Increased Lysis of Patient CD10-Positive Leukemic Cells by T Cells Coated With Anti-CD3 Fab' Antibody Cross-Linked to Anti-CD10 Fab' Antibody

By Kazuo Oshimi, Takashi Seto, Yoko Oshimi, Michihiko Masuda, Ko Okumura, and Hideaki Mizoguchi

An anti-CD3 Fab' × anti-CD10 Fab' bispecific hybrid F(ab')2 antibody (Ab) was generated. This bispecific Ab had a molecular mass of 100 to 110 Kd, and the capacity to react with both CD3+ T cells and CD10+ acute lymphoblastic leukemia (ALL) cells. We studied whether cytotoxic T lymphocytes (CTLs) could lyse patient CD10+ ALL cells after addition of the bispecific Ab. As effector CTLs, interleukin-2 (IL-2)-stimulated peripheral blood mononuclear cells (PBMCs) and CTL clones were used. When IL-2-stimulated PBMCs were assayed for cytotoxicity to 51Cr-labeled CD10+ ALL cells, their activity was shown to be markedly enhanced by the addition of the bispecific Ab. Most of the CTL clones established lacked cytotoxicity for CD10+ ALL cells, but addition of the bispecific Ab induced a significant level of cytotoxicity. CTLs derived from ALL patients also showed significant cytotoxicity for autologous CD10+ ALL cells after addition of the bispecific Ab. However, this Ab did not affect the cytotoxicity of CTLs when CD10+ leukemic cells were used as the targets. These findings suggest that the bispecific Ab can be used for immunotherapy in patients with CD10+ ALL.

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MATERIALS AND METHODS

Characteristics of patients and leukemic cells. Leukemic cells from six patients were used as target cells in the cytotoxicity assays. Patients 1 through 4 had acute lymphoblastic leukemia (ALL) with a CD10+ 19+ 20+HLA-DR+ phenotype, and patient 5 had ALL with a CD10+ 19+ 20+HLA-DR+ phenotype. Patient 6 had acute nonlymphoblastic leukemia (ANLL)-M1, and the leukemic cells were CD10-. In all patients, leukemic cells were obtained at a time when they comprised more than 90% of the PB white cells. In patients 1 through 4, the percentage of CD10+ cells among PBMCs was 92%, 78%, 91%, and 74%, respectively.

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 will be protected.

Preparation of the bispecific Ab. The method described previously was modified as follows. The anti-CD3 monoclonal Ab (MoAb), OKT3, was obtained from Ortho Pharmaceutical Inc (Raritan, NJ) and the anti-CD10 MoAb, J5, came from Coulter Immunology (Hialeah, FL). F(ab')2 fragments of these anti-CD3 and anti-CD10 Abs were prepared by overnight digestion at 37°C with pepsin (Sigma, St Louis, MO; 1/8 wt/wt) in 0.1 mol/L CH3COONa buffer (pH 4.5). The reaction was stopped by the addition of 0.02 mol/L NaHPO4 to increase the pH to 7.7. F(ab')2 fragments were then purified by fast protein liquid chromatography on a column of Mono Q HR5/5 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 20 mmol/L sodium phosphate containing a 0 to 0.5 mol/L NaCl gradient (pH 7.7). The fraction containing the F(ab')2 fragments was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Fast System, Pharmacia). F(ab')2 fragments (0.44 mg) of the anti-CD3 MoAb were subjected to reduction with 0.5 mmol/L DTT (Sigma) for 30 minutes at pH 7.5. This generated Fab' fragments with free SH groups (Fab'-SH) that were separated from the excess reagents on a Sephadex G-25 column (Pharmacia) in 0.1 mol/L sodium phosphate containing 1 mmol/L EDTA (pH 7.5). The F(ab')2 fragments (0.44 mg) of the anti-CD10 Ab were also reduced with 0.5 mmol/L DTT and the reaction was then stopped by the addition of DTT at a final concentration of 5 mmol/L. The nitrogenous acid derivatives of the anti-CD10 Fab' fragments (Fab'-S-NB) were also separated from the excess reagents and reaction products by a Sephadex G-25 column and the same buffer. Fab'-SH and Fab'-
S-NB fragments were then mixed at a 1:1 ratio, and incubated overnight at room temperature to produce the bispecific Ab. This bispecific Ab was then purified by a TSK-gel G3000SWXL (Toso, Tokyo, Japan) in 0.1 mol/L sodium phosphate containing 0.15 mol/L NaCl (pH 7.2). The fraction containing the reconstituted F(ab')2 fragments was checked by SDS-PAGE, and the F(ab')2 content (0.26 mg) was determined by measuring the absorbance at 280 nm. The bispecific Ab was obtained with a yield of 30% after purification.

