Role of Low-Affinity Fc Receptors in Antibody-dependent Tumor Cell Phagocytosis by Human Monocyte-derived Macrophages

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ABSTRACT

Human monocyte-derived macrophages (MDM) mediate efficient antibody-dependent cellular cytotoxicity (ADCC) against a variety of human tumor cell types in the presence of an anti-tumor monoclonal antibody. We have recently shown that the mechanism of this ADCC in our in vitro system involves phagocytosis of intact tumor cells. Some forms of macrophage ADCC have been reported to be inhibited by serum immunoglobulin, which competes with monoclonal antibodies for binding to macrophage ADCC have been reported to be inhibited by serum immunoglobulin, which competes with monoclonal antibodies for binding to high-affinity Fc receptor (FcRI). In this study we investigated the role of the three macrophage FcR-γ in antibody-dependent tumor cell phagocytosis. Hybridoma cells bearing surface antibody directed against either of the two low-affinity Fc receptors (FcRIII) were efficiently phagocytosed by MDM, compared to hybridomas bearing irrelevant antibody. Soluble anti-receptor antibodies against FcRII and FcRIII were able to inhibit ADCC but only when both antibodies were simultaneously present. These data suggest that either low-affinity Fc receptor is capable of functioning independently to mediate phagocytosis of tumor cells. Consistent with a mechanism involving the low-affinity receptors rather than FcRI, antibody-dependent phagocytosis occurred in the presence of human serum, purified human IgG, and irrelevant murine antibody. Greater than 75% of the MDM in our culture system were able to ingest tumor cells when a suitable target was available. Optimal phagocytosis occurred at monoclonal antibody concentrations of 10–100 ng/ml. Like other forms of macrophage phagocytosis, ingestion of tumor cells required the presence of divalent cations (either Ca²⁺ or Mg²⁺) and an intact actin cytoskeleton (as indicated by sensitivity to cytochalasin D). Because FcRI is normally occupied in vivo by serum immunoglobulin, the participation of low-affinity FcR in tumor cell phagocytosis is potentially important in establishing the in vivo applicability of this efficient form of cytolytic activity.

INTRODUCTION

Human monocytes and macrophages have long been postulated to play a role in host defense against malignancy (1), although the exact nature of the interaction between tumor cells and macrophages has not been fully defined (2, 3). One way that macrophages may kill tumor cells is through ADCC. We have shown that macrophages derived from human monocytes by in vitro culture with rhM-CSF mediate very efficient ADCC in the presence of murine and human chimeric monoclonal antibodies (4, 5). We recently reported that the mechanism of ADCC by these MDM in our system appears to involve phagocytosis of intact tumor cells rather than the release of oxidative intermediates or other soluble cytotoxic factors (5). Phagocytosis, although a characteristic function of macrophages, has not usually been considered a significant mechanism of tumor cell killing (6), despite the fact that the phenomenon has been well documented in the literature (7–11). However, phagocytosis may prove to be an important form of macrophage anti-tumor cytotoxicity, especially since many human tumor cells are relatively insensitive to the better-known mediators of macrophage killing such as tumor necrosis factor and reactive oxygen intermediates (12, 13).

Macrophages are potentially important effector cells in in vivo immunotherapy. A number of the recombinant cytokines now available for clinical trials can enhance macrophage function. We reported that rhM-CSF infusion in primates enhances in vitro anti-tumor ADCC by MDM (14), and other agents such as granulocyte-monocyte colony-stimulating factor and interleukin-3 may prove to have similar effects (15). Macrophages, unlike other effector cells, are constitutively present in most tumors (2). In fact, many tumor cells appear to secrete chemoattractants which actively recruit monocytes to tumor sites (16, 17). For all of these reasons, macrophages may be important in immunotherapeutic regimes involving anti-tumor ADCC.

