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J Immunol 2003; 171:5124-5129; doi: 10.4049/jimmunol.171.10.5124
http://www.jimmunol.org/content/171/10/5124

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Overexpression of the protooncogene HER-2/neu is observed in approximately one-third of breast cancers and a varying proportion of ovarian, gastric, and other epithelial tumors. HER-2/neu expression is an independent risk factor for early relapse and death in breast cancer (1). The combination of the mAb trastuzumab (Herceptin) directed against HER-2/neu and chemotherapy improved the clinical outcome for patients with HER-2/neu-overexpressing metastatic breast cancers (2). HER-2/neu forms heterodimers with other members of the epidermal growth factor receptor family, e.g., epidermal growth factor receptor, HER-3 or HER-4 (3). HER-2/neu is a tyrosine kinase signaling molecule and its overexpression results in constitutive and coreceptor-independent kinase activation. We have previously demonstrated that isolated polymorphonuclear neutrophils (PMN)\(^1\) are potent effector cells against a wide range of malignancies in vitro (4–6). In contrast to T cells, the cytotoxicity of PMN is dependent on the presence of Abs binding to the target cells. Usually, PMN-mediated Ab-dependent cellular cytotoxicity (ADCC) is measured using 3-h \(^{51}\)Cr release assays (7, 8) with high E:T cell ratios up to 1000:1 (9). To recruit cell-mediated effector mechanisms, Abs must interact with Ig FcRs, which are classified as Fc\(\gamma\)R, Fc\(\alpha\), or Fc\(\epsilon\)-receptors depending on their specificity for IgG, IgA, or IgE, respectively (10). Fc\(\gamma\)R are grouped according to their affinity into low affinity receptors—named Fc\(\gamma\)RII (CD32) and Fc\(\gamma\)RIII (CD16)—and the high affinity Fc\(\gamma\)RI (CD64) (11). In vivo Fc\(\gamma\)R-bearing cells like NK cells, monocytes, or PMN are able to infiltrate tumors and to cross-link mAb bound to the surface of tumor cells (12). The myeloid form of Fc\(\gamma\)RII, e.g., the Fc\(\gamma\)RIIa expressed on PMN, contains an immunoreceptor tyrosine-based activation motif in its cytoplasmic domain, whereas the Fc\(\gamma\)RIIb isoforms, e.g., expressed on B cells display immunoreceptor tyrosine-based inhibitory motifs. A genetic polymorphism in the extracellular ligand binding domain of Fc\(\gamma\)RIIa determines reactivity with human IgG2, murine IgG1, and rat IgG2b isotypes (11). The two alleles differ in a single amino acid at position 131. The arginine-containing allele (R131) displays an increased affinity for murine IgG1, when compared to the allotype with histidine in this position (H131). Binding of ligands to Fc\(\gamma\)RI (CD89), Fc\(\gamma\)RII, and Fc\(\gamma\)RIIIa initiates intracellular signaling by the FcR common \(\gamma\)-chain, containing immunoreceptor tyrosine-based activation motifs. We have recently demonstrated that Fc\(\gamma\)RIIa triggers PMN-mediated cytotoxicity even better than the established activating molecules Fc\(\gamma\)RI and Fc\(\gamma\)RIIIa (13). The pivotal role of Fc\(\gamma\)R for the tumoricidal activities of mAbs has recently been demonstrated in knock-out mice, in which the signaling machinery of Fc\(\gamma\)R was genetically disrupted (14). Recent studies in lymphoma patients showed an association between the Fc\(\gamma\)RIIIa isotype and the clinical and molecular responses to CD20-directed therapy with mAbs (15).

