Ph.D.THESIS

THE ROLE OF ANTIBODY-MEDIATED CELLULAR CYTOTOXICITY (ADCC) IN THE MECHANISM UNDERLYING THE ACTION OF TRASTUZUMAB

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INTRODUCTION

The ErbB2 and trastuzumab

Overexpression of ErbB2 (HER2), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, occurs in 20-30% of invasive breast cancers and is associated with poor prognosis and rapid relapse. Since cancer cell growth is often driven by highly overexpressed oncogenes, even late stage tumors can be successfully treated with therapies directed against the overexpressed oncoprotein. The finding that some of the anti-ErbB2 antibodies can inhibit the growth of cancer cells overexpressing ErbB2 on their surface was a breakthrough in anticancer therapy, and led to the development of trastuzumab (Herceptin®), a recombinant humanized monoclonal antibody against the extracellular domain of ErbB2. Trastuzumab has a remarkable anti-tumor effect, and is currently used world-wide for the treatment of breast cancer. Although the mechanisms underlying the action of trastuzumab are still not fully understood, several molecular effects have been observed. *In vitro* trastuzumab treatment leads to: *i.* down-regulation of cell surface ErbB2; *ii.* inactivation of the MAPK and phosphatidylinositol 3'-kinase (PI3K) pathways; *iii.* cell cycle arrest in G1; *iv.* induction of apoptosis; *v.* increase in HLA-I restricted antigen presentation of ErbB2. Furthermore, in *in vivo* models trastuzumab decreased the microvessel density of breast cancer xenografts.

In addition to the direct effects on cancer cells, several lines of evidence suggested that antibody-dependent cellular cytotoxicity (ADCC) plays an important role in the anti-tumor activity of trastuzumab. Clynes, *et al*. demonstrated that the activity of trastuzumab on breast cancer xenografts was attenuated in knock-out mice lacking activating FcγRIII receptors. Furthermore, administration of trastuzumab lacking functional FcR binding capability resulted in similarly attenuated response in wild type mice. In addition, Spiridon, *et al*. found that trastuzumab-$F(ab')_2$ and a mixture of $F(ab')_2$ fragments of three anti-ErbB2 IgGs exerted significantly reduced responses compared to those of trastuzumab IgG and the mixture of the three anti-ErbB2 IgGs in mice xenografted with trastuzumab-sensitive breast cancer cells.

Trastuzumab-resistance

Despite encouraging clinical results, some cancers are primarily resistant to trastuzumab, and a majority of those initially responding become resistant during prolonged treatment. The mechanisms of trastuzumab resistance have not been fully elucidated. The following mechanisms have been implicated: *i.* autocrine production of EGF-related ligands; *ii*. activation of the IGF-I receptor pathway; *iii.* masking of the trastuzumab epitope by MUC4, a cell surface sialomucin; *iv.* loss of PTEN function; *v.* impaired ADCC reaction:

Although Clynes *et al.* suggested that the effect of trastuzumab is mainly mediated via ADCC, only a few works have investigated the role of impaired ADCC in trastuzumab resistance. Mimura *et al.* reported that trastuzumab-mediated ADCC against transforming growth factor-β (TGF-β) producing esophageal cancer cells was enhanced by treatment with a selective inhibitor of TGF-β. Kono *et al.* showed that capability of trastuzumab-mediated ADCC was significantly reduced in NK cells from advanced gastric cancer patients compared to healthy individuals. NK cell dysfunction was correlated to the down-regulation of CD16ζ, whose phosphorylation has a key role in the NK cell-mediated ADCC. Furhermore, *in vitro* interleukin-2 treatment of NK cells could normalize CD16ζ expression and increase trastuzumab-mediated ADCC.

Possible mechanisms of trastuzumab resistance have been studied in detail using the breast cancer cell line JIMT-1 established from the pleural metastasis of a patient who was clinically resistant to trastuzumab. JIMT-1 cells are resistant to trastuzumab *in vitro*, and also *in vivo*, if therapy is initiated 45 days after establishing xenografts.

