Intracellular Domains of Target Antigens Influence Their Capacity to Trigger Antibody-Dependent Cell-Mediated Cytotoxicity

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Ab-mediated signaling in tumor cells and Ab-dependent cell-mediated cytotoxicity (ADCC) are both considered as relevant effector mechanisms for Abs in tumor therapy. To address potential interactions between these two mechanisms, we generated HER-2/neu- and CD19-derived chimeric target Ags, which were expressed in experimental tumor target cells. HER-2/neu-directed Abs were documented to mediate effective ADCC with both mononuclear cells (MNCs) and polymorphonuclear granulocytes (PMNs), whereas Abs against CD19 were effective only with MNCs and not with PMNs. We generated cDNA encoding HER-2/CD19 or CD19/HER-2 (extracellular/intracellular) chimeric fusion proteins by combining cDNA encoding extracellular domains of HER-2/neu or CD19 with intracellular domains of CD19 or HER-2/neu, respectively. After transfecting wild-type HER-2/neu or chimeric HER-2/CD19 into Raji Burkitt’s lymphoma cells and wild-type CD19 or chimeric CD19/HER-2 into SK-BR-3 breast cancer cells, target cell lines were selected for high membrane expression of transfected Ags. We then investigated the efficacy of tumor cell lysis by PMNs or MNCs with CD19- or HER-2/neu-directed Ab constructs. MNCs triggered effective ADCC against target cells expressing wild-type or chimeric target Ag. As expected, PMNs killed wild-type HER-2/neu-transfected, but not wild-type CD19-transfected target cells. Interestingly, however, PMNs were also effective against chimeric CD19/HER-2-transfected, but not HER-2/CD19-transfected target cells. In conclusion, these results demonstrate that intracellular domains of target Ags contribute substantially to effective Ab-mediated tumor cell killing by PMNs. The Journal of Immunology, 2002, 168: 3275–3282.

Antibodies offer the possibility to increase specificity and efficacy of oncologic therapy (1). For example, a HER-2/neu-directed Ab in combination with chemotherapy prolonged survival of patients with HER-2/neu-overexpressing breast cancer, compared with patients treated with chemotherapy alone (2). Similarly, lymphoma patients treated with a combination of chemotherapy and a CD20 Ab survived longer compared with control patients receiving chemotherapy only (3). Abs against many other target Ags are currently under active investigation (4), including Abs against B cell-related Ags such as CD19. However, despite these promising clinical data, we are still surprisingly ignorant about clinically relevant mechanisms of action of most Abs (5). Studies in genetically modified mice, in which the signaling machinery of FcRs was disrupted by knock-out of the common FcR γ-chain, indicated an important role for FcR-mediated effector mechanisms (6, 7). In contrast, Ab-mediated signaling in tumor cells is supposed to play an important role for the ability of Abs to induce tumor regression (8, 9). For example, therapeutic efficacy of idiotype Abs in clinical trials was correlated with their capacity to trigger signal transduction in respective patients’ tumor samples (10).

NK cells are often considered as the most relevant effector population for cell-mediated effects of therapeutic Abs, but their lytic capacity is inhibited by killer cell inhibitory receptors as long as tumor cells express HLA class I molecules (11). In contrast, results from animal models suggested that G-CSF-primed polymorphonuclear granulocytes (PMNs), the most numerous cytotoxic FcR-expressing cells, contributed substantially to the therapeutic efficacy of mAbs against lymphomas or melanomas (12, 13). However, previous experiments with PMN effector cells demonstrated unexplained differences of B cell-related Abs to serve as targets for Ab-dependent cell-mediated cytotoxicity (ADCC) against B lymphoma cells. Thus, Abs against HLA class II or related epitopes, such as Lym-1, Lym-2, 1D10, or invariant chain (CD74), were highly effective in recruiting PMNs as effector cells, whereas CD19 Abs were ineffective in this regard (14–17). Against solid tumor cells, Abs against HER-2/neu or epidermal growth factor receptor (EGFR) were among the best examples that PMNs may contribute to Ab efficacy (18, 19). To address potential explanations for these Ag-dependent differences in PMN-mediated ADCC, we generated chimeric target Ags by combining extracellular and intracellular domains of HER-2/neu and CD19 molecules, respectively, creating chimeric HER-2/CD19 and CD19/HER-2 proteins (Fig. 1).

