Clinically Effective Monoclonal Antibody 3F8 Mediates Nonoxidative Lysis of Human Neuroectodermal Tumor Cells by Polymorphonuclear Leukocytes

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ABSTRACT

Most studies of antibody-dependent cellular toxicity (ADCC) by polymorphonuclear leukocytes (PMN) have supported oxidative lytic processes. This may be because the studies used nonhuman or nonneoplastic cells that were highly sensitive to reactive oxygen species or were small enough to be phagocytosed by PMN. We therefore investigated whether oxygen radicals participate in PMN cytotoxicity toward human neuroectodermal solid tumor cells sensitized by 3F8, which is an anti-ganglioside GD2 murine IgG3 monoclonal antibody with documented anticancer activity in humans. A 4-48 hr release assay was used to assess tumor cell lysis by hydrogen peroxide, superoxide, and hypochlorite. Nine of 11 GD2(+) human melanoma and neuroblastoma cell lines had equal or greater resistance to these oxidants as compared to a GD2(-) human carcinoma line (SKBr3-III) found by others (and confirmed by us) to be significantly more resistant to oxidative lysis than a murine cell line (P388D1), representative of those commonly used in cytotoxicity assays. To facilitate detection of oxidant-mediated lysis, subsequent studies of 3F8-mediated ADCC used GD2(+) targets that were relatively sensitive and others that were relatively resistant to oxygen radicals. Normal PMN and PMN obtained from children with chronic granulomatous disease, which do not generate reactive oxygen species, were equally effective in ADCC. Granulocyte-macrophage colony-stimulating factor, which primes oxidative responses of normal but not of chronic granulomatous disease PMN, enhanced ADCC by both kinds of PMN. During ADCC of 3F8-sensitized targets, with or without granulocyte-macrophage colony-stimulating factor, GD2(-) "innocent bystander" tumor cells (including P388D1) were not lysed, a finding consistent with unimportant extracellular release of cytotoxic mediators. Finally, antioxidant and antimyeloperoxidase moieties did not block ADCC. We conclude that oxidants are not key factors in 3F8-mediated lysis by PMN of human neuroectodermal tumor cells.

INTRODUCTION

Monoclonal antibodies that selectively bind to tumor-associated antigens may mobilize the destructive capacity of leukocytes for cancer treatment. Mononuclear cells are the effectors studied in most investigations of monoclonal antibody-mediated cytotoxicity against human neoplastic cells. PMN also perform antineoplastic ADCC (1-18), but, to date, few reports (5, 15-17) document this process against human solid tumor cells, particularly with clinically applicable monoclonal antibodies. Most studies of ADCC by PMN rely on model systems that may not be relevant for monoclonal antibody-mediated immunotherapy of solid tumors in humans, since they use heterologous antibodies (1-12, 19-24) or nonhuman, nonneoplastic, or hematological targets (1-4, 6-14, 18-24). Many of these targets are highly sensitive to reactive oxygen species or are small enough to be phagocytosed by PMN. In contrast, human solid tumor cells may be too large for phagocytosis by PMN, as we have previously reported (25) and may have a resistance to oxidative lysis significantly exceeding that of murine cells (26), which are commonly used targets in ADCC assays. It is reasonable to postulate that monoclonal antibody-mediated lysis by PMN of human solid tumor cells may involve mechanisms different from those operative in the typical model systems used to explore the cytotoxicity of PMN.

