 1). Patients no. 1 through 8 had AML with a CD13+ phenotype and patients no. 9 through 11 had AML with a CD13- phenotype. Patient no. 12 had ALL and the leukemic cells were CD13-. In all patients but two, leukemic cells were obtained from the peripheral blood, and in patients no. 9 and 10 leukemic

cells were obtained from bone marrow. From seven patients PBMC were obtained when the patients were in complete remission.

Approval was obtained from the Institutional Review Board for these studies. Patients were informed that blood samples were obtained for research purposes and that their privacy would be protected.

Preparation of the BsAb. A method described previously[10,11] was modified as follows: the anti-CD3 monoclonal antibody (MoAb), OKT3, was obtained from Ortho Pharmaceutical Inc (Raritan, NJ), and the anti-CD13 MoAb, My7, came from Coulter Immunology (Hialeah, FL). F(ab′)₂ fragments of each MoAb were purified by high-performance liquid chromatography, on a MonoQ HR5/5 column (Pharmacia Fine Chemicals, Uppsala, Sweden). F(ab′)₂ fragments of the anti-CD3 MoAb were subjected to reduction with dithiothreitol (DTT; Sigma, St Louis, MO), and Fab′-SH was separated. The F(ab′)₂ fragments of the anti-CD13 MoAb were also reduced with DTT, the reaction was stopped by the addition of 5,5-dithiobis (2-nitrobenzoic acid), and the nitrobenzoic acid derivatives of the anti-CD13 Fab′ fragments (Fab′-S-NB) were obtained. Anti–CD3-Fab′-SH and anti–CD13-Fab′-S-NB fragments were then mixed to produce the hetero-BsAb. The resulting reaction mixture containing the hetero-BsAb was then separated on a TSKgel G3000 SWXL column (Tosoh, Tokyo, Japan). The fractions containing the hetero-F(ab′)₂ fragments were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), concentrated with Ultracent 30 (Tosoh), and further purified by chromatography on an HCA-column (Mitsui Toatsu Chemicals, Tokyo, Japan). The hetero-F(ab′)₂ content was determined by measuring the absorbance at 280 nm.

The generated BsAb was characterized by methods previously described.[10,11] The product was confirmed by SDS-PAGE to be almost

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Table 1. Characteristics of Patients and Leukemic Cells

Patient No.	FAB Classification	White Blood Cells (10⁹/L)	Leukemic Cells in PBMC (%)	CD13⁺ Cells in PBMC (%)
CD13⁺				
1	M1	141	98	80
2	M1	110	96	91
3	M2	44	95	70
4	M2	38	84	87
5	M4	25	97	93
6	M4	140	98	97
7	M4	80	87	86
8	M5b	28	93	66
CD13⁻				
9	M2	510	88	5
10	M2	329	80	4
11	M5a	97	95	3
12	L2	15	89	1

In patients no. 9 and 10 the data are those of bone marrow and the leukemic cells were obtained from bone marrow.

Abbreviation: FAB, French-American-British.

pure, showing a single band of 100 to 110 Kd. The BsAb reacted with both CD3⁺ T cells and CD13⁺ AML cells, indicating that this BsAb was bifunctional. To rule out the existence of contaminating homodimers of Fab' fragments, a target-absorption test was performed.[11] The absorption of the BsAb by CD3⁺ T cells concomitantly reduced the fluorescence intensity for anti-CD13⁺ reactivity by 90%, and vice versa. Absorption by CD13⁻ ALL cells did not diminish the anti-CD3 or anti-CD13 reactivity. These results indicated that the BsAb was indeed a true hetero-F(ab')₂ fragment.

Generation of effector cells. A method described previously[5,11] was modified as follows. PBMC were obtained with Ficoll-Conray density gradients, and suspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, 100 U/mL penicillin, and 10 μg/mL streptomycin. Allogeneic human AB serum was used for this complete medium, because preliminary experiments showed that the allogeneic AB serum and the autologous serum from PBMC donors induced a similar level of cytotoxic activity in IL-2-stimulated PBMC, and that the level of cytotoxic activity obtained was superior to that obtained after the addition of fetal calf serum (FCS). To generate effector cells, IL-2 (Shionogi Pharmaceutical Co Ltd, Osaka, Japan; specific activity 1.0 × 10⁷ U/mg protein) was used at 1,000 U/mL because, as shown previously, concentrations of 250 to 50,000 U/mL of IL-2 induced a similar maximum level of LAK activity for leukemic cells, and concentrations of 500 to 5,000 U/mL of IL-2 were most effective for the proliferation of PBMC.[5] IL-7 (Immunex Corp, Seattle, WA; specific activity 3.0 × 10⁷ U/mg protein) was used at 300 U/mL for the stimulation of PBMC. In the preliminary experiments, concentrations of 10 to 1,000 U/mL of IL-7 were examined, and PBMC proliferation and the induction of cytotoxic activity was observed to occur in a dose-dependent manner. Because 300 U/mL of IL-7 was equal to 1,000 U/mL of IL-2 in terms of protein concentration, this concentration of IL-7 was selected for use.

