Bispecific-Armed, Interferon γ-Primed Macrophage-Mediated Phagocytosis of Malignant Non-Hodgkin’s Lymphoma

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To show that macrophages can be effectively targeted against malignant B cells, bispecific antibodies (BsAb) were constructed from two antibodies having specificity for the high-affinity Fc receptor for IgG (FcγRI/CD64) and the B-cell differentiation antigens CD19 and CD37. Using a flow cytometry-based assay and confocal imaging, we show that these constructs mediated significant phagocytosis of B lymphocytes by macrophages that could be enhanced with interferon γ (IFNγ) and IFNγ in combination with macrophage colony-stimulating factor. BsAb-dependent phagocytosis was triggered through FcγRI and could be blocked only by using Fab′, fragments from the parent molecule or by cross-linking FcγRI. BsAb-dependent phagocytosis was not blocked by antibodies to the other Fc receptors, FcγRII and FcγRIII. Because these antibody constructs bind to an epitope outside the FcγRI ligand binding site, we show that autologous serum, polyclonal IgG, and monomeric IgG did not block BsAb-dependent phagocytosis, whereas autologous serum and the IgG fractions blocked parent molecule monoclonal antibody-dependent phagocytosis due to the avid binding of monomeric IgG to FcγRII. Finally, BsAb-mediated phagocytosis was effective against the malignant B cells of patients with mantle cell lymphoma, prolymphocytic leukemia, and chronic lymphocytic leukemia. Based on these studies, we propose that BsAbs may provide an effective means of immunomodulation for patients with B-cell malignancies.

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DESPITE IMPROVEMENTS in complete remission rates due to the use of high-dose combination chemotherapy with or without bone marrow and stem cell support, the majority of patients with non-Hodgkin’s lymphoma (NHL) will die of their disease. Therefore, immunologic approaches to NHL have been studied intensively in recent years. Anti-idiotypic antibodies, unlabeled monoclonal antibodies (MoAbs), and antibodies conjugated to various toxins or radioisotopes have shown promise. However, transient responses, inefficiency of killing, or toxicity have slowed their application in the clinical setting. An approach that retains the specificity of MoAb therapy and that enhances target cell destruction without toxicity would have great therapeutic potential. This would particularly benefit those patients who could not tolerate additional organ toxicity due to age, immunodeficiency, or previous damage from standard chemotherapeutic regimens. Bispecific antibodies (BsAbs) may provide such a method to harness an effector population and redirect cytotoxicity to a malignant cell target while minimizing systemic toxicity.

BsAbs are constructed such that two antibodies are joined to form a single molecule having specificity for both the effector cell and the target cell. Trigger molecules on cytotoxic effector cells include the T-cell receptor, CD2, CD3, and the Fc receptors for IgG (FcγR). One focus of tumor-directed BsAb studies that has shown promise both in vitro and in vivo has involved targeting lymphocytes (T cells via anti-CD3 or anti-CD2 or natural killer [NK] cells via anti-CD16) as effector cells. Like the cytotoxic T lymphocyte or NK cell, the macrophage (MØ) may also serve as a cytotoxic effector cell that can be directed by BsAb. MØ constitutively express and can mediate killing through all three classes of FcγR (FcγRI, FcγRII, and FcγRIII) either by phagocytosis or external killing. In fact, several clinical trials are now in progress in which BsAbs are being used to direct monocyte, MØ, and/or activated neutrophil killing of human tumors in vivo.

The studies presented here focus on BsAb-mediated MØ phagocytosis through FcγRI. Because of the avid binding of monomeric IgG to FcγRII, antitumor cell-directed MoAb may be competitively inhibited in vivo from binding to this receptor. To circumvent this problem, MoAbs (such as MoAb32 and MoAb22) that bind to FcγRII outside its ligand binding site are being used in the construction of BsAbs targeting FcγRI. Bispecific antibodies (BsAbs) constructed with these MoAbs are able to effectively mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis of tumor cells even in the presence of human serum. In this report, we use BsAbs specific for FcγRI and the pan-B-cell markers CD37 and CD19 to show that activated MØ can be effectively targeted to mediate phagocytosis of patient-derived malignant lymphocytes even in the presence of nonspecific human IgG.

