

ANTIBODY-DEPENDENT ANTITUMOR CYTOTOXICITY BY
HUMAN MONOCYTES CULTURED WITH RECOMBINANT
MACROPHAGE COLONY-STIMULATING FACTOR

Induction of Efficient Antibody-mediated Antitumor Cytotoxicity
not Detected by Isotope Release Assays

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Macrophage colony-stimulating factor (M-CSF)¹ is one of several cytokines that control the differentiation of monocytes and macrophages. In human and murine bone marrow cultures it stimulates the proliferation and differentiation of macrophage progenitors (1, 2), and it has been shown to exert a similar effect on murine pulmonary macrophages in vitro (3). Murine peritoneal macrophages exposed to M-CSF exhibit increased cytotoxicity against certain tumor cell targets, especially in the presence of other T cell-derived factors (4-6), and undergo morphologic and biochemical alterations indicative of cellular activation (7-9).

Macrophages possess the ability to kill certain tumor cells in vitro, and cytotoxicity is enhanced by activation with endotoxin, IFN- γ or other cytokines (reviewed in reference 10). It has been more difficult to demonstrate significant antitumor cytotoxicity in fresh unstimulation monocytes, but granulocyte/macrophage CSF (GM-CSF) (11) and M-CSF (12) have been reported to induce tumor cell killing in monocytes after a brief culture period. When cultured with human serum (13-15) or cytokines (16, 17), monocytes undergo in vitro differentiation into large, macrophage-like cells. Both macrophages and monocytes are capable of mediating antibody-dependent cellular cytotoxicity (ADCC) using murine mAbs against human tumor cells (18-23). One possible mechanism of macrophage ADCC is phagocytosis (24, 25). Since most conventional cytotoxicity assays rely on the release of an intracellular radioisotope after target cell death or injury, it is possible that phagocytosis, by masking this isotope release, may cause such assays to underestimate cytotoxicity. In this study we investigated the ability of human monocytes cultured in M-CSF to mediate antitumor ADCC using a novel colorimetric assay unaffected by the mechanism of cell killing.

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¹ *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; GM-CSF, granulocyte/macrophage colony-stimulating factor; G_{D2}, disialoganglioside G_{D2}; M-CSF, macrophage CSF; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium.

Antiganglioside mAbs were used against human neuroblastoma and melanoma cell lines.

Materials and Methods

Cell Lines. SKMel-1 and SKMel-28 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). SKMel-31 was provided by Dr. Alan Houghton, Memorial Sloan-Kettering Cancer Center. LAN-1 was obtained from Dr. Robert Seeger, and M-14 from Dr. Reiko Irie, both of UCLA, Los Angeles, CA. LA1-15n was provided by Dr. June Biedler, Memorial Sloan-Kettering. Cell lines were propagated in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin G, and 100 μ g/ml streptomycin (BS-RPMI). Cell lines used for in vitro assays were >90% viable by trypan blue exclusion.

Monoclonal Antibodies. The murine mAb 3F8 (IgG₃, anti-G_{D2}), developed in our laboratory, has been previously described (26). F(ab)₂ fragments were prepared from purified 3F8 as described (27). R24 (IgG₃, anti-G_{D3}) was the generous gift of Dr. Alan Houghton, and the antibody 10-11 (anti-G_{M2}) was the gift of Dr. Philip Livingston, Memorial Sloan-Kettering. HSAN1.2 was the gift of Dr. Patrick Reynolds, UCLA. Other antineuroblastoma antibodies derived in our laboratory include 3A5 (IgG_{2b}), 5F7 (IgG₃), both against a glycolipid antigen other than G_{D2} and 5F11 (IgM anti-G_{D2}). 3G8 (anti-CD16) was generously provided by Dr. Jay Unkeless, Mt. Sinai Medical School, New York, NY. Leu19 and FITC-conjugated Leu11 (anti-CD16) were purchased from Becton Dickinson & Co., (Mountain View, CA). Antimonocyte antibody MY4 (anti-CD14) was generously provided by Dr. James Griffin, Harvard Medical School, Boston, MA. Anti-Ia (clone L243) was obtained from ATCC.

Cytokines. Recombinant human M-CSF (8×10^5 U/mg) was generously supplied by Genetics Institute, Cambridge, MA. Endotoxin (purified *Escherichia coli* LPS) was obtained from Sigma Chemical Co. (St. Louis, MO).

Separation and Culture of Peripheral Blood Monocytes. Blood was obtained from healthy volunteer donors, anticoagulated with 20 U/ml heparin, and centrifuged on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) for 20 min at 200 *g*. The mononuclear cell fraction was washed twice, and spun over a cushion of 36% Percoll (Pharmacia Fine Chemicals) in HBSS at 400 *g* for 5 min to remove platelets. The cells were enriched for monocytes using a modification of the method of Gmelig-Meyling and Waldmann (28), by centrifugation over a single-step gradient of 47% percoll at 1,000 *g* for 20 min. The interface layer contained >95% of the starting monocyte population, and was typically 40% monocytes by size and granularity on flow cytometry. These cells were suspended in BS-RPMI and allowed to adhere for 1–2 h in 96-well flat-bottomed tissue culture plates (Falcon Labware, Oxnard, CA) at a concentration of 1.6×10^5 cells per well. The plates were washed vigorously in RPMI plus 0.1% human serum albumin using a multichannel pipette. Typically, $3\text{--}4 \times 10^4$ cells (about half of the starting monocyte population) remained adherent after washing, as determined by flow cytometry on the nonadherent cells and enumeration of adherent cells detached with EDTA. The adherent cells were >95% monocytes by morphology using Wright's stain. Cell counts after activation were typically $3\text{--}5 \times 10^4$ cells per well, and all cells were monocyte/macrophages by morphology.

