

# Phagocytosis of Breast Cancer Cells Mediated by Anti-*MUC-1* Monoclonal Antibody, DF3, and Its Bispecific Antibody<sup>1</sup>

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## ABSTRACT

Human epithelial mucin, *MUC-1*, is commonly expressed in adenocarcinoma including 80% of breast cancers. *erbB-2* is overexpressed in ~30% of breast cancers. Expression of *MUC-1* and *erbB-2* may be partially overlapping but discoordinate. Therefore, combined use of antibodies directed against these two antigens might increase the number of patients who benefit from immunotherapy. Monoclonal antibody (MAb) DF3 recognizes the *MUC-1* tandem repeat. We investigated phagocytosis and cytolysis of cultured human breast cancer cells by monocyte-derived macrophages mediated by MAb DF3 and its bispecific antibody (BsAb) DF3xH22 with the second epitope directed against the Fc component of phagocytic cells.

Purified monocytes from healthy donors were cultured with granulocyte macrophage colony-stimulating factor with or without IFN- $\gamma$ . antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC) assays were performed with these macrophages and *MUC-1*-expressing target cells (ZR75-1) in the presence of MAb DF3 and BsAb DF3xH22. ADCP was measured by two-color fluorescence flow cytometry using PKH2 (green fluorescent dye) and R-phytoerythrin (RPE) (red)-conjugated MAb against human CD14 and CD11b and was confirmed by confocal microscopy. ADCC was measured by <sup>51</sup>Cr release assay. Immunohistochemical staining studies of *MUC-1* and *erbB-2* were performed on 67 primary breast cancer tissues.

Expression of *MUC-1* and *erbB-2* was partially overlapping but discoordinate in 67 consecutive breast cancers. Both MAb DF3 and BsAb DF3xH22 mediated ADCP. However, ADCP mediated by MAb DF3 was greater than that mediated by BsAb DF3xH22. ADCC as detected by <sup>51</sup>Cr release was not seen with either antibody. The addition of IFN- $\gamma$  to monocyte-derived macrophage cultures inhibited ADCP compared to granulocyte macrophage colony-stimulating factor alone.

Given the partially overlapping but discoordinate expression of *MUC-1* and *erbB-2* in breast cancer, therapy directed toward both antigens should be considered. MAb DF3 and the BsAb DF3xH22, can effectively mediate phagocytosis of *MUC-1*-expressing target cells. Further investigations are needed to determine whether this antibody-induced phagocytosis results in long-term specific T-cell activation against *MUC-1*.

## INTRODUCTION

The human epithelial mucin, *MUC-1*, is a member of a large family of glycoproteins heterogeneously expressed on the apical surface of normal epithelial cell types, including those of the breast (1). In many human adenocarcinomas, *MUC-1* is aberrantly overexpressed throughout the cytoplasm, as well as on the cell surface, in an underglycosylated form (2, 3).

The murine MAb<sup>3</sup> DF3 recognizes glycosylated *MUC-1* core peptide antigen DF3 (4). The DF3 epitope is expressed in ~80% of

human breast cancers (1). Immunoperoxidase staining with MAb DF3 distinguishes malignant and benign breast lesions. In nonmalignant tissues, DF3 staining is detectable on the apical surface of glandular cells, whereas in malignant tissues staining is observed throughout the entire cellular membrane and within the cytoplasm (5). The level of antigen expression has been shown to correlate with degree of breast tumor differentiation, estrogen receptor status, and clinical outcome (1).

MAb DF3-P was generated against a dodecamer peptide present in the tandem repeat of the DF3 protein (VTSAPDTRPAGSTAP-PAHG) (3). MAb DF3-P reacts with underglycosylated precursors of DF3 antigen. In contrast to MAb DF3, immunoperoxidase staining with MAb DF3-P exhibits more specific reactivity with breast cancer cells (3). Because of the specificity and overexpression of the DF3/*MUC-1* epitope in breast cancer, MAb DF3 and MAb DF3-P might have therapeutic potential as an immunological approach.

MAbs directed against tumor antigens can mediate lysis of tumor cells either through a complement-mediated mechanism or by antibody-induced tumor destruction mediated by Fc receptors on immune effector cells. Thus, the therapeutic activity of MAbs is dependent on the activity of the host immune system, which may be defective in cancer patients. BsAb may help overcome this limitation of MAbs by redirecting and triggering the appropriate effector cells (6).

