

# Phagocytosis of Tumor Cells by Human Monocytes Cultured in Recombinant Macrophage Colony-Stimulating Factor

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## Summary

Macrophages and cultured human monocytes can mediate efficient antibody-dependent cytotoxicity (ADCC) against human tumor cells using monoclonal antibodies (mAbs). The mechanism of this killing is usually assumed to involve secreted factors (reactive oxygen intermediates, tumor necrosis factor, or other cytotoxic factors) leading to target cell lysis. In this study, we present evidence that phagocytosis of intact target cells is the principal mechanism of antitumor cytotoxicity in our *in vitro* model of ADCC by cultured monocytes. Human monocytes cultured in recombinant human macrophage colony-stimulating factor ingested up to 100% of fluorochrome-labeled melanoma and neuroblastoma target cells, in the presence of an appropriate antitumor mAb. Electron microscopy demonstrated phagocytosis of intact tumor cells by cultured monocytes during ADCC. All of the radionuclide in radiolabeled target cells was taken up by monocytes during phagocytosis. By preventing the release of radioisotope tracers, phagocytosis thus prevents the detection of this very efficient form of cytotoxicity by most conventional assays.

Under appropriate conditions, human monocytes are capable of differentiating *in vitro* into large, metabolically active, macrophage-like cells (1, 2). We have recently reported that such monocytes, cultured for 7–14 d in human serum or recombinant human macrophage CSF (rhM-CSF),<sup>1</sup> mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) against human neuroblastoma and melanoma cell lines (3). At that time, we observed that several commonly used isotope-release assays either significantly underestimated or entirely failed to detect this efficient form of ADCC. In this study, we investigate the hypothesis that phagocytosis might be the mechanism of antitumor ADCC by cultured monocytes, thus preventing release of radionuclide.

## Materials and Methods

**Cell Lines.** Human tumor cell lines SKMel-1 (melanoma) and SK-BR-3 (breast carcinoma) were obtained from the American Type Culture Collection, Rockville, MD. LA-N-1 (neuroblastoma) was the gift of Dr. Robert Seeger, UCLA, Los Angeles, CA. A431 (epidermoid carcinoma) was the gift of Drs. Hideo Masui and John Mendelsohn of Memorial Sloan-Kettering Cancer Center. LS180 (colon carcinoma) was obtained from Dr. Deborah Young, Genetics Institute, Cambridge, MA. All cell lines were propagated in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with

10% heat-inactivated bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin G, and 100 µg/ml streptomycin (BS-RPMI). Cell lines used in the assays were always >90% viable by trypan blue exclusion.

**mAbs.** The mAb 3F8 (murine IgG3, anti-GD2) was developed in our laboratory, and has been previously described (4). Antibody R24 (murine IgG3, anti-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. Hideo Masui and John Mendelsohn, Memorial Sloan-Kettering Cancer Center (5). NRCO4 (murine IgG3, anti-colon carcinoma) was the gift of NeoRx Corporation, Seattle, WA (6). A mouse-human chimeric antibody ch14.18 (human IgG1 Fc region, anti-GD2), derived from the anti-GD2 murine monoclonal 14.18 (7–9), was the gift of Damon Biotech, Needham Heights, MA. PE-conjugated anti-CD14 (Leu-M3) and anti-CD11b (Leu-15) were purchased from Becton Dickinson & Co. (Mountain View, CA).

**Cytokines.** rhM-CSF ( $2 \times 10^6$  U/mg) was generously supplied by Genetics Institute. It was >99% pure by PAGE, and contained <0.01 endotoxin units (EU)/ml at the working dilution used.

