Decreased Survival of Human Breast Cancer Cells Expressing HER2/neu on In vitro Incubation with an Anti-HER2/neu Antibody Fused to C5a or C5a_{desArg}

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Abstract

Treatment of human epidermal growth factor receptor 2 (HER2/neu)-expressing breast cancer patients with a monoclonal antibody (mAb) directed against HER2/neu improves the outcome of chemotherapy. In cases in which remission is observed, antibody-dependent cell-mediated cytotoxicity (ADCC) seems to be one of the main mechanisms of anti-HER2/neu mAb action, implicating Fcγ receptors (FcγRs) in this tumoricidal activity. In vitro and in vivo studies have revealed that anti-HER2/neu-mediated ADCC is mainly accomplished by polymorphonuclear granulocytes (PMN). C5a, a cleavage product of the complement component C5, modulates FcγR expression via upregulation of activating and downregulation of inhibitory FcγRs. C5a also recruits PMNs to sites of inflammation and increases PMN survival. To enhance the recruitment and activation of C5a receptor–bearing cells into the tumor microenvironment, we developed antibody fusion proteins composed of a human IgG3 anti-HER2/neu antibody genetically fused to C5a [anti-HER2/neu IgG3-(C5a)] or to its derivative, C5a_{desArg} [anti-HER2/neu IgG3-(C5a_{desArg})]. Both fusion proteins were expressed, properly assembled, and secreted by murine myeloma cells, and displayed chemotactic activity on human PMN. Under comparable conditions, anti-HER2/neu IgG3-(C5a_{desArg}) increased the survival of PMN more efficiently than anti-HER2/neu IgG3-(C5a) or C5a_{desArg}. Surprisingly, incubation of the fusion proteins with breast cancer cells that overexpress HER2/neu (SK-BR-3) induced cell death at a dose at which the anti-HER2/neu IgG3 antibody was innocuous. In the presence of human peripheral blood leukocytes as effector cells, both fusion proteins induced tumor cell death more efficiently than anti-HER2/neu IgG3. These data suggest that anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a_{desArg}) fusion proteins possess novel properties that could be useful in cancer immunotherapy.

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Introduction

The human epidermal growth factor receptor 2 (HER2/neu) is a 185 kDa membrane glycoprotein with intrinsic kinase activity (1). On ligand binding, other members of the human epidermal growth factor receptor family (HER1, HER3, and HER4) form heterodimers with HER2/neu and initiate intracellular signaling that leads to cell proliferation. In the context of cell cycle control defects, overexpression of HER2/neu can result in dimerization, intracellular signaling, uncontrolled cell growth, and tumorigenesis (2).

Her2/neu, a protooncogene that has been found amplified in a variable proportion of many different types of carcinomas, is associated with poor prognosis and resistance to chemotherapy and radiotherapy. Approximately 25% of all breast cancers exhibit overexpression of the HER2/neu protein (3). Adjuvant therapy with the anti-HER2/neu monoclonal antibody (mAb) trastuzumab improves the outcome of chemotherapy in such cases. Although the benefits of this combined therapy are well recognized, the disease-free interval and overall survival rates are nonetheless low (4). One experimental approach for improving the efficacy of mAb has been to fuse immunomodulatory molecules such as certain cytokines to therapeutic antibody using molecular biology techniques (5, 6). These antibody fusion proteins seek to target immunostimulating properties of cytokine(s) into the tumor microenvironment, enhancing the positive effects of the therapy, but avoiding the adverse side effects observed after the systemic administration of some of those cytokines at high doses (7, 8).
The mechanism of trastuzumab action that best correlates with remission responses in breast cancer patients is antibody-dependent cell-mediated cytotoxicity (ADCC; refs. 9, 10). In vitro studies show that anti-HER2/neu-mediated ADCC against HER2/neu-positive breast cancer cell lines is mainly accomplished by polymorphonuclear granulocytes (PMN; ref. 11). C5a, a soluble peptide released after cleavage of C5 during activation of the complement cascade, is a potent chemoattractant and activator of PMN. C5a recruits PMN to sites of inflammation and prolongs their survival (12, 13). Serum carboxypeptidases remove the carboxy-terminal arginine of C5a, converting it into C5adesArg. Human C5adesArg preserves most biological properties of C5a, but to a great extent loses its spasmodic/anaphylatoxin activity (14).

We hypothesized that fusing C5a or C5adesArg to an anti-HER2/neu antibody could enhance the latter’s anti-carcinoma activities via the activation, attraction, and retention of immune C5a receptor (C5aR)-bearing cells into the tumor microenvironment. Our objective in the present work was therefore to create such fusion proteins and test their capacity to attract and activate human PMN, as well as to interfere with tumor cell survival.