Since Ochoa et al[16] and Schmidt-Wolf et al[17] found that the addition of anti-CD3 MoAb or various types of cytokines generated cytotoxic cells with potent antitumor activity in long-term cultures, we followed similar procedures. First, 50 ng/mL of anti-CD3 MoAb OKT3 was added to IL-2-containing culture medium during the initial 48 hours. On day 12 100 U/mL of IL-1β (Genzyme, Boston, MA) was added, and on day 14 the cells were tested for cytotoxic activity.[16] Second, 1,000 U/mL of interferon γ (IFN-γ) (Biogen, Boston, MA) alone

was added on day 0. After 24 hours of incubation, 50 ng/mL of anti-CD3 MoAb, 1,000 U/mL of IL-2, and 100 U/mL of IL-1β were added. On day 14 the cells were tested for cytotoxic activity.[17]

Cytofluometric analysis. Cytokine-stimulated PBMC and AML blasts were stained by indirect immunofluorescence with the following MoAbs and analyzed with an Epics Profile Analyzer (Coulter)[5]: anti-CD3 MoAb OKT3, anti-CD4 MoAb OKT4, anti-CD8 MoAb OKT8 (Ortho), anti-CD16 MoAb Leu-11b (Becton Dickinson, San Jose, CA), and anti-CD13 MoAb My7. The presence of Fcγ receptor I (FcγRI) (CD64), FcγRII (CD32), and FcγRIII (CD16) on AML cells was detected by direct staining with fluorescein isothyocyanate-labeled MoAb 32.2, IV.3 (Medarex, Inc, West Lebanon, NH), and Leu-11a (Becton Dickinson), respectively.

Assay for cytotoxic activity. Leukemic cells were labeled with 300 to 400 μCi of Na₂⁵¹CrO₄ for 2 hours, washed, and used as targets in a 4-hour radioisotope-release cytotoxicity assay, as previously described.[5,11,18] The BsAb was added during the cytotoxicity assay, at a final concentration of 0.1 μg/mL, unless otherwise stated. The effector-to-target (E:T) ratios were 20:1, 10:1, and 5:1. Addition of only the BsAb to the target cells did not affect ⁵¹Cr release. The results of the cytotoxicity assay were expressed as lytic units (LU) and mean percent cytotoxicity. One LU was defined as the number of effector cells required to lyse 30% of the target cells. Because PBMC were subcultured repeatedly, the calculation of LU/culture (LU/C) was based on the initial number of PBMC in a cultured well and was multiplied to reflect the total number of cells that would have been recovered from the initial culture.

Morphologic observations by electron microscopy. Effector cells were patient PBMC cultured for 14 days with 1,000 U/mL of IL-2 and the initial 48-hour addition of anti-CD3 MoAb. Target AML cells (5 × 10⁶) and effector cells (1 × 10⁷) were suspended in RPMI 1640 containing 10% human AB serum and 1,000 U/mL IL-2 with or without the addition of 0.1 μg/mL BsAb. After centrifugation and 2-hour incubation, the pelletted cells were fixed with 1/2 strength Karnovsky's fixative at 0°C for 2 hours, postfixed in osmium tetroxide, dehydrated with ethanol, and embedded in Poly/Bed (Polyscience, Inc, Warrington, PA), following which the specimens were routinely processed for electron microscopy.

Blast colony assay. The colony assay for AML blast progenitors was performed by a previously described method.[19] As a colony-stimulating factor (CSF), we used 20% phytohemagglutinin (PHA)-stimulated leukocyte-conditioned medium (PHA-LCM) or 10 U/mL of granulocyte-macrophage (GM) CSF. GM-CSF (specific activity 4.7 × 10⁶ U/mg protein) was provided by Dr David Golde (UCLA School of Medicine, Los Angeles, CA) and Dr Gordon Wong (The Genetics Institute, Cambridge, MA). Cells obtained from colonies showed characteristics of leukemic blasts and not of T lymphocytes.[19] To determine the effect of cytokine-stimulated PBMC and the BsAb or other MoAb on blast colony formation, various numbers of effector cells were mixed with AML cells in the presence or absence of MoAb, incubated for 4 hours at 37°C, and then suspended in the methyl-cellulose-containing medium for colony growth. Effector cells were patient PBMC cultured with 1,000 U/mL of IL-2 for 14 days with the initial 48-hour addition of anti-CD3 MoAb.

Colony assay for normal progenitor cells. The colony assay for normal granulocyte-macrophage and mixed-cell colonies was performed according to the method described by Fauser and Messner[20] with modification. Briefly, bone marrow mononuclear cells (1 × 10⁵/dish) were suspended in 1 mL of Iscove's modified Dulbecco's medium (JR Scientific, Woodland, CA) containing 0.9% methylcellulose, 30% FCS, 1% bovine serum albumin, 2 × 10⁻⁵ mol/L 2-mercapto-ethanol (Sigma), 100 U/mL of recombinant human IL-3 (Kirin-Brewery Co Ltd, Tokyo, Japan; specific activity 7.94 × 10⁷ U/mg), 10 ng/mL of stem cell factor (Amgen Biologicals, Thousand Oaks, CA), and 2 U/mL of recombinant human erythropoietin (Snow Brand Milk Products Co, Tokyo; specific activity 1.4 × 10⁵ U/mg). Colonies
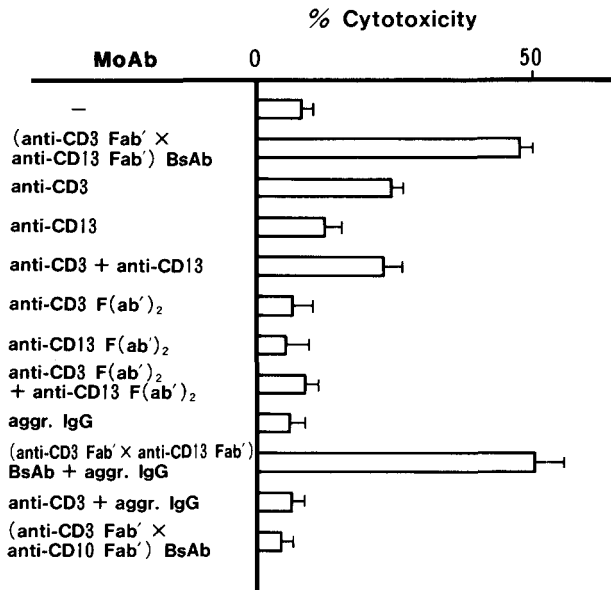
% Cytotoxicity

