

## Monocyte-mediated Lysis of Acute Myeloid Leukemia Cells in the Presence of the Bispecific Antibody 251 × 22 (Anti-CD33 × Anti-CD64)<sup>1</sup>

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### ABSTRACT

Immunotherapy using bispecific antibodies (BsAb) to direct immune effector cells toward target tumor cells has been shown to be effective in a number of studies. Several immune trigger molecules have been characterized. Among them, FcγRI appears to play an important role in antibody-dependent cellular cytotoxicity. It is expressed mainly on monocytes, macrophages, and neutrophils under certain clinical situations. The expression of FcγRI can be regulated by a variety of cytokines, primarily by IFN-γ. Recent studies have shown that granulocyte-colony-stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) can increase the number of the FcγRI-positive monocytes, increase the expression of FcγRI on circulating neutrophils after *in vivo* infusion, and greatly enhance the cytotoxic activity of circulating neutrophils. CD33 is a glycoprotein expressed on the cell surface of mature monocytes, myeloid progenitor cells, and myeloid leukemic blasts, but not on the earliest hematopoietic progenitor cells and other normal tissues. Herein, we report the construction of a BsAb, 251 × 22, by conjugating an anti-CD33 mAb (mAb 251) to an anti-FcγRI mAb (mAb 22). The BsAb 251 × 22 is capable of enhancing the cytotoxicity of several leukemia cell lines by cytokine-activated monocytes. Our data also show that G-CSF- and GM-CSF-stimulated monocytes can mediate cytotoxicity of target leukemia cells comparable to that of IFN-γ-stimulated monocytes. The expression of FcγRI on monocytes after 24-h *in vitro* incubation with G-CSF and GM-CSF was increased, although not significantly. Prolonged incubation of monocytes with G-CSF for 48 h significantly increased the FcγRI expression. Because humanized anti-CD33 and anti-FcγRI mAb are available, and because GM-CSF and G-CSF have been used widely for patients after chemotherapy to stimulate the recovery of myeloid hematopoiesis, additional clinical development of this project is feasible. A BsAb comprised of

humanized anti-CD33 and anti-FcγRI could have clinical application in the treatment of myeloid leukemia, especially in the management of minimal residual disease.

### INTRODUCTION

Specific tumor cell lysis can be achieved by applying BsAbs<sup>3</sup> to direct immune effector cells toward tumor cells. This novel immunotherapeutic approach has been the focus of numerous *in vitro* and *in vivo* studies (1-4). Several immune-triggering molecules, including FcγRI, FcγRII, FcγRIII, and T-cell receptor-associated molecules, have been shown to play important roles in various immune responses. FcγRI (CD64) is expressed mainly on the surface of monocytes, macrophages, and neutrophils under certain clinical conditions (5-7). CD64 expression can be modulated by various cytokines, primarily IFN-γ (8-10). Previous studies have shown that *in vivo* administration of G-CSF increases the FcγRI expression of neutrophils (8), whereas administration of GM-CSF *in vivo* can increase the number of circulating FcγRI-positive monocytes (10). GM-CSF has been shown to stimulate neutrophil phagocytosis (6, 8), and to enhance ADCC by monocytes and granulocytes (5-7). To use monocytes as immune effector cells, the BsAb should be capable of binding to FcγRI with high affinity and to a specific antigen on a tumor cell surface. Such a BsAb could direct monocytes toward tumor cells and elicit the lysis of target cells. CD33 is a glycoprotein with restricted expression on myeloid progenitor cells and leukemic blasts, but not on other normal tissue and early hematopoietic progenitor cells (11-13). Clinical trials targeting mAb to CD33 on leukemia cells have shown no significant toxicity to normal body tissues (14).

We have reported previously the synthesis of a BsAb 251 × 3G8 (anti-CD33 × anti-FcγRIII). This BsAb was capable of augmenting the cytotoxicity of natural killer cells against CD33-positive AML cell lines and fresh cells from patients (15). In this study, we describe a new BsAb 251 × 22 (anti-CD33 × anti-FcγRI) that can significantly increase the cytotoxicity of CD33-positive leukemia cells by cytokine (IFN-γ, G-CSF, and GM-CSF)-activated monocytes.

