Absolute Requirement of CD11/CD18 Adhesion Molecules, FcRII, and the Phosphatidylinositol-Linked FcRIII for Monoclonal Antibody-Mediated Neutrophil Antihuman Tumor Cytotoxicity

By Brian H. Kushner and Nai-Kong V. Cheung

We have previously shown that 3F8, a murine IgG, monoclonal antibody (MoAb) specific for the ganglioside G\(\text{d}_3\), mediates tumor cell kill in vitro and in vivo. We now describe receptor requirements of polymorphonuclear leukocytes (PMN) in 3F8-mediated cytotoxicity (ADCC) of human G\(\text{d}_3\)(+) melanoma and neuroblastoma cell lines. PMN from a child with leukocyte adhesion deficiency (LAD) were devoid of ADCC. MoAb to CD11b, CD11c, and CD18 each efficiently blocked ADCC by normal PMN. In contrast, a panel of different MoAbs to CD11a had no significant inhibitory effect on ADCC, a finding consistent with the low-to-absent expression of the CD11a ligand, intercellular adhesion molecule-1, on the target cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly increased the expression of CD11b, CD11c, and CD18 on normal PMN, decreased the expression of Fc receptors (FcR), and enhanced ADCC by normal but not by LAD PMN. MoAbs to FeRII and FeRIII each efficiently blocked ADCC; anti-FeRI MoAb had no effect. Flow cytometry using anti-FeRII MoAb versus anti-FeRIII MoAb did not show cross competition, suggesting that inhibition of ADCC was not a steric effect resulting from FeRII proximity to FeRIII. PMN deficient in FeRIII (obtained from patients with paroxysmal nocturnal hemoglobinuria) and PMN depleted of FeRIII by treatment with elastase or phosphatidylinositol (PI)-specific phospholipase C produced low ADCC, supporting a role for the PI-linked FeRIII. Thus, optimal ADCC using human PMN, human solid tumor cells, and a clinically active MoAb (conditions that contrast with the heterologous antibodies and nonhuman or nonneoplastic targets used in most models of PMN ADCC) required CD11b, CD11c, FeRII, and the PI-linked FeRIII. Furthermore, in this clinically relevant system, GM-CSF enhancement of antitumor PMN ADCC correlated with increased expression of CD11/CD18 molecules.

3F8 IS A MURINE IgG, monoclonal antibody (MoAb) that is well-suited for targeted immunotherapy of cancer because (1) its ganglioside G\(\text{d}_3\) target antigen is highly restricted to neuroectodermal tissues and is genetically stable; (2) it has excellent tumor localization in patients; and (3) in vitro, it mediates human tumor cell destruction by human complement and by human lymphocytes, cultured monocytes, and polymorphonuclear leukocytes (PMN). This constellation of features, which may account for regressions of G\(\text{d}_3\)(+) tumors in patients treated with 3F8, has been documented in very few MoAbs. For example, most studies of antibody-dependent cellular cytotoxicity (ADCC) use mononuclear cells as effectors, in part because few MoAbs (clinically applicable or otherwise) are known to mediate ADCC of human tumor cells by PMN. Yet, in vivo, PMN are the predominant circulating leukocyte and are activated and increased in number by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF). Studies showing PMN ADCC of human tumor cells mediated by heterologous antibodies are also few in number. Other studies of antitumor PMN ADCC have used heterologous antibodies and nonhuman, usually lymphohematopoietic, targets. The cytotoxic mechanisms in these model systems may differ from those operative in PMN ADCC of human solid tumor cells that not only are large in size, and hence may not be readily phagocytosed by PMN, but are also relatively resistant to reactive oxygen species.