MATERIALS AND METHODS

Cell preparation. Mononuclear cells were obtained by leukopheresis of normal, healthy donors and enriched for monocytes by cold aggregation, as previously described. Cells from leukopheresis packs were separated on Ficoll-Hypaque (Histopaque 1077; Sigma Chemical Co, St Louis, MO), washed three times with RPMI 1640 (GIBCO-BRL, Grand Island, NY), and resuspended in complete medium (CM), consisting of RPMI supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 0.1 mmol/L nonessential amino acids, 0.1 mmol/L sodium pyruvate, 2 mmol/L fresh glutamine (GIBCO-BRL), and 50 μg/mL gentamicin (Whittaker Bioproducts, Walkersville, MD). Suspended cells were placed in 15-mL polypropylene tubes and rotated to induce monocyte aggregation at 8 rpm for 30 minutes at 4°C on a Labquake (Labindustries, Ammandale, NJ). Submitted August 31, 1995; accepted January 10, 1996.

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Berkeley, CA). The aggregated cells were sedimented at 1g on ice for 10 minutes, gently resuspended in 2 mL of RPMI, and layered onto an equal volume of ice-cold fetal calf serum (FCS). After sedimentation for 15 minutes on ice, the lower phase was collected, washed twice in Versene buffer (GIBCO), and washed twice in CM. Purity was confirmed by Wright stained cytocentrifuge preparations and ranged between 85% to 95% monocytes, with the remainder being lymphocytes.

Monocyte-derived MØ (MDMØ) were obtained by culturing monocytes as a nonadherent suspension in Teflon beakers (Savillex, Inc, Minnetonka, MN) for 8 to 10 days at a concentration of 4 × 10^6 cells/mL in RPMI 1640 (Sigma) supplemented with 50 mmol/L HEPES, 2 mmol/L fresh glutamine, and 2.5% autologous serum. Venous blood was collected separately as a source of autologous serum.

Malignant lymphocytes were obtained from the peripheral blood of consenting patients with circulating lymphoma cells or chronic lymphocytic leukemia (CLL). Malignant lymphocytes were purified on Ficoll-Hypaque and frozen in a solution consisting of 45% RPMI, 45% FCS, and 10% dimethyl sulfoxide (Fisher, Fair Lawn, NJ) and stored at −80°C until needed. P24 was from a patient with mantle cell lymphoma, P49 was from a patient with prolymphocytic leukaemia, and P61 through P64 were from patients with CLL. Raji cells, derived from a Burkitts lymphoma, were obtained from ATCC (Rockville, MD).

MoAbs. All MoAbs were of murine origin. The development and properties of MoAbs 22 (IgG1) and 197 (IgG2a), which both recognize the high-affinity FcγRII², were MoAb IV.3 (IgG2b), which recognizes FcγRIII,² have been previously reported. Hybridomas producing antibodies to the B-cell differentiation antigens used in this study included the anti-CD19 MoAb FMC-63 (IgG2a; kindly provided by Dr Heddy Zola, Child Health Research Institute, Flinders University, Adelaide, Australia),³ and the anti-CD37 MoAb WR17 (IgG2a; gift of Dr Martin Glennie, Tenovus Research Laboratory, Southampton General Hospital, Southampton, UK).³ MoAbs were purified from hybridoma supernatant by ion exchange high-performance liquid chromatography (HPLC) on a (protein-pak 5PW) diethyl aminoethyl (DEAE) column (Waters Chromatography Division, Millipore, Milford, MA).

Human IgG1 was obtained from one of the serum samples by ion-exchange chromatography using Affi-Gel Blue (BioRad, Richmond, CA). The source for polyclonal Ig was commercially available Gamimune N (Miles, Inc, Elkhart, IN).