### MATERIALS AND METHODS

mAbs. mAb 251 (anti-CD33) was developed by E. D. Ball (16). Purified 251 and the anti-FcγRI mAb (mAb 22) were

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<sup>3</sup> The abbreviations used are: BsAb, bispecific antibody; GM-CSF, granulocyte-macrophage colony-stimulating factor; SATA, *N*-succinimidyl-*S*-acetylthioacetate; Sulfo-SMCC, sulfosuccinimidyl 4 (*N*-maleimidomethyl) cyclohexane-1-carboxylate; G-CSF, granulocyte colony-stimulating factor; AML, acute myeloid leukemia; MFI, mean fluorescence intensity; ADCC, antibody-dependent cellular cytotoxicity.

kindly provided by Medarex, Inc. (Annandale, NJ); both are murine IgG1 mAbs. SCCL-1, an IgG2a murine mAb, directed against the transferrin receptor, was used in this study as a positive control for ADCC via an unconjugated mAb (17). F(ab')<sub>2</sub> fragment of mAb 251 was prepared by pepsin digestion and purified using a protein A column (Pharmacia Biotech, Inc., Piscataway, NJ).

**Production of BsAb 251 × 22.** Conjugation chemicals SATA and Sulfo-SMCC were obtained from Pierce Chemical Co. (Rockford, IL). Hydroxylamine was purchased from Sigma Chemical Co. (St. Louis, MO). SATA (50 mM) was freshly prepared in 100% dimethylformamide and Sulfo-SMCC (50 mM), in PBS (pH 7.4). SATA was mixed with mAb 251 in a final molar ratio 10:1. After 1-h reaction at room temperature, nonreacted SATA was removed by size-exclusion centrifugation through a Centricon 30 apparatus (Amicon, Inc., Beverly, MA). The free sulfhydryl group was generated by deacetylation with hydroxylamine at room temperature for 2 h. Excess hydroxylamine was removed by a Centricon 30 apparatus. At the same time, the Sulfo-SMCC was reacted with mAb 22 in a final molar ratio of 20:1 at room temperature for 2 h. The maleimide-activated mAb22 was separated from unreacted Sulfo-SMCC by centrifugation through a Centricon 30 apparatus. The final conjugation was carried out by mixing equal molar amounts of both mAbs at room temperature overnight. The concentration of BsAbs was determined using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Richmond, CA). The purity of the BsAbs was verified by SDS-PAGE.

**Cell Lines.** HL-60 and KG-1a cell lines were obtained from American Type Culture Collection (Rockville, MD). The NB4 cell line was kindly provided by Dr. M. Lanotte (Paris, France). Cell lines were maintained in RPMI 1640 medium containing 10% FCS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (50 µg/ml) (GIBCO-BRL, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. All cell lines were checked periodically for *Mycoplasma* contamination using the DNA hybridization method (Gen-Probe, San Diego, CA). HL-60 and NB4 cells are strongly positive for CD33, whereas KG-1a cells are negative for CD33.

**Preparation of Effector Cells.** Monocytes were freshly separated from the peripheral blood of normal donors after they gave informed consent. After Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation, the mononuclear cells were collected and washed twice. The cells were incubated with mAb OKT3 (American Type Culture Collection) and mAb 6G7 (anti-CD45RA)<sup>4</sup> for 1 h at 4°C and washed twice. Dynabeads M-450 coated with sheep antimouse IgG (DYNAL, Great Neck, NY) were added at a final ratio of 20:1 and incubated at 4°C for 1 h. The lymphocytes were depleted using a handheld magnet. The procedure was repeated twice. The purity of monocytes was analyzed by flow cytometry, and the morphology of the cells was verified by microscopic examination of Wright-Giemsa-stained cytospin preparations. The separated monocytes contained more than 95% CDT4-positive cells, less than 1% CD3-positive cells, and less than 1% CD56-positive cells. Then, the

monocytes were incubated with 200 units/ml IFN-γ (Genentech, San Francisco, CA), 10 ng/ml G-CSF (Immunex, Seattle, WA), or 10 ng/ml GM-CSF (Immunex) for 24 h before the cytotoxicity assay. Monocytes incubated in medium without cytokines were included in each assay as the control.