Fig 1. Specificity of the BsAb in enhancement of cytotoxic activity of IL-2–stimulated PBMC. PBMC from a normal donor were cultured with IL-2 for 7 days and assayed for their cytotoxicity against the CD13+ AML cells of patient no. 1 at an E:T ratio of 20:1. Various types of MoAbs were added, at a final concentration of 0.1 μg/mL, during the cytotoxicity test. Aggr. IgG denotes aggregated IgG added at a final concentration of 0.15 mg/mL during the cytotoxicity test.

were enumerated after 14 days of culture. Autologous PBMC were stimulated with IL-2 and anti-CD3 MoAb for 14 days and added to the assay medium as described above.

RESULTS

Specificity of the BsAb in cytotoxic activity enhancement. The PBMC of a normal donor were cultured for 7 days with 1,000 U/mL IL-2, and then assayed for their cytotoxicity against patient no. 1 CD13+ AML cells, at an E:T ratio of 20:1, after the addition of different types of MoAbs. As shown in Fig 1, addition of the BsAb markedly enhanced cytotoxicity, and addition of an MoAb other than anti-CD3 did not enhance cytotoxic activity. Addition of the intact anti-CD3 MoAb, OKT3, moderately enhanced cytotoxicity; this enhancement seemed to be caused by anti-CD3–redirected cytotoxicity for FcγR+ AML target cells. The following factors led us to this conclusion: (1) patient AML cells expressed both FcγRI and FcγRII, as detected by anti-CD64 and anti-CD32 MoAbs, respectively (data not shown); (2) the addition of anti-CD3 F)ab')₂ MoAb did not enhance cytotoxic activity; and (3) the addition of aggregated IgG inhibited anti-CD3 MoAb-mediated but not BsAb-mediated enhancement of the cytotoxicity (Fig 1). When AML cells of patient no. 8 were used as target cells, similar findings were obtained; marked enhancement of cytotoxicity by the anti-CD3 Fab' × anti-CD13 Fab' BsAb, moderate enhancement of cytotoxicity by the anti-CD3 MoAb and inhibition of anti-CD3 MoAb-induced cytotoxicity by addition of aggregated IgG (data not shown). Effector cells induced from PBMC of another normal donor showed similar results for patients' no. 1 and 8 CD13+ AML cells (data not shown). The BsAb,

added at various concentrations of up to 1 μg/mL during the cytotoxicity assay, enhanced cytotoxic activity in a dose-dependent manner. Higher E:T ratios, of up to 40:1, induced higher cytotoxic activity in the presence or absence of the BsAb, and the level of cytotoxicity reached plateau when assayed with an E:T ratio of up to 160:1 (data not shown).

The BsAb enhances the cytolytic activity of normal donor IL-2–stimulated PBMC against CD13+ but not CD13⁻ leukemic cells. PBMC from two normal donors were cultured with IL-2 for 7 days and their cytotoxicity was assayed against CD13+ and CD13⁻ leukemic cells in the presence or absence of the BsAb. As shown in Fig 2, IL-2–stimulated PBMC exhibited low but significant levels of cytotoxicity against patient leukemic cells. Although the results suggested that CD13⁻ AML cells were more susceptible to IL-2–stimulated PBMC than CD13+ AML cells, repeated experiments did not support the difference: the susceptibility of CD13+ AML cells from five patients did not differ from that of CD13⁻ AML cells from five patients (data not shown).

Addition of the BsAb markedly enhanced cytotoxicity for CD13+ target cells but not for CD13⁻ target cells. However, the degree of cytotoxicity enhancement by the BsAb did not correlate with the percent positivity of CD13 antigen on the target cells. As in our previous study with anti-CD3 Fab' × anti-CD10 Fab' BsAb,[11] we performed a competitive inhibition test with unlabeled target cells, and the results showed that in the presence of the BsAb cytotoxic activity was significantly inhibited by unlabeled CD13+ cells but not by at least a 100-fold excess of unlabeled CD13⁻ cells (data not shown). This finding indicates that a small number of CD13+ malignant cells present among a large population of CD13⁻ nonmalignant cells was efficiently lysed by IL-2–stimulated PBMC after addition of the BsAb.