BsAb production. The BsAbs, anti-CD19xMoAb22, anti-CD37xMoAb22, and 520C9xMoAb22, were constructed as described by Glennie et al.²¹ using the bifunctional cross-linking reagent, ortho-phenylenediamine (o-PDM; Sigma), to join Fab' fragments via stable thioether linkages (Fig 1). Individual antibodies were first digested with pepsin (Pierce, Rockford, IL) at pH 3.5 to Fab'(ab')₂ fragments according to the method of Parham.²² Fab'(ab')₂ fragments were subsequently reduced to Fab'-thiols by the addition of 10 mmol/L mercaptoethanolamine (Sigma) for 30 minutes at 30°C and purified on a Sephadex G-25 (Pharmacia, Piscataway, NJ) column equilibrated in 50 mmol/L sodium acetate, 0.5 mmol/L ethylenediaminetetraacetic acid, pH 5.3, at 4°C. Available SH groups on the MoAb22 Fab'-thiol were maleimidated by incubating it for 30 minutes with one-half volume of o-PDM (12 mmol/L) dissolved in dimethyl formamide (Pierce) and prechilled in a methanol ice bath. The MoAb22 Fab'-maleimide was separated from free o-PDM on Sephadex G-25 as above. BsAbs were chemically linked by collecting the MoAb 22 Fab'-maleimide as it came off the G-25 column into its partner’s Fab'-thiol. A 1:1 molar ratio was used to favor thiol and maleimide reaction while minimizing the reoxidation of thiol groups. The reactants were immediately concentrated under nitrogen to the starting volume using a Diaflo membrane in an stirred cell ultrafilter (Amicon, Beverly, MA) at 4°C. After 18 hours, the pH was adjusted to 8.0 with 1 mol/L Tris-HCl, pH 8.0. The final product was then reduced with 10 mmol/L mercaptoethanolamine and alkylated with 25 mmol/L iodoacetamide (Sigma). The final Fab' × Fab' conjugates were separated from unreacted Fabs and other products by HPLC on a TSK G3000SW sizing column (The Nest Group, Southboro, MA) equilibrated with phosphate buffer, pH 7.2.

BsAb purity was assessed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Product was further subjected to a bifunctional immunosassay to ascertain that both moieties were linked and functional. Briefly, BsAbs were incubated with Raji cells, which express both CD19 and CD37, and then washed, followed by the addition of a soluble fusion protein consisting of the extracellular domain of human FcγRI and human IgM heavy chain (sFcγRI-M) obtained from transiently transfected COS cells. Flourescein isothiocyanate (FITC)-labeled goat-anti-human IgM was added and binding was assayed by flow cytometry. Only BsAb bound to Raji cells and the sFcγRI-M would remain in the assay and be FITC-positive. Both BsAbs were positive, whereas the parental antibodies (MoAb22, anti-CD19, and anti-CD37) were negative in this assay.

Phagocytic assay. A modification of the two-color flow cytometric assay as described by Munn et al.²³ was used to assess BsAb-mediated phagocytosis of malignant lymphocytes. On day 1, MDMØ effectors were harvested from Teflon beakers by vigorous pipetting, washed free of human serum, and resuspended in CM. MDMØs were counted and transferred to sterile, polycarbonate (Starstedt, Pennsauken, NJ) at a density of 3 to 5 × 10^6 effectors per tube in a volume of 200 μL. MØ were incubated overnight in CM (control) or with 160 U of human recombinant interferon γ (rIFNγ; generously provided by Genentech, South San Francisco, CA) alone or in combination with 100 ng/mL macrophage colony-stimulating factor (M-CSF; a generous gift of Immunex Corp, Seattle, WA) as indicated.

Fig 1. Preparation of BsAbs using o-PDM. Fab'(ab')₂ fragments of both antibody partners were prepared and reduced to Fab'-thiol fragments. All of the available thiol groups on one of the antibodies (MoAb22) were then maleimidated with an excess of o-PDM. This results in the vicinal dithiols complexed with o-PDM (R) and one of the -SH groups cross-linked with o-PDM, making available a free thiol. The Fab'-maleimide was then reacted with the thiol group on the other Fab' (anti-CD19 or -CD37) at a 1:1 molar ratio under conditions favoring thiol and maleimide reaction while minimizing the reoxidation of thiol groups. The final products were reduced and alkylated (Q) before gel filtration to remove any homodimeric Fab'(ab')₂ fragments that may have formed by reoxidation of hinge region thiol groups.
At the same time, malignant lymphocytes were quickly thawed in a 37°C water bath and labeled with PKH-26 (Zynaxis, Inc, Malvern, PA), a red fluorescent lipophilic dye that stably inserts into the cell membrane, as previously described.\(^3\) Washed cells were resuspended in supplied diluent and immediately combined with an equal volume of PKH-26 in diluent. Final staining conditions were 1 x 10^7 cells/mL in 5 μmol/L PKH-26. After 5 minutes at room temperature, an equal volume of FCS was added to stop the staining reaction. Stained cells were washed twice with CM, transferred to T25 culture flask, and incubated in CM at 37°C overnight to eliminate any spontaneous leakage of dye during the phagocytosis assay.