Adherent cells were cultured for 9–12 d in 150 μ l of medium: either 10% BS-RPMI supplemented with growth factors, or 20% human serum in RPMI. Human serum was obtained from volunteer donors, allowed to clot at 37°C for 30 min, centrifuged, and frozen at –70°C until used. Medium containing human serum was changed every 3–4 d. All media and factors used in the culture of monocytes 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.

ELISA for Cytotoxicity. Target cells were added directly to the 96-well plates containing cultured monocytes at 0.5, 1, 2, or 4×10^4 targets per well in 100 μ l of BS-RPMI (giving nominal E/T ratios of 8, 4, 2, and 1 respectively). Antibody (or medium) was added in 25 μ l of BS-RPMI. For 24-h assays, antibody was added at the beginning of the assay. For 72-h

assays, the target cells were allowed to adhere overnight before the addition of antibody, since G_{D2} and G_{D3} may play a role in the attachment of some cell lines (29). Control wells receiving antibody but no monocytes typically had 5–20% fewer cells at the end of 72 h, when compared with targets incubated in medium alone. To adjust for any antiproliferative effects of antibody, all cytotoxicity data were calculated based on parallel controls containing target cells cultured under identical conditions of antibody, medium, and cytokines. After incubation, the plates were centrifuged at 400 g for 5 min to recover any detached cells, the supernatant was aspirated, and the plates were air-dried and fixed with glutaraldehyde (1% vol/vol in PBS) for 5 min. After thorough washing with PBS using an automated plate washer (Dynatech Laboratories, Alexandria, VA), the plates were incubated with 10% BS in PBS for 1 h at 37°C to block nonspecific binding of antibody, then washed and incubated with an mAb specific for the target cells. For SKMel-1 and SKMel-28, the primary antibody was R24 (IgG₃, anti- G_{D3}) at a 1:500 dilution of ascites; for the other targets, 3F8 (IgG₃, anti- G_{D2}) was used at 2 μ g/ml in BS-PBS. After washing, peroxidase-conjugated goat anti-mouse IgG₃ (Fisher-Biotech, Pittsburgh, PA) was added in BS-PBS, and bound peroxidase was measured as previously described (26). Optical density (OD) was read as absorbance at 490 nm using a Dynatech automated plate reader (UV-700) connected to an IBM-AT computer. For every data point, two parallel controls were performed: OD of tumor targets cultured without effector cells, and OD of effector cells cultured without targets (typically <5% of the signal from targets alone). The percentage of surviving target cells was calculated using the formula: Percent surviving = $100 \times \{[\text{OD}(\text{targets} + \text{effectors}) - \text{OD}(\text{effectors alone})] / [\text{OD}(\text{targets alone})]\}$. This was converted to percent cytotoxicity using the formula: percent cytotoxicity = $100\% - \text{percent surviving targets}$. Determinations were performed in duplicate and the mean was reported.

Radionuclide Release Assays. Target cells were labeled with either ^{51}Cr (100 $\mu\text{Ci}/10^6$ cells) in 500 μl medium for 1 h, or [^3H]TdR (50 $\mu\text{Ci}/10^6$ cells) in 10 ml medium for 18 h. Labeled target cells were washed and added to M-CSF-cultured monocytes as described above, and incubated for 24 h at 37°C. The plates were centrifuged and 100 μl of supernatant was removed for counting in either a gamma counter (^{51}Cr) or liquid scintillation counter (^3H). Total release was determined by lysing labeled tumor cells with 0.1% Triton (Sigma Chemical Co.); background release was measured using target cells without effectors. Cytotoxicity was calculated using the formula: Percent cytotoxicity = $100 \times \{[\text{cpm}(\text{targets} + \text{effectors}) - \text{cpm}(\text{targets alone})] / [\text{cpm}(\text{triton treated targets}) - \text{cpm}(\text{targets alone})]\}$.

Proliferation Assays. Target cells, antibody, and M-CSF-cultured monocytes were co-incubated for 72 h as described above, then detached with 2 mM EDTA (30 min, at 37°C) diluted 1:64 in fresh medium, and allowed to proliferate for 8–10 d (until the control wells had reached confluent tumor growth). As controls, targets were incubated with 3F8 alone or with effectors alone, then treated as above. Plates were screened for proliferating target cells using the MTT-formazan reduction assay described by Mosmann (30). The wells were also examined for individual tumor colonies, which were readily visible using a phase-contrast inverted microscope.

Immunofluorescence and Flow Cytometry. M-CSF-cultured monocytes were co-incubated with target cells and 3F8 antibody as described, then detached with 2 mM EDTA. With vigorous pipetting all the cells detached and were >90% viable by trypan blue exclusion. The cell suspension was stained with a mixture of five mAbs to neuroblastoma (5F7, 5F11, 10-11, 3A5, and HSA1.2). Five controls were cultured and stained in identical fashion: macrophages alone (incubated with and without antibody), target cells alone (with and without antibody), and targets plus macrophages without antibody. After washing, the cells were incubated with biotinylated goat anti-mouse IgG (ICN Immunobiologicals, Lisle, IL), followed by avidin-phycoerythrin (Becton Dickinson & Co.). Monocytes were counterstained with Leu11-FITC. (The target cell lines used were unreactive with Leu11-FITC.) Stained cells were analyzed using a Becton Dickinson & Co. FACScan flow cytometer.

Cultured monocytes were also stained with mAbs Leu19, OKM1, L243, 3G8, and MY4, followed by FITC-conjugated goat anti-mouse IgG. Irrelevant antibodies (IgG₁) were used as negative controls.