**Separation and Culture of Peripheral Blood Monocytes.** Our monocyte culture technique has been described previously (3). Briefly, heparinized whole blood from normal volunteer donors was centrifuged over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). The mononuclear cells were spun over a cushion of 36% Percoll (Pharmacia Fine Chemicals) to remove platelets, then enriched for monocytes by centrifugation over a single-step gradient of 47% percoll. The interface layer (>95% of the monocyte population, typically 40% pure) was suspended in BS-RPMI ( $2\text{--}2.4 \times 10^6$  cells/ml, 100 µl/well) and allowed to adhere to serum-

<sup>1</sup> Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; rhM-CSF, recombinant human macrophage CSF.

coated 96-well flat-bottomed tissue culture plates for 1–2 h. The plates were washed vigorously using a multichannel pipette. Adherent cells (>95% monocytes by flow cytometry) were cultured for 9–12 d in 150  $\mu$ l of BS-RPMI medium supplemented with 200 U/ml rhM-CSF, with changes of medium as necessary. Cell counts after culture were typically  $3\text{--}5 \times 10^4$  cells per well; >95% were monocyte/macrophages by morphology and flow cytometry. All media and additives used in monocyte culture were screened for endotoxin using a limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) and contained <0.03 EU/ml in the concentrations used.

**Cytotoxicity Assay.** Target cells (SKMel-1) were added directly to the cultured monocytes, at 1 or  $2 \times 10^4$  targets per well in 100  $\mu$ l of BS-RPMI (giving approximate E/T ratios of 4:1 and 2:1, respectively). Antitumor antibody R24 was added at a final concentration of 2  $\mu$ g/ml. After 0, 4, 8, and 24 h, the cells were detached with 2 mM EDTA (45 min at room temperature) and harvested with vigorous pipetting. Target cells were stained with antitumor antibody 3F8 F(ab')<sub>2</sub> fragment biotin conjugate plus avidin-fluorescein. Monocytes were counterstained with a cocktail of PE-conjugated antibodies (anti-CD14 plus anti-CD11b). The ratio of residual target cells (3F8<sup>+</sup>, CD14/CD11b<sup>-</sup>) to effector cells (3F8<sup>-</sup>, CD14/CD11b<sup>+</sup>) was determined by flow cytometry. This ratio was converted into an actual number of target cells using the formula: target cell number = (percent targets/percent effectors)  $\times$  effector cell number. Within a single experiment, we assumed that effector cell number was constant for each sample, since one sample represented the average of 16 wells, and all wells were seeded and cultured as identical replicates.

**Radionuclide Release Assays.** Target cells (LA-N-1) were labeled with either <sup>51</sup>Cr (100  $\mu$ Ci/10<sup>6</sup> cells) in 500  $\mu$ l medium for 1 h, or <sup>111</sup>In (25  $\mu$ Ci/10<sup>6</sup> cells) in 1 ml medium for 15 min. Labeled target cells were added to cultured monocytes along with 3F8 (2  $\mu$ g/ml) and incubated for 18 h at 37°C. The plates were centrifuged, and 100  $\mu$ l of supernatant was removed for gamma counting. Radioactive medium was washed from the wells by three cycles of adding 200  $\mu$ l fresh medium, centrifuging, and removing 200  $\mu$ l. This procedure was followed so that nonadherent cells would not be disturbed. Residual targets were lysed with 200  $\mu$ l human complement serum (diluted 1:2 in HBSS) plus excess 3F8 antibody (10  $\mu$ g/ml) for 2 h at 37°C, and 100  $\mu$ l of supernatant was removed for counting. Finally, the monocyte monolayer was washed free of residual medium, the cells were lysed with 250  $\mu$ l of 0.1% triton (Sigma Chemical Co., St. Louis, MO), and 100  $\mu$ l was removed for counting. Specific release was calculated as:  $100 \times [(\text{observed release} - \text{spontaneous}) / (\text{total} - \text{spontaneous})]$ . Total release for the 24-h ADCC assay was determined by detergent lysis of labeled target cells. Spontaneous release during ADCC was 25–40% for chromium and 5–10% for indium. Total release for the complement incubation was based on the maximum complement-releasable counts from control target cells. Total complement release was typically 80–85% of detergent-releasable counts; trypan blue dye exclusion showed that >95% of tumor cells were dead after treatment with complement. Spontaneous release during the complement lysis step was <5%.