3F8 is a murine IgG3 monoclonal antibody that is directed against the tumor-associated ganglioside GD2 (27). One mechanism underlying its anticancer activity in humans (28) may be its capacity to mediate tumor cell destruction by PMN. Our initial studies of this phenomenon produced preliminary results implicating nonoxidative lytic systems against neuroblastoma (16). This finding was unexpected since it contrasted with the widely accepted view, based on a multitude of studies (2, 4, 8, 12, 20-23), that reactive oxygen species are major lytic intermediaries in ADCC by PMN. However, a later study using another clinically promising anti-GD2 monoclonal antibody (14.18) also pointed to nonoxidative lysis by PMN of neuroblastoma (17). In the current study, we first defined the susceptibility of human GD2(+) neuroectodermal solid tumor cells to lysis by preformed oxygen radicals and then used this information when exploring whether oxidants participate in 3F8-mediated ADCC by PMN against human melanoma as well as neuroblastoma. The results show that, in this ADCC system, solid tumor targets, including cell lines that are relatively sensitive and others that are relatively resistant to oxidative lysis, are efficiently destroyed by a process in which oxidants do not play a major role.

MATERIALS AND METHODS

Monoclonal Antibody. The murine anti-GD2 monoclonal antibody 3F8 (IgG3) was prepared in our laboratory as described before (27). Optimal 3F8 concentrations for ADCC were previously established (16); in the current study, 3F8 was used at a final concentration of 2 µg/ml except where indicated.

Cell Lines. The following were from the American Type Culture Collection (Rockville, MD): the CEM, MOLT-4, and Raji GD2(-) human lymphoma lines; the K562 GD2(-) human erythroleukemia line; the SKBr3-III GD2(-) human carcinoma line; the P388D1 GD2(-) murine macrophage line; the SKNC and SKNSH GD2(-) human neuroectodermal lines; and the HT144 and SKMel-1 GD2(+) human melanoma lines. The GD2(+) human neuroblastoma lines, LAN-1 and LAN-5, were from Dr. R. Seeger (University of Southern California, Los Angeles, CA). The LAN-1 neuroblastoma subclones, LA-1-5s, LA-1-6s, and LA-1-21n, and the human melanoma lines, SKMel-28 [GD2(-)] and SKMel-31 [GD2(+)], were provided, respectively, by Drs. J. Biedler and A. Houghton (both of Memorial Sloan-Kettering Cancer Center, New York). The M14 GD2(+) human melanoma line was from Dr. R. Irie (University of California, Los Angeles). The GD2(+) human...
neuroblastoma lines, IMR-6 and NMB-7, were from Dr. R. K. Liao (McMaster University, Hamilton, Ontario, Canada). Cell lines were propagated in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated calf serum (HyClone Laboratories, Logan, UT), 2 mmol/liter glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin, but all experiments were performed using RPMI 1640 with 0.5% human serum albumin.

Effector Cells. PMN were isolated from heparinized peripheral blood by Ficoll-Paque gradients (Pharmacia Fine Chemicals, Piscataway, NJ) followed by 3% dextran separation and hypotonic RBC lysis. PMN made up >95% of the resulting effector cell population by Wright-Giemsa stain.

Special Materials. The following were obtained from Sigma Chemical Co. (St. Louis, MO): aminotriazole; catalase; cytochrome c; H2O2; L-methionine; mannitol; potassium cyanide; erythrocyte superoxide dismutase; sodium azide; and sodium dodecyl sulfate. Potassium superoxide and sodium hypochlorite were from Aldrich Chemical Co. (Milwaukee, WI). Recombinant GM-CSF was from Genetics Institute (Cambridge, MA) and was used as described previously (16).

Chromium-51 Release Assay. Tumor cells were labeled with sodium chromate (51Cr) (Amersham, Arlington Heights, IL) at 100 μCi/10^6 cells at 37°C for 1 h. After the cells were washed, loosely bound 51Cr was leaked for 1 h at 37°C. After further washing, 5 x 10^5 target cells/well were admixed with PMN, monoclonal antibody, GM-CSF, and/or other reagents in 96-well polystyrene round-bottomed plates (Sarstedt, Federal Republic of Germany); the final volume was 250 μl/well, and assays were performed in duplicate or triplicate. The plates were incubated at 37°C for 4 h and then centrifuged at 400 x g for 5 min; the released 51Cr in the supernatant was counted in a gamma counter (Packard Instruments, Downers Grove, IL). Specific lysis was calculated using the formula

