JOURNAL OF HEMATOTHERAPY 1:85–94 (1992)
Mary Ann Liebert, Inc., Publishers

Initial Trial of Bispecific Antibody-Mediated Immunotherapy of CD15-Bearing Tumors: Cytotoxicity of Human Tumor Cells Using a Bispecific Antibody Comprised of Anti-CD15 (MoAb PM81) and Anti-CD64/FcγRI (MoAb 32)

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ABSTRACT

The high-affinity receptor for IgG, FcγRI, expressed on monocytes and interferon-γ (IFN-γ)-stimulated neutrophils, is a trigger molecule for cell-mediated cytotoxicity. We have prepared murine monoclonal antibodies (MoAb 22 and MoAb 32) that bind to FcγRI outside the ligand binding site and thus bind to and trigger cytotoxicity that is not competed by other immunoglobulins. Because of these properties, it seemed that these MoAbs would be very useful for the development of bispecific antibodies (BsAb) for targeting normal cellular immune defense mechanisms as a new form of immunotherapy for treatment of cancer. BsAbs incorporate into a single molecule the binding specificities of two different antibodies, and, thus, can be used to target myeloid cells to tumors, ensure activation of cellular cytotoxic mechanisms, and target cell lysis and/or phagocytosis. BsAbs were prepared using anti-FcγRI MoAb and an anti-myeloid cell MoAb, PM81, reactive with the CD15 antigen, for studies of antibody-dependent cellular cytotoxicity. Conjugates were made by cross-linking sulfhydryl groups of Fab fragments of MoAb 32 or 22 (both IgG_1) and sulfhydryl groups added to intact PM81 (an IgM) using *N*-succinimdyl-acetyl-*S*-thioacetate (SATA). The resulting product was purified by high-performance size-exclusion chromatography. The ability of the BsAbs to mediate attachment of human monocytes to tumor target cells was confirmed in a microtiter well assay of binding of MTT-labeled U937 cells (a human FcγRI-bearing cell line) to SKBR-3 (PM81-reactive breast carcinoma) target cells. The ability of the BsAbs to mediate killing of HL-60 promyelocytic leukemia cells was studied using a 6-hour Chromium-51 release assay. Effector cells were monocytes obtained by cytopheresis and cultured for 18 hours with IFN-γ. Monocytes alone caused minimal killing (5–20%), monocytes plus BsAb caused moderate killing (20–50%), and monocytes plus BsAb plus human serum resulted in maximal killing (50–80%). Experiments were performed to test the ability of the BsAb to purge bone marrow

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of small numbers of leukemia cells using bone marrow mononuclear phagocytes treated for 18 hours with IFN-γ prior to adding target cells. Without the addition of human serum as a source of complement, a 90% depletion of clonogenic HL-60 cells could be demonstrated. With human complement, up to 95% depletion was seen. Thus, this BsAb possessed the ability to lyse tumor cell targets by two different mechanisms, complement and cell-mediated lysis. In a Phase I clinical trial, 4 patients with $CD15^+$ tumors were treated with up to 48 mg of this BsAb with no toxicity. Of this group, the patient with acute myelogenous leukemia experienced a transient 30–60% reduction in circulating leukemic blast cells during each of six infusions over a 2-week period. Although *in vitro* assays indicated maximal effectiveness between 1 and 10 μg/ml of this BsAb, *in vivo* reduction in circulating cell counts was observed when peak serum concentrations were as low as 50 ng/ml. As such, this BsAb may be useful for *in vivo* therapy of high-risk tumors, especially after induction of remission or after bone marrow transplant, *i.e.*, for treatment of minimal residual disease in patients with CD15-expressing tumors, including acute myeloid leukemia, small cell carcinoma of the lung, and colon and breast carcinoma.

INTRODUCTION

ALTHOUGH MURINE MONOCLONAL ANTIBODIES (MoAb) directed to tumor-associated antigens have considerable therapeutic potential, there are also several limitations to their use *in vivo* (Dillman, 1989). These include lack of host cellular or humoral effector mechanisms, immune response to the foreign protein, and delivery of adequate amounts of the MoAb to the tumor. For a MoAb to be an effective anticancer agent, it is necessary that it work in concert with an effector mechanism such as complement (Stepan *et al,* 1984) or a cellular effector, as in antibody-dependent cellular cytotoxicity (ADCC) (Ortaldo *et al,* 1987). Many potentially useful MoAbs are not able to use these effector mechanisms because they are not of the appropriate subclass of immunoglobulin. It has become clear that murine Ig of the IgG_{2a} and IgG_3 subclasses are the most effective at mediating ADCC by virtue of their ability to bind to the high-affinity Fc receptor, FcγRI (Lubeck *et al,* 1985). Murine IgG_1 and IgG_{2b} MoAb mediate ADCC by human effector cells far less efficiently, if at all (Van de Winkel *et al,* 1991). In addition, human complement is not as capable of mediating cytotoxicity with murine MoAb as guinea pig or rabbit complement. Thus, only MoAbs of the IgG_{2a} and IgG_3 subclasses have shown activity during *in vivo* therapy, presumably through ADCC mediated by FcγRI-bearing cells (Steplewski *et al,* 1988).