**PKH2 Labeling.** Target cells were washed in PBS and labeled with PKH2 dye (Zynaxis Cell Science, Malvern, PA) at 2  $\mu$ M in 1 ml of the supplied diluent per  $5 \times 10^6$  cells. The labeled cells were washed three times and cultured overnight to reduce non-specific leaking of dye during the assay. Target cells were 95–99% viable after the leaking incubation. Labeled target cells were added to rhM-CSF-cultured monocytes along with antitumor mAb ( $\sim 1\text{--}2$   $\mu$ g/ml, depending on the antibody preparation), then harvested after

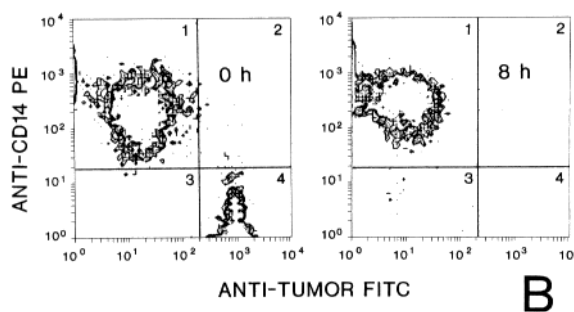
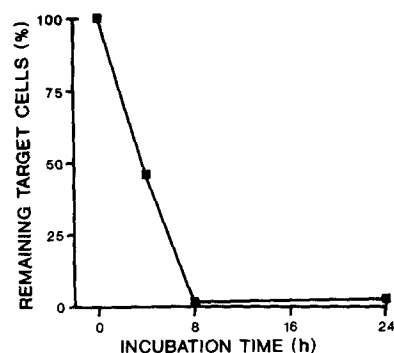
0, 4, 8, or 24 h. Monocytes were counterstained with anti-CD14/anti-CD11b PE as described above, and the cells analyzed by cytofluorometry. The number of remaining target cells was calculated using the formula: target cell number = [(percent targets + percent double positive)/(percent monocytes + percent double positive)]  $\times$  effector cell number. Target cells were defined as PKH2<sup>+</sup> and CD14/CD11b<sup>-</sup>. Monocytes were defined as CD14/CD11b<sup>+</sup> and PKH2<sup>-</sup>. Double-positive cells were counted as both a target cell and a monocyte. Percent phagocytosis was calculated as:  $100 \times [(\text{percent double positive} / (\text{percent targets} + \text{percent double positive}))]$ . To look for target cells passively bound to monocytes without undergoing phagocytosis, we viewed some of the experiments under epifluorescence illumination in a hemacytometer. Any target cell found in close apposition to a monocyte was scored as a possible conjugate.

## Results

When cultured monocytes were incubated with tumor cells in the presence of antitumor mAb, the number of cells bearing tumor-associated surface antigen declined rapidly (Fig. 1 A). Two-color staining (Fig. 1 B) showed that antigen-positive target cells were eliminated without the emergence of an antigen-negative population. The ganglioside antigens used in these assays were chosen, in part, because they showed little modulation under the influence of antibody. Once all the antigen-positive cells had been eliminated, there were no viable tumor cells detectable using a modified clonogenic assay (data not shown), confirming that tumor cytotoxicity was complete.

Although the target cells were efficiently eliminated by ADCC, we observed no release of radioactive chromium or indium during the process. To determine the fate of the unreleased tracer, we incubated radiolabeled tumor cells with cultured monocytes and antibody for 24 h, then selectively lysed any remaining target cells with complement. After ADCC, <2% of the original radionuclide was recoverable by complement lysis, even though none had been released during ADCC (Fig. 2 B). All of the sequestered tracer was recovered when the monocyte monolayer was solubilized with detergent. In contrast, radionuclide from control target cells incubated with monocytes but without antibody was >95% complement releasable (Fig. 2 A). Our interpretation of these data was that the tracer had been phagocytosed by the monocytes during ADCC, rendering it immune to complement but releasable by detergent. An alternative explanation of the data might be that the target antigen had modulated off the cell surface during ADCC, thus preventing fixation of complement. Experiments such as the one shown in Fig. 1 B proved that this was not the case. To determine whether the uptake of radionuclide occurred before or after target cell death, we released the chromium from labeled target cells by freeze-thawing, then incubated the cell lysate with cultured monocytes. Under these conditions, free radionuclide was not taken up at all. Therefore, we concluded that the monocytes were not lysing the target cells extracellularly and subsequently ingesting the liberated tracer.