The current study focused on receptor requirements of 3F8-mediated PMN ADCC of G\(\text{d}_3\)(+) human melanoma and neuroblastoma cells. Although CD11/CD18 molecules mediate a wide range of adhesion-dependent activities of PMN, no report to date has described the involvement of this complex in PMN ADCC of human tumor cells. The same holds for Fc receptors (FcR), despite the well-established necessity of FcR in ADCC. Of the three reported types of FcR, the low-affinity FeRII and FeRIII predominate on resting PMN. While FeRII has a well-documented role in many PMN activities, the importance of the more abundant FeRIII, including in ADCC, is uncertain, given its linkage to PMN via a phosphatidylinositol (PI) moiety. This form of linkage, in contrast to the polypeptide anchor of FeRII, lacks a transmembrane connection to the cytoplasm and may limit the capacity of FeRIII to transmit intracellular signals for PMN function. We now demonstrate a critical role in a clinically relevant model of PMN ADCC, not only for FeRII and all subunits of the CD11/CD18 complex, except CD11a, but also for the PI-linked FeRIII.

MATERIALS AND METHODS

MoAbs. Purified 3F8 (anti-G\(\text{d}_3\); IgG\(_\text{1}\)) was prepared in our laboratory as described, and was used at a final concentration of 2 \(\mu\text{g/mL}\), unless otherwise stated. A110 (anti-decay accelerating factor [DAF]) was the gift of Dr M.E. Medof (Case Western Reserve University, Cleveland, OH). 197 (anti-FcRI; IgG\(_\text{2}\)), prepared by hollow-fiber tubing, was the gift of Dr M.W. Fanger.
Rothlein (Boehringer Ingelheim Pharmaceuticals, Inc, Ridgefield, CT); IgG1; R15.7 (anti-CD18; IgG1); and R1.1 (anti-View, CA) and 3.9 (anti-CD11c; IgG1) as ascites, was obtained from Becton Dickinson Laboratories (Mountain View, CA) and J. Ritz (Dana-Farber Cancer Center, Boston, MA). Purified IB4 (anti-CD11b; IgG2a) was the gift of Dr T. Springer (Harvard University, Boston, MA). The following were obtained as hybridomas from the American Type Culture Collection (ATCC; Rockville, MD): IV.3 (anti-FcRII; IgG2a), TS1/22 (anti-CD11a; IgG2), and BBM.1 (anti-β2-microglobulin; IgG2a). 3G8 (anti-FcRIII; IgG1) was kindly provided as a hybridoma by Dr J. Unkeless (Mt Sinai Medical School, New York, NY). Hybridomas were grown as ascites in BALB/c mice, and partially purified by precipitation in 45% ammonium sulfate at 0°C, followed by dialysis against phosphate-buffered saline.

Cell lines. LAN-1 and IMR-6 (both neuroblastoma) were gifts, respectively, of Dr R. Seeger (University of Southern California, Los Angeles, CA) and Dr R.K. Liao (McMaster University, Ontario, Canada). SKMel-1 (melanoma) was obtained from the ATCC. Cell lines were propagated in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin, though in parallel assays GM-CSF enhanced ADCC by 22% to 270% for all antigens, except FcRII, for which the SD from the mean was ±12% to ±16%. P values were derived using a Student's t-test for paired differences to compare untreated versus treated aliquots of PMN. Examination of the data showed no extreme values that would positively influence this statistic.

RESULTS

Requirement for CD11/CD18 adhesion molecules. Experiments reported elsewhere25 showing no lysis of "innocent bystander" Gp39(-) tumor cell targets suggested a requirement for physical attachment between effector and target cells in 3F8-mediated PMN ADCC, which is a nonphagocytic process (Munn D.H., Kushner B.H., unpublished observations, April 1990). In the current study, results using LAD PMN established the importance of CD11/CD18 molecules in this system. LAD PMN, which were confirmed by immunophenotyping to be devoid of the entire CD11/CD18 complex, mounted no detectable ADCC (Table 1). The addition of GM-CSF did not alter this result, even though in parallel assays GM-CSF enhanced ADCC by normal PMN, as previously observed,3 and increased the expression of CD11/CD18 on normal PMN (see below).

To identify which CD11/CD18 subunits were required for 3F8-mediated PMN ADCC, subunit-specific MoAbs were added to the ADCC assay (Fig 1). MoAbs to CD11b (LM2/1, Mol), CD11c (LeuM5, 3.9), and CD18 (R15.7, IB4, TS1/18) each produced efficient concentration-dependent ADCC. These results are representative of three similar studies.