In this paper, we describe an approach to overcoming some of the limitations of certain MoAbs for *in vivo* therapy. We have prepared murine MoAbs (MoAb 22 and MoAb 32) that bind to FcγRI outside the ligand binding site, and thus their ability to bind to and trigger cytotoxicity through this receptor is not competed by other immunoglobulins. Bispecific antibodies (BsAbs) were previously shown to mediate ADCC of red blood cells that was not inhibited by nonimmune human IgG (Shen *et al,* 1986a). We now report use of BsAbs prepared by chemical linkage using Fab fragments of anti-FcγRI MoAb 32 or 22 and a whole IgM anti-myeloid cell MoAb, PM81, reactive with the CD15 antigen, for studies of ADCC *in vitro* and therapy. This BsAb was able to mediate ADCC of HL-60 leukemia cells. Moreover, when testing for the ability of human plasma to block this effect, we found an enhancement of killing mediated at least partly by human complement. Thus, this BsAb lysed tumor cells by two different mechanisms, complement and cellular-mediated lysis. In a Phase I clinical trial, 4 patients with $CD15^+$ tumors were treated with up to 48 mg of this BsAb with no toxicity, and in one of these patients who had acute myelogenous leukemia and could be evaluated, there was a transient 30–60% reduction in circulating leukemic blast cells during each of six infusions over a 2-week period. Thus, this BsAb may be useful for treatment of minimal residual disease in patients with CD15-expressing high-risk tumors.

MATERIALS AND METHODS

Cells

HL-60 and U937 cells (American Type Culture Collection (ATCC), Rockville, MD) were cultured in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) as previously described (Ball *et al,* 1983). SKBR-3 cells (ATCC) were cultured in DMEM + 10% FBS. Monocytes were purified as previously described (Shen *et al,* 1986b). Briefly, mononuclear cells were harvested from the peripheral blood of normal donors by leukapheresis. The monocytes were further purified by separation over Histopaque (Sigma Chemical Co., St. Louis, MO), and incubation with rotation at 4°C (Shen *et al,* 1986b). The resulting aggregates were allowed to sediment and then sedimented again through FBS. The resulting preparations were >95% monocytes as assessed by morphological examination of Wright's-Giemsa-stained cytospins and expression of CD14.

The hybridomas PM81 (Bell *et al,* 1983), 32 (Anderson *et al,* 1986), and 22 (Guyre *et al,* 1989) were tested for mycoplasma, sterility, and murine viruses by the MAP test and found to be free of contamination. The hybridomas were cultured in MF-1 medium (modified Excell-300, J.R. Scientific) containing 0.5% FBS.

Production of MoAbs

Antibodies were produced using hollow-fiber technology, with a serum-free, low-protein media feed stream. Each MoAb was purified from harvests of antibody-rich supernatant from a hollow-fiber cartridge using high-performance liquid chromatography (HPLC). The MoAb was purified using a semipreparative anion exchange (DEAE) column with a combined pH and salt gradient. The gradient was optimized for each MoAb to achieve a product of greater than 90% purity, with extremely low endotoxin (0.48–0.96 EU/ml or 0.42–0.84 EU/mg antibody) and DNA contamination levels (<5 pg/mg).

Flow cytometry

Briefly, cells (10^6) were incubated for 1 hour with varying concentrations of MoAb or BsAb (in the presence of normal human IgG (10^{-5} moles/liter), Sigma), washed with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.05% sodium azide (PBA), and further incubated with fluorescein isothiocyanate (FITC)-coupled, affinity-purified $F(ab')_2$ goat anti-mouse (GAM) Ig (Caltag, Inc., S. San Francisco, CA) for 1 hour at 4°C. Cells were then washed once in PBA and resuspended in PBA containing 1% paraformaldehyde (Sigma). The cells were analyzed on the Ortho Systems 50H flow cytometer (Ortho Diagnostics, Westwood, MA) interfaced to a 2150 computer using a logarithmic amplification scale.