The BsAb enhances the cytolytic activity of patient IL-2–stimulated PBMC against autologous CD13+ AML cells. PBMC obtained from six patients were stimulated with 1,000 U/mL of IL-2 and cytotoxicity against autologous AML cells
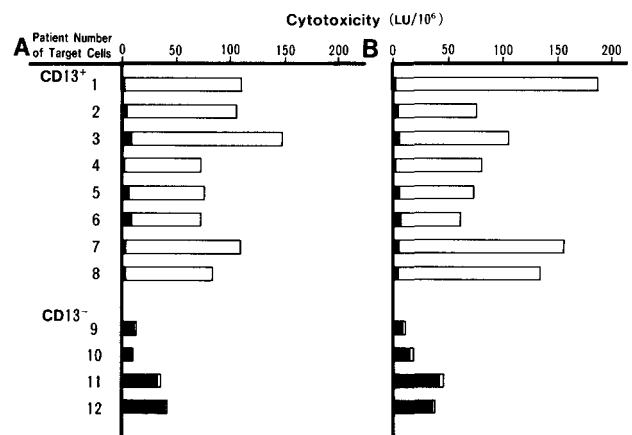
Fig 2. The BsAb enhances the cytolytic activity of normal donor IL-2–stimulated PBMC against CD13+ but not CD13⁻ leukemic cells. PBMC from two normal donors (A and B) were cultured with IL-2 for 7 days and assayed for their cytotoxicity against CD13+ AML cells and CD13⁻ AML and ALL cells in the absence (■) or presence (□) of 0.1 μg/mL BsAb.

was assayed on day 7. As shown in Fig 3, IL-2–stimulated PBMC lysed autologous AML cells to a small but significant extent, and with the addition of the BsAb all CD13$^+$ AML patient cells showed enhanced cytotoxic activity for autologous AML cells. However, compared with the cytotoxic activity of normal donors, patient effector cells, except those obtained from patient no. 6, showed lower levels of cytotoxicity enhancement against autologous AML cells after addition of the BsAb. In patient no. 7 with CD13$^+$ AML cells a similarly low level of cytotoxicity enhancement was obtained after addition of the BsAb, and in patient no. 10 with CD13$^-$ AML cells cytotoxicity was not enhanced after addition of the BsAb (data not shown).

Then, we investigated whether the addition of various types of cytokines or anti-CD3 MoAb-enhanced PBMC proliferation and cytotoxic activity, as reported by other investigators.[16,17,21-23] Addition of anti-CD3 MoAb at the initial 48 hours of IL-2 culture or addition of IL-7 did not affect the PBMC proliferation in three normal donors or four AML patients on day 7 or 8 (data not shown). However, when assayed on day 14, addition of anti-CD3 MoAb at the beginning of culture significantly enhanced the IL-2–mediated PBMC proliferation in normal donors and patients, whereas IL-7 had little effect on PBMC proliferation. Further addition of IFN-γ or IL-1β at the beginning of or during culture (see Materials and Methods) did not affect the cellular proliferation rate (data not shown).

When cytotoxic activity was assayed in three normal donors after PBMC culture with these cytokines or anti-CD3 MoAb, PBMC cultured for a longer term (2 weeks) exhibited higher levels of cytotoxic activity on a per culture basis (LU/C) after addition of the BsAb in both IL-2 and IL-2 plus anti-CD3 MoAb cultures as compared with PBMC cultured for a short term (1 week) (data not shown). Of particular interest was the finding that IL-2 plus anti-CD3 MoAb treatment induced a markedly higher level of cytotoxicity on a per culture basis in 2-week culture. However, the level of cytotoxicity induced by IL-7 was lower than that of cytotoxicity induced by IL-2 alone or IL-2 plus anti-CD3 MoAb. Addition of IFN-γ or IL-1β to these culture conditions did not affect cytotoxic activity (data not shown).

When patient PBMC were cultured with IL-2, IL-2 plus anti-CD3 mAb, or IL-7 for 1 or 2 weeks, we found that treatment with IL-2 alone and IL-2 plus anti-CD3 MoAb markedly enhanced 2-week cultured PBMC cytotoxicity on a per culture basis (LU/C) after addition of the BsAb (Fig 3). However, the levels of patient PBMC cytotoxicity obtained after 2 weeks of culture still tended to be lower than that of normal donors. Based on these results, further experiments were performed with cytotoxic cells generated after a 2-week culture of PBMC with 1,000 U/mL IL-2 and the initial 48-hour addition of anti-CD3 MoAb.

Ultrastructural changes of AML cells. Figure 4 shows electron micrographs of AML cells incubated with autologous cytotoxic cells and the BsAb. Cytotoxic cells from patient no. 8 were incubated with autologous CD13$^+$ AML cells for 2 hours in the IL-2–containing medium with or without addition of the BsAb and were then processed for electron microscopy. Without addition of the BsAb the proportion of AML cells undergoing changes leading to death was lower
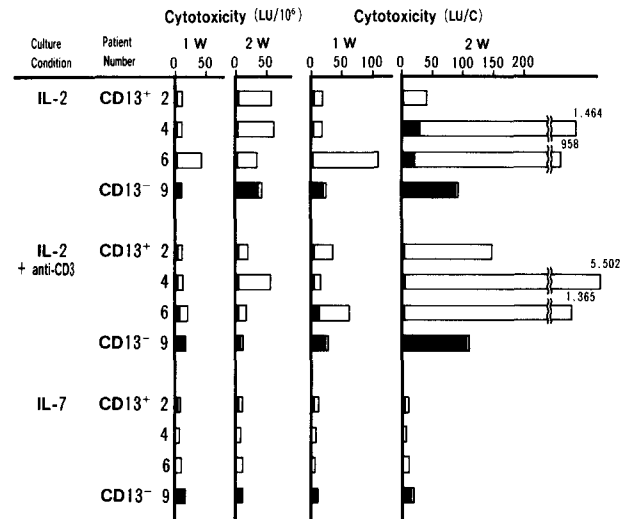