Results

Radionuclide-release Assays Underestimate Cytotoxicity by Cultured Monocytes. Neuroblastoma and melanoma target cells growing in tissue culture can be distinguished from cultured monocytes on the basis of morphology. In preliminary experiments, visual inspection with phase contrast microscopy had suggested that target cell eradication was nearly complete after co-incubation with cultured monocytes and antitumor antibody (3F8). However, this degree of cytotoxicity was not evident when two conventional radionuclide-release assays (^3H]TdR and ^{51}Cr) were used to detect target cell lysis. Fig. 1 shows parallel 18-h ADCC assays using both methods on two different target cell lines. The effector cells were the same in all assays (monocytes cultured for 10 d in 100 U/ml M-CSF), as was the antibody (3F8). The chromium release assay did not show significant killing of either target cell line, and the thymidine release assay gave inconsistent results depending on the target cell line used. However, when we assayed for surviving tumor cells using a novel ELISA method, we found a level of cytotoxicity that correlated well with visual inspection, but that was much higher than predicted by either isotope release method (also shown in Fig. 1).

The technique used to detect surviving target cells was a variation of the conventional ELISA, with surviving tumor cells (glutaraldehyde fixed) used as the adsorbed antigen, and tumor-specific anti- $\text{G}_{\text{D}2}$ or anti- $\text{G}_{\text{D}3}$ mAbs used as the primary antibody (see Materials and Methods). Due to the fact that cultured monocytes showed high nonspecific binding of most antibody subclasses, we found that only IgG_3 antibodies worked satisfactorily in our assay. Our most frequently used target (SKMel-1) expressed both $\text{G}_{\text{D}2}$ and $\text{G}_{\text{D}3}$ on >96% of cells, so the ADCC (co-incubation) assay could be performed using 3F8 (anti- $\text{G}_{\text{D}2}$) antibody, and the ELISA using an unrelated anti- $\text{G}_{\text{D}3}$ antibody (R24). Flow cytometry on SKMel-1 and other melanoma and neuroblastoma cell lines had previously shown identical patterns of reactivity with both antibodies after 72 h of culture alone or with either antibody, indicating that these antigens did not modulate or comodulate in the presence of antibody. Our other target cell lines expressed primarily either $\text{G}_{\text{D}2}$ or $\text{G}_{\text{D}3}$ alone, so the same antibody was used for both ADCC and ELISA. Since no modulation of these antigens was observable by flow cytometry, and no population of antigen-negative cells emerged after exposure to antibody, we considered this approach acceptable. (Further proof that our ELISA method actually reflected target cell death rather than antigen modulation was provided by the flow cytometry and tumor outgrowth experiments described below.)

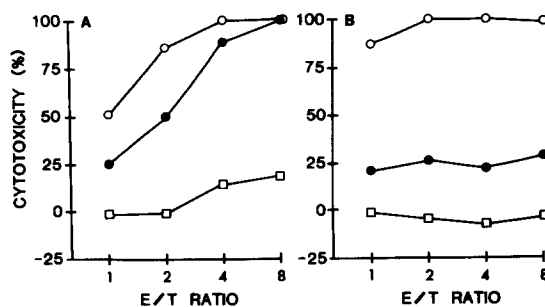


FIGURE 1. Underestimation of ADCC by radionuclide release assays. Human peripheral blood monocytes were cultured for 11 d in minimal medium with 100 U/ml M-CSF, then used as effector cells in one of three parallel ADCC assays: ELISA (open circles), thymidine release (solid circles), or chromium release (open squares). All assays contained 3F8 antibody (2 $\mu\text{g}/\text{ml}$) and were incubated for 18 h. Cytotoxicity was calculated as described in Materials and Methods. (A) SKMel-31 target cells; (B) SKMel-1 target cells.

The ELISA typically showed a 5–20% variation in cell number between antibody-treated and untreated cells in the absence of effector cells; this was attributable to a mild growth-inhibitory effect of antibody (especially 3F8) on some target cell lines. To control for this variation we always calculated cytotoxicity relative to target cells incubated under identical conditions of antibody, medium, and M-CSF concentration. There was close agreement between measurements of target cell number by ELISA and by MTT reduction ($r = 0.997$), even after 72 h incubation with antibody. The ELISA could reliably detect $2\text{--}4 \times 10^2$ tumor cells per well, while control wells typically contained $2\text{--}8 \times 10^4$ tumor cells after 72 h. Thus, 1% surviving target cells (99% cytotoxicity) could be detected by our ELISA method.

To prove that cultures without detectable antigen were truly free of tumor cells, we performed the experiment shown in Fig. 2. Neuroblastoma cells and cultured monocytes were co-incubated for 72 h, harvested with EDTA, and stained with a mixture of 5 antibodies against neuroblastoma (see Materials and Methods). Monocytes were identified with Leu11 (anti-CD16). After co-incubation without antibody (E/T ratio 4:1), 19% of the combined cell population bore tumor surface antigens; when 3F8 was present during the incubation <2% of cells bore tumor markers (the limits of detection for this assay). Identical results were obtained when 3F8 alone was used to identify tumor cells, instead of the antibody cocktail.