**Flow Cytometric Analysis.** FITC- or phycoerythrin-conjugated anti-CD3, anti-CD13, and anti-CD56 were purchased from Becton Dickinson Immunocytometry System (San Jose, CA). FITC-conjugated mAb 22 (anti-FcγRI) was provided by Medarex, Inc. (Annandale, NJ). For evaluation of FcγRI expression, monocytes before and after cytokine incubation were directly stained with this panel of mAbs. For evaluation of immunconjugate binding, monocytes, as well as cells from HL-60, KG-1a, and NB4 cell lines, were incubated with 1 µg/ml BsAb 251 × 22 for 1 h. The cells were washed twice and incubated with FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG (H + L) (Caltag, South San Francisco, CA) for 30 min. All of the samples were analyzed by FACScan (Becton, Dickinson). FITC- or phycoerythrin-conjugated mouse isotypic immunoglobulins were used as controls.

**Cytotoxicity Assay.** The assay was performed in 96-well round-bottomed microtiter plates (Rainin Instrument Co., Woburn, MA). The target cells were washed once with RPMI 1640 medium and incubated with 100 µCi sodium [<sup>51</sup>Cr] chromate (New England Nuclear, Boston, MA) for 1 h at 37°C. After being washed several times, the cells were resuspended in RPMI 1640 medium containing 10% FCS to a concentration of 1 × 10<sup>5</sup>/ml. Monocytes serving as effector cells were suspended in RPMI 1640 medium to a final concentration of 1 × 10<sup>7</sup>/ml. Effector cells (100 µl) were added to the first row of cells, and a serial dilution was performed with equal volumes of RPMI 1640 medium. Then, 100 µl target cells were added in the wells to yield final E:T ratios of 50:1, 25:1, and 12:1. In a standard assay, 0.2 µg immunconjugate was finally added to yield a final immunconjugate concentration of 1 µg/ml. SCCL-1, a mouse IgG2a mAb directed against transferrin receptor and capable of binding to the FcγRI through its Fc domain (17), was included in each assay as a positive control to measure the activity of the monocytes. Several other controls with E:T ratios of 50:1 also were incorporated in each assay, including incubation of target cells with the immunconjugate alone, incubation of target and effector cells with no antibody, incubation of target and effector cells with the unconjugated parental antibodies, and incubation of effector cells with CD33-negative leukemia cells in the presence of BsAb 251 × 22. In each assay, a 20-fold excess of unconjugated mAb 22 or mAb 251 along with the immunconjugate were incubated together with the target and effector cells to determine whether tumor cell lysis could be blocked by either of the parental antibodies. Cytotoxicity assays also were performed to compare the cytotoxicity mediated by the BsAb 251 × 22, mAb 251, and F(ab')<sub>2</sub> fragment of mAb 251 at various E:T ratios. To determine whether the presence of human immunoglobulin could interfere with the redirected tumor cell killing, some cytotoxicity assays were performed in 100% human AB serum. After incubation at 37°C for 4 or 18 h, the microplates were centrifuged, and the supernatant was collected for the determination of <sup>51</sup>Cr release. Maximum lysis was achieved by the addition of 100 µl 5% NP40 to 100 µl target cells. The percentage of cell lysis was calculated as: (experi-

<sup>4</sup> J. H. Zhou, unpublished observations.



Table 1 The binding of mAb 22, mAb 251, and BsAb 251 × 22 to target and effector cells<sup>a</sup>

	mAb 251		mAb 22		BsAb 251 × 22	
	% Positive	MFI	% Positive	MFI	% Positive	MFI
HL-60	99.5	126.4	64.6	16.6	99.6	151.0
NB4	89.2	54.6	54.2	12.9	89.1	56.7
KG-1a	Negative	Negative	Negative	Negative	Negative	Negative
Monocyte	81.6	27.0	79.0	43.9	94.0	101.3

<sup>a</sup> Flow cytometric analysis by indirect staining of cells with mAb 251 (0.5 µg/ml), mAb 3G8 (0.5 µg/ml), or BsAb 251 × 22 (1 µg/ml), then with FITC-conjugated goat F(ab')<sub>2</sub> antimouse IgG (H + L). Isotype-matched murine mAb controls were included in all analyses.

Table 2 Effect of cytokine stimulation on the expression of FcγRI on monocytes<sup>a</sup>

Cytokine	Positivity (%)	MFI	No. of experiments
No cytokine	84 ± 3	54 ± 3	3
IFN-γ	87 ± 9	150 ± 33 <sup>b</sup>	3
G-CSF	88 ± 6	72 ± 18	3
GM-CSF	85 ± 11	70 ± 28	3

<sup>a</sup> Flow cytometric analysis of the expression of FcγRI by direct staining of monocytes with FITC-conjugated mAb 22. Monocytes were separated from normal peripheral blood and incubated with indicated cytokine for 24 h before the analysis.