Fig 3. Effects of cytokines and anti-CD3 MoAb on patient PBMC cytotoxicity. PBMC obtained from four patients were cultured with IL-2, IL-2 plus anti-CD3 MoAb, and IL-7 as described in Materials and Methods. Cytotoxic activity against autologous AML cells in the absence (■) or presence (□) of the BsAb was assayed on days 7 and 14.

and most AML cells remained intact. However, with the addition of the BsAb apoptotic bodies or condensation of nuclear chromatins, such as crescent-shaped aggregates lining the nuclear membrane, were found. These changes are compatible with those described as apoptosis.[24-27] However, it was not clear whether necrosis of the cytoplasm was secondary to apoptosis or was the primary process. Some effector cells bound to target AML cells which appeared to be killed. AML cells incubated alone or with the BsAb did not show apoptotic changes. Cytotoxic cells generated from patient no. 4 PBMC also induced similar apoptotic changes in autologous CD13$^+$ AML cells after addition of the BsAb (data not shown).

Blast colony assay. Cytotoxic activity was measured by a blast colony assay. Figure 5A (patient no. 4), B (patient no. 8), and C (patient no. 10) show that effector cells inhibited colony formation by autologous AML cells and that addition of the BsAb further inhibited colony formation of CD13$^+$ (patients no. 4 and 8) but not CD13$^-$ (patient no. 10) AML cells. Figure 5D (patient no. 8) and E (patient no. 10) show that anti-CD3 or anti-CD13 MoAbs had little effect on colony formation when added with or without effector cells. Effector cells cultured alone in the presence or absence of PHA-LCM or GM-CSF did not form colonies and addition of the BsAb to the AML cell culture did not affect the number of colonies.

Colony assay for normal progenitor cells. The effect of cultured PBMC on autologous mixed cell and GM colony formation was examined. As shown in Fig 6, addition of effector cells at a higher E:T ratio of 5:1 inhibited colony formation of both mixed cell and GM colonies in the presence of the BsAb. However, the degree of inhibition was significantly smaller than that for the blast colonies. Anti-CD3 or anti-CD13 MoAbs did not affect colony formation (data not shown).
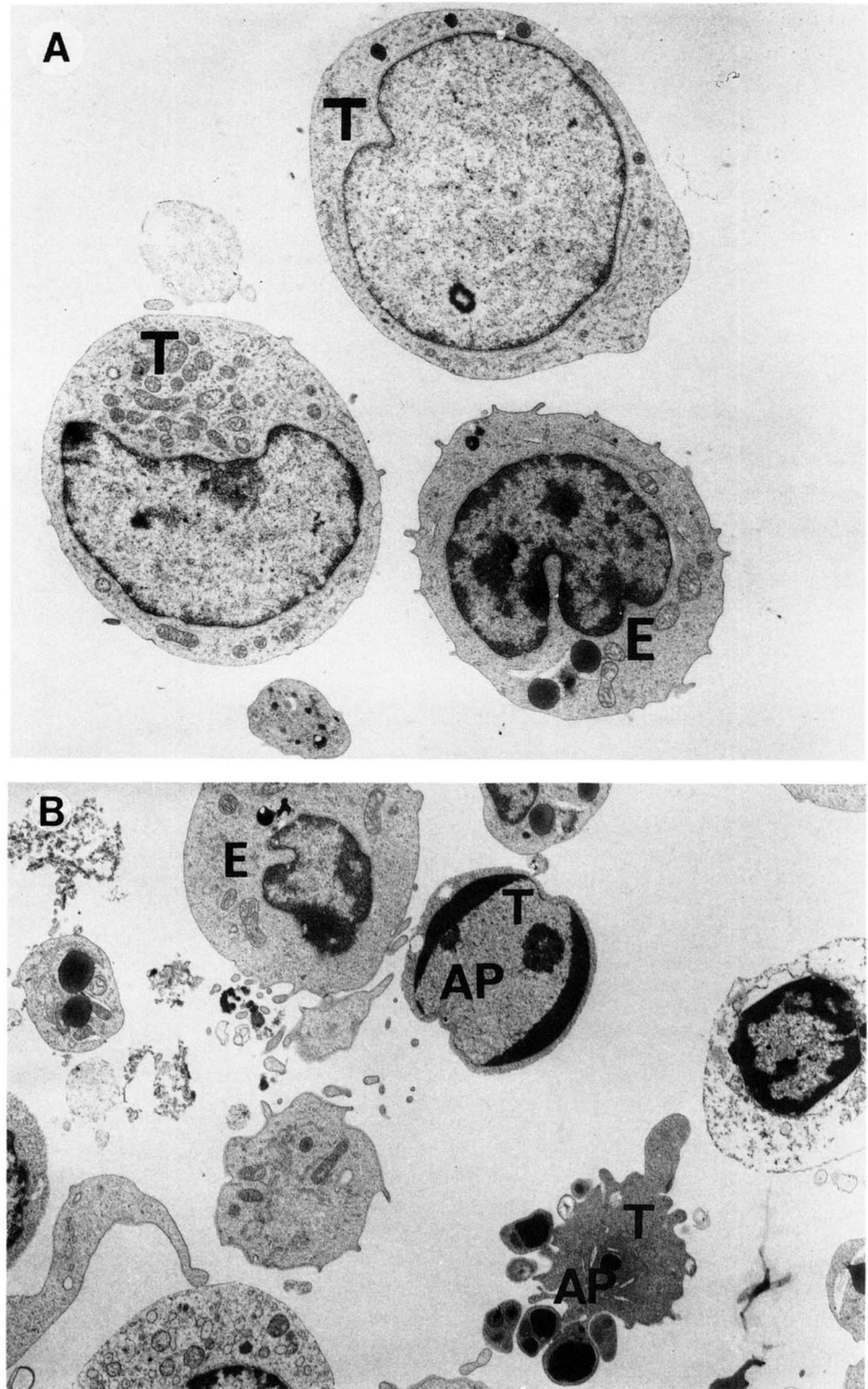
Fig 4.   Electron micrographs of IL-2 plus anti-CD3 MoAb-stimulated PBMC and AML cells. Effector cells from patient no. 8 were incubated for 2 hours with autologous AML cells, at an E:T ratio of 2:1, in the absence or presence of the BsAb and processed for electron microscopy. Effector cells and AML cells were incubated in the absence of the BsAb (A), and in the presence of the BsAb (B). E denotes effector cells; T, target AML cells; AP, apoptotic bodies (original magnification × 6,800).