\[
% \text{lysis} = 100\% \times \frac{\text{observed cpm} - \text{background cpm}}{\text{total cpm} - \text{background cpm}}
\]

where cpm are cpm of 51Cr released. Total releasable radioactivity was measured after lysis with 5% sodium dodecyl sulfate, and background release was defined as radioactivity released by target cells incubated in medium alone. The background was 10~28% for the neuroblastoma cell lines and 7~15% for the other lines. The LD₅₀ of preformed H₂O₂, superoxide, or hypochlorite was calculated by interpolation, as previously performed (29). Lytic units were calculated as previously described (16).

RESULTS

Resistance of GD₂⁺ Cell Lines to Preformed Oxidants. If reactive oxygen species are important effector molecules in ADCC against GD₂⁺ human solid tumor cells, one might expect the latter to be readily lysed by preformed oxidants. However, a prior study (26) of GD₂⁻ human solid tumor samples documented a relatively high resistance to H₂O₂ as compared to the SKBr-I-III human carcinoma line; three GD₂⁺ lines had equal or greater resistance to H₂O₂ than K562 to H₂O₂; and only one GD₂⁺ line (LA-1-21n) was approximately as sensitive as P388D₁ to H₂O₂ (Figs. 1A and 2). The H₂O₂ LD₅₀ for SKBr-I-III and P388D₁ were 10⁻³ M and 5 x 10⁻³ M, respectively, which are comparable to those reported by others using a similar assay system (26). One (SKMel-1) of four melanoma lines was significantly less resist-
cytolysis was not affected by Superoxide dismutase (300 units/ml) in blastoma lines, LAN-1, IMR-6, NMB-7, and LA-1-5s had similar sensitivities to H2O2; LAN-5 and LA-1-6s were significantly more resistant than these four cell lines (P = 0.008); and only the LAN-1 subclone LA-1-21n was highly sensitive to H2O2. Catalase (2000 units/ml) inhibited H2O2 (10⁻² M)-induced cytolysis by >95%.

Similarly, 9 of 11 GD2(+) cell lines had equal or greater resistance to superoxide and to hypochlorite as compared to SKBr1-III (Fig. 1, B and C). Superoxide (10⁻² M)-induced cytolysis was not affected by superoxide dismutase (300 units/ml) but was decreased 30 ± 8% (SEM) by cytochrome c (200 μg/ml) and 85 ± 10% by catalase (2000 units/ml). This last finding, plus the LD₅₀ (Fig. 1B) and the dose-response curves (not shown) of superoxide, which resembled those for H2O₂, suggested that superoxide-induced cytolysis largely resulted from the dismutation of superoxide to H₂O₂.

Hypochlorite-induced lysis (4000 units/ml) of tumor cell targets was 90 ± 5% inhibitable by methionine (1 mM) which specifically scavenges this molecule. All but one of the GD2(+) cell lines were significantly (P ≤ 0.012) more resistant to hypochlorite than P388D₁ and CEM (Fig. 1C), cell lines reported to be highly sensitive to this oxidant (30, 31).

Thus, the sensitivity or resistance to oxidative lysis of a given GD2(+) cell line appeared to be a consistent phenomenon irrespective of the type of cell used. Cell lines that were relatively sensitive to H₂O₂ (e.g., LA-1-21n, SKMel-1) were also relatively sensitive to superoxide and hypochlorite, while cell lines that were relatively resistant to H₂O₂ (e.g., LAN-5, SKMel-31) were relatively resistant to superoxide and hypochlorite as well. In contrast, the hierarchy of sensitivity of the GD2(+) cell lines to preformed oxidants (Fig. 1, A–C) differed from the hierarchy of sensitivity to ADCC (Fig. 1D). For example, among the neuroblastoma lines, NMB-7 stands out as relatively more sensitive to ADCC than to lysis by preformed oxidants. Among the melanoma lines, SKMel-31 is more sensitive to ADCC than HT144 or M14, whereas the latter are more sensitive to lysis by oxygen radicals. The relatively high resistance to oxidative lysis of the melanoma and neuroblastoma cell lines, and their different relative sensitivities to lysis by preformed oxidants and to ADCC, suggest that reactive oxygen species may not be critical effectors in 3F8-mediated destruction of GD2(+) human neuroectodermal solid tumor cells by PMN.