On day 2, MDM0 were washed twice to remove cytokine and resuspended in fresh CM. BsAb and stained lymphocyte targets were sequentially added to each microtube and routinely incubated in a final volume of 200 μL at 37°C for 60 minutes. Unless otherwise indicated, the final BsAb concentration was 0.1 μg/mL and an effector to target ratio of approximately 10 to 1 was used. In some experiments, MDM0 were preincubated for 10 minutes with blocking antibodies at a final concentration of 200 μg/mL before adding BsAb and targets. At the completion of each experiment, microtubes were immediately placed on ice.

Phagocytosis was assayed by flow cytometry after labeling the M0 with FITC-conjugated anti-CD14 (Medarex, Inc, Annandale, NJ). Briefly, the contents of each microtube were transferred to 96-well polystyrene plates (Costar, Cambridge, MA) and washed in FCM buffer (phosphate-buffered saline [PBS], pH 7.2, with 1% bovine serum albumin, 0.1% sodium azide, and 40 μg/mL disodium ethylenediamine-tetraacetic acid; Sigma). Cells were labeled for 30 minutes on ice with anti-CD14-FITC (20 μg/mL) in the presence of human IgG (6 mg/mL; Sigma) to reduce nonspecific antibody binding. They were washed three times with FCM buffer, fixed with 1% EM grade formaldehyde (Polysciences, Inc, Warrington, PA) in PBS, and stored refrigerated no longer than 1 week before analysis.

Labeled cells were analyzed on a FACSCan (Becton Dickinson, San Jose, CA). Linear forward scatter versus logarithmic side scatter displays were used to set a broad gate that eliminated small debris and large aggregates before collection of list mode data. FITC-labeled, CD14+ M0 were detected using logarithmic amplification in the FL1 channel (530/30 bandpass filter). PKH-26-positive target lymphocytes were evaluated using logarithmic amplification in the FL2 channel (585/42 bandpass filter). For each sample, a minimum of 10,000 events, excluding dual-negative cells, were analyzed. The percentage of cells in each quadrant was determined by integration of dual-positive events in quadrant 2 (upper right). After incubation for 1 hour without BsAb (Fig 2B), approximately 14% of the target cells had shifted to quadrant 2, representing CD14+ MDM0 that had nonspecifically ingested PKH-26-stained targets. Addition of either the anti-CD19xMoAb22 (Fig 2C) or the anti-CD37xMoAb22 (Fig 2D) BsAb significantly increased phagocytosis, shifting the proportion of target cells from quadrant 4 to quadrant 2. In this particular experiment, after 1 hour of incubation at an effector to target ratio of 10:1 and BsAb concentration of 0.1 μg/mL, 51% (Fig 2C, anti-CD19xMoAb22 and 53% (Fig 2D, anti-CD37xMoAb22) of the target cells were phagocytized. Routinely, the amount of BsAb-mediated phagocytosis was calculated by subtracting the amount of nonspecific phagocytosis from the total phagocytosis, for results of 38% and 39%, respectively, in this example.