Materials and Methods

Cell lines

U-937 (a human monocytic cell line expressing the C5 gene), THP-1 (a human monocytic leukemia cell line expressing Fcγ receptors), SK-BR-3 and MCF-7 (two human, mammary adenocarcinoma cell lines that express HER2/neu), and MDA-MB 468 cells (a human, mammary adenocarcinoma cell line that does not express HER2/neu) were purchased from the American Type Culture Collection (ATCC). TAUN (an anti-HER2/neu light chain producer transfectoma) was previously derived from the murine myeloma cell line P3X63Ag8.653 (15).

Construction of recombinant genes encoding antibody-fusion proteins

The human C5a gene was cloned as a cDNA by reverse transcriptase-PCR as previously described (16), using total RNA from U-937 cells, and the oligonucleotides A, B, and C shown in Table 1. The expected amplicon of 221 bp was purified (Gen Extraction Kit, Clontech) after separation by electrophoresis in 2% agarose, and cloned into a TA vector (pCR2.1, Invitrogen). DNA sequencing of multiple independent clones confirmed the identity and accuracy of the cloned gene.

The cloned C5a gene was modified by PCR as follows: (a) A short DNA sequence encoding the last 11 amino acids of the human Ig γ3 chain (not including the stop codon) was added at its 5′ terminus using the oligonucleotide D (Table 1). This sequence contains recognition sites for the restriction endonucleases SalI and SphI. (b) The carboxyl-terminal arginine codon was subtracted (only for the C5adesArg version) and a TAG stop codon was added at its 3′ terminus using the oligonucleotides E and F. Both oligonucleotides also incorporate a recognition site for the endonuclease EcoRI to the PCR products. C5a and C5adesArg PCR products were cloned and analyzed as indicated above.

Details of the strategy followed for preparing the recombinant genes encoding the fusion proteins and the expression vectors carrying them are provided in Supplementary Fig. S1. Briefly, the recombinant anti-HER2/neu Ig γ3 heavy chain used was prepared previously (15), and contains a human HER2/neu-specific VH domain obtained from the ScFv C6MH3-B1 developed (17) and kindly provided by Dr. James D. Marks (University of California, San Francisco, CA). The gene encoding this anti-HER2/neu γ3 heavy chain was retrieved as an XbaI/BamHI fragment from vector pAH3552 (15) and cloned into pACNR1180 (18). The pACNR1180 derivative obtained was used for transferring C5a or C5adesArg genes downstream of the anti-HER2/neu γ3 gene. To do that, C5a and C5adesArg PCR products were first transferred as SalI/EcoRI fragments into the plasmid 3447KS+, a pBluescript II derivative that harbors a piece of genomic DNA containing the human Ig γ3 heavy chain 3′ untranslated region (UTR), immediately upstream of the 3′UTR of γ3. To carry out the gene fusions, the C5a′/3′UTR or C5adesArg′/3′UTR DNAs constructs were then retrieved from plasmid 3447KS+ derivatives as SphI/BamHI fragments and inserted into the pACNR1180 derivative replacing the original 3′UTR. After fusion, chimeric genes were moved into the expression vector pAH1792 (19) as XbaI/BamHI fragments.

Expression and characterization of recombinant proteins

TAUN cells (15) producing the cognate anti-HER2/neu light chains were used to transfect the plasmids containing anti-HER2/neu γ3/C5a or γ3/C5adesArg genes using clonectin (Clontech) as recommended. Stable transfectants were selected in the presence of 20 mmol/L histidinol (Sigma) and culture supernatants were screened using human Ig γ chain- and C5adesArg-specific enzyme-linked immunosorbent assays (ELISA; OptiTEA, BD Pharmingen). Selected transfectomas were grown in roller bottles, and the fusion proteins were purified from tissue culture supernatant using Protein G-Sepharose 4B (Pharmacia) affinity chromatography. An IgG3 anti-HER2/neu antibody (15) with the same specificity as the fusion proteins was included as a control during the structural and functional assays described below. The IgG content of the fusion protein preparations was estimated by quantitative IgG-specific ELISA, using recombinant human IgG3 as standard.

Structural and functional assays

Molecular composition and size of the purified proteins were studied by SDS-PAGE/Coomassie blue staining and Western blot analyses, carried out under nonreducing and reducing conditions, and using antihuman IgG (Sigma) or antihuman C5adesArg (BD-Pharmingen) antibodies.
Fusion Proteins in Cancer Immunotherapy