Metastasis development

Death of most cancer patients is caused by metastatic spread of cancer cells from the primary tumor to distant organ. It has been suggested that approximately 10^6 tumor cells per gram of tumor tissue per day shed from the *primary tumor* into the bloodstream. Escape of tumor cells occurs primarily across the wall of mosaic tumor blood vessels composed of endothelial and tumor cells. Chang *et al.* suggested that approximately half of the tumor cells contacting the vessel lumen is shed in a day. Although a great number of tumor cells move to the circulation and become *circulating tumor cells*[*] (CTC), only a minority of them can form metastasis. Luzzi *et al*. showed

[*] We use the terminology suggested by P. Paterlini-Brechot.

that the formation of metastasis is a highly inefficient process: only 0.02% of CTCs are able to form metastasis. In the bloodstream a large fraction of the CTCs die quickly due to killing by the immune system, hemodynamic forces or apoptosis evoked by loss of cell attachment and cell-matrix connections. Some CTCs extravasate into distant organs and persist there as *disseminated tumor cells*[*] (DTC), which may be dormant and so partially resistant to cytotoxic chemotherapeutic agents. A very small proportion of extravasated CTCs (2%) are capable of dividing and forming *micrometastases*[*], and an even smaller fraction (0.02%) can evolve to a full-fledged *metastasis*. A *circulating multicellular tumor aggregate* (CMTA) is a group of attached cells shed from the primary tumor, which reach the circulation and migrate collectively. The presence of CMTAs in the blood is a marker of high metastatic potential.

Although metastasis formation from CTCs and DTCs is a highly inefficient process, the chance of metastasis formation can increase, since breast tumor size reach a typical clinically detectable size of 2-3 cm (diameter) in 1-3 years, as shown by studies analyzing serial screening mammograms. The presence of CTCs in primary and metastatic breast cancer is associated with poor prognosis. It has been published that ~50% of metastatic breast cancer patients had more than five CTCs in 7.5 ml blood; the presence of more than 5 CTCs was a strong independent predictor of poor outcome. Bone marrow micrometastases were detected in 30% of stage I-III breast cancer patients, who had larger and higher grade tumors, and more often had lymph node metastases. The presence of micrometastases was an independent predictor of poor outcome.

ErbB2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients. The presence of ErbB2-positive CTCs has been associated with larger tumor size, negative estrogen receptor (ER) status, poor histological differentiation, and lymphovascular invasion. Interestingly, it has been reported that nearly one-third of patients whose primary tumors were negative for ErbB2 had CTCs with amplified ErbB2 (determined by FISH). In addition, ErbB2-positive DTCs can be detected in patients with ErbB2 negative primary tumors (determined by immunohistochemistry). Wülfing *et al.* observed a discrepancy between the detection of ErbB2-positive CTCs and the ErbB2 score of the corresponding primary tumor. Furthermore, no

correlation was found between the ErbB2 staining score of the primary tumor and the presence of ErbB2-positive micrometastases in the bone marrow. Trastuzumab has a remarkable anti-tumor effect, and is now widely used for the treatment of breast cancer. After initial usage in metastatic breast cancer patients, subsequent studies of adjuvant trastuzumab therapy showed significant benefit in primary breast cancer as well. It has also been shown that trastuzumab reduced the number of CTCs and bone marrow micrometastases in chemotherapy-resistant breast cancer patients.

SPECIFIC AIMS

Our major goal was to investigate the trastuzumab-resistance in the JIMT-1 – SCID mouse model system. We ask the following questions:

It was published that JIMT-1 cells are resistant to trastuzumab *in vitro*, and also *in vivo*, if therapy is initiated 45 days after establishing xenografts. Has trastuzumab any inhibitory effect on the JIMT-1 tumor growth if the trastuzumab administration is started earlier than it was in the paper of Tanner *et al.* (on day 45).

Has trastuzumab any inhibitory effect on the JIMT-1 tumor growth if the trastuzumab administration is started immediately after the tumor inoculation?

We wanted to compared the *in vivo* tumor inhibithory effect of trastuzumab-F(ab')$_2$ and trastuzumab.

How can trastuzumab-F(ab')$_2$ and trastuzumab influence the ErbB2 level of the JIMT-1 – xenografts?

We wanted to re-established sub-cell lines from the xenografts of the JIMT-1 inoculated and trastuzumab or saline treated mice.

We wanted to investigate the sensitivity of trastuzumab sentitive and resistant cell lines to trastuzumab-mediated ADCC *in vitro*.

Can we detect circulating and disseminated tumor cells (CTC and DTC) in the blood and bone marrow of JIMT-1 inoculated mice?

How can trastuzumab influence the number of CTSs and DTCs?

MATERIALS AND METHODS

Cells. JIMT-1, the ErbB2 positive human breast cancer cell line was grown in Ham's F-12 / DMEM (1:1) supplemented with 10% FCS, 60 units/L insulin and antibiotics. SKBR-3 and BT-474 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and cultured according to specifications.