**RESULTS**

**Phagocytosis.** The mechanisms by which the macrophage can accomplish ADCC are numerous including the release of oxidative intermediates, cytotoxic factors such as tumor necrosis factor α and interleukin-1, and phagocytosis.\(^9\) Although many investigators have shown efficient macrophage-mediated ADCC of tumor cells by standard chromium release assay,\(^36,37\) Munn et al\(^34\) have made a compelling case, which has been confirmed in our own studies (data not shown), that phagocytosis is the predominant form of macrophage-mediated antibody-dependent tumor cytotoxicity. A flow cytometric method was used to measure BsAb-mediated tumor cell phagocytosis by MDM0 (Fig 2). Immediately after the addition of target cells to MDM0 (time 0, Fig 2A), all target cells (PKH-26 stained malignant lymphocytes) were located in quadrant 4 (lower right) of the contour plot and all effector cells (anti-CD14—FITC-labeled MDM0) were located in quadrant 1 (upper left). Negligible phagocytosis occurred at time 0, as evidenced by the lack of dual-positive events in quadrant 2 (upper right). After incubation for 1 hour without BsAb (Fig 2B), approximately 14% of the target cells had shifted to quadrant 2, representing CD14+ MDM0 that had nonspecifically ingested PKH-26-stained targets. Addition of either the anti-CD19xMoAb22 (Fig 2C) or the anti-CD37xMoAb22 (Fig 2D) BsAb significantly increased phagocytosis, shifting the proportion of target cells from quadrant 4 to quadrant 2. In this particular experiment, after 1 hour of incubation at an effector to target ratio of 10:1 and BsAb concentration of 0.1 μg/mL, 51% (Fig 2C, anti-CD19xMoAb22 and 53% (Fig 2D, anti-CD37xMoAb22) of the target cells were phagocytized. Routinely, the amount of BsAb-mediated phagocytosis was calculated by subtracting the amount of nonspecific phagocytosis from the total phagocytosis, for results of 38% and 39%, respectively, in this example.

**Kinetic analysis of BsAb-mediated phagocytosis.** In a standard experimental design, effectors and targets were mixed in the presence of BsAb and incubated for 60 minutes. To confirm that, under these conditions, the flow cytometric assay was measuring phagocytosis of target cells by MDM0 and not conjugate formation between the target and effector cells without internalization, representative samples were examined by light and fluorescent microscopy. For example, in an experiment with the anti-CD19xMoAb22 BsAb, 100 dual-positive cells from each of 6 samples were examined.
Of these 600 cells, 88.5% ± 1.3% showed evidence of phagocytosis, whereas the remainder appeared to be conjugates. Similar results were obtained with the anti-CD37xMoAb22 BsAb, ie, 88.0% ± 0.9%. Routinely, greater than 85% of the dual-positive MDMØ exhibited evidence of tumor cell ingestion.

The kinetics of BsAb-mediated phagocytosis of malignant lymphocytes was examined next by flow cytometry and confocal microscopy. Targets, stained with PKH-26, and effectors were combined in the presence of BsAb and incubated 30, 60, or 360 minutes before stopping the reaction by placing the samples on ice. Samples were labeled with anti-CD14 FITC and analyzed by flow cytometry. In the absence of BsAb, nonspecific phagocytosis increased from 6% at 30 minutes to 29% at 360 minutes. Maximum BsAb-mediated phagocytosis occurred by 60 minutes (anti-CD19xMoAb22, 41%; anti-CD37xMoAb22, 50%), and the additional phagocytosis detected at 360 minutes was attributable entirely to the increased nonspecific phagocytosis.

Dual-positive cells from each time point were sorted by flow cytometry and sectioned by confocal microscopy. Representative images, through the MDMØ center, are shown in Fig 3. The images depict clearly the process of target cell binding, target cell ingestion, and target cell destruction over a 6-hour period. After 30 minutes, 60% of the target cells examined in this manner were bound to the surface of effector cells, whereas the other targets were either completely ingested or in the process of being internalized. After 60 minutes, 86% of targets had been phagocytized and appeared as either an intact cell (33%) or as decomposed material.

Fig 2. Flow cytometric assay for phagocytosis of malignant lymphocytes by MDMØ. Malignant lymphocytes were stained with the red fluorescent lipophilic dye PKH-26 and MDMØ were labeled with FITC-conjugated anti-CD14. Quadrant 1 (upper left), MØ only; quadrant 2 (upper right), double-positive MØ associated with malignant lymphocytes; quadrant 4 (lower right), malignant lymphocytes only. Effectors and targets were combined and (A) immediately placed on ice or incubated for 1 hour (B) without BsAb, (C) with anti-CD19xMoAb22, or (D) with anti-CD37xMoAb22.

Fig 3. Confocal assay for phagocytosis of malignant lymphocytes by MDMØ. MØ (labeled with the green fluorochrome FITC), malignant lymphocytes (stained red with PKH-26), and BsAb were combined. Confocal sections were examined after 30, 60, and 360 minutes and show the process of target cell binding, ingestion, and destruction.
BsAb-MEDIATED PHAGOCYTOSIS OF NHL

Ab-MEDIATED PHAGOCYTOSIS OF NHL 3817

J -0- anti-CD37xMoAb22

Fig 4. BsAb-mediated phagocytosis was effective over a broad concentration range. MDM0 were incubated with malignant lymphocytes and logarithmic dilutions of anti-CD19xMoAb22 or anti-CD19xMoAb22 for 1 hour. Phagocytosis was measured by flow cytometry as described in Materials and Methods.