The human IgG Fc $\gamma$ RI is expressed primarily on monocytes, macrophages, and IFN- $\gamma$ -activated granulocytes (7). The cellular distribution of Fc $\gamma$ RI is restricted to immune cells that mediate ADCC. Humanized MAb 22 is directed against an epitope on the Fc $\gamma$ RI that is distinct from the ligand binding site and is not blocked by nonspecific immunoglobulin (8). Thus, bispecific molecules constructed from this antibody bind Fc $\gamma$ RI in the presence of saturating concentrations of IgG and may exploit the cytotoxicity of Fc $\gamma$ RI under physiological conditions.

BsAb against *erbB-2* mediate phagocytosis and cell killing of *erbB-2*-overexpressing cell lines (9, 10). Phase I trials of one such antibody, MDX-H210, have been reported (11). However, *erbB-2* is overexpressed in only 25–30% of breast cancers, representing a more poorly differentiated subgroup that is less likely to express higher levels of estrogen receptors than *erbB-2*-negative tumors (12). Since *MUC-1* expression is associated with better differentiated estrogen receptor-rich breast cancers, a strategy designed to use antibodies against both antigens might be more clinically useful than one directed against either antigen alone (1, 13). In this study, we demonstrate that the expression of these two antigens is partially overlapping but discoordinate in breast cancer and we have investigated *in vitro* MDM-mediated phagocytosis and cytolysis induced by MAb DF3-P, MAb DF3, and its BsAb DF3xH22.

## MATERIALS AND METHODS

**Cell Lines.** ZR75-1, MCF-7, BT-20, T-47D, SKBR-3 (human breast cancer cell lines), and U-937 (human histiocytic lymphoma cell line) were purchased from American Type Culture Collection (Manassas, VA).

antibody; Fc $\gamma$ R, Fc receptor  $\gamma$  for IgG; MDM, monocyte-derived macrophage; GM-CSF, granulocyte macrophage colony-stimulating factor; RPE, R-phytoerythrin.

Received 7/21/00; accepted 3/15/01.

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<sup>1</sup> Supported in part by NIH Grant CA64057 and by the Lombardi Cancer Center Flow Cytometry and Microscopy Shared Resource Facilities, U. S. Public Health Service Grant P30-CA-51008.

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<sup>3</sup> The abbreviations used are: MAb, monoclonal antibody; ADCP, antibody-dependent cellular phagocytosis; ADCC, antibody-dependent cellular cytotoxicity; BsAb, bispecific

**Antibodies.** MAb DF3 (murine IgG1) was prepared from mouse ascites as previously described (5). BsAb DF3xH22 was prepared using methods previously described for other BsAbs by Medarex, Inc. (Annandale, NJ) (9). MAb DF3-P (murine IgG2a) was prepared as described elsewhere (3). MAb 22 (murine IgG1, anti-Fc $\gamma$ RI) and MAb IV3 (murine IgG2b, anti-Fc $\gamma$ RII) were also provided by Medarex, Inc. Murine IgG1 and IgG2a (Sigma, St. Louis, MO) were used as isotype controls. Human IgG was obtained from Sigma.

**Fluorescent Dye.** PKH2 Green Fluorescent Cell Linker kit (PKH2), a nonspecific, lipophilic and green fluorescent dye, was purchased from Sigma. RPE-conjugated anti-CD11b and anti-CD14 were purchased from Dako (Glostrup, Denmark).

**Cytokines.** Human recombinant GM-CSF was purchased from BioSource International (Camarillo, CA). Human recombinant IFN- $\gamma$  was purchased from Sigma.

**Immunohistochemical Analysis of *MUC-1* Expression.** Sixty-seven Formalin-fixed, paraffin-embedded blocks containing primary infiltrating breast cancer tissues were obtained from the Department of Pathology at Georgetown University Hospital. Immunohistochemical staining of estrogen and progesterone receptors was previously performed for each of these tissues. Five-micrometer tissue sections were used for immunohistochemical studies.

Immunohistochemical analysis of *MUC-1* was performed using the Optimax Plus 2.0 automated cell-staining system (Biogenex Laboratories, San Ramon, CA). The biotin-streptavidin-peroxidase (Biogenex Laboratories) method was used as previously described (14). Briefly, tissues were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with H<sub>2</sub>O<sub>2</sub>. The sections were then incubated with normal goat serum. Anti-*MUC-1* MAb DF3 (1 mg/ml) was applied for 30 min. The slides were rinsed and sequentially incubated with biotin-goat antimouse serum for 20 min followed by streptavidin-horseradish peroxidase for 20 min. Following multiple washes, peroxidase activity was visualized after reaction with diaminobenzidine for 10 min. After counterstaining with Mayer's hematoxylin, dehydrating, and mounting under coverslips, the slides were scored for the percentage of invading tumor cells with cytoplasmic staining (5).