Stimulation of PBMCs with IL-2. PBMCs were stimulated with IL-2 as described previously.14 Heparinized PB was obtained from patients and normal donors. To minimize the effects of leukemic cells and chemotherapy on PBMC stimulation, blood from patients was taken at a time when leukemic cells were not present in the PB, and at least 4 weeks after cessation of chemotherapy. PBMCs were separated with Ficoll-Conray density gradients, washed twice, and suspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (Flow, McLean, VA), 1,000 U/mL of recombinant human IL-2 (Shionogi, Osaka, Japan; specific activity 1.2 x 10^6 U/mg of protein), 100 U/mL of penicillin, and 10 μg/mL of streptomycin. PBMCs (2 x 10^5) were cultured in a 24-well tissue culture plate (Linbro, Hamden, Conn) in 2 mL of the above medium at 37°C in humidified air with 5% CO2. After 5 days of culture, cells were harvested, washed once, and assayed for their cytotoxicity.

Generation of CTL clones. CTL clones were generated as described previously.15 In brief, PBMCs from normal donors and patient 1 were plated on a feeder layer (composed of irradiated allogeneic PBMCs plus an Epstein-Barr virus-transformed B cell line, FMO) in V-bottomed 96-well plates at 0.6 cells per well with 1,000 U/mL of IL-2 and lymphocyte-conditioned medium. Phytogamaglutinin-P (DIFCO, Detroit, MI) was used at 4 μg/mL to provide an initial mitogenic stimulus. Cloned CTLs were maintained with 1,000 U/mL of IL-2, irradiated allogeneic PBMCs, and irradiated FMO cells, and the cytotoxicity of CTL clones was assayed 5 days after replating the cultures with these three ingredients. Surface markers of the CTL clones were examined using indirect immunofluorescence. Expression of T-cell receptor-αβ (TCR-αβ) chains and TCR-γδ chains was detected by the MoAbs WT31 and anti-TCRδ, respectively.

Assay for cytotoxic activity. A 5-hour 51Cr-release test was used to determine cytotoxic activity.14 Effector cells were either IL-2-stimulated PBMCs or CTL clones, and the bispecific Ab was added during the cytotoxicity assay at a final concentration of 5 μg/mL or less. Target cells were leukemic cells isolated from the patients. Freshly isolated leukemic cells were suspended in 90% fetal calf serum (FCS) and 10% dimethyl sulfoxide, frozen in a programmable freezer, and stored in liquid nitrogen until use. For the labeling of target cells in the cytotoxicity assay, 300 to 400 μCi of Na251CrO4 (Japan Isotope Association, Tokyo, Japan) was incubated with 10^6 pelleted cells for 2 hours. Labeled cells were then washed three times, and used as target cells at 5 x 10^5 cells per microculture well. The effector-to-target (E:T) ratio was 10:1 unless otherwise stated, and all experiments were performed in triplicate. After 5 hours of incubation, the supernatant fluid was harvested and the radioactivity was determined with a gamma counter. The percent cytotoxicity was calculated by the following formula: (experimental 51Cr release – spontaneous 51Cr release) / (maximum 51Cr release – spontaneous 51Cr release) x 100, where spontaneous release was the 51Cr release from 5 x 10^5 labeled target cells incubated alone in the medium, and maximum release was that from labeled target cells incubated alone in water containing 5% detergent. Addition of only the bispecific Ab to the target cells did not affect 51Cr release. The results of the cytotoxicity tests were expressed as the mean percent cytotoxicity. The standard deviation of percent cytotoxicity was less than 5% in most of the experiments. In some experiments, the results were expressed as lytic units (LU) per 10^6 cells, with 1 LU being the number of effector cells required to cause 50% lysis of the target cells.