The HER-2/neu protooncogene encodes a 185-kDa transmembrane tyrosine kinase, which belongs to the EGFR family (20, 21) and is overexpressed on different epithelial tumors, including...
breast and ovarian cancer (22). Because no direct ligand has been characterized to date, HER-2/neu is considered an "orphan" receptor. However, HER-2/neu can be transactivated through heterodimerization with other EGFR family members and appears to be their preferred heterodimerization partner (23). HER-2/neu is an active tyrosine kinase that is constitutively autophosphorylated when overexpressed (20). In addition to transphosphorylating other HER receptors, it activates the mitogen-activated protein kinase as well as the phosphoinositol-3 kinase pathway (21). Thereby, HER-2/neu activation leads to transformation of cells, tumor cell growth, cytokine resistance, changed epithelial cell morphology, and increased vascular endothelial growth factor production (24).

CD19 is a 95-kDa glycoprotein of the Ig superfamily, which is exclusively expressed on B lymphocytes. CD19 is a key member of a multimeric cell surface signal transduction complex, which includes CD21 (CR2), CD81 target of antiproliferation Ab-1, and CD225 (Leu 13) (25, 26). CD19 plays an important role in regulating B cell activation and proliferation and in the development of humoral immune responses. The CD19 protein contains two Ig-like domains and a 242-aa cytoplasmic domain, which includes nine tyrosine residues that are targets for rapid phosphorylation after B cell receptor and/or CD19 ligation. By providing docking sites for signaling molecules, CD19 engagement leads to the induction of multiple downstream effector events, including activation of the mitogen-activated protein kinase pathways, intracellular Ca^{2+} mobilization, and phospholipase C phosphorylation. Tyrosine-phosphorylated CD19 interacts with src family protein tyrosine kinases (Lyn, Lck, and Fyn), the Vav adapter protein, phosphoinositol-3 kinase, and phospholipase C-y (26). The cell surface density of CD19 appears to establish cellular signal transduction thresholds, because B cells from CD19-deficient mice are hyporesponsive to transmembrane signals and generate only modest immune responses (27, 28), whereas B cells from mice that overexpress CD19 are hyperresponsive to T cell-dependent Ags (28, 29).

Considering the structural and functional similarities between HER-2/neu and CD19, it was unexpected to observe significant differences in the abilities of HER-2/neu and CD19 to trigger Ab-mediated tumor cell killing. The primary objective of the studies presented here was to investigate the particular roles of the extracellular and intracellular domains of CD19 and HER-2/neu for ADCC by PMNs or mononuclear effector cells (MNCs). For this purpose, we created HER-2/CD19 and CD19/HER-2 chimeric target molecules and compared them with wild-type HER-2/neu and wild-type CD19 regarding their capacity to trigger ADCC. Results from these experiments suggest an important contribution of intracellular target Ag domains in Ab-based immunotherapy.
Materials and Methods

**mAbs and Ab constructs**

HER-2/neu-directed Abs L87 (mouse IgG1 (mlgG1)) and 2ERB19 (mlgG1) against extracellular domains or 3B5 (mlgG1) against the intracellular region of CD19, were obtained from NeoMarkers (Fremont, CA) and from Oncogene Research Products (Boston, MA), respectively. Humanized HER-2/neu Ab 4D5 (hereceptin, human IgG1 (hIgG1)) was from Hoffmann-LaRoche (Basel, Switzerland). Abs A77 (FcRIII, CD16; mIgG1), 3G8 (FcRII, CD16; mIgG1), and 520C9 (HER-2/neu, mlgG1) were from Medarex (Annandale, NJ). CD19 Abs J4.119 and RFB9 (both mlgG1) were from Beckman Coulter (Brea, CA) and Dr. M. Glennie (Tenovus Research Laboratory, Southampton, U.K.), respectively. Mouse/human chimeric HLA class II Ab F3.3 (human IgG1) was generated by M. Dechant in our laboratory from the original hybridoma (14), which was provided by Dr. M. Glennie.