For tumor outgrowth experiments, we performed an ADCC assay as described above, then harvested the cells with EDTA, diluted them 1:64 in fresh medium, and allowed residual target cells to proliferate for 8–10 d. By this point the two controls

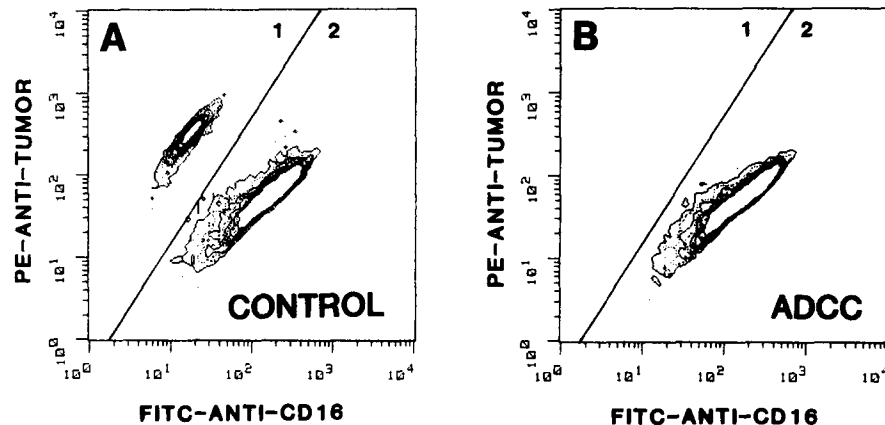


FIGURE 2. Two-color immunofluorescent staining and flow cytometry demonstrate the absence of tumor cells after ADCC by M-CSF-cultured monocytes. Human peripheral blood monocytes were cultured for 10 d in minimal medium with 100 U/ml M-CSF. Neuroblastoma target cells (LA1-15n, E/T - 4:1) were added and co-incubated for 72 h, either without (A) or with (B) 2 $\mu\text{g}/\text{ml}$ of 3F8 antibody. As controls, target and effector cells were cultured separately under identical conditions. All cells were harvested with EDTA and stained with a mixture of five antitumor antibodies indirectly linked to PE. Macrophages were counterstained with Leu11 (anti-CD16)-FITC. The regions as drawn contained 98% of the tumor cell controls in region 1 (whether cultured with or without 3F8), and 98% of macrophage controls in region 2. (A) After co-cultivation without 3F8, 19% of the cells harvested fell within region 1. When 3F8 was present during the co-incubation <2% of cells fell in region 1 (B). The figure shows one of three similar experiments using two different cell lines.

(target cells incubated with antibody but without effector cells, and target cells incubated with effectors but without antibody) had grown to confluence, while no tumor cell colonies had grown from the wells originally containing tumor cells, monocytes, and antibody. These experiments suggest that the absence of detectable tumor-associated antigens in our assay is reliable evidence that viable tumor cells have been eliminated.

M-CSF Supports Survival and Differentiation of Monocytes in Minimal Medium. Monocytes cultured in human serum underwent the morphologic changes characteristic of *in vitro* differentiation (13–15); they became large, spreading granular cells. Metabolic activity, as measured by mitochondrial reduction of MTT, increased steadily over the period of culture. Monocytes cultured in human serum usually showed some ADCC against tumor targets, but there was substantial variation in ADCC activity between lots of human serum. The effects of recombinant M-CSF on cultures containing human serum was difficult to assess given this variability, but with repeated experiments a pattern of enhancement by M-CSF, over and above the effects of serum alone, became apparent. To observe the action of M-CSF on monocyte ADCC in isolation from the unpredictable effects of human serum, we used a minimal medium consisting of 10% heat-inactivated bovine serum in RPMI 1640.

Figure 3 shows that, by itself, this medium did not support metabolic activation in culture or the development of ADCC; in fact, most monocytes were dead by day 12 in bovine serum alone. However, when supplemented with 100 U/ml of recombinant M-CSF this medium permitted reproducible survival and differentiation of monocytes. Fig. 3 *A* compares MTT reduction by monocytes cultured in human and bovine serum, with or without added M-CSF. The increased metabolic activity with human serum seen in this experiment was a reproducible feature of these cultures, but did not correlate with ADCC activity. Fig. 3 *B* shows parallel experiments (using the same effector cells) measuring ADCC against SKMel-1. The figure shows

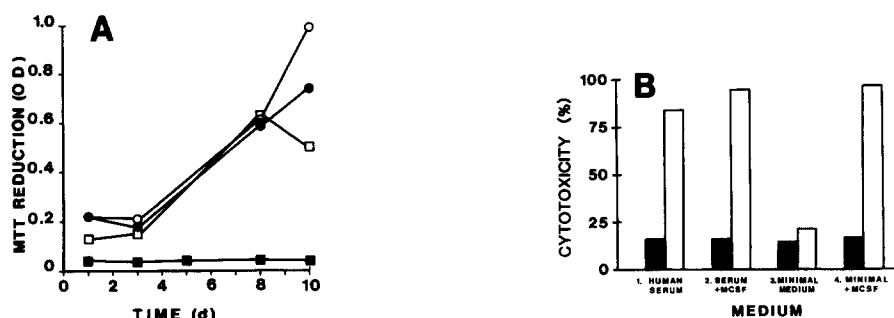


FIGURE 3. Effects of *in vitro* culture on metabolic activity and ADCC by human monocytes. (A) Human peripheral blood monocytes were cultured for 2–10 d in either 20% human serum or minimal medium, with and without 100 U/ml M-CSF. Replicate cultures were assayed on days 1, 3, 8, and 10 for metabolic activity by MTT reduction. (Open circles) Human serum with M-CSF; (solid circles) human serum alone; (open squares) minimal medium with M-CSF; (solid squares) minimal medium alone. (B) Monocytes were cultured as above for 10 d, then assayed for cytotoxicity against SKMel-1 (E/T = 1:1) as described in Materials and Methods. (Open bars) with 3F8 antibody (2 μ g/ml); (solid bars) without antibody. (1) human serum alone; (2) human serum with M-CSF; (3) minimal serum alone; (4) minimal medium with M-CSF.

one experiment in which ADCC by monocytes cultured in human serum was high. Other experiments showed little or no ADCC with human serum alone. The average ADCC was $42 \pm 34\%$ for human serum alone, $86 \pm 44\%$ for human with M-CSF, $19 \pm 10\%$ for minimal medium alone, and $93 \pm 6\%$ for minimal medium with M-CSF (average of 7-10 experiments with the target cell line at an E/T ratio of 4:1).