RESULTS

Characterization of the bispecific Ab. The products of the reaction between Fab'-SH and Fab'-S-NB were fractionated, and an elution profile was obtained. The earlier peak was that of the F(ab')2 molecules, which represented between 20% and 30% of the final product, and the later peak included the single Fab' molecules (data not shown). Material eluting in the F(ab')2 peak was ascertained to be nearly pure by SDS-PAGE, and showed a single band (100 to 110 Kd) with the same size as F(ab')2 fragments of J5 IgG obtained by digestion with pepsin (data not shown). These characteristics were similar to those of other bispecific F(ab')2 heterodimers previously generated.11,12

To demonstrate that the eluted F(ab')2 fragments were heterodimers and not homodimers, their reactivity with both CD3+ T cells and CD10+ ALL cells was investigated by indirect immuno-fluorescence (Fig 1). The results showed that the F(ab')2 fragments were bifunctional; that is, they reacted not only with CD3+ T cells but also with CD10+ ALL cells (Figs 1A and B). As has been reported for other bispecific F(ab')2 heterodimers,12 the reactivity of our bispecific Ab with CD3+ T cells or CD10+ ALL cells was found to be depressed when compared with the corresponding intact Abs by flow cytometry. Next, a target-absorption test was performed to rule out the existence of contaminating homo-F(ab')2 fragments. This test showed that absorption of the hetero-F(ab')2 by a CD3+ CTL clone concomitantly diminished the anti-CD10 reactivity (Figs 1C and D) and vice versa (data not shown). Absorption by CD10+ ANLL cells did not diminish the anti-CD3 or anti-CD10 reactivity (data not shown). These results indicated that the antibody...
was indeed a true hetero-F(a,b‘) fragment, rather than a mixture of homoconjugated F(a,b‘) fragments.

Induction of cytotoxicity for CD10+ ALL cells in the presence of the bispecific Ab. Figure 2 shows the cytotoxicity of IL-2-stimulated PBMCs and a CTL clone for CD10+ ALL cells obtained from patient 1. PBMCs from two normal donors (Figs 2A and B) were cultured for 5 days with 1,000 U/mL of IL-2. The PBMCs stimulated with IL-2 were shown to possess a substantial level of cytotoxicity against CD10+ ALL cells, and the addition of the bispecific Ab further enhanced their cytotoxicity. However, the addition of anti-CD3 Ab, anti-CD10 Ab, or both Abs did not enhance cytotoxicity, nor did the addition of anti-CD3 F(a,b‘) Ab, anti-CD10 F(a,b‘) Ab, or their combination. A CTL clone with the phenotype of TCR-αβ+, CD3+4-8+ and an unknown target specificity was established from a normal donor, and this clone by itself did not show cytotoxicity for CD10+ ALL cells (Fig 2C). Addition of the bispecific Ab induced cytotoxicity for ALL cells, while the addition of other Abs did not induce any cytotoxicity. Similar results were obtained using CD10+ ALL cells from patient 2 as targets (data not shown).

Bispecific Ab was added at various concentrations to assess the changes in cytotoxicity of the IL-2-stimulated PBMCs from two normal donors and the CTL clone. Figure 3 shows that the maximum level of cytotoxicity was obtained when effector cells were incubated with 1 to 5 µg/mL of the bispecific Ab. Higher E:T ratios induced a greater cytotoxicity (Fig 4), and the cytotoxicity levels almost reached a plateau at an E:T ratio of 20:1.

Kinetics of the development of cytotoxicity by IL-2-stimulated PBMCs. PBMCs from a normal donor were cultured for various periods of time with 1,000 U/mL of IL-2 to induce cytotoxicity against CD10+ ALL cells. Cytotoxic activity was found to peak on days 5 to 7 of culture and then to decline thereafter (Fig 5). When the bispecific Ab was added during the cytotoxicity assay, PBMCs cultured for longer periods showed a higher cytotoxicity and culture of PBMCs from another normal donor gave similar results (data not shown).

When PBMCs were cultured for 5 days in the presence of various concentrations of IL-2 (1 to 10,000 U/mL), 1,000 and 10,000 U/mL of IL-2 induced the maximum level of cytotoxicity against the CD10+ ALL cells of patients 1 and 2, and the magnitude of cytotoxicity enhancement by the addition of the bispecific Ab was highest at these concentrations of IL-2 (data not shown).