**Immunohistochemical Analysis of *erbB-2* Expression.** Tissue sections were prepared from the same blocks as those used for *MUC-1* analysis, and a similar method of immunohistochemical staining was used to evaluate expression of *erbB-2*. Briefly, a monoclonal anti-*erbB-2* CB11 (CMC602; Cell Marque, Austin, TX) diluted 1:25 was applied for 1 h after the slides were deparaffinized and blocked for nonspecific reactivity. *erbB-2* immunopositivity was estimated as a percentage of invasive tumor cells in which any membranous activity was observed at a magnification of  $\times 100$  (15).

**Indirect Immunofluorescence Flow Cytometry.** Cells were incubated with the appropriate primary murine MAb at saturating concentrations followed by FITC-conjugated goat antimouse F(ab')<sub>2</sub> serum (Pierce, Rockford, IL). Each incubation was performed for 60 min at 4°C. After the final wash, the cells were fixed with 2% formaldehyde-PBS. Cells were analyzed by flow cytometry with a FACStar<sup>PLUS</sup> (Becton Dickinson, San Jose, CA).

**Preparation of MDM from Peripheral Blood.** Mononuclear cells were obtained from leukopheresis of healthy donors by the Blood Bank at the NIH (Bethesda, MD). Monocytes were purified by double density separation with Ficoll-Paque and Percoll (Pharmacia Biotech, Piscataway, NJ) (16). Briefly, mononuclear cells were separated over Ficoll-Paque at 400  $\times g$  for 20 min at 18°C. The interface layer was collected and washed with PBS three times. Fifteen milliliters of cell suspension were layered onto 15 ml of 46% Percoll (13.75 ml Percoll, 1.25 ml of 1.5 M NaCl, and 15 ml PBS; osmolarity, 285 mOsm) and centrifuged at 400  $\times g$  for 20 min at 18°C. The interface layer was again collected and washed with PBS. The mononuclear cells were then cultured in T-75 tissue culture flasks (Falcon, Franklin Lakes, NJ) at a concentration of 1.0–2.0  $\times 10^6$ /ml with GM-CSF (200 units/ml) for 5–7 days. In some experiments, IFN- $\gamma$  (1–200 units/ml) was added to GM-CSF on days 1–5. The purity of monocytes was routinely 80–90% with the remaining cells being lymphocytes, as determined by Diff-Quick staining (Dade Diagnostics of Puerto Rico, Inc., Aguada, PR).

**ADCP Assay.** ADCP assays were performed as previously described (10). After 6–7 days of culture, MDM were harvested, resuspended in RF2 medium (RPMI 1640 supplemented with 2% FCS), and incubated overnight in 96-well round-bottomed polypropylene cluster plates (Costar, Cambridge, MA) at a concentration of 1  $\times 10^5$  viable cells in 100  $\mu$ l/well. Appropriate target cells (ZR75-1, MCF-7, or BT-20) were stained with PKH2 green fluorescence dye

according to the manufacturer's instructions. The next day, 1  $\times 10^4$  PKH2-labeled target cells and the appropriate antibody (MAb DF3, MAb DF3-P, BsAb DF3xH22, or mouse IgG1 isotype control) were added to the well of cultured MDM and incubated in a final volume of 200  $\mu$ l at 37°C for 24 h. Following the incubation, MDM and target cells were harvested with EDTA-PBS and transferred to 96-well V-bottomed plates (Costar). The plates were centrifuged and the supernatant was aspirated. Cells were counterstained with a 100- $\mu$ l mixture of RPE-conjugated anti-CD11b, anti-CD14 (Dako), and human IgG, mixed and incubated for 60 min on ice. The cells were washed and fixed with 2% formaldehyde-PBS.

Two-color flow cytometric analysis was performed with a FACStar<sup>PLUS</sup> under optimal gating. PKH2-labeled target cells (green) were detected in the FL-1 channel (emission wavelength, 530 nm) and RPE-labeled MDM (red) were detected in the FL-2 channel (emission wavelength, 575 nm). Residual target cells were defined as cells that were PKH2<sup>+</sup>/RPE<sup>-</sup>. Dual-labeled cells (PKH2<sup>+</sup>/RPE<sup>+</sup>) were considered to represent phagocytosis of targets by MDM. Phagocytosis of target cells was calculated with the following equation: percent phagocytosis = 100  $\times$  [(percent dual positive)/(percent dual positive + percent residual targets)]. All tests were performed in duplicate or triplicate and the results are expressed as mean  $\pm$  SD.