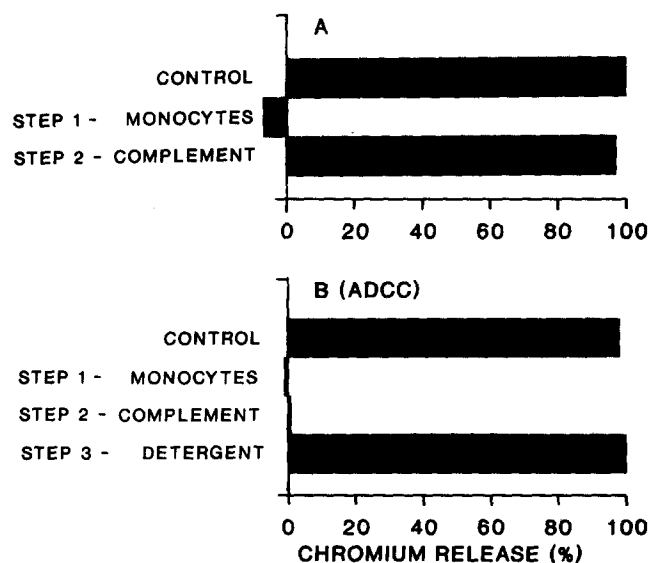
Transmission electron micrographs of cultured monocytes and tumor cells during ADCC showed intact target cells within monocyte phagosomes (Fig. 3). To quantitate this phenomenon, we prelabeled target cells with the fluorochrome



**Figure 1.** Disappearance of tumor-associated antigen during ADCC by cultured monocytes. Human monocytes were cultured for 11 d in rhM-CSF, as described in Materials and Methods, then SKMel-1 melanoma target cells and antitumor antibody (R24) were added. (A) After 0, 4, 8, and 24 h, the cells were harvested, and target cells were stained with fluorescein-linked antitumor antibody 3F8. Monocytes were counterstained with anti-CD14 and anti-CD11b PE, and the number

of surviving target cells (expressed as a percentage of the starting number of target cells) was determined by flow cytometry. (B) Two-color contour plots of the cells at the start of incubation (0 h), and after 8 h in the presence of opsonizing antibody. After ADCC, the target cell population disappeared from region 4, and no population of antigen-negative target cells emerged in region 3. Data are from one of six similar experiments; E/T ratio, 2:1.

PKH2, a green-fluorescent vital dye that binds tightly to cell membranes and shows little transference from cell to cell (10). Monocytes were counterstained with anti-CD14/anti-CD11b PE. During ADCC, dye-labeled target cells were rapidly taken up by cultured monocytes, often retaining their shape for a period of time after phagocytosis (Fig. 4).