Macrophages express three FcRs for IgG: the high-affinity receptor, FcRI, and the two low-affinity receptors, FcRII and FcRIII. FcRI avidly binds monomeric human IgG (Kₐ, approximately 2 × 10⁶ M⁻¹), and therefore the binding of monoclonal antibody to this receptor is competitively inhibited by serum immunoglobulin. FcRII and FcRIII, with affinity constants 100- to 1000-fold lower, are not inhibited by monomeric serum IgG (18). Thus, it is important in the context of in vitro immunotherapy to establish which of these receptors participate in anti-tumor ADCC. There is evidence from studies involving phagocytosis of bacteria and RBCs to suggest that any of the three receptors could potentially be active in tumor cell phagocytosis (19). However, we know of no report which addresses the role of the Fc receptors in phagocytosis of nucleated human cells. Therefore, in this study we characterized the FcR and other conditions required for monoclonal antibody-dependent tumor cell phagocytosis by monocyte-derived macrophages.

MATERIALS AND METHODS

Cell Lines. The SKMel-1 (human melanoma) and SK-BR-3 (human breast carcinoma) lines were obtained from the American Type Culture Collection, Rockville, MD. LA1–15n (human neuroblastoma) was the generous gift of Dr. June Biedler, Memorial Sloan-Kettering Cancer Center. Murine hybridomas OKT3 (anti-CD3), OKM1 (anti-CD11b), and IV.3 (anti-FcRII) (20) were obtained from the American Type Culture Collection. Hybridoma 3G8 (anti-FcRIII) was the generous gift of Dr. Jay Unkeless, Mt. Sinai Medical Center, New York (21). A431 (human epidermoid carcinoma of the lung) was the generous gift of Drs. Hideo Masui and John Mendelsohn, Memorial Sloan-Kettering Cancer Center. LS180 (human colon carcinoma) was obtained from...
Dr. Deborah Young, Genetics Institute, Cambridge, MA. Cell lines were grown in RPMI-1640 medium supplemented with 10% bovine serum, penicillin, streptomycin, and L-glutamine and used in log phase growth.

**Antibodies.** Antibodies 3F8 (murine IgG3, anti-GD2) and 3G6 (murine IgM, anti-GD2) were derived in our laboratory and have been previously described (22). R24 (murine IgG3, anti-distialanganglioside GD3) was the gift of Dr. Alan Houghton, Memorial Sloan-Kettering Cancer Center. Antibody 528 (murine IgG2a, anti-epidermal growth factor receptor) was the generous gift of Drs. Masui and Mendelsohn (23). NRCA4 (murine IgG3, anti-colon carcinoma) was the gift of NeoRx Corporation, Seattle, WA (24). Antibodies IV.3 (anti-FcRII) and 3G8 (anti-FcRIII) were prepared from Balb/c ascites by precipitation in 40% saturated ammonium sulfate at 0°C, followed by dialysis against phosphate-buffered saline. Antibody 197 (anti-FcRI), prepared in hollow-fiber dialysis tubing, was the generous gift of Dr. Michael Fanger, Dartmouth Medical Center (25). Phycoerythrin-conjugated anti-CD14 (LeuM3) and anti-CD11b (Leu15) were purchased from Becton Dickinson, Mountain View, CA.

**Cytokines.** Recombinant human M-CSF was the gift of Genetics Institute, Cambridge, MA. It had a molecular mass of 90 kDa and was >99% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Separation and Culture of Peripheral Blood Monocytes.** Our human monocyte culture technique was described previously (4). Briefly, heparinized whole blood was centrifuged over Ficoll-Paque (Pharmacia, Piscataway, NJ), and the mononuclear cells were spun over 36% Percoll (Pharmacia) to remove platelets and then enriched for monocytes by centrifugation over 47% Percoll. The interface layer (>95% of the monocyte population, typically 40% monocytes and 60% lymphocytes) was suspended in bovine serum-RPMI-1640 medium (2–2.4 x 10^6 cells/ml, 100 μl/well) and allowed to adhere to human serum-coated 96-well flat-bottomed culture plates. After 90 min at 37°C the plates were washed vigorously and adherent cells (usually 90% monocytes by flow cytometry) were cultured for 7–10 days in 150 μl of X-Vivo 10 serum-free medium (Whittaker Bioproducts, Walkersville, MD) supplemented with 200 units/ml rhM-CSF. Cell counts after culture were typically 2–4 x 10^4 cells/well; usually 90% were monocyte/macrophages by surface immunophenotype. Media and additives used in