**ADCC Assay.** A <sup>51</sup>Cr release assay was performed in a modified fashion as previously described (9). Briefly, target cells (ZR75-1 or SKBR-3) were labeled with <sup>51</sup>Cr using 100  $\mu$ Ci/1  $\times 10^6$  cells for 1 h and washed three times. MDM, target cells, and antibodies were added simultaneously to individual microtiter plate wells. Incubation times for ADCC ranged from 4 to 16 h. After incubation and centrifugation, supernatants were harvested and analyzed for radioactivity in a gamma counter. Cytotoxicity was calculated by the following formula: percent lysis = 100  $\times$  [(experiment cpm – target leak cpm)/(detergent lysis cpm – target leak cpm)]. All assays were performed in triplicate.

## RESULTS

**Immunohistochemical Analyses of *MUC-1* and *erbB-2* Expression in Breast Cancer Tissues.** Sixty-seven cases of invasive breast cancer were evaluated for *MUC-1* and *erbB-2* expression by immunohistochemistry. The pathology was reviewed by a single breast pathologist (B. S.). When  $\geq 50\%$  of tumor cells was arbitrarily chosen as a cutoff for considering samples "positive" for marker expression, 59/67 (88%) of the samples were positive for *MUC-1* and 7/67 (9%) for *erbB-2*. If  $\geq 10\%$  was considered as the cutoff, 60 of 67 (90%) samples stained positive for *MUC-1* and 12 of 67 (18%) stained positive for *erbB-2*. (Table 1) Of the 60 samples that stained  $\geq 10\%$  cell positive for *MUC-1*, 12 also had  $\geq 10\%$  cells stained positive for *erbB-2* (18.6%). All of the 12 that were positive for *erbB-2* were also positive for *MUC-1*. Forty-two percent of these double positives (5/12) stained  $>90\%$  positive for both antigens. Only 7 of the 67 patients (10%) were negative for both *MUC-1* and *erbB-2*, and 48 (72%) were positive only for *MUC-1*. A linear regression of the staining characteristics of the samples (Fig. 1) demonstrates that expression of these markers was partially overlapping but discoordinate. Indeed, statistical analyses demonstrated little or no correlation between expression of these markers ( $r^2 = 0.00279$ ,  $P = 0.67$ ).

**Binding of Antibodies with Target and Effector Cells.** Reactivity of MAb DF3 and BsAb DF3xH22 with a variety of cell lines was

Table 1 *MUC-1* and *erbB-2* expression in 67 consecutive cases of breast cancer as determined by immunohistochemistry

		<i>erbB-2</i> Staining <sup>a</sup>		Total
		+	-	
<i>MUC-1</i>	+	6 [12]	53 [48]	59 [60]
Staining	-	1 [0]	7 [7]	8 [7]
Total		7 [12]	60 [55]	67

<sup>a</sup> Open numbers represent the number of samples considered positive using  $\geq 50\%$  as a cutoff; numbers in brackets signify the number of samples considered positive using  $\geq 10\%$  as a cutoff.

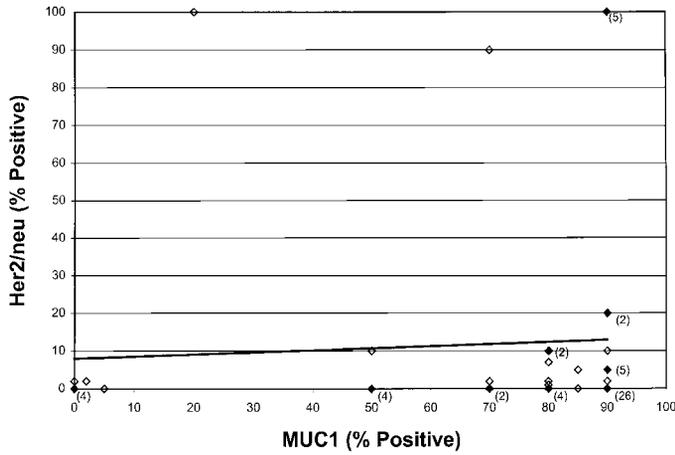


Fig. 1. Correlation of *MUC-1* and *erbB-2* expression. Sixty-seven samples of breast cancer tissues were stained with biotin-streptavidin-peroxidase using MAb DF3 as anti-*MUC-1* and CB11 as anti-*erbB-2*. ♦, more than one case, as indicated by the parenthetical numbers; ◇, single cases ( $r^2 = 0.00279$ ,  $P = 0.67$ ).