No Evidence for Extracellular Release of Tumoricidal Quantities of Oxygen Radicals during ADCC. Many studies have found that PMN use oxidative lytic mechanisms in ADCC (2, 4, 8, 10, 13, 20–23). In an attempt to detect involvement of oxygen radicals in 3F8-mediated lysis by PMN of GD2(+) human neuroectodermal tumor cells, we used PMN with defective oxidative metabolism, GD2(−) “innocent bystander” cells, and antioxidant moieties.

ADCC studies were performed using PMN from normal volunteers, from three patients with X-linked CGD (and unable to generate oxygen radicals), and from two patients with X-linked CGD who were receiving recombinant interferon γ which partially corrects the oxidative defect in patients with X-linked CGD (32). In order to facilitate the detection of differences in effector cell cytotoxic capacities, the studies included target cells that were relatively sensitive (e.g., SKMel-1) or relatively resistant (e.g., SKMel-31) to oxidative lysis (Fig. 1). No differences were observed among the different PMN populations in ADCC against four melanoma lines and two neuroblastoma lines. For example, in the experiment depicted in Fig. 3, PMN from a child with CGD (not receiving interferon) produced greater kill than normal PMN of three cell lines and less kill than normal PMN of three other cell lines. Fig. 3 also shows that ADCC by both the normal PMN and the abnormal PMN was augmented when the assay was performed in the presence of GM-CSF, a cytokine that is not known to correct the oxidative defects of PMN from children with CGD.

In the “innocent bystander” experiments (Table 1), targeted GD2(−) cells included the murine P388D₁ cell line that we (Figs. 1 and 2) and others (26, 31) found to be relatively sensitive to lysis by H₂O₂ and hypochlorite, and two lines, K562 and Raji, that are lysed by PMN in the presence of antiserum (13, 14) and are damaged by H₂O₂ released from pharmacologically stimulated PMN (33–35). Additional GD2(−) targets in these studies were two neuroectodermal lines, SKNMC and SKNSH, and two lines that express GD3 (a ganglioside precursor of GD2), SKMel-28 and MOLT-4. No significant specific lysis of the GD2(−) bystander cells occurred whether or not GM-CSF was included in the assay (Table 1). Although these experiments cannot rule out the presence of local concentrations of oxidants sufficiently high for a cytolytic effect, the results are consistent with an unimportant role for the extracellular release of cytotoxic mediators.

Additional support for this conclusion comes from experiments using scavengers or inhibitors of oxygen radicals. To facilitate detection of oxidant-mediated lysis, GD2(+) targets included cell lines that are relatively sensitive to oxidative lysis and to ADCC by PMN. Scavengers or inhibitors of H₂O₂ (catalase, 2000 units/ml), superoxide (superoxide dismutase, 300 units/ml; cytochrome c, 200 μg/ml), hydroxyl radicals (mannitol, 50 mM), and halides (methionine, 1 mM) had no effect on 3F8-mediated ADCC by PMN against LAN-1,
Table 1  Absence of bystander kill during 3F8-mediated ADCC by PMN

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<th>Bystander experiments</th>
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<td><strong>3Cr-labeled GD2(−) bystanders</strong></td>
<td><strong>Percentage specific</strong></td>
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<td>SKNSH</td>
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* Identical results with and without GM-CSF.
* Results with GM-CSF (2 ng/ml) present during the assay.