(67%) in the effector cells. By 360 minutes, no conjugates were seen in any of the images and 80% of the double-positive images showed internalized debris, whereas the remaining images were of intact phagocytized targets.

Titration curve. Figure 4 shows the effective concentration range of BsAb required for phagocytosis. Optimal BsAb-mediated phagocytosis plateaued over a broad concentration range. Maximum phagocytosis with anti-CD19xMoAb22 and anti-CD37xMoAb22 occurred at a concentration of 0.01 and 0.1 µg/mL, respectively. Large amounts of either BsAb were less effective, because at these concentrations both effector target cells were saturated with BsAb without cross-linking their receptors. In other studies, we mixed both BsAbs over a wide concentration range and determined that at lower, but not higher, concentrations their combined effects were additive (data not shown).

Effect of cytokines. The effect of cytokines on BsAb-mediated phagocytosis was examined next. In these experiments, MDMO were cultured free of cytokines or were exposed to either IFNγ (40 U/mL) alone or IFNγ in combination with M-CSF (100 ng/mL) for 18 hours. Before the phagocytosis assay, MDMO were washed free of cytokines to eliminate the potential tumoricidal effects of IFNγ. Fresh M-CSF (as indicated), BsAb anti-CD37xMoAb22 at 1.0 µg/mL, and malignant lymphocyte targets were then added in sequence to the MDMO effectors. Results (Fig 5) show that prior activation with IFNγ alone or IFNγ with M-CSF significantly (P < .05) enhanced phagocytosis as compared with those effectors treated with neither IFNγ nor M-CSF. Moreover, IFNγ priming with M-CSF enhanced phagocytosis to a greater degree than did IFNγ alone.

Blocking studies. In a series of blocking studies, we confirmed that BsAb-mediated phagocytosis was mediated through FcyRI and was not blocked by autologous serum, polyclonal IgG, or monomeric IgG1. MDMO were incubated with malignant lymphocytes and either whole MoAb or BsAb for 1 hour. In the presence of autologous serum, polyclonal IgG, and monomeric human IgG1, MoAb-mediated phagocytosis was competitively inhibited because both CD19 and CD37 MoAbs are of an isotype (IgG2a) whose Fc domain preferentially binds to FcyRI. Autologous serum, polyclonal IgG, and monomeric human IgG1 did not block BsAb-mediated phagocytosis because MoAb22 recognizes an epitope outside the ligand binding domain. This indicates that BsAbs constructed with MoAb22 will be able to circumvent the inhibitory effect that human IgG has on FcyRI-dependent functions in vivo.

Fig 5. IFNγ and IFNγ with M-CSF priming of Mo enhances BsAb-mediated phagocytosis. In this experiment, Mo without IFNγ or M-CSF priming, with IFNγ priming alone, and with IFNγ plus M-CSF priming were incubated for 6 hours with anti-CD37xMoAb22 and target lymphocytes derived from a patient with prolymphocytic leukemia at a effector:target ratio of 10:1. Phagocytosis was measured by flow cytometry as described in Materials and Methods. *P < .05 versus nonspecific (no BsAb) phagocytosis; †P < .05 versus no IFNγ or M-CSF.

Fig 6. Phagocytosis with BsAbs constructed from MoAb22 was mediated through FcyRI and was not blocked by autologous serum, polyclonal IgG, or monomeric IgG1. MDMO were incubated with malignant lymphocytes and either whole MoAb or BsAb for 1 hour. In the presence of autologous serum, polyclonal IgG, and monomeric human IgG1, MoAb-mediated phagocytosis was competitively inhibited because both CD19 and CD37 MoAbs are of an isotype (IgG2a) whose Fc domain preferentially binds to FcyRI. Autologous serum, polyclonal IgG, and monomeric human IgG1 did not block BsAb-mediated phagocytosis because MoAb22 recognizes an epitope outside the ligand binding domain. This indicates that BsAbs constructed with MoAb22 will be able to circumvent the inhibitory effect that human IgG has on FcyRI-dependent functions in vivo.
blocked BsAb-mediated phagocytosis with either BsAb. This indicates that BsAbs constructed from MoAb 22 will be able to circumvent the inhibitory effect that human IgG has on FcγRI-dependent function in vivo and suggests that receptor cross-linking, and not occupancy of the ligand binding site, is the principal signal required to initiate FcγRI function.