Table 1. Primer design for cloning the human C5a gene

<table>
<thead>
<tr>
<th>Name</th>
<th>(Id)*</th>
<th>Sequence</th>
<th>Target region in template</th>
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<tr>
<td>Primers for cloning the human C5a gene</td>
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<tr>
<td>C5a cDNA</td>
<td>(A)</td>
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<td>Nucleotides 2281-2300 of the human C5 mRNA (exon 18)</td>
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<tr>
<td>C5a sense</td>
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</tr>
<tr>
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<td>(C)</td>
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<td>Nucleotides 2245-2265 of the human C5 cDNA (exon 17)</td>
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<td>Primers used to modify the C5a gene</td>
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<tr>
<td>C5a/C5aDesArg sense</td>
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<td>Nucleotides 2044-2057 of the human C5 gene (exon 16)</td>
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<tr>
<td>C5aDesArg antisense</td>
<td>(E)</td>
<td>5’GGGAATTCCTATACTCCATGACATGTTTTATBGAGATTTAGACGGGAG</td>
<td>Nucleotides 2224-2265 of the human C5 gene (exon 17)</td>
</tr>
<tr>
<td>C5a antisense</td>
<td>(F)</td>
<td>5’GGGAATTCCTACCTCTCCAAATTGCA</td>
<td>Nucleotides 2224-2262 of the human C5 gene (exon 17)</td>
</tr>
</tbody>
</table>

NOTE: Primer A was designed to hybridize to a sequence in the human C5 mRNA located 3’ from the sequence encoding the C5a fragment. Primers B and C were designed to hybridize to sequences containing the first (B) or last (C) seven codons of the C5a fragment. Primer D contains the first 15 nucleotides of primer B at its 3’end, and it incorporates a DNA sequence containing the last 11 codons of the human y3 gene (bold), a SapI site (underlined), and a protected (GGG) SalI site (double-underlined). Primer E was designed to hybridize to the last 13 codons (not including the AGG triplet encoding the carboxy-terminal Arg) of the C5a fragment, and it incorporates a stop codon (bold) and a protected (GGG) EcoRI site (underlined). Primer F was designed to hybridize to the last 14 codons of the C5a fragment, and it incorporates a stop codon (bold) and a protected (GGG) EcoRI site (underlined).

*Id = primer identification.
†According to Wetzel, 2000 (ref. 41).

Freshly isolated, human peripheral blood leukocytes (PBL) were used as a source of PMN (C5aR-positive cells) in a series of functional assays set to evaluate binding and biological activities of the fusion proteins. Venous blood from healthy donors, 3.9% sodium citrate, and 6% Dextran 500 (Pharmacia) solutions were gently mixed and centrifuged at 100 × g (Laboratorios Behrens). The leukocyte-rich plasma was aspirated, and WBC were allowed to sediment for one hour at room temperature, at volume ratios of 1:0.10:0.20, respectively. RBC were collected by centrifugation at 100 × g and suspended in PBS (21). All solutions were prepared using pyrogen-free materials and water (Laboratorios Behrens).

HER2/neu, C5aR, and FcγR binding. Binding of the fusion proteins to HER2/neu was studied by flow cytometry. HER2/neu-expressing SK-BR-3 cells were suspended in culture supernatants containing the fusion proteins or controls, and incubated at 4°C for one hour with rotation. After washing the cells, bound fusion proteins were detected using a goat anti-human IgG conjugated to phycoerythrin (BD-Pharmingen). The percentage of positive cells was determined using a FACScalibur (Beckton Dickinson).

Binding of the fusion proteins to the C5a receptor (C5aR/CD88) was also evaluated by flow cytometry. Culture supernatants containing the fusion proteins were tested for their capacity to diminish the binding of a blocking anti-C5aR antibody (rabbit anti-human CD88, BD-Pharmingen) to human PBL. Briefly, cells incubated in culture supernatant as described above were washed, and then labeled with anti-CD88 and goat anti-rabbit IgG conjugated to FITC (BD-Pharmingen). To prevent binding of the antibody fusion proteins through FcγRs, PBL were incubated with purified human immunoglobulins (Sandoz SA) before incubation in culture supernatants. The percentage of positive cells was determined by flow cytometry in the region (forward versus side scatter plot) corresponding to the PMN population.

 Binding of the fusion proteins to FcγRs was studied using flow cytometry and human THP-1 cells as FcγR-positive cells. THP-1 cells, incubated in culture supernatant containing the fusion proteins as described above, were made to react with a recombinant human IgG3 antibody (21) conjugated to FITC (BD-Pharmingen). The capacity of the culture supernatant to diminish the binding of the recombinant human IgG3-FITC conjugate to THP-1 cells was measured as indicated above.

Chemotactic activity. The capacity of the fusion proteins to attract human PBL was assayed using a Boyden chamber protocol with modifications (22). Briefly, wells of the bottom chamber of a microplate (ChemoTx, Neuprobe) were filled with 29 µL of purified fusion protein or controls and covered with an 8-µm-pore-size filter.
Twenty-five microliters of a CalceinAM-loaded PBL suspension containing 4 \times 10^6 cells/mL were applied onto each position of the filter. The microplate was incubated at 37°C, in 5% CO2 for 4 hours. Fluorescence in the lower chamber was read at 485/530 nm using a GENios plate reader (TECAN). The migration ratio was calculated by dividing the estimated number of fluorescent cells in each well by the number of fluorescent cells that migrated spontaneously (control).