**Fig. 1.** Phagocytosis of cultured human tumor cells by monocyte-derived macrophages. MDM (red fluorescence) and fluorochrome-labeled tumor cells (green fluorescence) were coincubated for 6 h in the presence of an anti-tumor monoclonal antibody. In these contour graphs, region 1 contains MDM alone, region 2 contains MDM which have phagocyted target cells, and region 4 contains unphagocyted target cells. A, antibody 3G6 (IgM) with target cells LA-N-1 (neuroblastoma); B, Ab528 (IgG2a) with A43 (epidermoid carcinoma); C, Ab528 with SK-BR3 (breast carcinoma); D, 3F8 (IgG3) with LA1–15n (neuroblastoma); E, R24 (IgG3) with SKMel-1 (melanoma); F, NRCA4 (IgG3) with LS180 (colon carcinoma). The antibody concentration was 2 μg/ml, and the measured ET ratio was 2:1–4:1 in all experiments.
monocyte culture were screened for endotoxin by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) and contained <0.03 units/ml at the concentrations used.

PKH2 Labeling. Target cells were labeled with PKH2 dye (ZynaxisCell Science, Malvern, PA) according to the manufacturer's directions. Labeled cells (except for the hybridoma lines) were usually cultured overnight to minimize spontaneous leakage of dye during the phagocytosis assay, and then target cells were added directly to cultures of MDM. Where appropriate, an anti-tumor monoclonal antibody was added with the target cells. Cultures were harvested after 6 h using EDTA (2 mM for 45 min at 4°C), monocytes were counterstained with a cocktail of phycoerythrin-conjugated anti-CD14/anti-CD11b, and the cells were analyzed by cytfluorometry. Any contaminating lymphocytes surviving after culture were excluded from analysis on the basis of forward and right-angle light scatter criteria.

Residual target cells were defined as cells which were PKH2 positive and CD14/CD11b negative. MDM were defined as any CD14/CD11b-positive cells. Double-positive cells were counted as both a phagocytosed target cell and a macrophage. Percentage of target cell phagocytosis was calculated as

\[
\% \text{ double positive} = \frac{\% \text{ residual targets} + \% \text{ double positive}}{\% \text{ double positive}} \times 100\%
\]

To look for target cells passively bound to monocytes without undergoing phagocytosis, we also analyzed some experiments by fluorescence microscopy; <1% of target cells were seen bound to MDM. To ensure that target cells were not dying in culture and subsequently being phagocytosed, we periodically assayed control target cells (incubated without monocytes) with trypan blue at the end of the 6-h assay. Human tumor cell lines were typically 90-95% viable and hybridomas were 80-90% viable.

Fc Receptor Inhibition. Monocytes cultured for 7 days in serum-free medium with rhM-CSF were incubated in situ with antibodies against FcRI (Ab197), FcRII (IV.3), or FcRIII (3G8), 20 μg/ml for 20 min at room temperature. They were washed once and incubated with purified goat anti-mouse IgG (Fisher Biotech), 5 μg/ml for 1 h at 37°C, to facilitate cross-linking and capping of bound receptors, then washed, and again exposed to the anti-FcR antibodies to prevent reexpression of the receptors. Targets and anti-tumor antibody were then added and assayed as described above.

Indirect Immunofluorescence. Monocytes, cultured for 7 days as described, were harvested with EDTA and stained with Ab197, IV.3, and 3G8, followed by fluorescein-conjugated goat anti-mouse F(ab')2 fragments. The negative control was an irrelevant IgG1 monoclonal antibody (2C9).