The capacity to mediate ADCC has been demonstrated in vitro for monocytes/macrophages, NK cells, as well as eosinophilic and neutrophilic granulocytes. PMN are increasingly recognized as an important effector cell population for rejection of malignant tumors in vivo (12, 16, 17). Furthermore, PMN were the predominant effector cell population for the killing of breast cancer cells in the presence of HER-2/neu Abs in vitro (4). However, the mechanism of target cell death remains elusive. Recent therapeutic advances in Ab-mediated HER-2/neu directed cancer therapy have renewed the interest in its mechanism (2).
A apoptotic cell death is characterized by a breakdown of the membrane lipid asymmetry, by the loss of matrix adhesion and mitochondrial membrane potential, by "boiling" of cytoplasm, condensation of chromatin, and finally by internucleosomal cleavage of the nuclear DNA (18). Exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane already occurs at an early stage of apoptosis and is detected by the binding of annexin V. In late stages, DNA fragmentation and loss of chromatin result in sub-G_1 DNA content of the dying cells. The latter can be detected by propidium iodide (PI) staining in the presence of detergent. In contrast, the conventional short-term 51Cr release assay measures release of cytosolic chromate ions, which can only be observed when the cytoplasmic membrane of the target cells are disrupted in either primary or secondary necrosis. The 51Cr release assay requires high E:T cell ratios, unlikely to occur in vivo. In the present study, we investigated ADCC at low E:T cell ratios. Under these more physiological conditions, we observed an Ab-dependent apoptosis of target cells (ADAC) depending on the presence of both PMN and tumor-specific Abs.

Materials and Methods

Cells and culture conditions

The human breast cancer cell lines SK-BR-3 and MDA-MB 453 were obtained from the American Type Culture Collection (Manassas, VA). Cells were kept in RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 3 mM g-l-glutamine (all from Life Technologies). Experiments reported in this study were in accordance with the Declaration of Helsinki. After informed consent, 10–20 ml of citrate anticoagulated peripheral blood was drawn from healthy volunteers for effector cell preparation.

Isolation of PMNs and mononuclear cells

Isolated neutrophils were obtained by a method slightly modified from that described in Ref. 19. Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (Seromed, Berlin, Germany), consisting of 70% and 62% Percoll. After centrifugation, neutrophils were collected between the two Percoll layers, and mononuclear cells from the plasma/Percoll interface. Remaining erythrocytes were removed by hypotonic lysis. Purity of neutrophils was determined by cytospin preparations and exceeded 95%, with few contaminating eosinophils and <1% mononuclear cells. Viability of cells tested by trypan blue exclusion was >95%.

mAb and Ab constructs

Murine whole Ab and F(ab')_2, against the proto-oncogene product HER-2/neu were 520C9 (mlgG1; Medarex, Annandale, NJ) (20). Control Ab was Th 69 (mlgG1) against CD7 (21). FITC-labeled F(ab')_2 of Ab to mouse IgG were from Cappel (Cocranville, PA).

For Ab A77 to FcoRI (mlgG1, CD89) (22) was kindly provided by Medarex, mAb AT10 (mlgG1) (23) and mAb 41H16 (mlgG2a) (24), both against FcyRII, were generous gifts from M. Glennie (Tenovus Research Laboratory, Southampton, U.K.) and B. Longenecker (University of Alberta, Edmonton, Alberta, Canada), respectively.

Bispecific Ab (FcoRI × HER-2/neu) was a generous gift from M. Glennie (Tenovus Research Laboratory, Southampton, U.K.). The bispecific Ab was produced by chemically cross-linking F(ab')_2 of mAb 520C9 (HER-2/neu), and A77 (FcoRI; CD89), as described elsewhere (25). The bispecific Ab showed binding to both E:T cells consistent with the specificity pattern of the parental Abs.

Allotyping for the R-H131 polymorphism of FcyRIIa

Phenotyping for the R131 and H131 alleles of the FcyRIIa was performed by quantitative indirect flow cytometry of peripheral blood monocytes (26). Cells were stained with mAb 41H16, which selectively recognizes the FcyRIIa-R131 alloform, or by mAb AT10 recognizing both FcyRIIa allofroms.