To definitively establish that both BsAbs were triggering phagocytosis through FcγRI, the following FcγR blocking agents were examined (Fig 7): MoAb22 F(ab’)_2, MoAb IV.3, MoAb 3G8, and MoAb 197. As expected, BsAb-mediated phagocytosis was blocked by MoAb22 F(ab’)_2 antibody fragments. These fragments target the same FcγRII epitope recognized by the MoAb22 arm of the BsAbs and confirms that the MoAb22 portion of both BsAbs was triggering phagocytosis. MoAb22 fragments did not block phagocytosis mediated by whole anti-CD37 or anti-CD19 (data not shown). Neither MoAb IV.3, which blocks FcγRII, nor MoAb 3G8, which blocks FcγRIII, interfered with antibody-mediated phagocytosis. Thus, phagocytosis mediated by these BsAbs and MoAbs is through FcγRII only. Finally, the ability of MoAb 197 to inhibit phagocytosis was examined. MoAb 197 will effectively cross-link all FcγRI on a cell by binding through its Fc domain to the ligand binding site and through its Fab domain to the external epitope. The external epitope recognized by MoAb 197 differs from the site recognized by MoAb22. In the presence of MoAb 197, phagocytosis by both BsAbs was effectively blocked, further confirming that they were triggering through FcγRI.

**Phagocytosis of patient-derived material.** The susceptibility of malignant B cells from patients with circulating lymphoma cells or CLL was also examined using the phagocytosis assay (Fig 8). Four samples were examined from patients with CLL (P61 through P64), and one each was from a patient with prolymphocytic leukemia (P49) and a patient with mantle cell lymphoma in a leukemic phase (P24). All patient-derived material expressed CD37 and CD19 on the surface of the malignant B cells. In all six samples, patient-derived lymphocytes were susceptible to a significant degree of BsAb-mediated phagocytosis by both BsAbs. Significantly more (P < .05) phagocytosis was observed with anti-CD37xMoAb22 than with anti-CD19xMoAb22.

**DISCUSSION**

Successful application of immunotherapeutic approaches against B-cell lymphoma necessitates a ubiquitous target molecule, a functional and easily accessible cytotoxic mechanism, and a means of directing the effector to the target. B cells carry a variety of surface molecules that may serve as targets for immune therapy. CD19, CD20, CD22, and CD37 and the variable regions of surface Igs and a variant of HLA-DR have been targeted in recent in vitro and in vivo studies examining the therapeutic potential of antibodies in B-cell lymphoma.6,8,9,12,13,16,17 Perhaps the most promising results to date have used a CD37-specific antibody carrying 131I as the effector molecule.14,16,17 Although effective against the tumor, this therapeutic modality has been associated with pancytopenia, in some cases requiring marrow rescue.15

Our approach has been quite different, using a tumor-
targeting molecule designed to direct M0 killing and/or phagocytosis of the tumor cells. The BsAb used in our investigations targeted lymphoma cells to a potent cytolytic trigger molecule, FcyRI/CD64, that is expressed exclusively by monocytes, M0, myeloid precursors, dendritic cells, and activated neutrophils.25,26,27 In a patient population that is frequently lymphopenic due to prior therapy or the disease itself and in which early MoAb studies have shown that immunotherapy is most effective in a minimal residual disease state,28,29 ie, immediately after chemotherapy or bone marrow transplant when most potential effector cells are unlikely to be available, the M0 has the advantage of accessibility and functional integrity. Tissue M0 have been shown to remain functional throughout transplant. As measured by the clearance of anti-D coated red blood cells, M0 are capable of supporting ADCC at day 0 of bone marrow transplantation, with increased capabilities during the first month of recovery.30 Furthermore, with respect to tumor killing, monocytes are fully functional even in the early posttransplantation period,31 ie, before day 100 posttransplantation. Thus, our experiments were designed to determine whether the theoretical advantage of using the M0 could be realized and improved on when armed with BsAb.