Xenograft tumors. The severe combined immunodeficiency (SCID) C.B-17 scid/scid (originated from the laboratory of Fox Chase Cancer Center, Philadelphia, PA) and nu/nmri nude mouse population (Harlan Netherland, Horst, Netherland) were housed in a pathogen-free environment. Only non-leaky SCID mice with murine IgG levels below 100 ng/ml were used in this study. Seven-week old female nude or SCID mice were given a single subcutaneous injection of 5×10^6 JIMT-1 cells suspended in 150 µl Hanks' A buffer and mixed with an equal volume of Matrigel (BD Matrigel™, BD Biosciences, Two Oak Park, Bedford, MA). Tumor volumes were derived as the product of the length, width and height of the tumor measured once a week with a caliper.

Trastuzumab and trastuzumab-F(ab´)$_2$ were administered at a dose of 5 µg/g and 25 µg/g, respectively, by weekly intraperitoneal (i.p.) injection. The 5-times greater amount of administered F(ab´)$_2$ was chosen based on the different half-lives of IgG and F(ab´)$_2$ *in vivo*. Control mice were treated with weekly i.p. injection of 100 µl physiologic saline (saline) or rituximab (a humanised anti-CD20 antibody) at a dose of 5 µg/g. Animals were euthanized by CO_2 inhalation. The experiments were done with the approval of the ethical committee of the University of Debrecen.

Establishment of sublines from JIMT-1 xenografts. The JIMT-1 X- and the JIMT-1 X+ cell lines were derived from the tumor of mice treated continuously with saline and trastuzumab, respectively. Animals were euthanized, the tumors were removed and cut into small pieces with a sterile blade, washed twice with sterile PBS, placed in culture dishes in medium containing F-12/DMEM (1:1) supplemented with 20% FCS, 60 units/L insulin and antibiotics. The medium of JIMT-1 X+ cells also contained 10 µg/ml trastuzumab (28). After 3 days, debris and dead cells were removed and the medium was refreshed. Confluent cultures were trypsinized and split at a ratio of 1:2, using the same medium but with only 10% FCS. JIMT-1 X+ cells have been grown in the presence of 10 µg/ml trastuzumab ever since the establishment of the cell line.

Antibodies. Herceptin® (trastuzumab) and Mabthera® (rituximab) were purchased from Roche Ltd. (Budapest, Hungary). Anti ErbB2 monoclonal antibody (mAb) 2C4 was a gift from Genentech Inc.,

South San Francisco, CA. The W6/32 (IgG2a) mAb with specificity for the heavy chain of human class-I HLA A, B, and C molecules was a kind gift from Francis Brodsky (University of California San Francisco, USA). Anti human ErbB1 monoclonal antibody (mAb) 528 (IgG2a) was produced by the hybridoma cell line 528 (HB-8509, ATCC, Manassas, VA). W6/32 and 528 antibodies were purified using protein A affinity chromatograpy. MAb recognizing all isoforms of mouse CD45 was produced from the supernatant of YBM42.2 hybridoma cell line (kindly provided by Denis R. Alexander, The Babraham Institute, Babraham, Cambridge, UK) and purified using protein G affinity chromatography. Monoclonal antibody (mAb) against ErbB2 (erbB2-76.5 IgG1) was produced from the supernatant of the erbB2-76.5 (kindly provided by Y. Yarden, Weizmann Institute of Science, Rehovot, Israel) and purified using protein A affinity chromatography.

Conjugation of whole antibodies and Fab fragments with fluorescent dyes. Covalent binding of AlexaFluor 488, AlexaFluor 546 and AlexaFluor 647 succinimidyl esters (Molecular Probes-Invitrogen, Eugene, OR) to the anti mouse CD45 antibody and the Fab fragments of 2C4, W6/32, 528 and erbB2-76.5 antibodies was carried out according to the manufacturer's instructions. The dye : protein labeling ratio was determined by spectrophotometry, and was approximately 3:1 and 1:1 in the case of whole IgGs and Fabs, respectively.

Isolation and labeling of circulating and disseminated tumor cells from the blood and the bone marrow of breast cancer xenografted SCID mice. The total blood volume of sacrificed SCID mice with JIMT-1 xenografts was collected, and the bone marrow from the femurs was washed out with PBS. Blood and bone marrow samples anticoagulated by heparin were centrifuged at 400 × *g* on a density gradient (Ficoll-Isopaque, 1.077 g/cm^3) for 35 min and cells were collected from the buffy coat [37; 38; 39]. Cells were washed twice in PBS with 1% bovine serum albumin (BSA) (pH 7.4) and labeled with a saturating concentration (10-20 μg/ml) of dye-conjugated antibodies in 100 μl PBS containing 1% BSA on ice, washed twice with PBS, fixed in 2% formaldehyde and placed into chambered coverglasses. Positive cells were counted.

For FISH analysis cells collected from the buffy coat were fixed with methanol: acetic acid (3:1), washed twice and carefully pipetted, in 5 μl volume onto slides.