DISCUSSION

Targeting of CTL to tumor cells by BsAb as a possible way of treating cancer has gained increasing interest in the last few years. Various forms of BsAb have been generated and tested not only in vitro but also in vivo and many promising results have been reported.[8-15]

In a previous study[11] we generated an anti-CD3 Fab' × anti-CD10 Fab' BsAb that efficiently enhanced cytotoxic activity of IL-2–stimulated PBMC against autologous and allogeneic CD10$^+$ ALL cells. In the present study we generated an anti-CD3 Fab' × anti-CD13 Fab' BsAb to target CD13$^+$ AML cells. For targeting AML cells with BsAb we selected
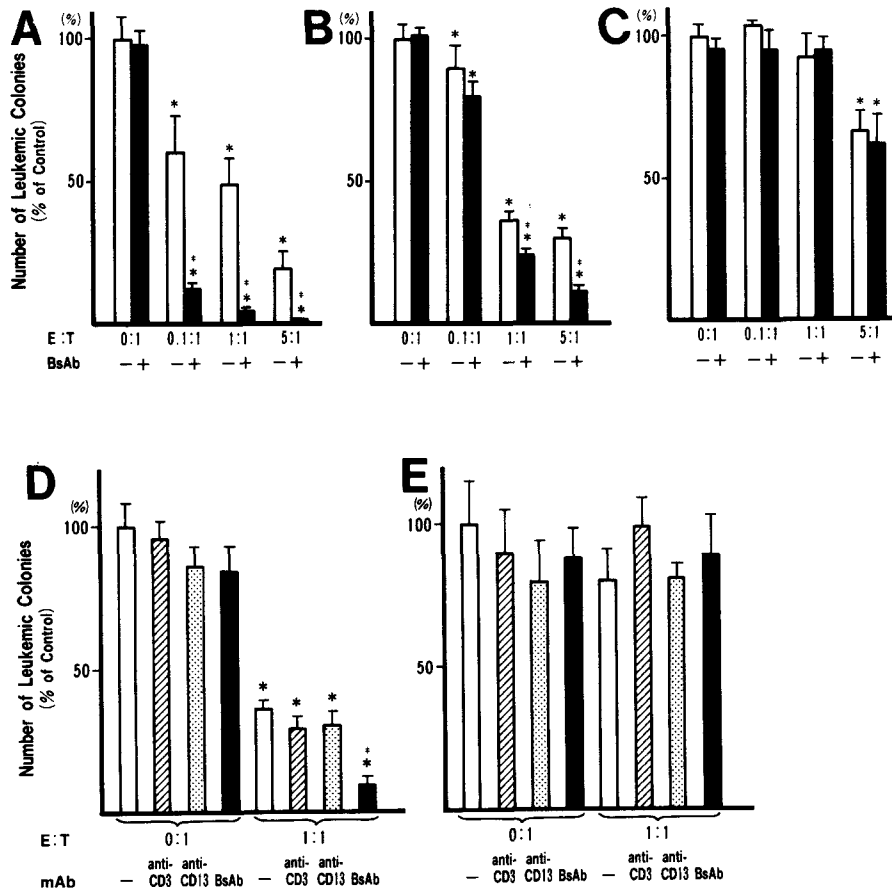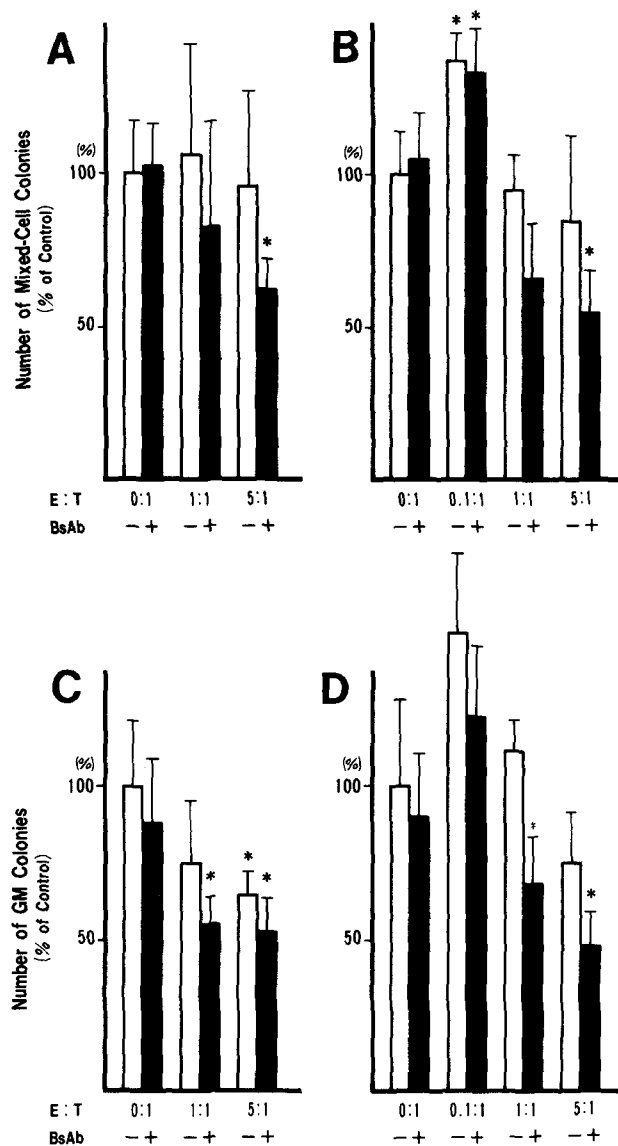
Fig 5. Effects of the stimulated PBMC and the BsAb on blast colony formation. PBMC from three patients were stimulated with IL-2 plus anti-CD3 MoAb for 2 weeks. Various numbers of cultured PBMC were mixed with autologous AML cells in the presence or absence of the BsAb or other MoAb, incubated for 4 hours at 37°C, and then suspended in methylcellulose-containing medium. After 7 or 10 days colonies were enumerated; the results are expressed as percentage of control colonies. The number of colonies in the control obtained after culture of AML cells alone without the addition of effector cells or antibodies were 180 ± 14 in patient no. 4 (A), 365 ± 27 in patient no. 8 (B), 79 ± 7 in patient no. 10 (C), 259 ± 20 in patient no. 8 (D), and 111 ± 17 in patient no. 10 (E). Experiments A to C and D and E were performed at different periods. The asterisk (*) denotes significant inhibition of colony formation as compared with the control culture. The double dagger (‡) denotes significant inhibition of colony formation after addition of the BsAb. Cytotoxic activity tested by ^{51}Cr-release assay in the absence or presence of the BsAb was <0.02 LU/10^6 and 52.1 LU/10^6 in patient no. 4, <0.02 LU/10^6 and 50.0 LU/10^6 in patient no. 8, and <0.02 LU/10^6 and <0.02 LU/10^6 in patient no. 10.