Specificity of the cytolysis induced by the bispecific Ab. PBMCs from six different donors were stimulated with IL-2 for 5 days, and their cytotoxicity was then assayed against CD10+ and CD10- leukemic cells in the presence or absence of the bispecific Ab. As shown in Fig 6, addition of the bispecific Ab enhanced cytotoxicity for CD10+ target cells, but not for CD10- target cells. It is of note that IL-2-stimulated PBMCs efficiently lysed autologous CD10+ ALL cells in the presence of the bispecific Ab, as shown in donor 1 (Fig 6A) and donor 2 (Fig 6D).

Various types of CTL clones were generated and their cytotoxicity for CD10+ ALL cells was examined (Fig 7). Clones 1 to 3 were TCR-αβ+, CD3+ clones derived from normal donors, and clone 4 was a TCR-γδ+, CD3+4-8+ clone derived from a normal donor. Clones 5 and 6 were the TCR-γδ+, CD3+4-8+ clones originally described as clones 8C9 and 6D6, respectively, in the previous report.15 Clones 7 to 10 were TCR-αβ+, CD3+ clones with either a CD4+8- or CD4-8+ phenotype that were derived from patient 1. It was found that these clones efficiently lysed CD10+ target
cells in the presence of the bispecific Ab, with clones 7 to 10 lysing autologous ALL cells (Fig 7A). Even without addition of the bispecific Ab, clone 5 showed strong cytotoxicity for two different CD10⁺ target cells, and the target speci-

Fig 6. The bispecific Ab enhanced IL-2-treated PBMC cytotoxicity for CD10⁺ but not CD10⁻ target cells. IL-2-stimulated PBMCs from different donors were assayed for their cytotoxicity against CD10⁺ and CD10⁻ leukemia cells at an E:T ratio of 10:1 in the presence [□] or absence [○] of the bispecific Ab. The target leukemia cells used as follows: (A) 92% CD10⁺ ALL cells from patient 1; (B) 78% CD10⁺ ALL cells from patient 2; (C) 91% CD10⁻ ALL cells from patient 3; (D) 74% CD10⁺ ALL cells from patient 4; (E) CD10⁺ ALL cells from patient 5; and (F) CD10⁻ ANLL cells from patient 6. Donor 1 was patient 1; donor 2 was patient 4; donor 3 was patient 5; and donors 4 through 6 were normal donors.

Fig 7. Cytotoxicity of CTL clones for CD10⁻ ALL cells. Cytotoxicity was assayed for CD10⁻ ALL cells from patient 1 (A) and patient 2 (B) at an E:T ratio of 10:1 in the presence [□] or absence [○] of 1 μg/mL of the bispecific Ab. CTL clone 1 was TCR-α⁺⁺, CD3⁺utters⁺, CD4⁻2⁻; clones 2 and 3 were TCR-α⁺⁺, CD3⁺utters⁺, CD4⁻2⁻; clones 4 and 5 were TCR-γδ, CD3⁺utters⁺, CD4⁻2⁻; clones 7 and 8 were TCR-α⁺⁺, CD3⁺utters⁺, CD4⁻2⁻; and clones 9 and 10 were TCR-α⁺⁺, CD3⁺utters⁺, CD4⁻2⁻. Clones 7 to 10 were established from patient 1.

We investigated whether or not a small number of CD10⁺ target cells present in a large population of CD10⁻ cells could be lysed by IL-2-stimulated PBMCs or CTL clones after addition of the bispecific Ab. Graded numbers of unlabeled CD10⁻ or CD10⁻ target cells were added, and the cytotoxicity assay against CD10⁺ target cells was performed. Figure 8 shows that, in the presence of the bispecific Ab, cytotoxicity

Fig 8. Competitive inhibition tests with unlabeled target cells. IL-2-stimulated PBMCs from a normal donor (A) and CTL clone 1 (TCR-α⁺⁺, CD3⁺utters⁺) (B) were assayed for their cytotoxicity against patient 1 CD10⁺ ALL cells at an E:T ratio of 10:1. Graded numbers of unlabeled patient 1 CD10⁺ ALL cells [□] or unlabeled patient 5 CD10⁻ ANLL cells [□] were added during the cytotoxicity assay in the presence of 1 μg/mL of the bispecific Ab. Unlabeled cells from patient 1 [□] or patient 5 [□] were added in the absence of the bispecific Ab.
of both IL-2-stimulated PBMCs and a CTL clone was significantly inhibited by unlabeled CD10+ ALL cells, whereas cytotoxicity was only marginally inhibited by unlabeled CD10- ANLL cells. Without the addition of the bispecific Ab, IL-2-stimulated PBMC cytotoxicity for CD10+ ALL cells was inhibited equally by unlabeled CD10+ ALL cells and CD10- ANLL cells.