<sup>b</sup> Significant increase ( $P < 0.05$ ) compared to that with no cytokine stimulation.

mental cpm – spontaneous release mean cpm) × 100 / (maximum release mean cpm – spontaneous mean cpm). Spontaneous release of <sup>51</sup>Cr from the target cells was less than 5% of maximum release in a 4-h assay and less than 15% in an 18-h assay. For dose-response assays, BsAb 251 × 22 was serially diluted and added; the E:T ratio in those assays was 50:1.

**Statistical Analysis.** Each experimental result in the cytotoxicity assays was obtained in triplicate and reported as the mean ± SD. The significance level was determined using the paired Student's *t* test when applicable.

## RESULTS

### Binding of BsAb 251 × 22 to Target and Effector Cells.

The binding of the two parental mAbs (mAb 22 and 251) and BsAb 251 × 22 to target and effector cells was studied by flow cytometric analysis. HL-60 and NB4 cells were strongly positive for CD33 and weakly positive for FcγRI. KG-1a cells were negative for both CD33 and FcγRI. The BsAb 251 × 22 bound to HL-60, NB4, and monocytes with similar percentages and mean fluorescence intensities, suggesting that the process of chemical conjugation did not alter the binding characteristics. The results of flow cytometric analysis are presented in Table 1. The BsAb 251 × 22 could be detected on the surface of 90% of HL-60 cells after 4-h incubation, suggesting that the BsAb 251 × 22 is not readily internalized after binding to CD33 molecules on HL-60 cell surface (18). The absence of antigenic modulation may be important for BsAb 251 × 22 to direct immune effector cells to target cells and to induce subsequent cytotoxicity of target cells. The fact that BsAb 251 × 22 does not bind to KG-1a cells allows us to use these cells as controls in the cytotoxicity assay to measure nonspecific tumor cell lysis, as well as to validate the specificity of the BsAb-mediated cytotoxicity.

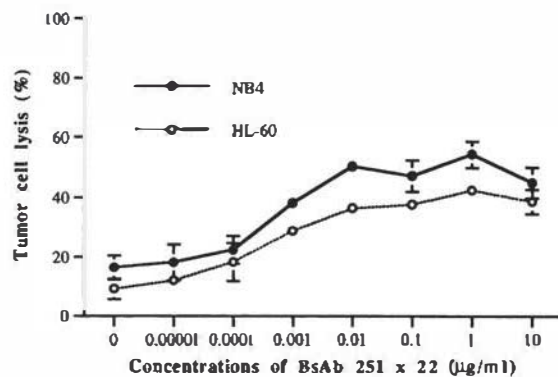


Fig. 1 Cytotoxicity of target cells by IFN-γ-activated monocytes in the presence of various concentrations of BsAb 251 × 22. A significant increase of target cell lysis was observed with 0.001–10 µg/ml BsAb 251 × 22. Bars, SE.

**Activation of Effector Cells by Cytokines.** It has been well documented that the incubation of monocytes with IFN-γ increases the expression of FcγRI on their surface and stimulates their cytotoxic activity. Several previous studies indicate that G-CSF and GM-CSF also can stimulate the expression of FcγRI on circulating monocytes and neutrophils after *in vivo* administration. Both of the cytokines have been shown to enhance the cytotoxicity of monocytes and neutrophils after *in vitro* incubation, although the FcγRI expression does not increase significantly. We studied the effect of G-CSF and GM-CSF incubation on the expression of FcγRI and cytotoxic activity of monocytes. Incubation of monocytes with 200 units/ml of IFN-γ for 24 h resulted in a significant increase of FcγRI expression, whereas G-CSF and GM-CSF incubation did not increase FcγRI significantly (Table 2). With prolonged incubation for 48 h, IFN-γ and G-CSF both increased FcγRI expression further (MFI = 218 ± 82 and 110 ± 33, respectively), whereas GM-CSF did not (MFI = 64 ± 16).

**Cytotoxicity of Target Cells in the Presence of BsAb 251 × 22.** The concentration dependence of BsAb 251 × 22 required for cytotoxicity was measured. The results of one typical assay are presented in Fig. 1. A significant increase in tumor cell lysis was observed from 0.001 µg/ml to 10 µg/ml BsAb 251 × 22. The presence of cytotoxicity at very low concentrations of BsAb 251 × 22 may be important for effective *in vivo* immunotherapy, because the concentration of BsAb may be low in tissues.