the anti-CD13 MoAb My7 to cross-link anti-CD3 MoAb OKT3. CD13 antigen is a cell surface glycoprotein and is identical to aminopeptidase N that is expressed not only on the surface of AML cells but also on the surface of normal hematopoietic cells of myeloid lineage, intestinal epithelium, renal tubular epithelium, and synaptic membranes of the central nervous system.[28-30] There are five reasons why we selected the anti-CD13 MoAb: first, there have been no known AML cell-specific antigens. Second, AML cells from most patients express CD13 antigen.[31-34] Third, AML patients with CD13+ blasts are reported to have a lower rate of complete remission to standard induction chemotherapy or a shorter survival than AML patients with CD13- blasts,[33,34] thus implying that a CD13-directed BsAb may be useful for these patients. Fourth, blast progenitors forming leukemic colony cells may also express the CD13 antigen.[35] Fifth, even though the CD13 antigen is expressed on various types of normal tissues, a CD13-directed BsAb will not cause serious

tissue damage when it is used ex vivo, ie, for purging CD13+ AML cells in autologous bone marrow transplantation.

Our present study clearly demonstrated that addition of the BsAb enhanced cytolysis of IL-2-stimulated PBMC against CD13+ but not CD13- AML cells. However, the susceptibility of leukemic cells did not correlate to the percent positivity of CD13 antigen on these cells (Table 1 and Fig 2). Although the cause of this discrepancy is not known, we had similar findings in other experiments: the susceptibility of CD10+ cell lines to CTL targeted with anti-CD3 Fab' × anti-CD10 Fab' BsAb did not correlate to the density of CD10 antigen expressed on these cell lines.[18] Thus, the susceptibility may partially depend on unknown factors inherently present in target tumor cells.