There were clear and reproducible morphologic differences between monocytes cultured in human serum and those cultured in minimal medium plus M-CSF. Human serum produced round, very granular cells. M-CSF produced less granular, more spindle-shaped cells. When both human serum and M-CSF were present, the monocytes had the morphology associated with human serum.

Monocytes Acquire the Ability to Mediate ADCC During Culture in M-CSF. Fresh monocytes (cultured <2 d) did not mediate significant cytotoxicity with or without antibody at the E/T ratios used in our assays. Fig. 4 shows the development of cytotoxicity over 10 d. A target cell line (SKMel-31) sensitive to both ADCC and antibody-independent killing is presented, to show that both antibody dependent and antibody independent killing emerge at about the same rate, although ADCC is more efficient. Other cell lines tested (SKMel-1, LAN-1) showed similar patterns. To more accurately measure cytotoxicity on each specific day, 24 h (rather than 72 h) incubations were used for the experiments in Fig. 4. Time-course experiments performed at 12, 24, 48, and 72 h of co-incubation had previously shown that killing reached a plateau by 12-24 h. Thereafter it appeared that surviving tumor cells proliferated at approximately the same rate as controls, since cytotoxicity (expressed as a percentage of control) did not change significantly over the next 48 h. Fig. 5 shows the response of monocytes to culture for 9-11 d in various concentrations of M-CSF. Antibody-independent killing varied considerably from donor to donor and from day to day, but did not exceed 75% even in the more sensitive cell lines. In contrast, ADCC was reproducibly high after culture in 100 U/ml of M-CSF, and usually reached 95-100% at E/T ratios of 2:1 or 4:1 by day 10.

Fig. 6 shows cytotoxicity as determined by our assay for each of the six different melanoma and neuroblastoma cell lines that we tested. Antibody-independent killing varied greatly between cell lines, but all lines were sensitive to ADCC, and all reached 90-100% cytotoxicity at an E/T ratio of 8:1 or less.

It should be noted that our assay measures cytotoxicity by comparing the number of surviving target cells present at the end of 72 h with the number of control target cells present after incubation without effectors. Therefore, it cannot differentiate be-

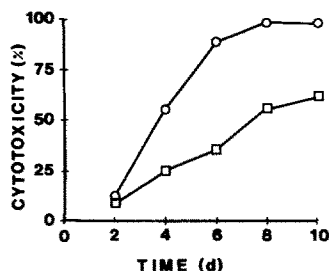


FIGURE 4 Time course for the development of monocyte cytotoxicity during culture in M-CSF. Human peripheral blood monocytes were cultured for 2-10 d in minimal medium with 100 U/ml M-CSF. Target cells (SKMel-31, E/T = 4:1) were added on days 2, 4, 6, 8, and 10, and co-incubated for 18 h. Cytotoxicity was measured by ELISA, as described in Materials and Methods. (Circles) with 3F8 antibody (2 μ g/ml); (squares) without antibody. This figure shows one of three similar experiments using two donors and two target cell lines.

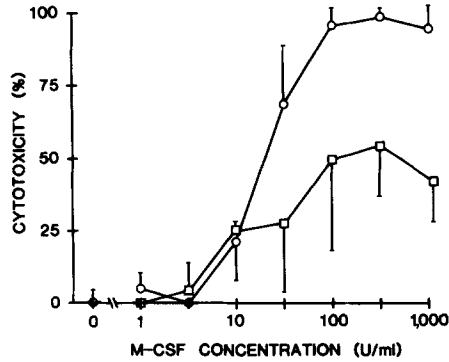


FIGURE 5. Dose-response relationship between M-CSF concentration and development of monocyte ADCC in culture. Human peripheral blood monocytes were cultured for 9–11 d in minimal medium plus various concentrations of M-CSF. Target cells (SKMel-1, E/T = 2:1) were added and co-incubated for 72 h. Cytotoxicity was determined by ELISA as described in Materials and Methods. (Circles) with 3F8 antibody (2 µg/ml); (squares) without antibody. The data represent the average of four experiments using two donors. The bars show standard deviation. Monocytes cultured without M-CSF were virtually all dead by the time of the assay. Wells that had originally contained monocytes without M-CSF did not support target cell proliferation as well as those containing medium alone (typically 15% growth inhibition), and the cytotoxicity figures have been adjusted to reflect this.

tween growth inhibition (cytostasis) and target cell killing. During 72 h target cells underwent at most two doublings. Thus, cytostasis at most might account for a 75% reduction in the number of surviving target cells relative to controls. Between 75 and 100%, however, cytostasis alone could not account for the observed reduction in target cells, and true target cell killing must be present. Our tumor outgrowth experiments (described above) had shown us that even a few surviving target cells would rapidly grow to confluence once removed from the influence of effector cells.