<table>
<thead>
<tr>
<th>Target</th>
<th>E:T</th>
<th>3F8 (μg/mL)</th>
<th>No GM-CSF</th>
<th>+GM-CSF (2 ng/mL)</th>
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<tbody>
<tr>
<td>LAN-1</td>
<td>Normal</td>
<td>25:1</td>
<td>2</td>
<td>52</td>
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<tr>
<td>LAD</td>
<td>25:1</td>
<td>2</td>
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<tr>
<td>LAD</td>
<td>100:1</td>
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These results are representative of three similar studies. Abbreviation: ND, not done.
dependent inhibition of ADCC, with complete or near complete abrogation of ADCC at 5 to 10 μg/mL. In contrast, three different anti-CD11a MoAbs (TS1/22, 2F12, G25.2) in concentrations up to 100 μg/mL had no effect on ADCC. A fourth anti-CD11a MoAb (R3.1) was inhibitory only at concentrations ≥25 to 50 μg/mL when SKMel-1 was the target, and at concentrations ≥50 μg/mL when LAN-1 was the target. This lack of effect of anti-CD11a MoAb may be explained by the low expression on melanoma of ICAM-1, which is a ligand for CD11a, and the absence of ICAM-1 on LAN-1, which was previously reported and which we documented using flow cytometry and the anti-ICAM-1 MoAb R1.1 (not shown).

We found variability in the ADCC inhibitory efficiency of different anti-CD11/CD18 MoAbs, a phenomenon described with non-ADCC adherence functions of PMN. Among the anti-CD11/CD18 MoAbs assayed in our system, the anti-CD18 MoAbs were the most efficient inhibitors (R15.7 more than IB4 and TS1/18; Fig 1), and the anti-CD11b MoAbs LM2/1 and Mo1 were more inhibitory than OKM-1 and OKM-10 (not shown). Thus, OKM-1 in concentrations up to 100 μg/mL produced less than 20% inhibition of ADCC against LAN-1, and inhibited ADCC against SKMel-1 by 50% or more only when present at 12.5 μg/mL or greater. In comparison to LM2/1 or Mo1, OKM-10 was less inhibitory when LAN-1 was the target, but equally inhibitory when SKMel-1 was the target. The results with OKM-1 and, to a lesser degree, with OKM-10 suggested that nonspecific steric effects did not account for the sensitivity of ADCC to the anti-CD11/CD18 MoAbs. This point was reinforced by the normal ADCC that occurred in the presence of the anti-β2-microglobulin MoAb BBM.1 in concentrations up to 100 μg/mL (not shown).

FcR requirements. Blocking MoAbs were used to identify the roles of the different FcR in 3F8-mediated PMN ADCC (Fig 2). The anti-FcRI MoAb 197 in concentrations up to 100 μg/mL had no effect on ADCC. This result was not unexpected because FcRI is present at very low density on PMN. In contrast, the anti-FcRII MoAb IV.3 and the anti-FcRIII MoAb 3G8 each completely abrogated ADCC at 1 μg/mL or less. Flow cytometry studies with anti-FcRII MoAb versus anti-FcRIII MoAb did not show cross competition (not shown), confirming prior reports of individual anti-FcR MoAb specificity, and suggesting that steric factors could not account for the respective ADCC blocking effects of IV.3 and 3G8. Thus, both FcRII and FcRIII appeared to be required for successful ADCC.

Because of evidence that the PI-linked FcRIII of PMN may not be critical for PMN function, including tumor cell kill, studies were performed to assess directly the require-
ment for FcRIII in 3F8-mediated PMN ADCC. Enzyme treatment significantly depleted PMN of FcRIII ($P = .044$), with little effect on the expression of non–PI-linked surface molecules (Table 2). In comparison to normal PMN, the FcRIII-deficient PMN performed poor ADCC in the presence of 3F8 (Table 2). These findings favored an FcRIII requirement for optimal lysis in this system.