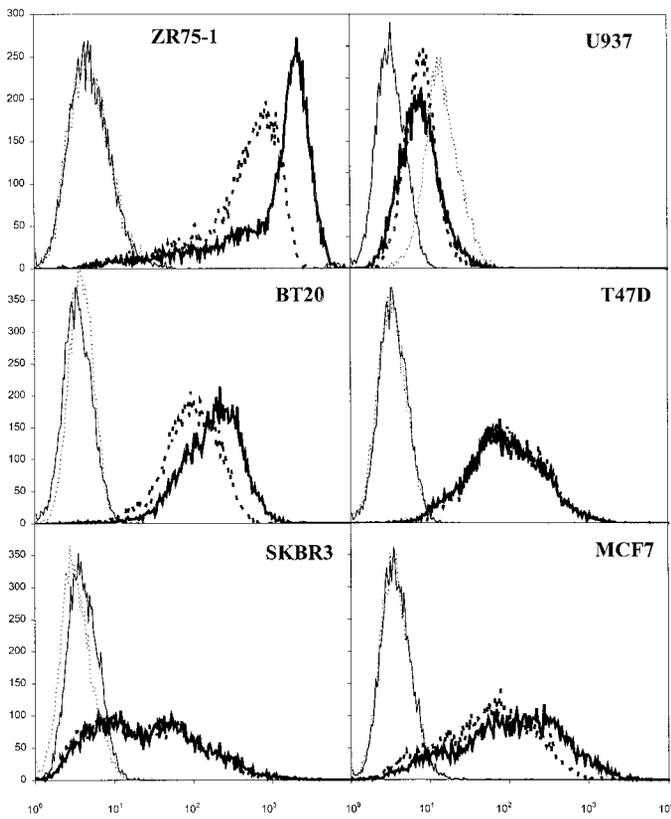


Fig. 2. Reactivity of MAb and BsAb antibodies with cultured human cancer cell lines that express different *MUC-1* levels. Human breast cancer cell lines ZR75-1, MCF-7, BT-20, T-47D, SKBR-3, and human lymphoma cell line U-937 were evaluated with the respective antibodies as depicted. Analysis by fluorescent flow cytometry is as described in "Materials and Methods." MAb DF3, —; BsAb DF3xH22, ----; MAb 22, ···; murine IgG1 as a negative control, -·-·-.

investigated using indirect immunofluorescent flow cytometry (Fig. 2). ZR75-1 highly expresses DF3 antigen, whereas MCF-7, BT-20, T-47D, and SKBR-3 express lower levels (17). U-937 expresses  $Fc\gamma RI$  as well as low levels of DF3 antigen (10). At saturating concentrations, BsAb DF3xH22 reactivity was observed with both ZR75-1 and U-937, but was lower for each cell line than with the parent MABs (MAb DF3 with ZR75-1, and MAb 22 with U-937), presumably attributable to loss of bivalency and/or steric constraints

that occur as a result of construction of the BsAb. No reactivity was detected between MAb 22 and any of the breast cancer cell lines.

**ADCP Mediated by MAb DF3, MAb DF3-P, and BsAb DF3xH22.** In our previous studies, we observed that MDM recovery is improved in the presence of GM-CSF (10). Therefore, we used MDM preincubated for 6–7 days with GM-CSF in subsequent assays. ADCP assays were performed with antibody concentrations ranging from 0.01 to 10  $\mu g/ml$  of BsAb DF3xH22, MAb DF3, and mouse IgG1 isotype control, using an E:T ratio of 10:1 and a 24-h incubation. Percent phagocytosis depended on the concentrations of the antibody in a dose-saturation and dose-dependent manner. Similar results were obtained in four separate experiments. Representative data using ZR75-1 as the target cell line are provided in Fig. 3A. In each experiment, ADCP for the isotype control antibody was always <20%. ADCP levels for BsAb DF3xH22 were lower than the levels obtained for its parent MAb DF3, but were always higher than the isotype control. A maximum ADCP level was obtained for MAb DF3 and BsAb DF3xH22 at concentrations of 1 and 10  $\mu g/ml$ , respectively. In contrast to MAb DF3 and BsAb DF3xH22, ADCP for MAb DF3-P was not different from that obtained with the control antibody or MAb H22 in three breast cancer cell lines (ZR75-1, MCF-7, and BT-20) even at concentrations up to 20  $\mu g/ml$ . Representative data are provided in Fig. 3B. This finding might be explained by decreased cell surface binding of MAb DF3-P with ZR75-1 and other DF3-positive