Antibody-dependent phagocytosis of tumor cell lines (breast, neuroblastoma, and melanoma) by MDM0 acting through the low-affinity Fc receptors (FcyRII and FcyRIII) has been shown by Munn et al.32 The high-affinity FcyRI has also been shown to trigger phagocytosis of human erythrocytes sensitized with IgG.33 Antibody-mediated external lysis of both malignant and nonmalignant target cells by FcyRI and FcyRII on the monocyte and all three FcyR on M0 is well documented. van de Winkl34 has shown that, although both FcyRI and II can mediate ADCC measured by 51Cr release, a much higher degree of target sensitization is necessary for FcyRII- than for FcyRI-mediated ADCC.

In the studies reported here, we examined the ability of two BsAbs to mediate phagocytosis of patient-derived malignant B cells. Both BsAbs were constructed by chemically linking the Fab portion of MoAb22, an antibody that reacts with the high-affinity FcyRI to the Fab portion of MoAbs recognizing the B-cell differentiation antigens CD19 and CD37. The widespread expression of both CD19 and CD37 on B-cell NHLs and chronic leukemias and their absence from hematopoietic cells, including platelets and stem cells, makes them especially suitable targets for therapy. In vitro, the anti-CD19xMoAb22 and the anti-CD37xMoAb22 BsAbs effectively mediated phagocytosis by MDM0 of patient tumor cells. Moreover, preincubation of M0 with IFNγ alone or IFNγ with M-CSF enhanced BsAb-mediated phagocytosis. As expected, phagocytosis by these BsAbs was not inhibited in the presence of whole serum, the polyclonal gammaglobulin fraction, or the monoclonic IgG1 fraction because MoAb22 reacts with FcγRI at an epitope outside the ligand binding site. This indicates that these and other BsAbs constructed with MoAb22 will be able to circumvent the inhibitory effect that human IgG has on FcγRI-dependent function in vivo. BsAb-mediated phagocytosis was blocked by antibodies that target or sterically hinder binding to the FcγRI epitope recognized by MoAb22. Independently, both BsAbs were effective against a number of patient-derived tumors.

Our experiments show that BsAb-mediated phagocytosis through FcγRI must be considered an important and efficacious killing mechanism of the M0 that may be potentially harnessed against malignant lymphoma cells. We have developed another BsAb, MDX-210, an Fab x Fab (520C9xMoAb22) construct combining specificity for human CD64 (FcγRI) and the HER-2/neu oncoprotein.24 In vitro data show that MDX-210 facilitates ADCC and phagocytosis of HER-2/neu-positive targets and stimulates release of monocyte/M0-derived cytokines. Based on these encouraging observations, phase I studies have been undertaken in women with breast or ovarian cancer and in men with prostate cancer. To date, treatment with this BsAb has been well tolerated. The most common toxicity was grade 1 to 2 fever and malaise. Hypotension also developed in some patients, persisting for 3 to 6 hours at lower doses and for up to 12 to 20 hours at higher doses.

Biologic effects of MDX-210 in these phase I studies included greater than 80% saturation of monocyte FcγRI receptors at doses greater than 3.5 mg/mL. Peak plasma levels in excess of 1 mg/mL were achieved, which is a substantially greater concentration than required for optimal monocyte/M0 activation in vitro. MDX-210 was immunologically active and induced increased plasma levels of TNFa, interleukin-6, granulocyte colony-stimulating factor (G-CSF), and neopterin. MDX-210 induced one partial response and one mixed response among 10 patients with breast cancer who were evaluable for response. Moreover, treatment with MDX-210 resulted in an increase in antitumor antibodies in the circulation of approximately 30% of the patients treated, suggesting a vaccine-like effect of this therapy.

Overall, the in vitro results presented here indicate that either anti-CD19 or anti-CD37 containing BsAb is a excellent candidate for clinical trials of NHL and may be particularly effective when used in combination with hematopoietic growth factors or ex vivo-activated myeloid cells. Moreover, the encouraging results of recent clinical trials using a similar BsAb justify undertaking a clinical trial using FcγRI-targeted BsAbs in patients with NHL with disease resistant to standard chemotherapeutic agents or with organ and marrow toxicity that precludes the use of standard agents.

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