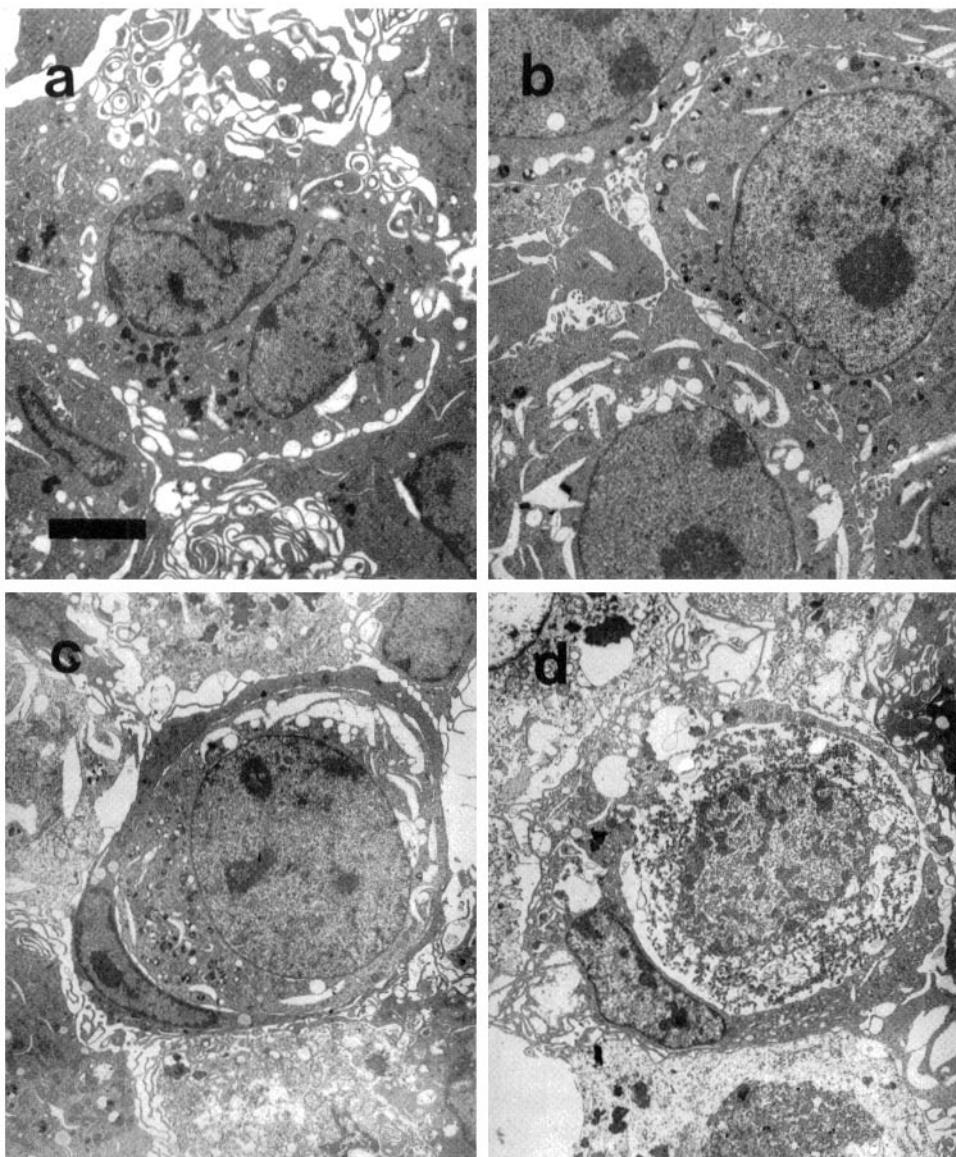
**Figure 2.** Internalization of  $^{51}\text{Cr}$  by cultured monocytes during ADCC. Human monocytes, cultured as in Fig. 1, were incubated with  $^{51}\text{Cr}$ -labeled neuroblastoma cells (LA-N-1), either without (A) or with (B) antitumor antibody 3F8. Control target cells were incubated without monocytes. After 24 h, the supernatant was removed for gamma counting, and the cell monolayers were washed to remove free  $^{51}\text{Cr}$ . The plates were centrifuged before and after washing to retain nonadherent cells. Residual target cells were lysed with human complement and additional antibody. Target cells were equally sensitive to lysis by complement, regardless of exposure to antibody during the 24-h incubation, as demonstrated by the control bars in both figures. After complement lysis, the monocyte monolayer in B was solubilized with detergent to release ingested chromium. The bars show specific release of radionuclide for each successive treatment. Data are from one of four experiments using  $^{51}\text{Cr}$ , and two experiments using  $^{111}\text{In}$ , all with similar results; E/T ratio, 4:1.