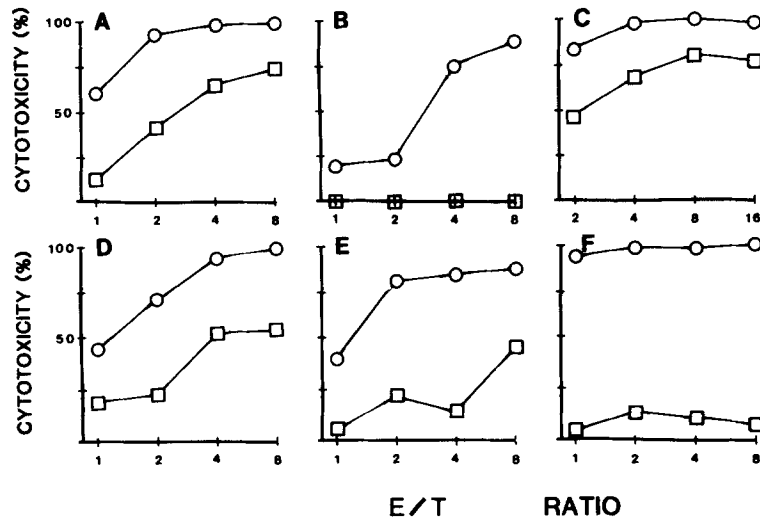


FIGURE 6. ADCC by M-CSF cultured monocytes against various melanoma and neuroblastoma target cell lines. Human peripheral blood monocytes were cultured for 9–12 d in minimal medium with 100 U/ml M-CSF. Targets were added in various concentrations to achieve the E/T ratios indicated. Parallel groups received either the anti-G_{D2} antibody 3F8, 2 µg/ml (circles), or medium alone (squares). R24 (anti-G_{D3}, 1:500 ascites) was used for the cell line shown in C. After 72 h the cultures were assayed for residual tumor cells by ELISA, as described in Materials and Methods. (A) SKMel-1 (average of four experiments); (B) M-14; (C) SKMel-28 (two experiments); (D) SKMel-31 (24-h incubation, three experiments); (E) LA1-15n; (F) LAN-1.

We thus felt that the region approaching 100% cytotoxicity was more interesting (and clinically relevant) than partial reductions in target cell number, and our assay was designed to detect small numbers of residual target cells.

ADCC Is Specific for Antibody-coated Target Cells and Requires the Fc Region. Fig. 7 shows the dose-response curve for ADCC by cultured monocytes at various concentrations of 3F8 antibody. A significant increase in cytotoxicity was detectable at 0.1 $\mu\text{g/ml}$ of 3F8, and the curve appears to reach a plateau at 1–3 $\mu\text{g/ml}$. These concentrations are quite similar to those that we have reported for ADCC by fresh and IL-2-activated lymphocytes (31), and for ADCC by granulocytes (32), using the same antibody and target cells. The experiments in this figure were performed at an E/T ratio of 2:1. ADCC did not occur in the presence of F(ab')_2 fragments of 3F8, even when tested at concentrations up to 10 $\mu\text{g/ml}$ (100 times the concentration at which ADCC was detectable with intact antibody).

To test whether ADCC by M-CSF cultured monocytes involved the release of cytotoxic factors into the surrounding medium, we co-incubated monocytes with two target cell lines simultaneously. One target expressed principally $\text{G}_{\text{D}3}$, and the other expressed principally $\text{G}_{\text{D}2}$. In one experiment (Table I), anti- $\text{G}_{\text{D}3}$ antibody was present during the incubation, resulting in 82–90% cytotoxicity for the $\text{G}_{\text{D}3}^+$ target. At the end of 72 h, surviving ($\text{G}_{\text{D}2}^+$) bystander cells were assayed by ELISA. Cytotoxicity to bystander cells was not increased by the presence of target cells undergoing ADCC. In fact, bystander killing was usually less in the presence of target cells plus antibody; possibly the large number of ADCC target cells served as competitive inhibitors for nonspecific bystander killing. When another $\text{G}_{\text{D}3}^-$ bystander cell (LAN-1) was used, we likewise observed no increase, rather a decrease, in bystander cytotoxicity during ADCC. In parallel experiments (also presented Table I), the roles of bystander and target cell were reversed by using an anti- $\text{G}_{\text{D}2}$ antibody for ADCC. Although the baseline level of bystander cell killing was higher (due to the sensitivity of SKMel-28 to antibody-independent cytotoxicity), there was no increase in bystander killing during ADCC. The absence of bystander cytotoxicity suggests that ADCC is not principally mediated by soluble factors released into the medium, nor by depletion of growth factors or nutrients.

In separate experiments, when supernatant was removed from ADCC cultures (monocytes and targets plus antibody) and added to fresh targets, there was no inhi-

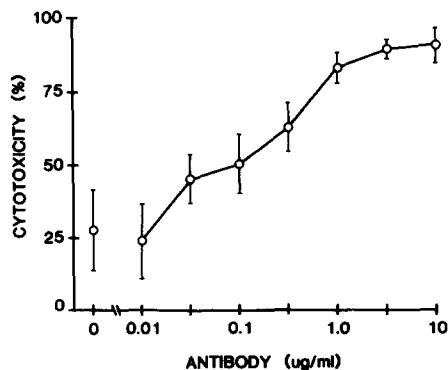


FIGURE 7. Dose-response relationship between 3F8 antibody concentration and ADCC by M-CSF-cultured monocytes. Human peripheral blood monocytes were cultured for 9–14 d in minimal medium with 100 U/ml M-CSF. Target cells (SKMel-1, E/T = 2:1) were added and co-incubated for 72 h in the presence of various concentrations of purified 3F8 antibody. Cytotoxicity was measured by ELISA, as described in Materials and Methods. The figure shows the mean of four experiments using two donors; the bars show standard deviation.