Bispecific antibody preparation and purification

BsAbs composed of PM81 conjugated to 32 Fab (or 22 Fab) were prepared by cross-linking the sulfhydryl of the 32 Fab fragment to sulfhydryl groups added to the PM81 MoAb. Sulfhydryl groups were added to PM81 using *N*-succinimidyl-acetyl-*S*-thioacetate (SATA). 32 was digested with pepsin to produce the $F(ab')_2$ fragment, which was then purified by high-performance size-exclusion chromatography. Reduction with mercaptoethylamine and reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) converted the $F(ab')_2$ fragment to a Fab fragment with endogenous sulfhydryl groups activated with thionitrobenzoic acid (Fab-TNB). The Fab-TNB was purified by gel filtration and assayed to make sure that there were enough TNB groups attached to the Fab to make a good coupling partner. An excess of 32 Fab-TNB was added to the PM81 SATA to drive the reaction to complete conversion to bispecific antibody. The bispecific antibody was then purified using high-performance size-exclusion chromatography. Endotoxin levels, as determined by the *Limulus* amebocyte lysate assay, were ≤1.74 EU/mg.

Cytotoxicity assays

HL-60 cells expressing CD15 on their surface were labeled for 1 hour at 37°C with 200 mCi of ^{51}Cr sodium chromate in normal saline (New England Nuclear, Boston, MA) and used as target cells. Effector cells were normal monocytes isolated from a leukopack as described and cultured overnight with 50 units/ml of IFN-γ. To quantify cytotoxicity of HL-60 cells, equal volumes of test reagents (control antibodies and conjugates), ^{51}Cr-labeled HL-60 cells, and effector cells were mixed in round-bottomed microtiter wells. Plates were incubated for 6 hours at 37°C, after which half of the supernatant was removed and counted for release of ^{51}Cr. Maximal lysis was obtained by addition of 2% sodium dodecyl sulfate in water. Percent cytotoxicity was calculated as 100× (counts released with effectors − spontaneous lysis) ÷ (maximal lysis − spontaneous lysis). In all experiments, tests were conducted in triplicate and the results are expressed as the mean ± SD.

MTT cell binding assay

An assay to determine bispecificity was developed that relies on the binding of a BsAb to CD15 on a target cell (SKBR-3 cells, American Type Culture Collection, Rockville, MD) and subsequently to FcγRI on model effector cells (U-937 cells, American Type Culture Collection, Rockville, MD) labeled with MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) (Green *et al,* 1984). This binding induces stable association between effector and target cells. SKBR-3 cells were plated at 4×10^4 cells per well into 96-well flat-bottomed plates (Costar) in DMEM + 50 μg/ml gentamicin + 10% FBS, and cultured for 24–48 hours. Culture media was then removed by rapid inversion, and antibodies (PM81 × 32, PM81, 32, PM81 + 32 or P3, a nonspecific murine IgG_1 MoAb) were added in a volume of 50 μl at the concentrations shown. The plate was rotated at 4°C for 30 minutes, and then each well was washed twice with 200 μl of PBS-BSA at 4°C. After washing, 4×10^5 MTT-labeled U-937 cells were added and the plate rotated for an additional 30 minutes at 4°C. MTT labeling of U-937 cells was accomplished by adjusting cells to 2×10^6 cells/ml in RPMI-1640 + 10% FBS and incubating at 37°C, 5% CO_2 for 1 hour with 0.5 mg/ml MTT. After incubation of MTT-labeled U-937 cells with the SKBR-3 monolayer for 30 minutes, each well was washed five times very gently with 200 μl of PBS-BSA. The plate was forcefully inverted between washes, without centrifugation, to remove nonadherent cells. After the last wash, 100 μl of 95% isopropanol + 0.04 *N* HCl was added to each well, the cells were thoroughly mixed by pipetting to dissolve the MTT reaction product, and the plate was read in an ELISA reader (Dynatech, MR650). Results are reported as O.D. units, and represent absorbance at 570 nm.

Colony-forming assay

Peripheral blood mononuclear cells from a normal donor were cultured for 18 hours in IFN-γ (100 units/ml) prior to addition of HL-60 cells (to 1% final concentration of HL-60 cells). This mixture of cells was then cultured for another 18 hours under a variety of conditions (with or without autologous plasma as a source of complement; with or without bispecific or monospecific MoAb, including the negative control MoAb, Thy-1) and then seeded into methylcellulose cultures with HL-60 conditioned media (as a source of growth factors for HL-60 cells) (Howell & Ball, 1985).