**Bispecific Abs (BsAbs)** FcRI × HER-2/neu, FcRI × HLA class II, FcRI × CD19, and FcγRI × HLA class II were produced in the laboratory from Dr. M. Glennie by chemically cross-linking (Fab')2, from trigger molecule Abs A77 (FcRIII, CD19) or 22 (FcγRII, CD64; mlgG1), 3G8 (FcRII, CD16; mlgG1), and 520C9 (HER-2/neu, mlgG1) were from Medarex (Annandale, NJ), CD19 Abs J4.119 and RFB9 (both mlgG1) were from Beckman Coulter (Brea, CA) and Dr. M. Glennie (Tenovus Research Laboratory, Southampton, U.K.), respectively.

**cDNA cloning and generation of chimeric target Ags**

Plasmids containing human CD19 or HER-2/neu cDNAs were from Drs. M. Glennie and J. G. J. van de Winkel (University of Utrecht, Utrecht, The Netherlands). cDNAs were amplified from corresponding plasmids by PCR, adding HindIII and the XhoI restriction sites at the 5’ and 3’ ends, respectively. cDNAs were then cloned into the pcDNA3.1 vector containing a CMV promoter and ampicillin and neomycin (G-418) resistance genes (Stratagene, La Jolla, CA), using the newly introduced restriction sites. Chimeric target molecules HER-2/CD19 and CD19/HER-2, containing the extracellular and transmembrane regions of HER-2/neu and the intracellular region of CD19 or the extracellular and transmembrane regions of CD19 and the intracellular region of HER-2/neu, respectively, were created by SOE-PCR (31) (see Fig. 1 for a schematic representation). Four primers were designed for generating each of the chimeric cDNAs (see Table I for sequences). For chimeric HER-2/CD19, primer A annealed at the 5’ end of HER-2/neu; primer D annealed at the 3’ end of the CD19 cDNA. Primers B and C were complementary to each other, overlapping at the respective fusion point of the two cDNA molecules. cDNA fragments encoding the extracellular and transmembrane domains of HER-2/neu, using primers A and B, and the intracellular region of CD19, using primers C and D, were amplified in different reactions. Then, purified PCR products were mixed to allow annealing of complementary regions and were cloned to the full-length chimeric cDNA by PuI polymerase (Stratagene). Finally, primers A and D were used to amplify the novel cDNA, and the chimeric cDNA was cloned into the pcDNA3.1 vector, as described above. The chimeric CD19/HER-2 construct was generated similarly, using primers E to H. All constructs were verified by sequencing using the ABI PRISM sequencing kit (Applied Biosystems, Foster City, CA) and were compared with nucleotide data X13132 and M11730 from GenBank for CD19 and HER-2/neu, respectively.

**Transfection of cell lines**

Raji (Burkitt’s lymphoma) and SK-BR-3 (HER-2/neu-positive breast cancer) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in R10 medium supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 4 mmol/L 1-glutamine (Invitrogen, Carlsbad, CA). Raji cells were transfected by electroporation with either wild-type HER-2/neu or chimeric HER-2/CD19 cDNA. SK-BR-3 cells were transfected with either wild-type CD19 or chimeric CD19/HER-2 cDNA, using the lipofectamine-plus transfection system (Invitrogen). Transfected cells were grown under selective pressure in 0.6 mg/ml geneticin (Invitrogen) and were repeatedly sorted for high membrane expression of target Ags on a FACS (MoFlo, Cytomation, Fort Collins, CO) using FITC-conjugated HER-2/neu (520C9) or PE-conjugated CD19 (J4.119) Abs. Expression of correct target Ags was proved by RT-PCR followed by sequencing, immunofluorescence, and immunoblotting.