TABLE I
Absence of Bystander Killing During Macrophage ADCC

| Target cell | Bystander cell | Antibody* | | Percent cytotoxicity | |
|-------------|----------------|----------------------|----------------------|----------------------|-----------------|
| | | Anti-G _{D2} | Anti-G _{D3} | Target cells | Bystander cells |
| SKMel-28 | — | — | — | 55 | — |
| SKMel-28 | — | — | + | 82 | — |
| — | SKMel-31 | — | — | — | 0 |
| — | SKMel-31 | — | + | — | 18 [†] |
| SKMel-28 | SKMel-31 | — | — | 61 | 0 |
| SKMel-28 | SKMel-31 | — | + | 90 | 12 |
| SKMel-31 | — | — | — | 0 | — |
| SKMel-31 | — | + | — | 93 | — |
| — | SKMel-28 | — | — | — | 33 |
| — | SKMel-28 | + | — | — | 45 [†] |
| SKMel-31 | SKMel-28 | — | — | 0 | 30 |
| SKMel-31 | SKMel-28 | + | — | 86 | 39 |

Peripheral blood monocytes were cultured in minimal medium with 100 U/ml M-CSF for 10 d. One of two target cell lines, SKMel-28 (G_{D3}⁺G_{D2}⁻) or SKMel-31 (G_{D3}⁻G_{D2}⁺), was added (E/T ratio 4:1), with or without antitarget cell antibody. In some wells, both cell lines were present throughout the 72-h assay. Cytotoxicity was determined by separately measuring residual G_{D2}⁺ and G_{D3}⁺ cells in replicate wells by ELISA, as described in Materials and Methods. The data are from one of three experiments with similar results.

* When R24 (anti-G_{D3}, 1:500 ascites) was present during the assay, SKMel-28 served as the target for ADCC and SKMel-31 was considered the bystander cell. When 3F8 (anti-G_{D2}, 2 μg/ml) was present, the roles were reversed.

† Some increase in bystander cell killing was observable in the presence of supposedly "irrelevant" antibody, which we attribute to low-level binding of antibody to the bystander cells via specific and nonspecific interactions.

bition of oxidative metabolism as measured by MTT reduction. This suggests that a soluble factor such as respiratory inhibition factor (33) does not play a major role in ADCC by M-CSF-cultured monocytes. In preliminary experiments, when monocytes from a patient with chronic granulomatous disease were cultured in M-CSF, they mediated significant ADCC (92% killing against SKMel-31 at an E/T ratio of 8:1).

Immunophenotype of M-CSF-cultured Monocytes. Monocytes were cultured for 10 d in M-CSF and assayed for the expression of several monocyte/macrophage surface antigens (Table II). Nonspecific binding of irrelevant antibodies to cultured monocytes was high (~10 times that seen with fresh monocytes), and made interpretation of weakly positive staining difficult. We arbitrarily defined cells with a fluorescence intensity >98% of the "negative" controls as being positive. However, cultured monocytes showed a single peak (not a bimodal distribution) for all the antibodies shown, suggestive of a relatively homogeneous population. Thus, the mean fluorescence for each antibody may be a more useful measurement of antigen expression, and it is included in Table II to facilitate comparison. Leu19 stained cultured monocytes no more strongly than did the irrelevant antibodies, suggesting that contaminating NK cells are not present in our cultures. Note that many of the activated monocytes expressed the CD16 (low-affinity Fc receptor, FcR_{III}) antigen, which is not found on fresh monocytes.

TABLE II
Immunophenotype of Monocytes Cultured in M-CSF

| Antibody | Antigen | Percent positive* | Relative mean fluorescence† |
|----------|-------------|-------------------|-----------------------------|
| Controls | | 2 | 1.0-1.6 |
| Leu19 | | 2 | 1.0 |
| 3G8 | CD16 | 53 | 2.89 |
| OKM-1 | CD11b (CR3) | 82 | 5.48 |
| L243 | Ia | 76 | 7.67 |
| MY4 | CD14 | 59 | 3.69 |

Monocytes were cultured for 10 d in minimal medium with 100 U/ml M-CSF, then harvested with EDTA and stained for analysis by flow cytometry. The data are from one of two experiments with similar results.

* Due to the large amount of nonspecific antibody binding by activated macrophages, positive cells were defined as those with a fluorescence intensity >98% of cells stained with the least reactive of the control antibodies (Leu19).

† Relative mean fluorescence, measured on an arbitrary 4-decade logarithmic scale, with the least reactive of the control antibodies defined as 1.0. All measurements were made on the same day at the same flow cytometer settings.

Discussion

We have examined the emergence of antitumor ADCC during the differentiation of monocytes in culture with human serum and recombinant M-CSF. We have established that M-CSF is able to support the differentiation and activation of monocytes, even in a minimal medium that alone does not support differentiation. After culture in M-CSF or human serum, monocytes acquire the ability to mediate efficient antitumor ADCC with two IgG₃ murine mAb. We have demonstrated efficient ADCC at E/T ratios as low as 1:1, with 90-100% killing usually reached at an E/T ratio of 4:1 or 8:1. This is significantly higher cytotoxicity than is generally reported in the literature for monocytes or macrophages.

To detect surviving target cells after monocyte ADCC, a modified ELISA method was used, in which surviving tumor cells served as the target antigen. Most tumor cell lines did not remain adherent during the wash steps of our ELISA, so to immobilize them we relied on fixation with glutaraldehyde. This was effective, but restricted our choice of antigens to those which survived fixation. Also, due to the high nonspecific binding of most murine antibody subclasses to activated macrophages, we found that only IgG₃ antibodies worked satisfactorily in the ELISA. We used antibodies against two glycolipid antigens, G_{D2} and G_{D3}, which are strongly expressed on various melanoma and neuroblastoma cell lines, and which are unaffected by glutaraldehyde. For cell lines that did not express both antigens, we used the same antibody for ADCC and ELISA (first confirming by flow cytometry that these antigens did not modulate, even after prolonged exposure to antibody).