ADCC

51Cr release assays were performed as described elsewhere (27). Briefly, target cells were labeled with 200 mCi 51Cr for 2 h. After extensive washing with RF10°, cells were adjusted to 10^5 per milliliter. PMN at the indicated E:T cell ratio, sensitizing Abs at a final concentration of 2 mCi/ml, and RF10° were added to round-bottom microtiter plates (Nunc, Roskilde, Denmark). Assays were started by adding 50 mCi of the target cells, resulting in a final volume of 200 mCi. After 3 h at 37°C, assays were stopped by centrifugation, and 51Cr release from triplicates was measured. Percentage of cellular cytotoxicity was calculated using the formula: specific lysis = (experimental cpm – basal cpm)/maximal cpm – basal cpm) × 100%.

Maximal 51Cr release was determined by adding perchloric acid to target cells at a final concentration of 3%. Basal release was measured in the absence of sensitizing Abs and effector cells. No Ab-independent cytotoxicity was observed with PMN as effector cells in the absence of targeting Abs.

For analyses of ADAC by flow cytometry and DNA binding, MLN and SK-BR-3 cells at a final concentration of 10^7 were added to 10^5 RF10° to yield an E:T cell ratio of 10:1 in a final volume of 1 ml. Assays were started by addition of mAb or of bispecific Ab at a final concentration of 2 mCi/ml, respectively. Assays were incubated for 18 h at 37°C in a humidified atmosphere containing 5% CO_2.

Immunofluorescence analyses

Phosphatidylserine exposure during apoptosis was detected by FITC-labeled annexin V (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Necrotic cells were detected by PI uptake. After a 20-min incubation, annexin V binding was measured with an EPICS Profile flow cytometer (Beckman Coulter, Brea, CA). PMN and target cells were distinguished by forward scatter vs side scatter (Fsc-Ssc) characteristics. Annexin V-positive and PI-negative cells were considered apoptotic.

Nuclear DNA content was analyzed by PI staining after permeabilization of the cytoplasmic membrane with detergent (28). Two to five volumes of PI-Triton staining solution (0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/ml PI) were added to the cell suspension. The DNA content was measured after an incubation at 4°C in the dark for 24 h. Because the nuclei of the SK-BR-3 target cells were hyperdiploid and displayed different morphology compared to PMN, they could easily be differentiated from the nuclei of the PMN by increased PI staining and by characteristic Fsc-Ssc.

Fluorescence laser scanning microscopy

The in situ end labeling of DNA strand breaks (TUNEL) generated during apoptosis was performed with the In Situ Cell Death Detection Kit, Fluorescein (Roche), according to the manufacturer’s instructions for cytopsin preparations. A TUNEL reaction mixture without TdT served as a negative control. After ADCC assay for 18 h at 37°C, PMN and SK-BR-3 cells were centrifuged onto microscope slides. Cells were air-dried and fixed with a solution of 4% paraformaldehyde in PBS at pH 7.4 for 30 min. After flushing with PBS, the slides were incubated with a mixture of PI (0.1% sodium citrate) and PI (1 mg/ml) for 30 min. The slides were rinsed twice with PBS and the area around the sample was dried. Fifty microliters of the TUNEL reaction mixture was added and the staining was allowed to proceed for 60 min at 37°C in the dark. The slides were washed three times in PBS and were examined by confocal laser scanning microscopy (Bio-Rad, Munich, Germany).

Statistical analysis

Results are expressed as means ± SEM. The statistical significance was calculated with the Student t test (Excel; Microsoft, Richmond, VA).

Results

High E:T cell ratios are required for lysis of SK-BR-3 cells in 51Cr release assays

HER-2/neu-positive SK-BR-3 breast cancer cells were incubated in the presence of the murine IgG1 mAb 520C9 (2 mCi/ml) with increasing PMN to target cell ratios. In 3-h 51Cr release assays, mAb-mediated relevant lysis only at E:T ratios above 40:1. Twenty-hour 51Cr release assays gave similar results (Fig. 1). No significant lysis was to be observed in the absence of either PMN or 520C9 (data not shown).