**Immunofluorescence analyses**

Target cell lines were incubated for 30 min with mAbs at 4°C, washed, and stained with dichlorotriazinyl amino fluorescein-labeled F(ab’2)2 of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were washed again and analyzed on an EPICS Profile flow cytometer (Coulter, Hialeah, FL). For each cell population, relative fluorescence intensity (RFI) was calculated as the ratio of mean linear fluorescence intensity of relevant to irrelevant, isotype-matched control Ab. For analyses of target Ag modulation, wild-type CD19- or chimeric CD19/HER-2-transfected SK-BR-3 cells and wildtype HER-2/neu- or chimeric HER-2/CD19-transfected Raji cells were incubated with Abs 520C9 (HER-2/neu), RFB9 (CD19), or isotype control, respectively, for 30 min on ice. After incubation at 37°C for 0 min, 30 min, 60 min, or 180 min, respectively, cells were washed and stained with dichlorotriazinylaminofluorescein-labeled F(ab’2)2 of goat anti-mouse IgG. The percentage fluorescence was calculated as the mean fluorescence intensity at x min at 37°C divided by the mean fluorescence intensity at 0 min at 37°C × 100.

**Immunoblot analyses**

Total cell proteins for immunoblot analyses were extracted in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor mixture (Roche, Indianapolis, IN). A total of 20 μg (SK-BR-3) or 40 μg (Raji) of protein lysate was resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Amersham, Little Chalfont, U.K.), and probed with HER-2/neu Abs (L87/2ERB19 1:500 or 3B5 1:5000). Immunoreactive proteins were visualized by chemiluminescence detection using HRP-conjugated goat anti-mouse IgGs (DAKO, Glostrup, Denmark) and ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions.

**Isolation of MNC and PMN effector cells**

After informed consent, 10–20 ml of peripheral blood was drawn from healthy volunteers or from patients receiving rG-CSF (5 μg/kg of body weight; Neupogen, Hoffmann-LaRoche), based on clinical indications. PMNs were isolated by a method as described (14). Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (62% weight; Neupogen, Hoffmann-LaRoche), based on clinical indications. PMNs were isolated by a method as described (14). Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (62% weight; Neupogen, Hoffmann-LaRoche), based on clinical indications. PMNs were isolated by a method as described (14). Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (62% weight; Neupogen, Hoffmann-LaRoche), based on clinical indications. PMNs were isolated by a method as described (14). Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (62% weight; Neupogen, Hoffmann-LaRoche), based on clinical indications.

| Table I. Primers for generation of chimeric target Ags and addition of respective restriction sitesa |
|-----------------------------------------------|
| **Primer** | **Sequence** |
| A | 5’-CCC CCC CCC AAG CTT CCA GTG AGC ACC ATG CAG C TG |
| B | 5’-TGG CTT CTT TCT TTT CTT C TT GAT GAG GAT CCC AAA GAC C |
| C | 5’-GTT CTT TGG GAT CTT CAT CAG GAG GAA AAG GGC G A |
| D | 5’-CCC CCC CCC TCT AGA CCA TGG TCG TCC CGA CCG GCT |
| E | 5’-CCC CCC CCC AAG CTT AGT CTT CCG ACC ACC ATG CCA CTT |
| F | 5’-CAA AGA GCC CTT GTC CTT AAG CAG CCA CGG CAG CAA G |
| G | 5’-CTCTT CTT CTC CCG TCG TCT CTT CAG CAC CAG GCC TCT TG |
| H | 5’-CCA CCC CCC TCT AGA TGG CCT CTT GTC TCA CAC TGG |

a Primer designations correspond to Fig. 1. Bold letters indicate HindIII restriction sites. XhoI restriction sites are underlined. Poly(C) overhangs were added for adequate binding of restriction enzymes.
Western blots of wild-type HER-2/neu, chimeric CD19/HER-2, or HER-2/CD19 fusion proteins. Whole cell lysates of transfected Raji or SK-BR-3 cells were adjusted to 100,000/ml. Effector cells (50 μl), sensitizing Ab constructs (all at 2 μg/ml), and R10° were added to round-bottom microtiter plates. For MNC effector cells, conventional Abs against HER-2/neu (hereceptin), HLA class II (chimeric F3.3), or CD19 (J4.119) were used, whereas killing by PMNs was analyzed in the presence of FcγRI × HER-2/neu or FcγRI × CD19 BsAb. In control experiments, FcγRI × HLA class II, FcγRI × HER-2/neu, or FcγRI × HLA class II BsAbs were used as indicated. Assays were started by adding labeled target cell suspension (50 μl), giving a final volume of 200 μl and an E:T cell ratio of 40:1 for Raji and 80:1 for SK-BR-3 cells. After 3 h at 37°C, assays were stopped by centrifugation, and 51Cr release from triplicate samples was measured in cpm. Percentage of cellular cytotoxicity was calculated using the following formula: % specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100, with maximal 51Cr release determined by adding perchloric acid to target cells (final concentration, 3%) and basal release measured in the absence of sensitizing mAb and effector cells. Only very low levels of Ab-mediated, noncellular cytotoxicity (without effector cells) were observed under these assay conditions (<5% specific lysis). Low levels of Ab-independent cytotoxicity (effectors without Abs) were seen with isolated MNCs.