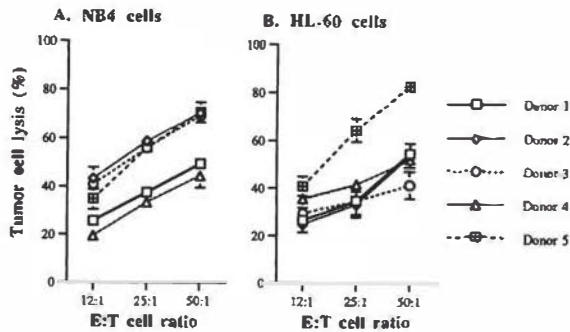


Fig. 2 Cytotoxicity of target cells by IFN- $\gamma$ -activated monocytes from five donors. The cytotoxicity is dependent on the E:T cell ratio in individual donors and varies greatly among different donors. Bars, SE.

The specific lysis of HL-60 and NB4 cells by IFN- $\gamma$ -activated monocytes is summarized in Fig. 2. The cytotoxicity was dependent on E:T ratios and incubation time. At an E:T ratio of 50:1, more than 70% of target cells were lysed in the presence of BsAb 251 × 22. Variations of cytotoxicity among individual donors and differences in susceptibility of target cells to lysis were observed. As shown in Fig. 2, monocytes from donor 5 had the highest cytotoxicity toward both HL-60 and NB4 cells, whereas monocytes from donor 3 had a much higher cytotoxicity toward NB4 cells (40–75%) than did HL-60 cells (25–45%). The expression of Fc $\gamma$ RI on monocytes after IFN- $\gamma$  incubation was comparable in all cytotoxicity assays, and the BsAb 251 × 22 used in these assays was derived from one conjugation. The effect of incubation time on the cytotoxicity assay was important. A comparison of cytotoxicity from a 4-h and an 18-h assay is presented in Fig. 3. In these assays, the background leak of  $^{51}\text{Cr}$  was less than 5% of the maximal release in the 4-h assay and less than 15% of the maximal release in the 18-h assay. The percentage of tumor cell lysis was significantly increased after an 18-h incubation. This observation, combined with the fact that a higher E:T ratio is required for cytotoxicity, suggests that the production of cytotoxic cytokines may be involved in target cell lysis.

**The Effect of Cytokine Activation on Cytotoxicity of Target Cells.** A series of assays was performed to compare the effects of cytokine incubation on the enhancement of cytotoxicity of target cells. In general, the cytotoxicity of HL-60 and NB4 cells by monocytes incubated with G-CSF and GM-CSF was comparable to that mediated by monocytes incubated with IFN- $\gamma$ . All three cytokines significantly increased the cytotoxic potency of monocytes. The results are summarized in Table 3.

#### Specificity of Cytotoxicity Mediated by BsAb 251 × 22.

To determine whether target cell lysis was induced specifically by the presence of BsAb 251 × 22, CD33-negative KG-1a cells were used as target cells in some cytotoxicity assays. Lysis of KG-1a cells in the presence of BsAb 251 × 22 was very low, ranging from 3.7%  $\pm$  1.2% (without IFN- $\gamma$  incubation) to 7.7%  $\pm$  1.0% (with IFN- $\gamma$  incubation). The specificity of target cell lysis can be shown further by blocking the cytotoxicity with excess concentrations of either parental antibody. As illustrated

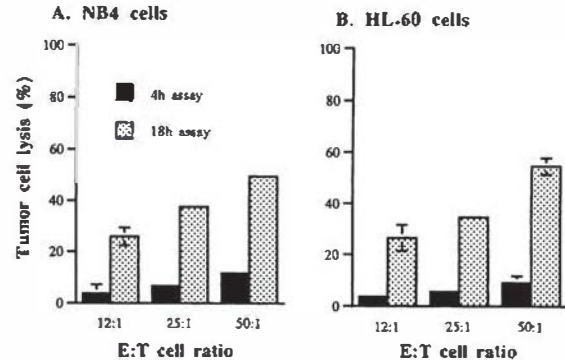


Fig. 3 Effect of incubation time on target cell lysis by IFN- $\gamma$ -activated monocytes from donor 2. A significant increase of target cell lysis was observed in 18-h assays. Bars, SE.