RESULTS

We used our flow cytometric assay to measure tumor cell phagocytosis by monocyte-derived macrophages. Target cells were stained with the vital dye PKH2, an intensely hydrophobic dye which inserts tightly into the cell membrane and shows little transference from cell to cell in culture (26). Stained target cells were added directly to cultures of MDM and incubated for 6 h in the presence of an anti-tumor monoclonal antibody. The cultures were harvested and the MDM counterstained with antibodies against CD14 and CD11b. In the contour plots shown in Fig. 1, region 1 contains MDM and region 4 contains unphagocytosed target cells. Region 2 contains MDM which have ingested a dye-labeled tumor cell, thus becoming doubly positive. Fig. 1 shows that murine monoclonal antibodies of the IgG3 and IgG2a subclasses mediated ADCC against a
were capable of mediating phagocytosis of tumor cells in our
(27) in which hybridomas bearing surface antibodies directed
(CD 16) (Fig. 3). To determine whether the low-affinity FcR
culture in our serum-free system it is unoccupied. We found
tested gave similar results.
and reached a plateau at 100-1000 ng/ml. The other antibodies
detectable phagocytosis at concentrations of 10 ng/ml or less
different antigens on different cell lines, the cytotoxicity curves
(25)]. Since all of these treatments effectively block
FcRI, our findings suggested that binding of anti-tumor anti-
body to FcRI was not obligatory for phagocytosis. Therefore,
in order to more accurately simulate the in vivo role of FcRII
and FcRIII, we selectively blocked FcRI with Ab197 in all subsequent experiments. As shown in Fig. 5, when FcRI was
blocked, blocking either FcRII or FcRIII individually had rel-
atively little effect on phagocytosis, ranging from no inhibition
to partial inhibition. However, when both FcRII and FcRIII
were blocked, we saw a consistent and significant decrease in
antibody-dependent phagocytosis, usually to a point close to
the background level of antibody-independent killing. These
data are consistent with the hypothesis that tumor cell phago-
cytosis can proceed via either FcRII or FcRIII independently,
as suggested by the hybridoma cell experiments above.

Because there was heterogeneity in FcR expression (espe-
cially in the case of FcRIII; Fig. 3), we wished to ascertain
whether there was a specific subset of MDM which mediated
ADCC. As is evident from Fig. 1, not all of the MDM in a
given culture participated in phagocytosis. However, the meas-
ured ET ratios in these experiments were between 2:1 and 4:1,
which means that not all MDM would have the opportunity to
encounter a target cell. To determine what percentage of MDM
were capable of mediating ADCC when target cell number was
not a limiting factor, we added an excess of target cells (up to
4 times as many targets as effectors). Fig. 6 shows that under
these conditions >75% of MDM were recruited for phagocy-
tosis. Even at the highest concentration of target cells used, the
geometry of the cultures imposed an upper limit on the number
of MDM exposed to a target. Since MDM do not form a perfect
monolayer, some macrophages may have been masked by over-
lying cells. Therefore, we cannot determine from these experi-
ments whether the minority of MDM which did not show
phagocytosis were incapable of doing so or simply did not come
in contact with a target. Nevertheless, we may conclude that
the large majority of MDM, rather than a small subset, appears

variety of human tumor cell lines, including lung, breast, and
colon carcinoma, neuroblastoma, and malignant melanoma.
These data are representative of experiments using 11 different
cell lines and a total of 8 antibodies (6 murine antibodies of the
IgG3 or IgG2a subclass and 2 mouse-human chimeric antibo-
dies of the human IgG1 subclass). In contrast, Fig. 1A shows
that a murine IgM antibody (against the same GD2 antigen
shown in Fig. 1D) did not mediate phagocytosis, even though
it bound strongly to the target cells by immunofluorescent
staining (data not shown). All of the cell lines tested underwent
some degree of spontaneous phagocytosis in the absence of
antibody (range, 10–53%), although ADCC was more efficient
in all cases.