**Activation and survival of PMN.** The increase in intracellular calcium concentration was measured to check whether incubation with the fusion proteins initiated an intracellular signaling cascade. Human PBL were loaded with 200 nmol/L FURA 2-AM (Molecular Probes) at 37°C for 30 minutes, and, after washing with PBS, changes in the concentration of intracellular calcium in response to the addition of the fusion proteins were detected using a spectrofluorometer (Photon Technology International). Excitation wavelengths were 340 nm and 380 nm, and emission was fixed at 510 nm. The maximal fluorescent ratio (R_{max}) was determined by the addition of 80 μg/mL digitonin, and the minimal fluorescent ratio (R_{min}) by subsequent addition of 80 mmol/L EGTA in 100 mmol/L Tris buffer, pH 7.4.

Upregulation of the integrin Mac-1 (CD11b) expression was used as an indicator of PMN activation (13). PBL (3 \times 10^8 cells per condition assayed) were incubated in culture medium containing purified fusion proteins or controls for 30 minutes at 37°C and 5% CO2. Afterwards, the cells were washed and suspended in PBS/1% human serum albumin (Grifols) containing a mouse anti-CD11b mAb (BD Pharmingen). The level of Mac-1 expression was determined by flow cytometry in the PMN region as indicated above, and using an antimouse Ig/G3-FITC conjugate (BD Pharmingen).

To test whether the C5a or C5a_{desArg} present in the fusion proteins preserved the capacity to increase the in vitro survival of PMN (12), PBL (3 \times 10^6 cells/mL) were incubated at 37°C, in 5% CO2 for 24 hours in the presence of the fusion proteins or controls, and then washed and suspended in PBS containing propidium iodide (50 μg/mL). Samples were incubated in the dark at room temperature for 15 minutes, and the PMN population was analyzed by flow cytometry as indicated above.

**Tumor cell proliferation**

The effect of the fusion proteins on tumor cell survival was tested in the presence or absence of leukocytes as effector cells (23). In brief, 10^6 human breast cancer cells per well were dispensed in 96-well plates. HER2/neu-highly positive SK-BR-3 cells were suspended in RPMI 1640 (Sigma) containing 10% heat-inactivated FCS (GIBCO) and 2 mmol/L L-glutamine (Sigma), HER2/neu-positive MCF-7 in DMEM plus 1.25% FCS and 2 mmol/L L-glutamine, and HER2/neu-negative MDA-MB 468 in DMEM plus 10% FCS and 2 mmol/L L-glutamine. After 18 hours, the culture supernatant was substituted for fresh medium containing the fusion proteins, fusion proteins plus PBL (10:1 effector:target ratio), or controls. Culture supernatants were aspirated off 96 hours later, culture wells were washed once with PBS, and the adherent cells were stained by adding 1 mg/mL MTS, inner salt/phenazine methosulfate (Sigma) in RPMI-1640 to each well. The plate was incubated at 37°C, in 5% CO2 for 1 hour and read at 490 nm in an ELISA reader (Biotek Instruments).

**Data analysis**

Statistical analyses were done using Prism 5.0 (GraphPad Software, Inc.). Flow cytometry data were analyzed by one-way ANOVA followed by multiple comparisons. In the C5aR binding experiments, the percent of positive cells in each individual sample was normalized to that of the control. The square root of these proportions was transformed to Arc-sin values prior to calculating the ANOVA. Similarly, the square root of the migration ratios in the chemotaxis experiments and the percent of dead PMN in survival assays were transformed to Arc-sin values prior to the ANOVA. Finally, in the experiments of tumor cell viability, tumor cell number was also normalized to that of the untreated control, and the resulting proportions were transformed as described, prior to the ANOVA. All multiple comparisons were done using Bonferroni test.