Statistical analysis

Group data are reported as mean ± SEM. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student’s t test. Significance was accepted when p < 0.05.

Experiments reported here were approved by the Ethical Committee of the University of Erlangen-Nürnberg (Germany), in accordance with the Declaration of Helsinki.

Results

Generation of experimental target cells

To investigate the influence of intracellular Ag domains for Ab-mediated cellular cytotoxicity, cDNAs for chimeric HER-2/neu and CD19 molecules were generated by SOE-PCR from wild-type HER-2/neu and CD19. For a schematic representation of target molecule generation, see Fig. 1. cDNAs for wild-type HER-2/neu or chimeric HER-2/CD19 (extracellular/intracellular domains) were then transfected into Raji Burkitt’s lymphoma cells, whereas SK-BR-3 breast cancer cells were transfected with wild-type HER-2/CD19 cDNA fragments.

ADCC assays

ADCC assays were performed as described (18). Briefly, target cells were labeled with 200 μCi 51Cr for 2 h. After washing three times with R10°, cells were adjusted to 100,000/ml. Effector cells (50 μl), sensitizing Ab constructs (all at 2 μg/ml), and R10° were added to round-bottom microtiter plates. For MNC effector cells, conventional Abs against HER-2/neu (hereceptin), HLA class II (chimeric F3.3), or CD19 (J4.119) were used, whereas killing by PMNs was analyzed in the presence of FcγRI × HER-2/neu or FcγRI × CD19 BsAb. In control experiments, FcγRI × HLA class II, FcγRI × HER-2/neu, or FcγRI × HLA class II BsAbs were used as indicated. Assays were started by adding labeled target cell suspension (50 μl), giving a final volume of 200 μl and an E:T cell ratio of 40:1 for Raji and 80:1 for SK-BR-3 cells. After 3 h at 37°C, assays were stopped by centrifugation, and 51Cr release from triplicate samples was measured in cpm. Percentage of cellular cytotoxicity was calculated using the following formula: % specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100, with maximal 51Cr release determined by adding perchloric acid to target cells (final concentration, 3%) and basal release measured in the absence of sensitizing mAb and effector cells. Only very low levels of Ab-mediated, noncellular cytotoxicity (without effector cells) were observed under these assay conditions (<5% specific lysis). Low levels of Ab-independent cytotoxicity (effectors without Abs) were seen with isolated MNCs.

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CD19 or chimeric CD19/HER-2. Cells were sorted for high membrane expression of transfected target Ags, which was demonstrated to be similar to endogenous HER-2/neu on SK-BR-3 or to endogenous CD19 on Raji cells, respectively (Fig. 2). Importantly, transfected HER-2/neu or HER-2/CD19 on Raji cells and transfected CD19 or CD19/HER-2 on SK-BR-3 demonstrated similar levels of modulation upon Ab binding, respectively (data not shown). Immunoblot analyses with Abs against intracellular or extracellular domains of HER-2/neu confirmed the expected size of wild-type or chimeric proteins in respectively transfected cells (Fig. 3).