PMN induce Ab-dependent apoptosis of SK-BR-3 cells

The mechanism of Ab-mediated cell death induced by PMN remains unclear. Therefore, we analyzed the induction of apoptosis in breast cancer cells during ADCC. Apoptosis of the SK-BR-3 cells was measured by binding of Annexin V (nonspecific or target cells after a 20-h incubation with PMN isolated from healthy donors.
In the presence of the murine IgG1 mAb 520C9, PMN of R/R131 individuals were significantly more cytotoxic than those of H/H131 donors

Two allelic forms of FcyRIIa are expressed on myeloid cells. Because chromium release induced by murine IgG1 has been demonstrated to be more efficient with PMN of R/R131 individuals (4), we examined whether this is also true for the induction of apoptosis. ADAC by PMN from three donors homozygous for FcyRIIa-R/R131 or FcyRIIa-H/H131 were compared in the presence of the mAb 520C9. As shown in Fig. 4, the viable nuclei of the tumor cells, measured by PI staining, was significantly (p < 0.001) reduced in the presence of PMN isolated from all donors. However, PMN from R/R131 individuals were significantly more cytotoxic than those from H/H131 donors (p < 0.001).

TUNEL confirmed apoptosis of breast cancer cells in ADCC

To further confirm apoptotic cell death of the target cells in ADCC, we used the TUNEL method. PMN and SK-BR-3 cells were co-incubated for 20 h in the presence or absence of the mAb 520C9. Subsequently, cells were permeabilized and stained with the TUNEL technique. Nuclear counterstaining was performed with PI. Stained cells were analyzed using laser scanning fluorescence microscopy. In the absence of targeting mAb, we detected only background levels of TUNEL-positive SK-BR-3 nuclei. In contrast, in the presence of the mAb 520C9 we observed numerous apoptotic breast cancer cells (Fig. 2C), thus confirming apoptotic death of the tumor cells. PMN could easily be differentiated from the tumor cells by their nuclear morphology. Apoptotic PMN showed bright TUNEL staining, whereas viable PMN were TUNEL-negative. Few contaminating eosinophilic granulocytes showed unspecific dUTP FITC staining (30).

Cytokeratin-18 cleavage confirmed apoptosis of breast cancer cells in ADCC

SK-BR-3 cells were cocultured with PMN at an E:T ratio of 10:1 for 42 h in the presence or absence of HER-2/neu mAb. Adherent cells were stained for a cytoplasmic neoepitope of cytokeratin-18, which is generated by caspase cleavage during late phase of apoptosis of epithelial cells (31). Apoptotic SK-BR-3 cells were almost exclusively observed in the presence of the HER-2/neu mAb. Cytoplasmic staining of apoptotic SK-BR-3 cells could easily be distinguished from rare PMN showing faint, unspecific surface fluorescence (Fig. 2, D–G).

Discussion

The aim of this study was to investigate the mechanism of Ab-dependent, PMN-mediated cytotoxicity against breast cancer cells. We used flow cytometry and fluorescence microscopy to investigate changes in target cells indicative for apoptotic vs necrotic cell death. Interestingly, we observed annexin V binding to PI-negative, apoptotic tumor cells during PMN-mediated ADCC. In addition, analyses of cellular DNA content, TUNEL staining, and detection of specific caspase-cleaved cytokeratin-18 fragments confirmed PMN-induced ADAC in breast cancer cells. Interaction between FcR on PMN and target cells was required for the induction of apoptosis. Conventional Abs targeting FcyRII (4), or bispecific Abs targeting FcR on PMN initiated apoptosis, whereas isotype controls and F(ab')2 of the HER-2/neu Ab had no apoptosis-inducing effect on breast cancer cells. The F(ab')2 of the mAb 520C9 even increased tumor cell survival, most likely due to stimulation of SK-BR-3 due to cross-linking of HER-2/neu molecules.