**Results**

**Construction, expression, and structural characterization of the fusion proteins**

The strategy followed for construction of the anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a_{desArg}) expression vectors is outlined in Supplementary Fig. S1. Standard procedures for DNA transfer into eukaryotic cells, briefly explained in Materials and Methods and detailed elsewhere (5), were followed to obtain transfectedomas secreting the fusion proteins. Clones secreting anti-HER2/neu IgG3-(C5a) or anti-HER2/neu IgG3-(C5a_{desArg}) fusion proteins were identified by ELISA and the proteins were examined by SDS-PAGE and Western blot. Anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a_{desArg}) have the expected molecular mass. SDS-PAGE (not shown) and Western blot (Fig. 1A) analyses under nonreducing conditions showed that anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a_{desArg}) possess a higher molecular mass than the anti-HER2/neu IgG3 antibody. Both fusion proteins, but not IgG3 anti-HER2/neu, were recognized by a C5a/C5a_{desArg}-specific antibody (Fig. 1B). SDS-PAGE analysis under reducing conditions showed that both fusion proteins split apart into two polypeptides of different sizes, a small one corresponding to the Ig light chain and a larger one corresponding to the Ig heavy chain-C5a (or -C5a_{desArg}) fusion polypeptide (not shown). Western blot analysis of the fusion proteins under reducing conditions and using an antihuman Ig antibody revealed a molecular mass of approximately 71 kDa corresponding to the γ3-(C5a) and γ3-(C5a_{desArg}) fusion polypeptides.
IgG3-(C5a desArg) clearly stained SK-BR-3 HER2/neu IgG3; or anti-C5a desArg antibodies as indicated. Arrows, position of the IgG3-(C5a) were subjected to SDS-PAGE and Western blot under nonreducing (A and B) or reducing (C) conditions using antihuman IgG or anti-C5a desArg antibodies as indicated. Arrows, position of the anti-HER2/neu IgG3 antibody or the fusion proteins.

The heavy chain of anti-HER2/neu IgG3 exhibited a molecular mass of about 60 kDa (Fig. 1C).

**HER2/neu, C5aR, and FcγR binding**

Fusion proteins bound specifically to HER2/neu, FcγRs, and C5aR at the cell surface. Culture supernatants containing anti-HER2/neu IgG3-(C5a) or anti-HER2/neu IgG3-(C5a desArg) clearly stained SK-BR-3 HER2/neu-positive cells (Fig. 2A) and significantly interfered (P ≤ 0.001; Bonferroni test, after an ANOVA F3,11 = 46.64; P ≤ 0.0001) with the binding of a specific antibody that interacts with the C5aR at the C5a-binding site on human PBL (Fig. 2B). Similarly, both fusion proteins significantly diminished the binding of a human IgG3 to FcγRs on THP-1 cells (Fig. 2C).

**Assessment of C5a bioactivity**

Anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a desArg) fusion proteins induced biological responses in C5aR (CD88)-expressing cells. In the chemotaxis assay, the purified fusion proteins attracted a proportion of PBL significantly greater (P ≤ 0.05 Bonferroni test, after an ANOVA F5,23 = 6.45, P ≤ 0.0013) than the controls, whereas the anti-HER2/neu IgG3 antibody alone was inactive (Fig. 3).

The purified fusion proteins also increased intracellular calcium concentration in cells expressing the C5aR, whereas anti-HER2/neu IgG3 did not (Fig. 4A). An increased expression of the integrin Mac-1 in granulocytes treated with the fusion proteins or C5a desArg was also observed, whereas Mac-1 expression remained unaltered in response to treatment with anti-HER2/neu IgG3 (Fig. 4B). Anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a desArg) also delayed the spontaneous death of granulocytes cultured in vitro for 24 hours (Fig. 4C). This delay was comparable with the effect observed when granulocytes were exposed to C5a desArg, but reached statistical significance only for the anti-HER2/neu IgG3-(C5a desArg) fusion protein (P ≤ 0.05 compared with negative control, Bonferroni test, after an ANOVA F4,19 = 5.67; P ≤ 0.017). Anti-HER2/neu IgG3 showed no activity in this assay.

**Effect of the fusion proteins on HER-2/neu-expressing tumor cells**

Light microscopy observation of SK-BR-3 cell cultures treated with anti-HER2/neu IgG3-(C5a) or anti-HER2/neu IgG3-(C5a desArg) clearly revealed a pronounced direct tumoricidal action of both fusion proteins on HER2/neu-positive tumor cells (not shown). Such an effect was not observed with the parental anti-HER2/neu IgG3 antibody. These findings were confirmed through a colorimetric assay that allowed a quantitative estimation of the tumoricidal effect (Fig. 5). Treatment of SK-BR-3 cells with anti-HER2/neu IgG3-(C5a) or anti-HER2/neu IgG3-(C5a desArg) at a concentration of 1 μg/mL significantly reduced (P ≤ 0.001, Bonferroni test, after an ANOVA F7,95 = 88.09; P ≤ 0.0001) tumor cell viability (Fig. 5A.1). This effect was not observed with the anti-HER2/neu IgG3 antibody alone or in combination with C5a or C5a desArg, and it was not due to nonspecific cytotoxicity, because culturing human granulocytes under similar conditions for 24 hours did not induce cell death, but instead prolonged cell survival (Fig. 4C). Moreover, no reduction in cell viability was observed (Fig. 5B and C) in cell lines with low (MCF-7) or no (MDA-MB 468) HER2/neu expression (ANOVA F5,17 = 0.7814; P = 0.5820 for MCF-7; and F5,14 = 0.7396; P = 0.612 for MDA-MB 468). At a 5-fold lower concentration (0.2 μg/mL), anti-HER2/neu IgG3-(C5a) preserved most of its intrinsic tumoricidal activity on SK-BR-3 cells (P ≤ 0.001, Bonferroni test, after an ANOVA F3,47 = 35.48; P ≤ 0.0001) whereas anti-HER2/neu IgG3-(C5a desArg) lost about 40% of it (P ≤ 0.05, Bonferroni test, after an ANOVA F3,47 = 35.48; P ≤ 0.0001; Fig. 5A.2).