SKMel-1 and SKMel-31. All antioxidants were used at concentrations that we (see above) and others (22, 34, 36, 37) found were inhibitory of cytolysis by preformed or by enzymatically generated oxidants, as well as by pharmacologically stimulated PMN. Similarly, no inhibition of ADCC against LAN-1, LAN-5, IMR-6, NMB-7, SKMel-1, SKMel-31, and M14 occurred when the myeloperoxidase inhibitors azide and cyanide were included in the assay at concentrations (1.0 mM for each reagent) that blocked cytotoxicity produced by the myeloperoxidase-hydrogen peroxide-halide combination in model systems (38). To ensure that these agents were not also enhancing the sensitivity of target cells to H<sub>2</sub>O<sub>2</sub> by depleting catalase and thereby obscuring a decrease in PMN cytotoxic capacity, PMN were incubated for 1 h with azide (1.0 mM) or with another myeloperoxidase inhibitor, aminotriazole (50 mM), and then washed prior to the ADCC assay. This procedure also did not affect cytotoxicity (not shown).

**DISCUSSION**

Monoclonal antibodies are potential targeting agents for exploiting cytotoxic leukocytes in cancer treatment. Identification of mechanisms underlying monoclonal antibody-mediated effects may suggest means for maximizing anticancer activity. A previous study documented that the clinically effective monoclonal antibody 3F8 mediates efficient lysis by PMN of GD<sub>2</sub>(+) human neuroectodermal tumor cells (16). The results of the current study confirm our earlier, preliminary findings which implicated nonoxidative processes in the destruction by PMN of neuroblastoma and, in addition, demonstrate that this conclusion applies to human melanoma targets as well.

Regarding the mechanisms underlying the cytolytic activity of PMN, abundant data support oxidative killing in model systems that use heterologous antibodies or target erythrocytes or nonhuman tumor cell lines (2, 4, 8, 10, 13, 20–23). However, the relatively high resistance to oxygen radicals of the GD<sub>2</sub>(+) human solid tumor cell lines and the lack of correlation between sensitivity to oxidative lysis versus sensitivity to ADCC (Fig. 1) are consistent with the possibility that oxidants are not major factors in 3F8-mediated lysis by PMN of these targets. After comparing H<sub>2</sub>O<sub>2</sub>-induced lysis of murine versus human cells, O’Donnell-Tormey et al. (26) also concluded that leukocyte oxidative capacities may be inadequate for lysing selected human solid tumor cells. Their study documented H<sub>2</sub>O<sub>2</sub> LD<sub>50</sub> for human carcinoma cells in the same range as those that we measured for human neuroectodermal cells, i.e., 10<sup>-3</sup> to 10<sup>-2</sup> M. These values exceed by 2 or more logs both the H<sub>2</sub>O<sub>2</sub> LD<sub>50</sub> of murine cells (including the P388D<sub>5</sub> line that served as a reference standard in our study) and the maximum of 12 x 10<sup>-5</sup> M of H<sub>2</sub>O<sub>2</sub> produced by 3 x 10<sup>5</sup> activated PMN/ml (39).

PMN from children with CGD were as efficient as normal PMN in 3F8-mediated ADCC against both melanoma and neuroblastoma targets. This finding strongly supports nonoxidative lytic mechanisms in this system, especially since the GD<sub>2</sub>(+) targets included cell lines that are relatively sensitive and others that are relatively resistant to oxidative lysis. These different populations were targeted in order to maximize the chances of detecting decreased ADCC by PMN with defective oxidative metabolism. A number of studies using erythrocytes or nonhuman cells as targets show decreased ADCC by PMN obtained from patients with CGD (2, 8, 20–23) and by PMN experimentally deprived of oxygen (4, 8, 13, 22). However, our results are in accord with a recent study describing equivalent or greater cytolytic potency of CGD PMN as compared to normal PMN in monoclonal antibody (14,18)-mediated ADCC against neuroblastoma (17). PMN from patients with CGD also mount normal ADCC against herpes simplex virus-infected human embryonic lung cells (19).