The polymorphisms of FcyRIIa (32, 33), FcyRIIb (34), FcyRIIa (15), and FcyRIIib (35, 36) have been demonstrated to have clinical implications and functional relevance. In the presence

ADCC results in apoptotic target cells with sub-G1 DNA contents

Due to their hyperdiploid karyotype, PI-stained G0/G1 and G2 nuclei of SK-BR-3 cells could easily be distinguished from euploid PMN nuclei (Fig. 3A). During apoptosis induced by irradiation with UV B, chromatin is degraded and apoptotic cells can be identified by their sub-G1 DNA content. In contrast, induction of necrosis (e.g., by treatment with methanol, ethanol, or heating for 30 min to 56°C) does not generate nuclei with sub-G1 DNA content (data not shown). In the presence of conventional HER-2/neu-directed mAb or bispecific Ab (FcR1 × HER-2/neu), SK-BR-3 cells underwent apoptosis, reflected by the disappearance of target cell nuclei with G0/G1-S-G2 DNA content (Fig. 3A). F(ab')2 of HER-2/neu-directed mAb or isotype controls had no effect on SK-BR-3 cell death. The latter results demonstrate the involvement of FcR in the induction of apoptosis. In accordance with our previous data obtained with the chromium release assay (13, 29), targeting FcR1 as cytotoxic trigger molecule on PMN induced significantly more apoptosis than targeting FcyR with conventional mAb (Fig. 3B). Similar results were obtained using the HER-/neu positive breast cancer cell line MDA-MB453 (Table I).
of the murine IgG1 Ab 520C9, apoptosis induction of SK-BR-3 cells was affected by the genetic polymorphism of FcR/H9253RIIa. Neutrophils isolated from FcR/H9253RIIa-R/R131 donors induced significantly higher levels of apoptosis than those isolated from FcR/H9253RIIa-H/H131 donors (Fig. 4). This FcR/H9253RIIa allotypic polymorphism has previously been shown to be relevant in several types of assays involving murine IgG1 Abs. These include anti-CD3-induced T-cell activation experiment. *, A highly significant increase in apoptotic cells compared to control without mAb, \( p < 0.001 \). Photomicrographs showing cocultures of PMN and SK-BR-3 stained with the TUNEL technique (C). PMN can easily be differentiated from SK-BR-3 by their nuclear morphology (upper left panel, PI staining). Apoptotic PMN (PA) show bright TUNEL staining, whereas viable PMN (PV) are TUNEL-negative (upper right panel). In the absence of targeting mAb (upper panels), no induction of TUNEL-positive SK-BR-3 nuclei has been observed (SV). In contrast, in the presence of the mAb 520C9 targeting HER-2/neu on the breast carcinoma cell line SK-BR-3, numerous apoptotic tumor cells (SA) have been recognized (bottom right panel). E, A contaminating eosinophilic granulocyte with unspecific dUTP FITC staining (30). PMN-induced cytokeratin-18 cleavage in SK-BR-3 is dependent on the presence of HER-2/neu Abs. SK-BR-3 cells were cocultured with PMN at an E:T ratio of 10:1 for 42 h in the presence (D1–3, E1–3) or absence (F and G) of the HER-2/neu mAb 520C9. Adherent cells were stained for a cytoplasmic neoepitope of cytokeratin-18, generated by caspase-mediated cleavage during the late phase of apoptosis of epithelial cells. Apoptotic SK-BR-3 cells (arrows) can easily be distinguished from rare unspecific surface binding of PMN (arrowheads). The space bar represents 20 \( \mu \)m.
cell proliferation, phagocytosis of IgG-coated erythrocytes (37), and HER-2/neu Ab-dependent 51Cr release with murine IgG1 Ab (4). Thus, the efficacy of murine IgG1 Abs in tumor therapy may also depend on the FcγRIIa allotype of the patients’ effector cells. Interestingly, apoptosis was seen with E:T cell ratios as low as 10:1, which reasonably occur in vivo. Targeting HER-2/neu, invasion of PMN into breast cancer metastases has been demonstrated in a phase I clinical trial (38).