Two-color cytofluorometry of PKH2-stained cells (Fig. 5) demonstrated that during ADCC the target cell population (region 4) progressively disappeared, while a new population of PKH2<sup>+</sup> monocytes emerged. These two populations represent, respectively, the unphagocytosed and phagocytosed target cells illustrated in Fig. 4, A and B. Because it was possible that antibody-coated tumor cells might bind to macrophages without undergoing phagocytosis, we also examined the stained cells under a fluorescence microscope, looking for the formation of monocyte-target cell conjugates. We found <1% of target cells bound to monocytes after the vigorous washing steps used in the staining process. The time course for the disappearance of PKH2-labeled target cells (Fig. 5 C) was similar to that seen for the elimination of cells bearing tumor-associated antigen (shown in Fig. 1 A).

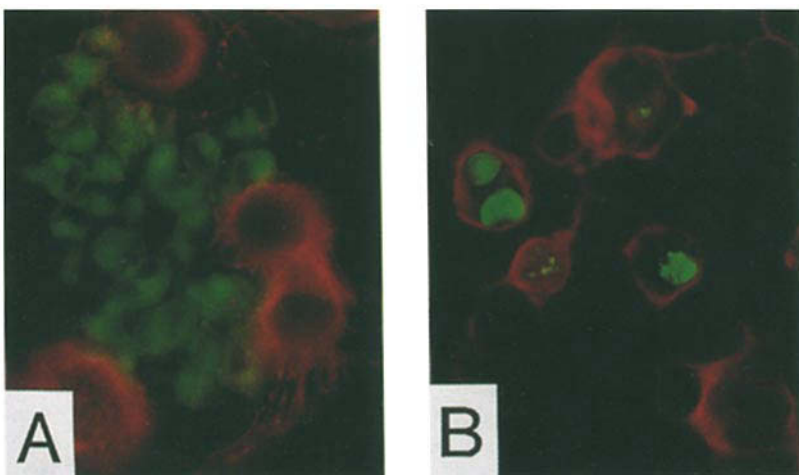
We observed antitumor phagocytosis with a variety of cell lines. Table I presents data from representative experiments using five different human tumor cell lines, and mAbs of the murine IgG3 and IgG2a and human IgG1 subclasses. Phagocytosis was measured as uptake of PKH2-labeled target cells, as shown in Fig. 5. These data are taken from several experiments using different lots of effector cells and various E/T ratios. They are included to show that the phenomenon of phagocytosis is not a restricted one, but are not intended to provide a comparison of relative sensitivity to ADCC.