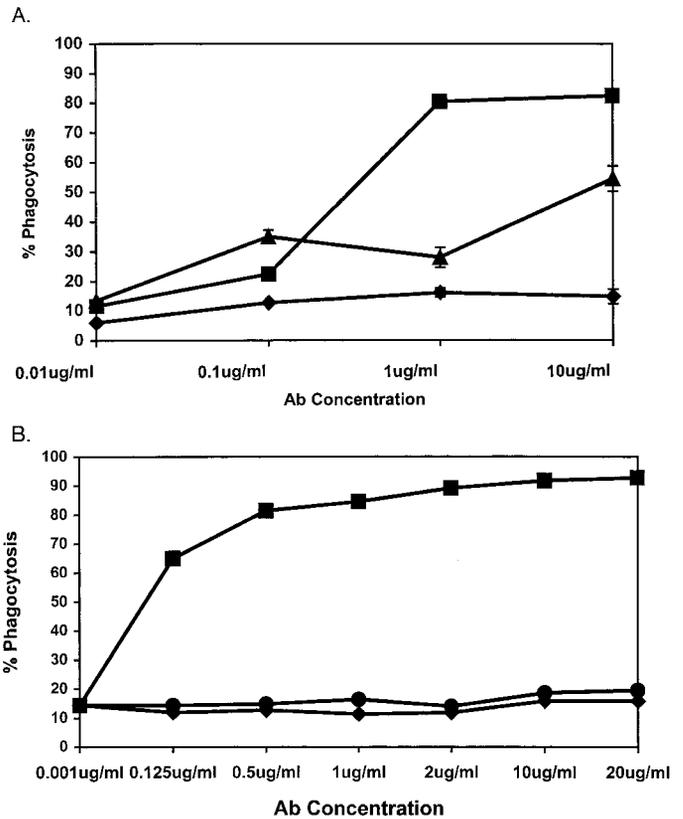


Fig. 3. ADCP mediated by MAb DF3, BsAb DF3xH22, and MAb DF3-P at increasing antibody concentration. A and B illustrate separate representative experiments. A, ADCP mediated by MAb DF3 and BsAb DF3xH22. ADCP assays were performed using the ZR75-1 cell line as target as described in "Materials and Methods" (assay duration time, 24 h; E:T ratio, 10:1). MAb DF3, ■; BsAb DF3xH22, ▲; isotype control immunoglobulin, ◆. Data points, mean of duplicate determinations of a representative experiment; bars, SD (error bars not seen in the figure are encompassed within the data points). B, ADCP mediated by MAb DF3-P at increasing antibody concentration. ADCP assays were performed as described in "Materials and Methods" using cell line ZR75-1 as target (assay duration time, 18 h; E:T ratio, 10:1). MAb DF3-P, ●; isotype control immunoglobulin, ◆. ADCP with MAb DF3 (■) was included as a positive control. Error bars not seen in the figure are encompassed within the data points.

breast cancer cell lines, as determined by flow cytometry [data not shown and as described previously (2)].

**Cytokine Effects of ADCP Assay.** We studied ADCP after preincubation of MDM with GM-CSF alone or GM-CSF + IFN- $\gamma$ . MDM were cultured for 6–7 days with GM-CSF; IFN- $\gamma$  was added to GM-CSF from days 1 to 5. In four different experiments, the addition of IFN- $\gamma$  inhibited phagocytosis of ZR75-1 cells mediated by both MAb DF3 and BsAb DF3xH22 when compared to GM-CSF alone. Representative data are provided in Fig. 4. Inhibition of antibody-mediated phagocytosis seemed to be maximal following 24 h of IFN- $\gamma$  priming. A similar inhibition was seen by increasing the dose of IFN- $\gamma$  from 10 to 200 units/ml (data not shown). Despite the inhibitory effect of IFN- $\gamma$  on ADCP, flow cytometry demonstrated that MDM cultured with IFN- $\gamma$  + GM-CSF expressed twice as many Fc $\gamma$ RI compared with MDM cultured with GM-CSF alone (data not shown). There was no significant change in the level of expression of Fc $\gamma$ RII on MDM primed with GM-CSF + IFN- $\gamma$  compared to that on MDM primed with GM-CSF alone.

**ADCC Mediated by MAb DF3 and BsAb DF3xH22.** ADCC assays were performed using MDM following 6–7 days of GM-CSF priming, with antibody concentrations ranging from 0.001 to 10  $\mu$ g/ml, using E:T ratios of 10:1 and 100:1, and 4- to 16-h incubation periods. In seven separate experiments using the chromium release assay, no significant cytotoxicity of target cells (ZR75-1 or MCF-7) mediated by MAb DF3 and BsAb DF3xH22 was observed when compared with the control antibody. Under similar chromium release assay conditions, a significant degree of cytotoxicity was evident using antibodies to *erbB-2* (MAb 520c9 and BsAb MDX-H210) and the *erbB-2*-overexpressing breast cancer cell line SKBR-3. Representative data are provided in Table 2.