In vivo *administration of bispecific antibody*

With the approval of the DHMC Committee for the Protection of Human Subjects, and after informed consent, 4 patients with $CD15^+$ tumors were treated in a Phase I study of BsAb infusions. The first 2 patients were treated with 0.065 mg/kg per dose and the second 2 patients with 0.125 mg/kg dose. The antibody dose was diluted in 500 ml of normal saline. Each patient was treated six times (three times per week over 2 weeks) with 6-hour infusions of antibody at a single dose level per patient.

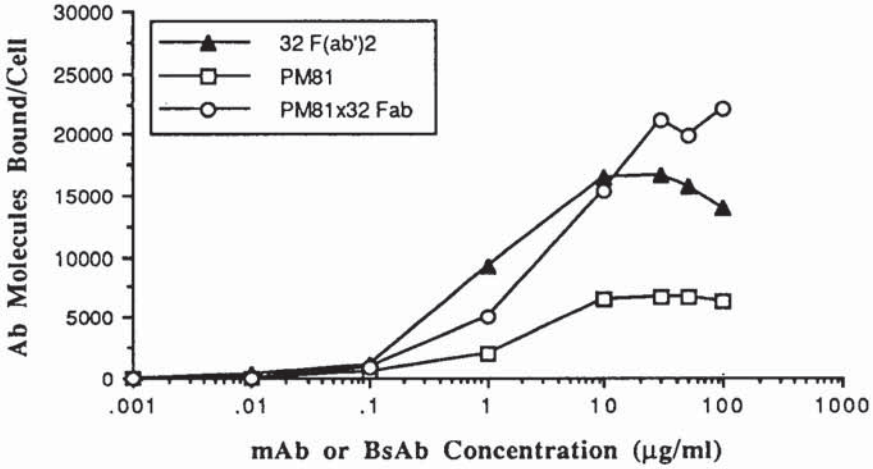

FIG. 1. Binding of BsAb to normal human monocytes. The binding of BsAb PM81 × 32 Fab, or the component MoAb, to freshly isolated human leukocytes was analyzed by flow cytometry (gated on light scatter to include only the monocytes) as described in Materials and Methods. Values are the mean for duplicate measurements of the number of second Ab molecules bound per cell.

RESULTS

Characteristics of BsAb

The ability of the BsAb to bind to antigen-positive target cells was assessed by flow cytometry. As shown in Fig. 1, the BsAb recognized approximately the same number of antigenic sites on normal monocytes as the constituent MoAb. However, the avidity of binding may have been slightly reduced by the chemical conjugation. The MTT cell-binding assay was used to demonstrate that the antibody was functioning as a true BsAb. This assay indicated that maximal attachment of the $CD64^+$ U937 cells to the $CD15^+$ SKBR-3 cells occurred at 5 μg/ml of BsAb and that both the PM81 × 32 and the PM81 × 22 BsAbs were similarly effective in mediating conjugate formation between these cells (Fig. 2).

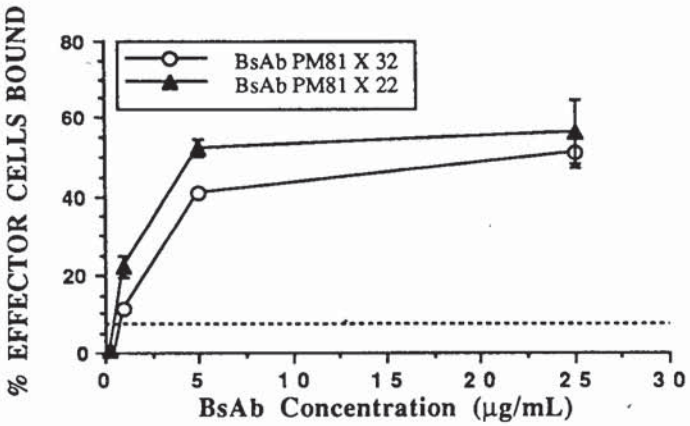

FIG. 2. Demonstration of the ability of the BsAb to mediate attachment of U937 cells ($Fc\gamma RI^+$) to SKBR-3 cells ($CD15^+$). MTT-labeled U937 cells were added to monolayers of SKBR-3 cells in the presence or absence of two different BsAb. Cell attachment was measured by spectrophotometry as described in Materials and Methods. Values are mean ± SD for triplicate measures of bound U937 cells. The broken line indicates the maximum binding in the absence of antibody or in the presence of the uncoupled component MoAb.

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