## Discussion

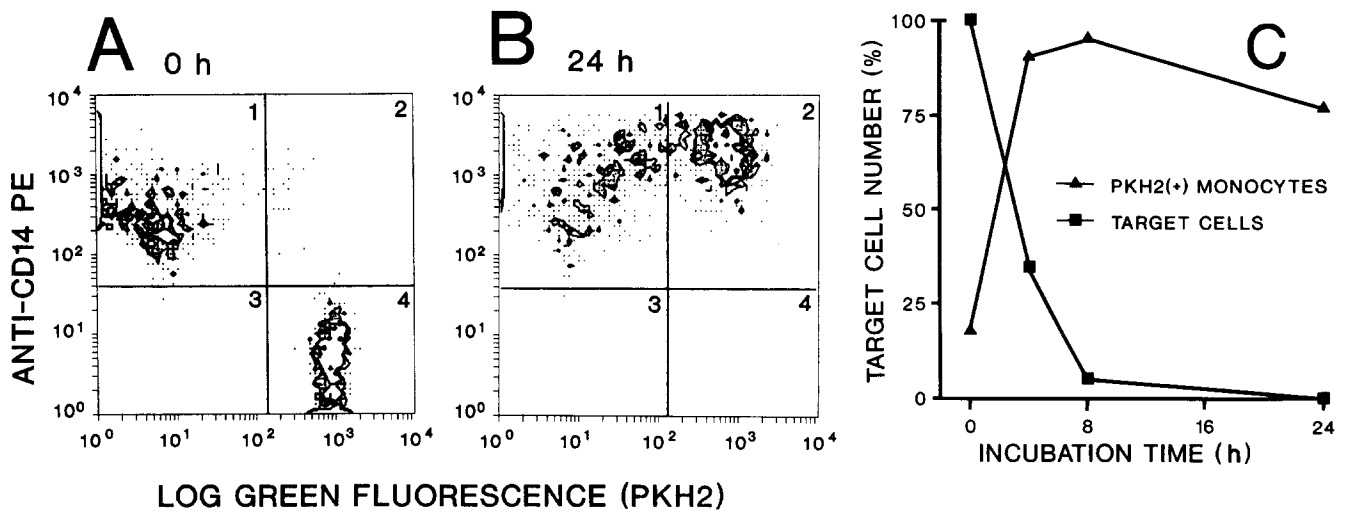
Several mechanisms of monocyte/macrophage antitumor cytotoxicity have been described. The most extensively studied of these have been the release of reactive oxidative intermediates (11-14), secretion of TNF (15-17), and elaboration of other soluble cytotoxic factors (18-21). Like many human tumor cell lines (22-24), the target cells used in our assays were relatively insensitive to both oxidative killing and TNF ( $\text{LD}_{50}$  for  $\text{H}_2\text{O}_2$ , >1,000  $\mu\text{M}$  as measured by  $^{51}\text{Cr}$  release;  $\text{LD}_{50}$  for TNF, >1,000 U/ml as measured by 48-h in vitro proliferation assay). In previous studies of bystander cell killing during ADCC, we had found no evidence of any secreted cytotoxic factor in our system (3). Thus, we turned to phagocytosis



**Figure 3.** Phagocytosis of antibody-coated tumor cells by cultured monocytes. Electron micrographs of cultured monocytes and melanoma target cells after 4-h co-incubation in the presence of antibody, as described in Fig. 1. (A) Cultured monocytes (bar, 5  $\mu\text{m}$ ); (B) tumor cells; (C) tumor cell inside monocyte phagosome; (D) cytolysis of phagocytized tumor cell.



**Figure 4.** Phagocytosis of fluorochrome-labeled target cells. Monocytes cultured in rhM-CSF were incubated for 8 h with neuroblastoma cells and antitumor antibody 3F8, as in Fig. 1. The target cells had been prelabeled with the fluorescent dye PKH2 (green); monocytes were counterstained with anti-CD14/anti-CD11b PE (red). (A) Control, no antibody; (B) with antibody.



**Figure 5.** Time course of tumor cell phagocytosis. PKH2-labeled neuroblastoma cells were incubated with cultured monocytes and antibody as described in Fig. 4, then harvested with EDTA. Monocytes were stained with anti-CD14/anti-CD11b PE. (A) At the start of incubation, region 1 contains the monocyte population and region 4 contains the unphagocytosed target cells. (B) After 24 h, phagocytosed target cells (as pictured in Fig. 4 B) are seen in region 2. (C) Total number of unphagocytosed target cells (squares) and PKH2<sup>+</sup> monocytes (triangles) over the course of a 24-h ADCC incubation. These data are from one of four similar experiments; E/T ratio, 2:1. In some experiments, samples were also scored for the formation of monocyte–target cell conjugates by fluorescence microscopy (<1% of target cells were found passively bound to monocytes).

as a potential mechanism of ADCC by rhM-CSF-cultured monocytes.

Antibody-directed phagocytosis is an important function of monocytes and macrophages. Ingestion of opsonized microorganisms, red cells, and platelets by macrophages have all been extensively studied. It is thus somewhat surprising that the role of phagocytosis in antitumor cytotoxicity has received relatively little attention. The phenomenon was well described 25 yr ago by Bennett et al. (25, 26), using mouse peritoneal macrophages and polyclonal antisera. However, since that time, we are aware of only three studies in the literature dealing with macrophage phagocytosis of malignant cells (27–29). Recent excellent and extensive reviews of macrophage antitumor cytotoxicity (30, 31) do not mention phagocytosis as a potential mechanism for ADCC, nor does a 1985 workshop report on assays for monocyte cytotoxicity (32).