in Table 4, the addition of a 20-fold excess of either parental antibody significantly inhibited the increment of cytotoxicity mediated by BsAb 251 × 22. As expected, the presence of human AB serum did not interfere with the cytotoxicity mediated by BsAb 251 × 22 because the binding of mAb 22 to Fc $\gamma$ RI occurs outside the Fc binding site. The cytotoxicity mediated by mAb SCCL-1 (an IgG2a mAb) was blocked significantly by human AB serum, presumably because the presence of serum immunoglobulin could compete with the Fc portion of the IgG2a SCCL-1 mAb for binding to Fc $\gamma$ RI (data not shown). An additional control compared the effects of the parental mAb 251 and its F(ab') $_2$  fragments to BsAb 251 × 22 on the cytotoxicity of leukemia target cells (Fig. 4). The cytotoxicity mediated by mAb 251 and its F(ab') $_2$  fragments was comparable with that mediated by monocytes alone, whereas BsAb 251 × 22 significantly enhanced the cytotoxicity of leukemia targets.

## DISCUSSION

Although the treatment of AML has been improved significantly by various new chemotherapeutic agents and regimens, relapse as a result of the persistence of minimal residual disease after chemotherapy remains a major problem for these patients. Efforts such as allogeneic bone marrow transplantation have been made to further improve the outcomes of the current therapy. In addition, autologous bone marrow transplantation has been used as consolidation therapy for patients with AML. Gene-marking experiments of bone marrow transplants in AML have suggested that residual leukemic cells in the transplant can contribute to the relapse of leukemia (18). Various purging techniques have been developed, including treatment of bone marrow with cytotoxic drugs, mAb plus complement, and immunomagnetic bead separation (19). All of these strategies have met with some measure of success. However, considerable numbers of patients still relapse because of either failure of the primary therapy to eliminate all disease *in vivo* or failure to purge all malignant cells from the autograft.

In this study, we examined an alternative strategy to eliminate residual leukemia cells by directing host immune effector



Table 3 Effect of cytokine incubation on monocyte-mediated cytotoxicity in the presence of BsAb 251 × 22<sup>a</sup>

E:T	Donor	Cytokine				
		IFN- $\gamma$	G-CSF	GM-CSF	No cytokine	
NB4 cells	12:1	3	40.9 $\pm$ 2.4	43.6 $\pm$ 1.1	41.5 $\pm$ 2.0	5.9 $\pm$ 0.5
		4	19.6 $\pm$ 0.4	24.2 $\pm$ 1.1	15.5 $\pm$ 0.5	3.3 $\pm$ 0.9
		5	34.9 $\pm$ 4.3	32.2 $\pm$ 5.5	ND <sup>b</sup>	7.1 $\pm$ 1.8
	25:1	3	65.8 $\pm$ 3.4	55.0 $\pm$ 3.4	56.5 $\pm$ 1.7	6.1 $\pm$ 1.5
		4	33.3 $\pm$ 0.6	32.7 $\pm$ 1.7	27.3 $\pm$ 0.7	5.5 $\pm$ 0.4
		5	56.0 $\pm$ 2.8	46.4 $\pm$ 2.1	ND <sup>b</sup>	10.2 $\pm$ 1.1
	50:1	3	69.0 $\pm$ 1.3	63.5 $\pm$ 0.7	61.7 $\pm$ 2.6	6.5 $\pm$ 1.2
		4	44.5 $\pm$ 4.8	45.8 $\pm$ 3.5	40.4 $\pm$ 4.4	5.3 $\pm$ 1.1
		5	70.5 $\pm$ 4.1	57.8 $\pm$ 2.5	ND <sup>b</sup>	13.9 $\pm$ 1.3
HL-60 cells	12:1	3	29.4 $\pm$ 1.8	26.2 $\pm$ 0.6	26.5 $\pm$ 0.9	4.4 $\pm$ 0.5
		4	35.4 $\pm$ 1.3	29.1 $\pm$ 1.1	28.2 $\pm$ 1.0	3.6 $\pm$ 0.9
		5	60.7 $\pm$ 4.1	53.9 $\pm$ 4.4	48.6 $\pm$ 4.9	0.6 $\pm$ 0.1
	25:1	3	41.4 $\pm$ 1.6	35.6 $\pm$ 2.0	37.3 $\pm$ 0.7	5.8 $\pm$ 0.7
		4	41.5 $\pm$ 1.7	34.0 $\pm$ 1.2	33.0 $\pm$ 2.1	5.2 $\pm$ 0.5
		5	74.5 $\pm$ 5.1	61.5 $\pm$ 4.4	59.4 $\pm$ 2.5	2.1 $\pm$ 0.2
	50:1	3	51.3 $\pm$ 0.5	46.0 $\pm$ 0.7	49.6 $\pm$ 1.4	6.3 $\pm$ 0.4
		4	52.0 $\pm$ 1.8	42.9 $\pm$ 1.0	36.7 $\pm$ 2.1	5.0 $\pm$ 0.8
		5	82.7 $\pm$ 3.5	78.0 $\pm$ 6.7	75.9 $\pm$ 1.3	10.2 $\pm$ 0.2