Although the number of patients tested was small (Figs 2 and 3), an important finding to be addressed is that, except for one patient, the level of LAK activity of patient IL-2-stimulated PBMC for autologous CD13+ AML cells was

Fig 6. Effects of the stimulated PBMC and the BsAb on colony formation of normal progenitor cells. PBMC from two normal donors were stimulated with IL-2 plus anti-CD3 MoAb for 2 weeks. Effector PBMC were mixed at various E:T ratios with target autologous bone marrow mononuclear cells in the presence or absence of the BsAb, incubated for 4 hours at 37°C, and then suspended in methylcellulose-containing medium. The results are expressed as percentage of control colonies. The number of mixed cell colonies in the control obtained after culture of bone marrow mononuclear cells without addition of effector cells or the BsAb was 32 ± 7 for donor no. 1 (A), 45 ± 5 for donor no. 2 (B); and control number of GM colonies was 40 ± 9 in donor no. 1 (C), and 45 ± 13 in donor no. 2 (D). The asterisk (*) denotes significant inhibition of colony formation as compared with the control culture. The double dagger (‡) denotes significant inhibition of colony formation after addition of the BsAb.

much lower than that of normal donors. LAK cell generation is reported to be defective in acute leukemia patients with active disease.[6,7] However, because our patients' blood samples were collected during a period of complete remission, it seems unlikely that disease activity affected LAK generation. Indeed, in our previous study patient LAK activity for au-

tologous leukemia or lymphoma cells tended to be lower than that of normal donors, even when patient blood was collected during complete remission.[5] We therefore investigated better methods for obtaining potent effector cells. We found, as has been reported by other investigators,[16,17] that the addition of anti-CD3 MoAb during the initial culture period induced marked proliferation of PBMC, which, in turn, led to the production of potent cytotoxic activity on a per culture basis (LU/C), even though cytotoxic activity on a per cell basis (LU/10[6]) did not differ significantly (Fig 3). Repeated restimulation with anti-CD3 MoAb may further expand the effector cell population.[12] Thus, when only weak cytotoxic activity is induced in short-term culture, anti-CD3 MoAb treatment may be useful for obtaining large numbers of effector cells in long-term culture.

IL-7 is another cytokine that induces LAK activity.[21-23] In this study we have confirmed an IL-7 effect on induction of cytotoxic cells, and we have shown that the addition of the BsAb further enhanced cytotoxic activity of IL-7–stimulated PBMC for autologous and allogeneic CD13^{+} AML cells (Fig 3). However, the level of cytotoxicity achieved by IL-7–stimulated PBMC was significantly lower than that achieved by IL-2–stimulated PBMC. The lower proportion of CD8^{+} cells found in IL-7–stimulated PBMC (data not shown) would explain its lower cytotoxicity. A cytotoxicity assay using sorted CD4^{+} and CD8^{+} effectors will be required to solve this issue.

We investigated the interaction of IL-2 plus anti-CD3 MoAb-stimulated PBMC and AML cells by electron microscopy, and we found many AML cells to have been killed in an apoptotic manner after addition of the BsAb (Fig 4). The mechanisms by which cytolytic lymphocytes lyse their targets have been the subject of intensive research. Two models have been posed.[24-27] One of them proposes that killer cells disrupt target cell membranes, which leads to osmotic cell lysis. This passive type of death has been described for complement- or perforin-induced death and is called necrosis. The second model calls for active participation by the target cells in their own death, the killer cells simply inducing a program for cell death or apoptosis in the targets. In cells undergoing necrosis, plasma and organelle membranes disintegrate and the cytoplasmic structure is disrupted, whereas the nucleus remains unaffected. The morphologic characteristics of apoptosis include vacuolation of the cytoplasm, condensation of the chromatin, and segmentation of the nucleus. CTL, natural killer cells, LAK cells, and antibody-dependent cellular cytotoxicity are known to cause both types of target cell lysis.[24-27] In our present study AML cells incubated with a combination of IL-2 plus anti-CD3 MoAb-stimulated PBMC and the BsAb showed morphologic changes characteristics of apoptosis, ie, formation of apoptotic bodies and condensation of nuclear chromatins. Because "fragmentin" in NK cells causes apoptosis only in the presence of perforin,[36] it is unknown whether the cytoplasmic necrosis found in some AML cells with condensed nuclei was secondary to apoptosis or occurred as the primary process. Although the mechanisms by which effector cells targeted with BsAb cause the apoptotic death of these cells remain to be clarified, this is, to our knowledge, the first morphologic observation of the manner in which patient AML blasts are killed by autologous IL-2 plus anti-CD3 MoAb-stimulated PBMC.

Addition of the BsAb to IL-2 plus anti-CD3 MoAb-stimulated PBMC further enhanced colony inhibition of CD13+ AML cells (Fig 5), suggesting that colony-forming blast progenitors express CD13 antigen.[35] The finding that colony formation was markedly inhibited by effector cells and the BsAb at lower E:T ratios indicates that blast colony assay was more sensitive than ^{51}Cr-release assay for examining the effects of IL-2-stimulated PBMC and the BsAb. CTL targeted with BsAb are reported to release cytokines such as TNF-β and IFN-γ.[37] Because these cytokines are known to inhibit colony formation by AML blast progenitors,[38,39] BsAb-targeted cytokine release from CTL in addition to the direct killing may explain why the colony inhibition assay was more sensitive than the ^{51}Cr-release assay.

In hematopoietic cells, other than being expressed by AML cells, the CD13 antigen is expressed by normal granulocytes, monocytes, and a small number of bone marrow mononuclear cells.[32-34] Because the number of GM and mixed cell colonies was not reduced by the addition of the BsAb, there might be a CD13- fraction of normal bone marrow progenitor cells that is able to form mixed cell and GM colonies, as was reported previously.[35] Because the degree of inhibition for these normal progenitor cells was much smaller than that for the leukemic blast colonies in the presence of IL-2 plus anti-CD3 MoAb-stimulated PBMC and the BsAb in the culture, we believe that this anti-CD3 Fab' × anti-CD13 Fab' BsAb is useful for ex vivo purging of CD13+ leukemic cells in autologous bone marrow transplantation.

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