Additional support for this conclusion came from studies using PMN from adults with PNH (Table 3). Immunophenotyping confirmed that these PMN, as reported, had very low expression of PI-linked surface molecules, including FcRIII and DAF. Similar to findings with PMN enzymatically depleted of FcRIII, PNH PMN were ineffective in ADCC as compared with normal PMN (Table 3).

**Effects of GM-CSF.** To gain further insight into the roles of FcR and the CD11/CD18 complex in 3F8-mediated PMN ADCC, studies were performed using GM-CSF. This cytokine enhances PMN ADCC and has obvious potential clinical relevance. GM-CSF significantly increased CD11b ($P < .001$), CD11c ($P = .007$), and CD18 ($P = .004$) expression and, as expected, enhanced 3F8-mediated PMN ADCC (Table 4). GM-CSF produced a less than significant decrease in both FcRII and FcRIII expression, but did not appreciably change the density of other PMN surface molecules. The inclusion of GM-CSF in the ADCC assay did not alter the relative inhibitory effects of the anti-CD11/CD18 MoAbs, although higher concentrations of these MoAbs were required to inhibit ADCC (not shown). Thus, while anti-CD11a MoAb had no effect on ADCC by GM-CSF–stimulated PMN, cytotoxicity curves (such as those in Fig 1) using MoAb to CD11b, CD11c, and CD18 were shifted to the right in the presence of GM-CSF. These results were consistent with the possibility that GM-CSF enhances 3F8-mediated PMN ADCC at least partly via upregulation of CD11b, CD11c, and CD18.

**DISCUSSION.** We have defined PMN surface receptors critical for PMN ADCC of human solid tumor cells. In the presence of 3F8, an MoAb that has antitumor activity in patients, ADCC is dependent on a physical interaction between PMN and G$_{d}$(+) human neuroectodermal cells that does not involve phagocytosis but requires most subunits of the CD11/CD18 complex, FcRII, and the PI-linked FcRIII. Several of these features differ from those associated with PMN ADCC in model systems using heterologous antibodies and nonhuman or nonneoplastic target cells.
The availability of LAD PMN allowed us to establish the importance of the CD11/CD18 complex in 3F8-mediated PMN ADCC. Inhibition studies using specific MoAbs showed that all subunits of the complex, except CD11a, were critical for normal PMN cytotoxicity with 3F8. Given the results with LAD PMN, the abrogation of 3F8-mediated PMN ADCC produced by MoAbs to CD18, the β subunit common to all three CD11 α subunits, might have been predicted. The same holds for the inhibitory effects of MoAb blockade of CD11b, which is the most abundant of the CD11 α subunits on PMN. Recent studies, however, suggest that CD11c also can function independently as an adhesion molecule. Our results suggest that either (1) PMN-target cell attachment via CD11b or CD11c is necessary but not sufficient for 3F8-mediated ADCC to proceed, or (2) in our system, when CD11b or CD11c is blocked, the other becomes nonfunctional. A redundancy of function among the CD11/CD18 molecules has previously been noted, and is supported, for example, by the relative structural homology between CD11b and CD11c.

No prior report has described the involvement of CD11/CD18 molecules in PMN ADCC of human solid tumor cells (as mediated by MoAbs or by heterologous antibodies). The sole previously reported study of ADCC by LAD PMN targeted herpes simplex virus-infected Chang liver cells. Findings in that study differed from our own in that defective ADCC was attributed to the absence of CD11a and CD11b, because anti-CD11a (TS1/22) MoAb alone or the combination of anti-CD11b (OKM-1) plus anti-CD18 (TS1/18) MoAbs blocked ADCC by normal PMN (anti-CD11b or anti-CD18 MoAb alone produced insignificant inhibition). The lack of effect of anti-CD11a MoAb on 3F8-mediated ADCC in CD11b-depleted PMN is consistent with the low-to-absent expression of the CD11a ligand ICAM-1 on the melanoma and neuroblastoma targets. The known counter-receptors for CD11b and CD11c include iC3b, fibrinogen, factor X, as well as ICAM-1; identification of the ligands for CD11b and CD11c in our system awaits further studies.