However, the mechanism of apoptosis induction by neutrophils remains elusive. It is still unclear whether PMN start an intrinsic cell death program in the target cells, or whether, in analogy to the killing by cytotoxic T cells and NK cells, the release of the contents of neutrophils into the target cells is required. Recently, complement-mediated apoptosis of malignant B cell lines, opsonized with CD20-directed mAb, has been demonstrated in the absence of effector cells (39). Human neutrophils contain a rapidly mobilizable pool of so-called secretory vesicles, which are distinct from the azurophilic and specific granules. They contain plasma-derived proteins (40), e.g. albumin, and are referred to as “easily mobilizable vesicles.” These secretory vesicles presumably also contain complement. Because PMN do not contain perforin, one may speculate that complement molecules released from secretory vesicles may permeabilize the cell membrane of target cells. This may enable the penetration of apoptosis-inducing enzymes into the cytosol of the target cells. PMN are continuously in a preapoptotic state (41). Therefore, apoptosis-inducing molecules, e.g., activated caspases, may be transferred into the target cell following cell surface contacts, and cytosolic communication after pore-forming or membrane fusion. Alternatively, an intrinsic cell death program, triggered by the engagement of cell surface molecules of the target cells, may operate.

At high E:T cell ratios, as required for chromium release assays, cells are struck with such a strong impact that energy supply quickly ceases, repair function declines, and necrosis ensues. However, less impact, e.g., at low E:T cell ratios, is sufficient to initiate apoptosis. However, the execution of the energy-consuming death program takes time. We conclude that induction of apoptosis and necrosis both contribute to Ab-dependent killing by PMN of target cells.

In the context of biological tumor therapy, induction of tumor cell apoptosis seems to be profitable for the host. Nevertheless, nonopsonized apoptotic cells have been described to exert potent immunosuppressive effects (42). Mice immunized with apoptotic tumor cells mounted a significantly reduced humoral immune response when compared with animals injected with viable tumor cells (43). Therefore, the generation of huge amounts of apoptotic cells may suppress the immune response of the host, thereby enabling the immune escape of the tumors. However, in these experiments, apoptosis was induced by irradiation with UV B in nonimmunized animals, whereas in the present study, tumor cell apoptosis was induced by the concerted action of PMN and tumor-specific mAb via ADAC. Therefore, opsonization with Abs of tumor cells, may target the dying cells to FcR and complement-receptor mediated phagocytosis by professional APC, e.g., dendritic cells. Additionally, the activation of PMN during target cell apoptosis induction may herald a “danger signal” (44) for the immune system. In accordance with this hypothesis, PMN have been demonstrated to be critical in some animal models of tumor rejection (12). Surprisingly, depletion of T cells in a murine model of tumor therapy with FcγRI-directed bispecific Abs recognizing the idiotype of a B cell lymphoma resulted in an complete loss of efficacy (17). In conclusion, we demonstrated that PMN are able to kill tumor cell targets via Ab-dependent apoptosis induction. The exact mechanism of this apoptosis pathway and its role during tumor immune responses remain to be defined.

**Acknowledgments**

We gratefully acknowledge the excellent technical assistance by S. Gehr and P. Heyder. Furthermore, we thank Dr. Martin Glennie from the Cancer Sciences Division, School of Medicine, General Hospital (Southampton, U.K.) for providing the bispecific Ab construct.

**References**


![Figure 4](http://www.jimmunol.org/)


22. Elsaesser, T. F., G. J. Launon, and B. M. Longenecker. 1983. A monoclonal antibody to the 63,000 m.w. molecule that is present on B lymphocytes and chronic lymphocytic leukemia cells but is rare on acute lymphocytic leukemia blasts. J. Immunol. 131:504.