In the presence of PBL as effector cells (cocultures), the absolute number of surviving tumor cells was slightly reduced probably due to some spontaneous killing of the targets by the effector cells. Nevertheless, compared with the untreated control, treatment of SK-BR-3 cells with anti-HER2/neu IgG3 antibody at 1 μg/mL led to a significant reduction (P ≤ 0.001, Bonferroni test, after an ANOVA F7,71 = 30.49; P ≤ 0.0001) of tumor cell viability. Similar reductions were observed for each coculture condition tested in which the anti-HER2/neu IgG3 antibody combined with C5a or C5a desArg or the fusion proteins were present (Fig. 6A.1). At 0.2 μg/mL (Fig. 6A.2), anti-HER2/neu IgG3 did not affect tumor cell viability, whereas anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a desArg) were still able to mediate the death of a significant percent of tumor cells (P ≤ 0.05 and P ≤ 0.001, respectively, Bonferroni test, after an ANOVA F7,71 = 30.49; P ≤ 0.0001).
In similar cocultures, treatment of MCF-7 cells (low expression of HER2/neu) with anti-HER2/neu IgG3-(C5a) at 1 μg/mL (Fig. 6B) led to a comparable cytotoxic effect \((P < 0.05\), Bonferroni test, after an ANOVA \(F_{5, 17} = 8.466; P = 0.0012\), but no effect was observed (Fig. 6C) on MDA-MB 468 cells (no expression of HER2/neu; ANOVA \(F_{5,17} = 2.665; P = 0.0763\)).

**Discussion**

In an attempt to boost the antitumoral properties of an antihuman HER2/neu mAb, we successfully produced two fusion proteins in which a recombinant human anti-HER2/neu IgG3 antibody was genetically fused to the molecule C5a or its derivative, C5a\(_{\text{desArg}}\). The presence of an antibody molecule covalently attached to the NH\(_2\)-terminus of C5a or C5a\(_{\text{desArg}}\) did not adversely affect their capacity to bind, activate, attract, and prolong the survival of human PMN. Likewise, the presence of C5a or C5a\(_{\text{desArg}}\) did not preclude the ability of the antibody moiety to bind antigen or interact with Fc\(\gamma\)Rs. Anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a\(_{\text{desArg}}\)) fusion proteins bound to the surface of PMN via C5aR, and induced intracellular calcium mobilization and upregulation of the integrin Mac-1 expression, both of which are indicators of PMN activation. In addition, under the conditions tested, both fusion proteins were capable of attracting PBL more efficiently than were free C5a or C5a\(_{\text{desArg}}\).

Anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a\(_{\text{desArg}}\)) fusion proteins delayed spontaneous death of human PMN in vitro. Interestingly, under comparable experimental conditions anti-HER2/neu IgG3-(C5a\(_{\text{desArg}}\)) induced the survival of a greater percent of PMN than did free C5a\(_{\text{desArg}}\). This difference may be due to a different carbohydrate content of the C5a\(_{\text{desArg}}\) moiety in the fusion protein versus free C5a\(_{\text{desArg}}\). Natural human C5a\(_{\text{desArg}}\) normally displays a glycan residue attached to the side chain of an asparagine located at position 64 of the polypeptide chain (24), which diminishes C5a\(_{\text{desArg}}\) biological activity (25). Because the fusion proteins we prepared were synthesized not by human but by mouse myeloma cells, it is possible that they lack or carry modified oligosaccharides (26), resulting in a more active form of the human C5a\(_{\text{desArg}}\) in the fusion protein.

Most importantly, when used at concentrations of 1 or 0.2 μg/mL, both fusion proteins interfered with the survival of HER2/neu-positive SK-BR-3 cells, an effect not observed with the anti-HER2/neu IgG3 antibody alone. This interference did not seem to be caused by nonspecific cytotoxicity, because it was not observed in tumor cell lines with low (MCF-7) or no (MDA-MB-468) expression of HER2/neu.
of HER2/neu. Moreover, under similar conditions the fusion proteins delayed, rather than prompted, human PMN death.