Our results with GM-CSF are consistent with an important role for CD11/CD18 in 3F8-mediated PMN ADCC, because GM-CSF enhancement of the latter occurred in conjunction with increased expression of CD11b, CD11c, and CD18. Although GM-CSF can affect the respiratory burst of PMN, two lines of evidence suggested to us that GM-CSF may act via CD11/CD18 in our system: (1) We had previously found that GM-CSF enhances 3F8-mediated ADCC by PMN with defective oxidative metabolism (obtained from patients with chronic granulomatous disease). (2) The time-course of GM-CSF enhancement of PMN ADCC that we and others had observed was more consistent with its rapid upregulation of PMN CD11/CD18, than with the more delayed onset of its activation of PMN oxidative metabolism.

In addition to its effects on the CD11/CD18 complex, GM-CSF decreased PMN expression of FcRII and FcRIII, a previously reported phenomenon. Studies cannot rule out the possibility that GM-CSF enhances 3F8-mediated PMN ADCC by non-CD11/CD18 effects, such as increasing the affinity of FcR or mobilizing nonoxidative cytolytic capacities.

Studies of the role of FcR in 3F8-mediated PMN ADCC underscored the importance of physical PMN-target attachment. Thus, anti-FcRII and anti-FcRIII MoAbs each efficiently inhibited ADCC. Furthermore, PMN enzymatically depleted of FcRIII and PMN PMN, which have low FcR expression, are ineffective in ADCC as compared with normal PMN. The results suggest that, as with CD11b and CD11c, the separate involvement of each low-affinity FcR is necessary but not sufficient for optimal 3F8-mediated PMN ADCC. The current report is the first to document a requirement for both FcRII and FcRIII in PMN ADCC against human (or nonhuman) tumor or nonneoplastic targets. Our findings are noteworthy because, although Fc-FcR interactions are the well-established basis of ADCC, the issue has been clouded by the discovery that FcRIII is anchored to the plasma membrane of PMN by a PI moiety and may therefore have a limited capacity for signal transduction (see below).

A requirement for the PI-linked FcRIII in PMN ADCC is evident in some but not all model systems. Thus, PMN lyse murine hybridoma cells bearing antibody directed against FcRII, but not those bearing antibody against FcRIII. In contrast, PMN lyse chick erythrocytes coated with antibody directed against FcRII or FcRIII; heteroaggregates of anti-FcRII and anti-2,4-dinitrophenyl antibodies mediate PMN destruction of 2,4,6-trinitrophenyl-modified RDM4 murine leukemia cells; and salivary PMN, which have very low expression of FcRIII, are ineffective in ADCC of herpes simplex virus-infected Chang liver cells. The latter two reports do not include data relating to other FcR, such as FcRII expression on salivary PMN or the effects on ADCC of anti-FcRII MoAb.

FcR requirements in PMN ADCC of human tumor cells have not been described before the present report. One attempt to do so was unsuccessful when PMN did not lyse JY human B-lymphoblastoid cells that were precoated with a murine IgG1 MoAb. This failure illustrates the difficulty in achieving ADCC of human tumor cells by unstimulated PMN; it also supports the hypothesis that PMN ADCC of human tumor cells, which are too large for phagocytosis by PMN and are relatively resistant to reactive oxygen species, involves cytotoxic mechanisms different from those in model systems using murine targets.

We have established that FcRIII is one of several PMN receptors required for 3F8-mediated PMN ADCC of human solid tumor (neuroectodermal) cells. It remains to be determined whether the PI-linked FcRII on PMN is generating an intracellular signal to promote PMN cytotoxicity, or is only serving as a receptor for tight binding to MoAb-coated tumor cells, while signal transduction is effected by FcRII. Evidence supporting an active role for the PI-linked FcRIII independent of FcRII includes reports that PMN FcRIII can mediate exocytosis of granule...
proteins, production of superoxide anion, and elevation of cytosolic calcium derived from intracellular stores.

ACKNOWLEDGMENT

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