**Correlation between target Ag density and tumor cell lysis**

Previous studies had demonstrated that PMNs effectively killed different HER-2/neu-expressing breast cancer cell lines with HER-2/neu-directed Abs. However, killing levels were found to correlate with HER-2/neu expression levels of respective cell lines (18). To investigate the influence of target Ag density independently from different cellular backgrounds, Raji cells were transfected with wild-type HER-2/neu. Cells with distinct HER-2/neu expression levels, determined by immunofluorescence, then served as targets in ADCC assays with MNC or PMN effector cells. Because PMNs and MNCs required different Ab constructs to trigger optimal tumor cell lysis (32), killing by MNCs was analyzed in the presence of a humanized IgG1 Ab (herceptin), whereas PMNs were tested with a FcαRI × HER-2/neu BsAb. As demonstrated in Fig. 4, killing by both MNCs and PMNs reached a plateau beyond certain levels of target cell sensitization. However, PMNs required higher target Ag densities (half maximal killing at a RFI of 14) compared with MNCs (half maximal killing at a RFI of 6). At the same E:T ratio, MNCs triggered higher levels of tumor cell killing compared with PMNs. In all additional experiments, transfected Ags were expressed at similarly high levels, which were in the range of plateau killing by PMNs.

**Effector-target cell conjugate formation**

To mediate ADCC, effector cells first need to adhere to their targets. Therefore, we investigated whether our tumor cell transfectants differed in their capacity to form effector-target cell conjugates (Fig. 5). In the presence of FcαRI × HER-2/neu BsAb, conjugate formation between PMNs and wild-type HER-2/neu- or chimeric HER-2/CD19-transfected Raji cells was similarly effective (28 ± 3% and 24 ± 5%, n = 3, respectively). Similarly, PMNs adhered equally well to wild-type CD19- or chimeric CD19/HER-2-transfected SK-BR-3 cells in the presence of FcαRI × CD19 BsAb (34 ± 8% and 24 ± 10%, n = 3, respectively). With all transfected tumor cells, only marginal conjugate formation (<3%) was observed in the absence of sensitizing Abs.

*Intracellular domains of target Ags influence their capacity to mediate ADCC by PMNs*

Next, we investigated the contribution of intracellular target Ag domains on ADCC by PMN and MNC effector cells. For these experiments, experimental tumor cells served as targets in ADCC assays. PMNs effectively killed wild-type HER-2/neu-transfected Raji cells, but only low levels of killing against endogenous CD19 or against chimeric HER-2/CD19 were observed (Fig. 6A). Furthermore, PMNs effectively killed SK-BR-3 cells via chimeric...
CD19/HER-2, but did not lyse wild-type CD19-transfected SK-BR-3 cells (Fig. 7A). In contrast, MNCs mediated similarly effective cytotoxicity against all investigated target Ags (Figs. 6B and 7B). The described differences between chimeric and wild-type Ag-mediated killing by PMNs were also observed at other E:T ratios ranging from 20:1 to 160:1 (data not shown). To ensure that transfection and selection did not significantly alter lysis susceptibility of target cells, ADCC against endogenous HLA class II on Raji or against endogenous HER-2/neu on SK-BR-3 cells was investigated. In these experiments, similar killing of experimental target cells was observed (Figs. 6 and 7).

To exclude that differences in PMN-mediated killing were FcγRI-specific, killing of Raji cells transfected with either wild-type HER-2/neu or chimeric HER-2/CD19 was also investigated in the presence of a FcγRI × HER-2/neu Bs/Ab. For these experiments, G-CSF-primed PMNs were used as effector cells, which express similar levels of FcγRI and FcγRI (32). Interestingly, PMNs were highly effective with both BsAbs against wild-type HER-2/neu-transfected Raji cells, but not against chimeric HER-2/CD19-transfected Raji cells (Fig. 8).