A possible mechanism of action for the direct anti-tumor cell activity of the fusion proteins may involve loss of HER2/neu from the tumor cell surface. Loss of HER2/neu from the cell surface is incompatible with long-term growth of cell lines with high levels of HER2/neu expression such as SK-BR-3 (27), whereas cell lines that express low levels of HER2/neu seem to be less dependent on HER2/neu expression for their survival (27). In fact, an increased rate of HER2/neu internalization is considered an important intrinsic mechanism of trastuzumab action in therapy (28, 29). Although we did not explore the mechanism through which the fusion proteins decreased tumor cell survival, it is plausible that fusion to C5a or C5adesArg interfered with recycling of the fusion protein-HER2/neu complex at the cell membrane. For instance, fusion of antibodies to certain cytokines (immunocytokines) alters their susceptibility to be cleaved by intracellular proteases (30). The fusion protein-HER2/neu complex may therefore have been cleaved by intracellular proteases at a higher rate than the anti-HER2/neu IgG3-HER2/neu complex. In future work, it will be important to evaluate the surface expression and the intracellular fate of HER2/neu during the course of the incubation of SK-BR-3 cells with the fusion proteins.

In the presence of effector cells, the anti-HER2/neu antibody and the fusion proteins mediated similar levels of tumor cell death in the cell line SK-BR-3, which overexpresses the HER2/neu antigen, when used at 1 μg/mL. Hence, under these conditions the antibody moiety seems sufficient to mediate ADCC and promote killing of the tumor cells. However, the fusion proteins were more efficient than the antibody alone when used at a concentration of 0.2 μg/mL. Interestingly, although anti-HER2/neu IgG3-(C5adesArg) lost some of its intrinsic capacity to interfere with tumor cell survival at this concentration, it was the most effective treatment in the presence of effector cells. This result may be related to the enhanced capacity of the anti-HER2/neu IgG3-(C5adesArg) fusion protein to delay PMN death. Experiments blocking the C5aR on effector cells prior to their encounter with the fusion proteins will be needed to assess the contribution of the fused C5a or C5adesArg to PMN action on tumor cells.

In MCF-7, only anti-HER2/neu IgG3-(C5a) (1 μg/mL) mediated tumor cell death. Having a low expression of HER2/neu, this cell line may not bind enough antibody molecules on its surface to mediate an effective ADCC. However, simultaneous engagement of FcγRs and C5aR through the anti-HER2/neu IgG3-(C5a) fusion protein may still induce activation of effector mechanisms and degranulation of PMN, promoting the killing of the target cells. In the case of anti-HER2/neu IgG3-(C5adesArg), although the treatment may be extending PMN half-life, the activation induced might not be sufficient to kill the target cells, because higher concentrations of C5adesArg than of C5a are required to induce PMN degranulation (31). In MDA-MB 468 tumors, with no expression of HER2/neu, no effector mechanisms could take place due to a lack of fusion protein binding to the target cell.

Human C5a and C5adesArg are active in mice (32), which will facilitate future studies of anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5adesArg) fusion proteins in an experimental animal model. In vivo, C5a modulates FcγR expression on monocytes/macrophages upregulating activating and downregulating inhibitory FcγRs (33, 34). Interestingly, the antitumoral effect of a melanoma-specific mAb was dramatically improved in mice knockout for the inhibitory FcγRIIB (35), underscoring the importance the balance between activating and inhibitory FcγRs has for the therapeutic effect of this kind of reagents. Similar results have been reported using other experimental models of FcγRIIB-deficient animals injected with tumor cells and treated with anti-HER2/neu (Herceptin) or anti-CD20 (Rituxan) therapeutic mAbs (35), suggesting that engagement to proper FcγRs may foster the antitumor antibody therapy. Hence, we would expect that the fusion of an anti-HER2/neu antibody to C5a or C5adesArg could tilt the balance towards an activating, ADCC-promoting antitumor antibody response.

One concern about the in vivo use of C5a or C5adesArg comes from their possible toxicity. Elevated levels of C5a observed in serum of patients with sepsis correlate with reduced hemolytic activity, multiorgan dysfunction, and poor clinical outcome (36). However, other than initial neutropenia followed by neutrophilia that
lasts less than two hours, low doses of C5a and C5adesArg do not produce any serious toxicity or adverse effects when injected i.v. in healthy rabbits (37). C5a toxicity observed in sepsis may thus be a consequence of the prolonged exposure to high levels of C5a, which is produced due to the continuous presence of bacteria in the blood (36). Avoiding prolonged exposure to systemic C5a at high concentrations may prevent undesirable outcomes.