<sup>a</sup> Monocytes were separated from three different donors. Percentage of target cell lysis is expressed as mean  $\pm$  SD of triplicates.

<sup>b</sup> ND, not done.

Table 4 Inhibition of BsAb 251 × 22-mediated cytotoxicity by its parental antibody<sup>a</sup>

Experiment	Inhibition (%)	
	mAb 251	mAb 22
1	90	95
2	86	96

<sup>a</sup> The concentration of BsAb 251 × 22 was 1  $\mu$ g/ml, and the concentration of each parental mAb was 10  $\mu$ g/ml. The E:T ratio in these experiments was 50:1. The cytotoxicity mediated by BsAb 251 × 22 was defined as 100%, and the cytotoxicity observed by mixing target cells with IFN- $\gamma$ -activated monocytes without any antibody was defined as 0%. The percentage of inhibition was calculated accordingly.

cells toward leukemia cells via a BsAb. Unconjugated mouse mAbs alone have been studied for their antitumor effects in patients with AML and other malignancies by ADCC and other effector mechanisms (20, 21). Unconjugated mAbs suffer from several disadvantages: one is that the binding of a mAb to Fc receptors on effector cells may be competed by circulating immunoglobulin. This nonspecific binding of circulating immunoglobulin to Fc $\gamma$ RI on immune effector cells can block the ADCC triggered by unconjugated antibody. This problem can be overcome by BsAb 251 × 22 because mAb 22 binds with high affinity to Fc $\gamma$ RI outside the binding site of Fc domain of immunoglobulins (22). BsAb 251 × 22 can bind and activate Fc $\gamma$ RI without the interference of the high concentration of circulating immunoglobulin. The other major disadvantage of mouse mAbs is that they may induce the formation of human antimouse antibody, thereby abolishing the therapeutic effect of the mAb. The development of recombinant humanized versions of mouse mAbs can overcome this problem. A humanized version of anti-CD33 mAb has been reported (14), and mAb 22

has been humanized as well.<sup>5</sup> Therefore, the construction of a humanized BsAb for immunotherapy targeting CD33 and CD64 is feasible.

Activation of immune effector cells by cytokines was crucial for the cytotoxicity of target cells. Monocytes without preincubation with cytokines had much less cytotoxicity than did those with preincubation. Although IFN- $\gamma$  incubation yielded the highest expression of Fc $\gamma$ RI, its application *in vivo* for this purpose has not been studied extensively. In this study, we compared the effect of IFN- $\gamma$ , G-CSF, and GM-CSF incubation on the expression of Fc $\gamma$ RI and cytotoxic activity of monocytes. After 24-h incubation, all three cytokines significantly enhanced the cytotoxicity of monocytes in the presence of BsAb 251 × 22. The cytotoxicity of target cells by monocytes incubated with G-CSF and GM-CSF is comparable that of these cells incubated with IFN- $\gamma$ . The expression of Fc $\gamma$ RI was significantly increased on monocytes incubated with IFN- $\gamma$  for 24 h, but not on monocytes incubated with G-CSF or GM-CSF for 24 h. However, the expression of Fc $\gamma$ RI was increased significantly on monocytes incubated with G-CSF for 48 h. It has been previously shown that incubation of monocytes with G-CSF or GM-CSF *in vitro* could increase their cytotoxic activity (5, 7, 9), and recent data showed that the expression of Fc $\gamma$ RI could be increased after infusion of G-CSF *in vivo* (9). It has also been reported that IFN- $\gamma$ -activated monocytes can be used for *in vitro* purging of clonogenic leukemic cells (23). These observations are important for clinical application of our immunotherapeutic approach, because both G-CSF and GM-CSF have been widely used after chemotherapy and/or bone

<sup>5</sup> R. Graziano, personal communication.

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