To ensure that no population of antigen-negative cells was escaping detection by ELISA, we used flow cytometry to reveal residual tumor cells. Using two-color immunofluorescence and a cocktail of antitumor antibodies, we could reliably detect >98% of the target cells and distinguish them from the effector cells. After a 72-h ADCC incubation, <2% residual tumor cells were detectable by flow cytometry. This high level of cytotoxicity suggested by ELISA and flow cytometry was further confirmed by the absence of proliferating tumor cells after ADCC.

It is noteworthy that two widely used indicators of target cell death, release of ^{51}Cr and release of $[^3\text{H}]\text{TdR}$, underestimated cytotoxicity significantly when compared with either visual inspection or immunologic detection of surviving tumor cells. The disparity was especially marked in the case of chromium release (0% specific lysis, vs. 100% cytotoxicity by ELISA), but thymidine release also consistently underestimated cytotoxicity. A similar phenomenon has been reported previously with the killing of mastocytoma target cells by endotoxin-activated rat liver macrophages (24). In that study, Gardner et al. observed no release of ^{51}Cr after macrophage cytotoxicity, and only partial release of $[^3\text{H}]\text{TdR}$ (with a flat dose-response curve to increasing E/T ratio very similar to our Fig. 1 B). They were able to show by electron microscopy that target cells were being phagocytized intact by activated macrophages, and that the chromium from the target cells remained within the macrophages after phagocytosis. The similarity of their data to ours suggests that phagocytosis may be one reason that radionuclide release assays appear to underestimate cytotoxicity by monocyte/macrophages for certain cell lines.

We did not observe killing of bystander cells during ADCC by cultured monocytes. Further, supernatant from ADCC cultures had no detectable effect on the ability of target cells to reduce MTT to formazan (a measure of metabolic activity). These observations suggest that soluble mediators released into the surrounding medium did not play a principal role in target cell killing. This is in agreement with published studies on ADCC by rat macrophages (34). However, it has also been reported that tumor necrosis factor may not need to be secreted in order to cause tumor cell death (35). It was interesting to us that our one donor with chronic granulomatous disease also showed efficient ADCC. Further experiments to elucidate the role of soluble factors and oxidative intermediates in ADCC by M-CSF cultured monocytes are in progress.

We found that most M-CSF-cultured monocytes expressed the low-affinity Fc receptor (FcRIII, CD16), and mediated ADCC with murine IgG₃ antibodies. FcRIII is not normally present on fresh human monocytes, but it is expressed on peritoneal and alveolar macrophages (36) and is acquired by monocytes after 7–9 of culture *in vitro* (37). It is the receptor used by lymphocytes and granulocytes for ADCC (38), and it has a high affinity for murine IgG₃ antibodies (39). Further experiments are in progress to elucidate the role of FcRIII in ADCC by cultured monocytes.

It is unclear whether monocytes cultured *in vitro* in human serum or M-CSF undergo true activation, or whether they merely follow a path of differentiation similar to that which they would follow *in vivo*. The ability of both serum and M-CSF to induce antitumor ADCC may reflect a common pathway of activation, possibly through M-CSF present in the human serum used, or secreted by the monocytes themselves. Rodents have detectable levels of M-CSF activity in serum (40), and although it has been more difficult to measure M-CSF in human serum, the effects of human serum and M-CSF in culture appear to be similar (16). The degree to which cultured monocytes resemble resting or activated tissue macrophages will require further studies to elucidate.

The ability of M-CSF cultured monocytes to use murine IgG₃ antibodies for efficient ADCC *in vitro* may have implications for *in vivo* immunotherapy. 3F8 and other antibodies of the IgG₃ subclass have been shown to mediate antitumor cytotoxicity through activation of complement (41), and through ADCC by granulo-

cytes (32), lymphocytes, and IL-2-activated lymphocytes (31). Phase I-II clinical trials are under way using 3F8 as a single agent, and have shown antitumor responses (complete and partial remissions) in patients with progressive and refractory neuroblastoma and melanoma (42). The observation that apparently complete target cell killing could often be achieved by ADCC at low E/T ratios was interesting to us. A long-term assay at a low E/T ratios may more realistically reflect the conditions prevailing in vivo than short-term assays using a large number of effector cells. Cells of the monocyte/macrophage lineage have been noted to be resident in many primary tumors and tumor metastases (as abundant as 50% of cells) (43, 44), and we have seen tumor infiltration with monocytes, lymphocytes, and mast cells in patients receiving 3F8 (unpublished results). The ability to target tumor-cytotoxic monocytes and macrophages using mAb may have therapeutic potential in the immunotherapy of malignant diseases.

Summary

Macrophage colony-stimulating factor (M-CSF) is known to stimulate proliferation of monocyte/macrophage progenitors and enhance in vitro antitumor cytotoxicity by murine macrophages. In this paper we have shown that recombinant human M-CSF causes human peripheral blood monocytes to differentiate in culture into metabolically active macrophage-like cells. These cells mediate very efficient antibody-dependent cellular cytotoxicity (ADCC) against human melanoma and neuroblastoma cell lines in the presence of two murine IgG₃ mAbs (3F8 and R24). They also mediate antibody-independent cytotoxicity (or cytostasis) to a lesser extent. Human serum had an inconsistent effect on ADCC, but often induced similar high levels of ADCC. Cytotoxicity was measured using a novel ELISA to detect surviving tumor cells after ADCC. Two conventional isotope-release assays (⁵¹Cr and [³H]TdR) underestimated or entirely failed to detect ADCC by M-CSF-activated monocytes. Optimal activation occurred with 100–300 U/ml of M-CSF, and required 9–11 d for completion. Most of the M-CSF cultured monocytes expressed the low-affinity Fc receptor (CD16). ADCC by cells of the monocyte/macrophage lineage using murine IgG₃ mAbs may have significance for the immunotherapy of human malignancies.

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