As a result of the contradictory data from the phagocytosis and the  $^{51}\text{Cr}$  release assays, we performed confocal microscopy of dual-labeled cells after ADCP. Direct visualization confirmed that the flow cytometric results used to quantify ADCP represented phagocytosis and not cellular adhesion. Sequential observations demonstrated effector and target cell binding, internalization of the target cell with a cell-in-cell pattern, and target cell destruction within the cytoplasm of MDM (data not shown). This sequence of events was identical to that observed previously with BsAb MDX-210-mediated phagocytosis (10).

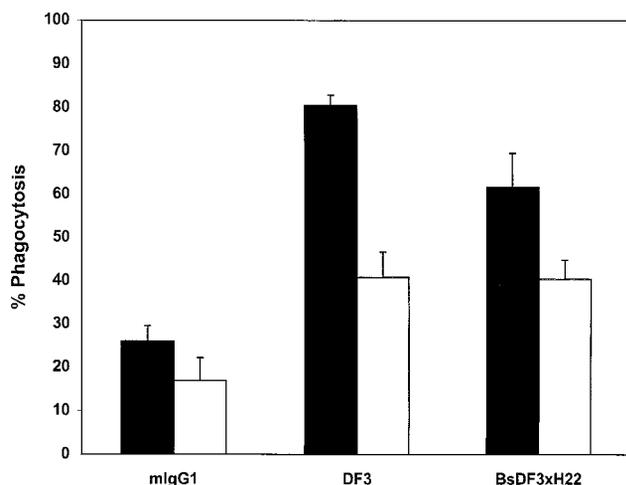


Fig. 4. Comparison of ADCP mediated by anti-*MUC-1* antibodies after preincubation of monocytes with GM-CSF or GM-CSF + IFN- $\gamma$ . Purified human monocytes were cultured in medium containing GM-CSF (200 units/ml) for 7 days or GM-CSF (200 units/ml) for 7 days + IFN- $\gamma$  (100 units/ml) added on day 6 for 24 h. ADCP assays were performed as described in "Materials and Methods" (antibody concentration, 10  $\mu$ g/ml; assay duration time, 24 h; E:T ratio, 10:1). GM-CSF, ■; GM-CSF + IFN- $\gamma$ , □. mIgG1, mouse IgG1.

Table 2 ADCC of cultured human breast cancer cell lines

Cell line	% Antibody-specific cytotoxicity			
	DF3 (%)	DF3 $\times$ H22 (%)	m520C9 (%)	m520C9 $\times$ H22 (%)
ZR75-1 <sup>a</sup>	4.4	3.8	2.5	NP <sup>b</sup>
MCF-7 <sup>a</sup>	4.3	5.1	NP	NP
SKBR-3 <sup>c</sup>	1.8	NP	63.6	53.1

<sup>a</sup> ZR75-1 and MCF-7 overexpress *MUC-1* and do not express *erbB-2*.

<sup>b</sup> NP, assay not performed.

<sup>c</sup> SKBR-3 overexpress *erbB-2* and do not express *MUC-1*.

## DISCUSSION

Therapy directed against *erbB-2* is now established for patients with *erbB-2*-overexpressing breast cancers (18, 19). However, since only 25–30% of patients have *erbB-2*-positive cancers, immunotherapeutic strategies directed toward *erbB-2* will exclude a sizeable fraction of women with this disease. Using immunohistochemical staining performed on sections from the same paraffin block in each tumor, we demonstrated that the expression of *MUC-1* and *erbB-2* in breast cancer among different patients and with tissue from individual patients is partially overlapping but discoordinate. The tumors of many patients expressed only *MUC-1* (72%). However, the majority of the specimens that expressed *erbB-2* (11 of 12) also expressed *MUC-1*, with 5 of these 12 (42%) staining >90% of cells for both markers. Thus, although *MUC-1* expression is associated with better differentiated pathology, high levels of *MUC-1* can also be found in less well-differentiated tumors. Nonetheless, lack of correlation between *MUC-1* and *erbB-2* signifies their partially overlapping but discoordinate expression. These data suggest that therapy directed toward both antigens would increase the number of patients who might benefit, compared with targeting either antigen alone. Moreover, immunotherapy directed against both antigens may improve the efficacy of the treatment for individual patients as well.

In this investigation, we have studied antigen-directed phagocytosis and cytotoxicity of cultured *MUC-1*-expressing human breast cancer cells by allogeneic MDM in the presence of either the parent antibody MAb DF3 or its BsAb DF3xH22. Both MAb DF3 and BsAb DF3xH22 induced a substantial increase over background in phagocytosis of ZR75-1 cells by MDM cultured with GM-CSF. The activity of MAb DF3 was greater than that of BsAb DF3xH22. ADCP and ADCC were related to cell surface antigen density. Indeed, the decreased phagocytosis mediated by BsAb DF3xH22 may reflect reduced surface binding of the antibody to its antigen on cancer cell lines when compared to its parent antibody MAb DF3. Decreased binding might be attributable to steric hindrance caused by the BsAb configuration or decreased affinity of the monovalent DF3 limb of the BsAb for its epitope as a result of biochemical manipulation (20).