In the present study, our results support the hypothesis that phagocytosis of intact target cells is the principal mechanism of *in vitro* antitumor ADCC by rhM-CSF-cultured monocytes. While it is quite possible that other, nonphagocytic (i.e., extracellular) mechanisms also operate, their contribution would appear to be minor in our system. This is shown by the isotope release assays, which indicate that few target cell deaths occur extracellularly, since very little radioisotope is released during target cell killing. If tumor cells were being killed or injured before phagocytosis, we should expect to see evidence of chromium release, especially since chromium once liberated from target cells is not taken up by cultured monocytes.

Tumor cell phagocytosis during ADCC is not a restricted phenomenon in our system. We observed phagocytosis, as demonstrated by uptake of fluorescent-labeled target cells, with

**Table 1.** Phagocytosis of Various Tumor Cell Targets Using Opsonizing Antibodies of Different Human and Murine Subclasses

Target cell	Tumor type	Antibody	Fc subclass	E/T	Phagocytosis
					%
LA-N-1	Neuroblastoma	3F8	Murine IgG3	4:1	92
SKMel-1	Melanoma	ch14.18	Human IgG1	2:1	55
LS180	Colon	NRCO4	Murine IgG3	1:1	44
A431	Epidermoid carcinoma	528	Murine IgG2a	4:1	89
SK-BR-3	Breast	528	Murine IgG2a	4:1	65

Human tumor cell lines were labeled with PKH2 dye and incubated for 24 h with cultured monocytes and antitumor antibody, as described in Materials and Methods. Phagocytosis was quantitated by two-color flow cytometry, using the definitions for phagocytosed (double positive) and unphagocytosed target cells established in Fig. 5. Percent phagocytosis was calculated as the ratio of phagocytosed target cells to total target cells. The E/T ratio is a measured value based on the relative number of targets and effectors after the 24-h co-incubation.

a variety of tumor types. Antibodies of the murine IgG2a and IgG3 subclasses, and a chimeric antibody with a human IgG1 Fc portion, were all able to direct efficient phagocytosis. Thus far, we have tested 26 cell lines and have seen antibody-dependent and/or antibody-independent phagocytosis in all of them (manuscripts in preparation).

We have demonstrated that cytotoxicity mediated by phagocytosis cannot be accurately measured by methods that rely on radionuclide release. In a limited number of experiments, we have seen some release of thymidine with selected cell lines, but indium and chromium release have consistently been negative. Other laboratories have used isotope-release assays and have reported seeing varying degrees of cytotoxicity. Several factors may contribute to the difference between our results and theirs. First, our system for culturing monocytes produces large, highly cytotoxic effector cells. With fresh monocytes, or monocytes cultured under other conditions, phagocytosis might play a less prominent role. Second, monocytes and macrophages stimulated with certain agents (e.g., lipopolysac-

charide or phorbol ester) may preferentially release cytotoxic substances that cause target cell lysis, rather than killing by phagocytosis. Third, if target cells are chosen that are particularly sensitive to extracellular lysis (e.g., by oxidative intermediates or TNF), the contribution of these mechanisms may appear larger. Finally, the cytotoxicity reported by others has usually been substantially less than we have observed, and the E/T ratio used generally much higher. It is possible that under these conditions, isotope release may indeed occur, but may be reflective of only a fraction of the actual cytotoxicity.

The degree to which our system of monocytes cultured in rhM-CSF reflects *in vivo* macrophage differentiation and cytotoxicity is not clear, but there is some evidence to suggest that cultured monocytes may be a reasonable *in vitro* model of macrophage differentiation (1, 2, 33, 34). If this is true, then the role of phagocytosis in macrophage antitumor cytotoxicity may prove to be more significant than has been previously thought.

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