Fig. 2 shows the relationship between antibody concentration
and phagocytosis for a representative IgG3 (3F8) and IgG2a
(Ab528) antibody. Since these antibodies were directed against
different antigens on different cell lines, the cytotoxicity curves
had somewhat different shapes, but both antibodies mediated
detectable phagocytosis at concentrations of 10 ng/ml or less
and reached a plateau at 100–1000 ng/ml. The other antibodies
tested gave similar results.

Cultured MDM expressed detectable levels of all three IgG
FcR: FcRI (CD64), FcRII (CD32), and low levels of FcRIII
(CD16) (Fig. 3). To determine whether the low-affinity FcR
were capable of mediating phagocytosis of tumor cells in our
system, we used an assay based on that of Graziano and Fanger
(27) in which hybridomas bearing surface antibodies directed
against Fc receptors served as targets for our phagocytosis
assay. Fig. 4 shows that hybridomas bearing antibody directed
against FcRII and FcRIII were efficiently phagocytosed, com-
pared to hybridomas against irrelevant antigens. To minimize
non specific phagocytosis of target cells via adhesion molecules
other than Fc receptors, we performed some of these experi-
ments (including the one shown in the Fig. 3) using effector
cells obtained from a patient congenitally deficient in the
CD11/CD18 family of adhesion molecules. Both normal and
CD18-deficient MDM showed efficient phagocytosis of hybrid-
omas directed against FcRII or FcRIII but not against CD3,
CD14, or CD11b.

We next attempted to inhibit antibody-dependent phagocy-
tosis by soluble antibodies against the various Fc receptors. We
first tested FcRI in isolation. Although this receptor is normally
occupied in vivo by serum immunoglobulin, after 10 days of
culture in our serum-free system it is unoccupied. We found

that phagocytosis was not significantly inhibited by human
serum, purified human IgG (up to 100 µg/ml), irrelevant murine
IgG2a, or IgG3 antibodies (100 µg/ml), or Ab197 [which blocks
FcRI function both by occupying the antibody-binding site and
by cross-linking the receptor and causing it to modulate off the
cell surface (25)]. Since all of these treatments effectively block
FcRI, our findings suggested that binding of anti-tumor anti-
body to FcRI was not obligatory for phagocytosis. Therefore,
in order to more accurately simulate the in vivo role of FcRII
and FcRIII, we selectively blocked FcRI with Ab197 in all subsequent experiments. As shown in Fig. 5, when FcRI was
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data are consistent with the hypothesis that tumor cell phago-
cytosis can proceed via either FcRII or FcRIII independently,
as suggested by the hybridoma cell experiments above.
capable of mediating phagocytosis during ADCC.

As we expected from the existing studies of macrophage phagocytosis, we found that phagocytosis of tumor cells required both a functioning cytoskeleton and the presence of divalent cations. Fig. 7 shows that cytochalasins B and D inhibited phagocytosis, as did treatment with EDTA, and that the effect of EDTA could be overcome by the readdition of either calcium or magnesium ions.

DISCUSSION

Anti-tumor ADCC by MDM is an efficient form of cytotoxicity. On a cell for cell basis, it is 10- to 100-fold more efficient than ADCC by lymphocytes, granulocytes, or lymphokine-activated killer cells using the same targets and antibodies (28, 29). We have previously shown that phagocytosis of intact tumor cells is the principal mechanism of ADCC by MDM in our system (5). In that study and in the present one, we demonstrated that antibody-dependent phagocytosis occurs with a variety of human tumor cell types and can be mediated by murine monoclonal antibodies of the IgG2a and IgG3 subclasses, as well as mouse-human chimeric antibodies of the IgG1 subclass. However, some forms of ADCC by MDM have been reported to be inhibited by human serum (30, 31). Presumably this reflects an obligate involvement of FcRI, since that receptor is competitively inhibited by serum immunoglobulin (32). Obviously, any mechanism which is abrogated by human serum would be of questionable significance in vivo. Thus it was important to ascertain the role of the low-affinity FcR in our system, since they are not inhibited by monomeric IgG.