GM-CSF is equally effective at augmenting 3F8-mediated ADCC by normal PMN and by PMN from children with CGD. This finding provides further evidence for nonoxidative cytotoxicity since, unlike recombinant interferon γ (32), GM-CSF is not known to improve the oxidative defect of CGD PMN. GM-CSF primes PMN for enhanced oxidative metabolism in response to membrane stimulants (40), causes PMN adherent to substrate to release H<sub>2</sub>O<sub>2</sub> (41), and increases the expression on PMN of CD11/CD18 adhesion molecules (25, 42). Our results with CGD PMN are consistent with data presented elsewhere (25) which suggest that up-regulation of CD11/CD18 on PMN may contribute to GM-CSF enhancement of 3F8-mediated ADCC. Apart from our own studies, GM-CSF effects on the capacity of CGD PMN to perform in monoclonal antibody-mediated tumor-cell destruction have not been previously reported; GM-CSF does induce significant serum-mediated lysis by these effectors against retrovirus-infected lymphoid cells (24).

The studies with PMN from patients with CGD cannot rule out either the presence of small subpopulations of highly effective PMN with intact oxidative function or the recruitment of nonoxidative cytolytic capacities not fully exploited in normal PMN. Furthermore, even though we documented that a panel of GD<sub>2</sub>(+) human neuroectodermal cell lines have a resistance to H<sub>2</sub>O<sub>2</sub> far exceeding the H<sub>2</sub>O<sub>2</sub> productive capacity of PMN, many reports describe pharmacological triggering of PMN cytotoxicity via the extracellular release of oxidants (29, 30, 33–35, 38). These considerations prompted us to perform experiments looking for either lysis of GD<sub>2</sub>(−) "innocent bystander" tumor cells or abrogation of 3F8-mediated ADCC by antioxidant moieties. Neither set of experiments produced evidence of oxidative lysis, even though tumor cell targets were chosen on the basis of characteristics that might facilitate detection of oxidant-mediated cytolytic activity. Thus, the
ADCC studies using antioxidant and antimielyperoxidase moieties included targets that are relatively sensitive to pre-formed oxidants and to ADCC, and the bystander studies included lymphohematopoietic cells lysed by low concentrations of oxygen radicals in other systems. It is noteworthy that bystander kill remained absent when GM-CSF was present in the assays, even though this cytokine, as noted above, primes the oxidative responses of PMN.

The results of the experiments using bystander cells and of those using a range of antioxidant moieties are consistent with, although not conclusive regarding an unimportant effector role for reactive oxygen species in 3F8-mediated tumor-cell destruction by PMN. Our earlier studies document the requirement of PMN-target physical interaction in this lytic process (16, 25). The absence of bystander kill does not rule out the functional presence of high local concentrations of oxygen radicals at sites of close contact between PMN and targets. Furthermore, proximity between effectors and targets may not allow penetration of antioxidant moieties into the space where cytotoxic molecules might act. Tight attachment has been invoked as an explanation for the fact that, with few exceptions (20), scavengers or inhibitors of oxygen radicals fail to affect ADCC by PMN, even when oxygen are active mediators of cell kill in the system studied (2, 4).

Differences in target cells other than resistance to oxidative injury may contribute to the divergent findings between the current study and prior studies of the mechanisms underlying ADCC by PMN. For example, PMN may phagocytose small targets such as erythrocytes. Under those circumstances, oxidative mechanisms might be operative since phagocytosis stimulates a respiratory burst. In contrast, one might predict that monoclonal antibody-mediated destruction by PMN of the much larger human neuroectodermal tumor cells, which we have shown is a nonphagocytic process (25), may involve different cytotoxic mechanisms. The conclusion of the current study is such indeed is the case, since the results indicate that, in the presence of 3F8, oxygen radicals are not important mediators of PMN cytotoxicity toward human melanoma and neuroblastoma cells.

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