Other anti-HER2/neu IgG antibody fusion proteins are currently being tested in preclinical protocols for antitumor vaccination or treatment. Vaccination of mice with the extracellular domain of HER2/neu combined with anti-HER2/neu IgG3-interleukin-2 (IL-2), IgG3-(IL-12), and/or IgG3–granulocyte macrophage colony-stimulating factor (GM-CSF) antibody fusion proteins results in significant protection against challenge with HER2/neu-expressing tumors (38). Moreover, the combination of anti-HER2/neu IgG3-(GM-CSF) with anti-HER2/neu IgG3-(IL-12) is an effective therapy in a mouse model of HER2/neu-positive tumors (39), inducing long-term protection against subsequent challenges with HER2/neu-positive tumors (40). The anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a desArg) fusion proteins described here could also be tested in some of these settings.

In summary, in vitro functional assays showed that IgG3 anti-HER2/neu-(C5a) and IgG3 anti-HER2/neu-(C5a desArg) fusion proteins attract, activate, and prolong the survival of human PMN. More importantly, treatment of SK-BR-3 cells with the fusion proteins substantially decreased tumor cell survival, an effect not observed with the antibody alone. In the presence of challenge with HER2/neu-expressing tumors (38). Moreover, the combination of anti-HER2/neu IgG3-(GM-CSF) with anti-HER2/neu IgG3-(IL-12) is an effective therapy in a mouse model of HER2/neu-positive tumors (39), inducing long-term protection against subsequent challenges with HER2/neu-positive tumors (40). The anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5a desArg) fusion proteins described here could also be tested in some of these settings.
human PBL, both fusion proteins mediated tumor cell death more efficiently than anti-HER2/neu alone, and IgG3 anti-HER2/neu-(C5a) did better than IgG3 anti-HER2/neu-(C5a) at mediating this effect. Taken together, these data suggest that anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5adesArg) fusion proteins possess novel properties that could be useful in cancer immunotherapy.

Figure 5. Viability of three tumor cell lines with different levels of expression of HER2/neu in the presence of the fusion proteins. The effect of anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5adesArg) fusion proteins on the relative survival of SK-BR-3 cells (high HER2/neu expression), MCF-7 cells (low HER2/neu expression), and MDA-MB-468 cells (no HER2/neu expression) was studied. SK-BR-3 (A1), MCF-7 (B), and MDA-MB 468 (C) cells were incubated for 96 hours with: 0, control medium; 1, anti-HER2/neu IgG3 (1 μg/mL); 2, anti-HER2/neu IgG3-(C5adesArg) or 3, anti-HER2/neu IgG3-(C5a) [both at 1 μg/mL, equivalent to 0.132 μg/mL of C5a or C5adesArg]; 4, C5adesArg or 5, C5a (both at 0.132 μg/mL); or the combination of anti-HER2/neu IgG3 with C5adesArg (1 + 4) or C5a (1 + 5). A2, relative survival of SK-BR-3 cells incubated for 96 hours with: 0, control medium; 1, anti-HER2/neu IgG3 (0.2 μg/mL); 2, anti-HER2/neu IgG3-(C5adesArg); 3, anti-HER2/neu IgG3-(C5a) [both at 0.2 μg/mL, equivalent to 0.026 μg/mL of C5a or C5adesArg]. Mean ± SD of three experiments. *, P ≤ 0.05; **, P ≤ 0.01 compared with control.

Figure 6. Viability of three tumor cell lines with different levels of expression of HER2/neu in the presence of the fusion proteins and effector cells. The effect of anti-HER2/neu IgG3-(C5a) and anti-HER2/neu IgG3-(C5adesArg) fusion proteins plus PBL on the relative survival of SK-BR-3 cells (high HER2/neu expression), MCF-7 cells (low HER2/neu expression), and MDA-MB-468 cells (no HER2/neu expression) was studied. SK-BR-3 (A1), MCF-7 (B), and MDA-MB 468 (C) cells incubated for 96 hours with PBL (10:1 effector:target) and: 0, control medium; 1, anti-HER2/neu IgG3 (1 μg/mL); 2, anti-HER2/neu IgG3-(C5adesArg) or 3, anti-HER2/neu IgG3-(C5a) [both at 1 μg/mL, equivalent to 0.132 μg/mL of C5a or C5adesArg]; 4, C5adesArg or 5, C5a (both at 0.132 μg/mL); or the combination of anti-HER2/neu IgG3 with C5adesArg (1 + 4) or C5a (1 + 5). A2, relative survival of SK-BR-3 cells incubated with PBL as indicated above and: 0, control medium; 1, anti-HER2/neu IgG3 (0.2 μg/mL); 2, anti-HER2/neu IgG3-(C5adesArg) or 3, anti-HER2/neu IgG3-(C5a) [both at 0.2 μg/mL, equivalent to 0.026 μg/mL of C5a or C5adesArg]. Mean ± SD of three experiments. *, P ≤ 0.05; **, P ≤ 0.01 compared with control.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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