Anderson et al. (19), using ox erythrocytes coated with anti-FcR antibodies, showed that all three FcR-γ are capable of mediating macrophage phagocytosis of RBCs. This excellent study, although providing conclusive evidence that each FcR can trigger a pathway leading to phagocytosis, leaves several questions unanswered where human tumor cells are concerned. It is not clear that phagocytosis of tumor cells occurs via the same mechanisms operative with erythrocytes. For example, Anderson et al., in the report cited, found that granulocytes, fresh monocytes, and MDM were all capable of phagocytosing RBCs. In contrast, we have seen no evidence in our studies that phagocytosis of human tumor cells occurs with any effector cell other than MDM. Furthermore, ADCC against human tumor cells requires that the opsonizing antibody interact with the FcR via its Fc portion rather than through its antigen-binding site, as would be the case when using anti-FcR antibodies. Therefore we undertook the present study to establish which FcR were involved in phagocytosis of tumor cells.

We found that both of the low-affinity FcR were capable of mediating phagocytosis independently. FcRII, which has been implicated in extracellular lysis of tumor cells and phagocytosis of RBCs (19, 27), was expressed on essentially all of our cultured MDM and mediated effective tumor cell phagocytosis. The expression of FcRIII on MDM was more heterogeneous. This receptor is not present on most fresh monocytes, but it is expressed during culture in vitro (33). It is the receptor which mediates ADCC by NK cells (34). Although it is present on granulocytes, it lacks the transmembrane region found in NK cells and thus is presumably unable to participate in signal transduction (35, 36). The FcRIII on macrophages is similar to the NK cell form (36, 37) and has been implicated in the clearance of antibody-coated erythrocytes in vivo (38). Young et al. (15) reported that FcRIII and FcRi are involved in antibody-dependent extracellular cytolyis by MDM (as demonstrated by thymidine release), and Shen et al. (39) reported that FcRIII can mediate extracellular lysis of hybridoma cells by fresh monocytes. In our system, despite the relatively low level of FcRIII expression on MDM, we were able to demon-
strate a clear functional role for this receptor in tumor cell phagocytosis.

The role of FcRI in our system was less clear. This may be due in part to the fact that the antibody preparations we used to inhibit FcRII and FcRIII, since they were derived from mouse ascites, may have contained irrelevant IgG2a and IgG3 antibodies which could nonspecifically inhibit FcRI. In 6 different inhibition experiments, we observed some degree of phagocytosis apparently mediated by FcRI in 3 experiments and no detectable effect in 3 experiments. Thus the role of FcRI remains to be clarified, and further studies are in progress. However, because this receptor is normally occupied by serum immunoglobulin in vivo, its functional role was of less interest to us than that of the low-affinity receptors.

The majority of MDM produced in our model of macrophage differentiation appears competent to mediate phagocytosis of tumor cells. As with other forms of phagocytosis, there was a requirement for an intact cytoskeleton, as indicated by sensitivity to the actin-inhibiting effects of the cytochalasins (40). Colchicine, which disrupts microtubules but reportedly does interfere with neutrophil phagocytosis (41), did not inhibit phagocytosis in our system. Divalent cations were required, and our results suggest that either calcium or magnesium can serve this function. This pattern of cation requirement for phagocytosis is in contrast to that described for extracellular lysis of tumor cells by monocytes and macrophages, which has been reported to be magnesium dependent but calcium independent (42).

This study shows that antibody-dependent tumor cell phagocytosis is an efficient form of cytotoxicity, which is applicable to a variety of cell types and antibodies. It can be mediated by either of the low-affinity FcR and thus occurs despite the presence of human serum and irrelevant immunoglobulin. Macrophages may prove to be an important effecter cell in tumor immunotherapy regimens involving monoclonal antibodies.

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TUMOR CELL PHAGOCYTOSIS VIA FcRII AND FcRIII


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