*In vivo* observations from clinical trials of BsAbs are consistent with induction of immune activation and proper targeting of these reagents against *erbB-2* (11, 21). Presumably, BsAb concentrate antigen onto antigen-presenting cells, increasing T-cell recognition and subsequent priming. Whether a BsAb against *MUC-1* is more effective than a monospecific antibody in triggering the immune response in an *in vivo* model warrants further investigation.

Because MAb DF3-P selectively reacts with breast cancer cells and exhibits little if any reactivity with normal mammary epithelium (2), we compared phagocytosis with this antibody to MAb DF3 and BsAb DF3xH22. Unlike MAb DF3, MAb DF3-P did not induce phagocytosis over control even at high concentrations (20  $\mu$ g/ml). The level of phagocytosis by MAb DF3-P correlates with the low level of expression of this immature precursor of glycoproteins on the surface of breast cancer cell lines, as demonstrated by flow cytometry. There-

fore, we did not further pursue studies with this MAb or its bispecific derivative.

Despite substantial phagocytosis of ZR75-1 cells mediated by MAb DF3 and BsAb DF3xH22, there was no cytolysis evident as determined by the absence of the release of intracellular chromium or lactate dehydrogenase (data not shown) after target cell death. We were therefore concerned that dual-labeled cells in the ADCP assay might merely reflect adhesion, and not true phagocytosis. Confocal microscopy studies subsequently demonstrated unequivocal phagocytosis, with cell-cell adherence followed by target ingestion with a cell-in-cell pattern. These data are consistent with those from previous studies that have shown that release of radioactive materials from target cells may underestimate cytotoxicity, when compared with either visual inspection or immunological detection of surviving target cells (22). Phagocytosis may be the principle mechanism of target cell killing by MDM in the presence of MAb DF3 or its BsAb in our *in vitro* model. Whether this short-term cytotoxicity results in activation of antigen-presenting cells and long-term antitumor immune response deserves further investigation.

GM-CSF enhances the viability of monocytes in culture as well as the phagocytic function of MDM on a cell-for-cell basis (10). In this investigation, we evaluated IFN- $\gamma$  as an additional priming cytokine. IFN- $\gamma$  is a potent activator of monocytes/macrophages and can enhance ADCC (23). In our studies, the addition of IFN- $\gamma$  to GM-CSF inhibited ADCP by MDM, when compared to MDM cultures in GM-CSF alone. This inhibitory effect was demonstrable even at low doses (10 units/ml). However, Fc $\gamma$ RI expression on MDM was increased by IFN- $\gamma$  exposure (data not shown). Thus, receptor-ligand binding is unlikely to account for the inhibition. These data are consistent with those from other investigators who have observed IFN-induced reduction of ADCP mediated by M-CSF-primed MDM (24). Nonetheless, observations regarding cytokine effects in this study pertain only to MDM. It is possible that *in vivo* administration of IFN- $\gamma$  in addition to GM-CSF will enhance interaction of other cellular components, such as CTLs, given the pleotropic effects of IFN- $\gamma$  in several cell types.

In conclusion, our data demonstrate partially overlapping but discoordinate expression of *MUC-1* and *erbB-2* in breast cancer tissues. Immunotherapy directed toward both antigens should be considered based on this observation. Data from our *in vitro* model demonstrate that MAb DF3 and BsAb DF3xH22 can effectively mediate phagocytosis of *MUC-1*-expressing breast cancer cells by MDM cultured in GM-CSF. However, we detected no cytotoxicity as determined by chromium release with these antibodies. Additional studies are needed to determine whether antibody-induced phagocytosis of tumor cells results in antigen presentation and long-term specific cell-mediated immune response against *MUC-1*.

## ACKNOWLEDGMENTS

We thank Dr. Susan Leitman for her help in collection of monocytes and Dr. Norio Azumi for his advice in immunohistochemical analysis of *erbB-2*. We also acknowledge the excellent advice and technical support for confocal microscopy from Drs. Susette Mueller and Maozheng Dai. BsAb DF3xH22 and MAb DF3-P were generated and supplied by Robert Graziano of Medarex, Inc., Annandale, NJ. We also thank the Fashion Footwear Association of New York/Shoes on Sale for their generous support.

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*Cancer Res* 2001;61:4061-4065.

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