Discussion

In this study, we demonstrate that intracellular domains of target Ags influence their capacity to trigger ADCC by PMN effector cells. Thus, transfected Ags containing the HER-2/neu intracellular domain triggered ADCC effectively, whereas Ags with the CD19 intracellular domain were ineffective in mediating killing. Interestingly, conjugate formation between effector and target cells was not affected by the intracellular domains of the transfected Ags. Although the exact function of the intracellular domains in ADCC is not defined, our results suggest an important link between FcγR-mediated effector mechanisms of Abs (5) and Ab-mediated signaling in tumor target cells (33). Thus, potential explanations for these differences between HER-2/neu and CD19 include differences in their capacity to initiate signal transduction. For example, the HER-2/neu intracellular domain itself has direct tyrosine kinase activity (20, 21), whereas CD19 must become tyrosine phosphorylated by other kinases before it associates with src family kinases Lyn, Fyn, and Lck (25, 26). In our experiments, we observed tyrosine phosphorylation of transfected HER-2/neu or chimeric CD19/HER-2, but not of transfected CD19 or chimeric HER-2/CD19, upon stimulation with vanadate (data not shown).
Furthermore, target Ags may differently internalize after Ab binding (34, 35). However, modulation of wild-type HER-2/neu and chimeric HER-2/CD19 on Raji cells, as well as wild-type CD19 and CD19/HER-2 on SK-BR-3, were similar. Alternatively, intracellular domains of HER-2/neu and CD19 may differently regulate recruitment of target Ags into lipid rafts (36, 37). This process may be involved in the formation of immunological synapses (38), which were critical for NK cell- (39) and PMN-mediated ADCC (40). Synapse formation also required interactions with the actin cytoskeleton of effector cells (41). However, it appears conceivable that recruitment of the target cell cytoskeleton also influences the outcome of ADCC and that the intracellular domains of HER-2/neu and CD19 differ in their capacity to interact with actin or related proteins. Thus, further studies are required to explore why target Ags with the HER-2/neu intracellular domain trigger ADCC more efficiently than Ags containing the CD19 intracellular domain.

Importantly, results with PMN effector cells were similar with either FcγRI- or FcγRI-directed BsAbs (Fig. 8), indicating that the observed differences between HER-2/neu and CD19 are not FcR-related. In contrast, MNC-mediated killing of tumor cells was not affected by the intracellular domains of target Ags, suggesting that killing mechanisms of PMNs and MNCs are fundamentally different. Recent evidence supported the role of cell-mediated mechanisms for the therapeutic efficacy of herceptin and rituximab in mice (7), but the most relevant effector cell population in this study was not defined. Clinical efficacy of rituximab was found to correlate with effector, and in particular NK, cell numbers (42), suggesting an important role for NK cells as effectors for Ab efficacy. This notion was supported by observations that MNC-mediated ADCC is independent from the selected tumor target Ag (14, 15) and that MNCs effectively trigger tumor cell lysis, even at low Ag expression levels. However, NK cell activity is tightly regulated as long as tumor cells express HLA class I Ags (11). In vivo, PMNs also may contribute substantially to Ab efficacy (12, 13) and, furthermore, may participate in the generation of tumor-directed active immune responses (43). At least for PMNs’ direct tumor killing capacity, selection of appropriate target Ags and sufficient expression levels appear critical. Results from studies presented here may help to more directly identify target Ags with the potential to recruit PMNs as effector cells for ADCC.

In animal models, CD19 Abs proved significantly less efficient than isotype-matched CD20 Abs (44), stimulating research to increase the therapeutic efficacy of CD19 Abs (45). Strong evidence that Ab-induced signaling in tumor cells is indeed relevant for Ab efficacy in patients came from studies with anti-idiotype Abs. In these experiments, Ab-triggered tyrosine phosphorylation in tumor samples was correlated with their clinical efficacy as anti-idiotype Abs (10). Our results underline the importance of intracellular domains of target Ags for ADCC by neutrophils, whereas MNCs were able to mediate effective cytotoxicity independently from the intracellular domain of the target Ag. Considering the high numbers of neutrophils compared with NK cells, at least in peripheral blood, these observations may influence the selection of target Ags for Ab trials. Provided that further studies confirm the observation that ADCC is a relevant mechanism of action for Abs in vivo (7) and that PMNs contribute to Ab efficacy in vivo (46), results from this study suggest inclusion of the capacity to trigger ADCC as an important selection criterion for novel target Ags in Ab-based immunotherapy.

Acknowledgments

We acknowledge the excellent technical assistance by S. Gehr and B. Bock, and Dr. P. Rohwer’s expertise in performing FACS on a MoFlo cell sorter. We thank Drs. J. G. J. van de Winkel and M. Glennie for generously providing valuable reagents.

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