**DISCUSSION**

In patients with hematologic malignancies, it is difficult to induce and expand CTLs specific to host leukemia or lymphoma cells. Natural killer (NK) cells are also unreactive to leukemic or lymphoma cells, unless they are activated by interferon or IL-2, or unless suppressor monocytes are removed from the effector cell population. However, although interferon-activated NK cells are able to lyse allogeneic leukemia and lymphoma cells, NK cells reactive to autologous tumor cells are difficult to induce. IL-2-treated NK cells, on the other hand, are potent mediators of lysis for both autologous and allogeneic leukemia and lymphoma cells. However, high E:T ratios are required to produce target cell lysis, and the level of cytotoxicity actually achieved tends to be low. In this article, we have shown that IL-2-stimulated PBMCs and CTL clones efficiently lysed autologous and allogeneic CD10+ ALL cells after the addition of an anti-CD3 Fab' × anti-CD10 Fab' bispecific Ab.

We used both IL-2-stimulated PBMCs and CTL clones as the effector cells. IL-2-stimulated PBMCs are known to include activated NK cells (CD3+16+56+) as well as a wide variety of CTLs (ie, CD3+4+8-, CD3+4-8+, and CD3+4-8+ T cells). Therefore, the CTL population of IL-2-stimulated PBMCs not only seemed to mediate cytotoxic activity but also produced a marked increase in cytotoxicity in the presence of the bispecific Ab. There are a number of other reports describing the use of IL-2-stimulated PBMCs as effector cells and the response to such PBMCs applied in combination with a bispecific Ab has seemed to be promising in clinical trials.

Kinetic studies of the development of cytotoxicity by IL-2-stimulated PBMCs (Fig 5) showed that the addition of the bispecific Ab enhanced PBMC cytotoxicity, and that, surprisingly, the level of cytotoxicity increased further with prolongation of the culture period, in contrast to the decline of PBMC cytotoxicity seen in assays performed without the addition of the bispecific Ab. IL-2 can be used to expand PBMC numbers in culture, so these results suggest that IL-2-expanded PBMCs could possibly be used as effector cells for specific targeting therapy after treatment with the bispecific Ab. As indicated in Fig 6, the PBMC cytotoxicity induced by IL-2 was lower for the two patients (donors 1 and 2) than for the normal individuals. In contrast, the enhancement of patient PBMC cytotoxicity by the addition of the bispecific Ab was equal to or higher than that seen for the PBMCs from normal individuals. Although a larger series of patients needs to be studied, these results suggest that IL-2-stimulated host PBMCs can be used as effector cells.

We used various types of CTL clones as the effector cells. Although detailed studies have not yet been reported on TCR-γδ+ cells, the bispecific Ab was shown to be effective in inducing cytotoxicity in both TCR-αβ+ and TCR-γδ+ clones (Fig 7). CTL clone 5 (TCR-γδ+, CD3+4-8+) exhibited marked cytotoxicity for ALL cells even without addition of the bispecific Ab. It would be of interest to know how this clone recognized the target cells, and the recognition mechanism and target cell specificity of this clone are now under investigation.

In hematologic malignancies, bispecific Abs may first be applied for the ex vivo purging of malignant cells in autologous bone marrow transplantation. In such a situation, a small number of malignant cells may be present among a large population of nonmalignant cells. Figure 8 shows that, even when a 100-fold excess of CD10+ cells was present, CD10+ cells were efficiently lysed by the addition of the bispecific Ab. Thus, this method of purging may be effective by itself and also has the potential to be used in combination with other methods, eg, immunotoxins, antibody plus complement treatment, or the in vitro application of chemotherapeutic agents.

We are currently attempting to cross-link the anti-CD3 Fab' Ab with other types of Abs, because this should make it feasible to lyse other types of leukemia cells.

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Increased lysis of patient CD10-positive leukemic cells by T cells coated with anti-CD3 Fab' antibody cross-linked to anti-CD10 Fab' antibody

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