Confocal microscopy. A Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss, Göttingen, Germany) was used to image samples. AlexaFluor 488 was excited with the 488 nm line of an argon ion laser, and its emission was detected through a 505-530 nm bandpass filter. AlexaFluor 546 and AlexaFluor 647 were excited with the 543 and 633 nm lines of a green and a

red He-Ne laser, respectively, and their emissions were measured between 560-615 nm and over 650 nm, respectively. Fluorescence images were taken as 1 micrometer optical sections using a 63x (NA=1.4) oil immersion objective. The transmission image was created with the 633 nm line of the He-Ne laser.

Fluorescence in situ hybridization (FISH) on circulating human tumor cells. In order to easily locate the circulating human JIMT-1 tumor cells in the peripheral blood and bone marrow of mice double target FISH was performed to discriminate mouse and human cells using DNA probes against mouse genomic DNA and human X chromosome. Briefly, total genomic DNA was extracted from normal mouse muscle using the DNeasy Blood and Tissue Kit (QIAGEN, Budapest, Hungary). One µg mouse genomic DNA was labeled with SpectrumRed-dUTP (Vysis Inc., Downers Grove, IL USA) using a nick translation kit (Vysis Inc.). Two hundred ng of labeled mouse DNA was mixed with 100 µg of Cot-1 DNA (Life Technologies, Inc., Gaithersburg, MD), precipitated and resuspended in 10 µl of hybridization solution (55% formamide, 10% dextran sulfate, 2x SCC) containing 1 µl of SpectrumGreen labeled human chromosome X probe (Vysis Inc.). The hybridization mixture was mixed well and denatured for 5 min at 70^{o}C, preannealed at 37 ^{o}C for 30 min and placed on the denatured mouse peripheral blood or bone marrow cells. Hybridization was carried out overnight at 37 ^{o}C. After hybridization, samples were processed as described [40]. To visualize nuclei 0.3 µg/ml DAPI (Vysis) was used in an antifade solution to prevent bleaching. Nuclei were scored for the number of chromosome X signals using fluorescent microscope (OPTON, Oberkochen, Germany) equipped with selective filters for the detection of FITC, SpectrumGreen, SpectrumOrange and DAPI. Three colour images were captured using a digital image analysis system (ISIS, MetaSystem, Germany).

Preparation and testing of trastuzumab-F(ab´)$_2$ fragment. 20 mg of trastuzumab IgG was dissolved in 20 mM acetate buffer (pH 4.5) and dialyzed 3 times into the same buffer and concentrated using Centricon-10 tubes (Millipore Corp., Bedford, MA). Digestion was performed by adding 0.5 ml immobilized pepsin (Pierce Biotechnology, Rockford) at 37 ℃ for 6 h. The reaction was stopped by 10 ml 2 M Tris-HCl (pH 8.2), digested trastuzumab was filtered through a 0.22 µm Millex filter (Millipore Corp.) to remove immobilized pepsin bound to agarose beads, and concentrated to 1 ml using Centricon-50 tubes (Millipore Corp.). Trastuzumab-F(ab´)$_2$ fragments and undigested trastuzumab were separated by high performance liquid chromatography using a column (10 × 800 mm) filled with Sephacryl® S-300 (Pharmacia LKB, Uppsala, Sweden) and an

eluant of 50 mM sodium phosphate (pH 7.0). Fractions of 0.5 ml were collected and tested using non-reducing SDS PAGE analysis. Fractions containing trastuzumab-F(ab´)$_2$ without contamination of undigested trastuzumab were merged, concentrated and stored at – 20 °C. The quality of trastuzumab-F(ab')$_2$ was tested on SKBR-3 cells; trastuzumab-F(ab´)$_2$ bound to the cells, competed with intact trastuzumab, and bound no Fc specific secondary antibodies (data not shown).

***In vitro* assay of drug sensitivity.** The effects of trastuzumab and trastuzumab-F(ab')$_2$ on the growth of JIMT-1, SKBR-3 and BT-474 cells were evaluated using the AlamarBlue method (TREK Diagnostic Systems, Inc. Cleveland, OH). Exponentially growing cells were harvested and plated in single wells of a 96-well flat-bottomed tissue culture plate at defined densities, ranging from 4500-8000 cells/well depending on the cell line. After overnight culture, the regular medium was exchanged to medium containing 0, 1, 10 or 100 µg/ml trastuzumab or trastuzumab-F(ab')$_2$. Cell viability was tested after 72 hours of treatment according to the manufacturer's instructions. Fluorescence was excited at 544 nm and emission was detected at 590 nm using a Wallac Victor2 plate reader (Perkin-Elmer, Turku, Finland).

Immunohistochemistry. Subcutaneous tumors were removed from anesthetized mice, covered with Shandon Cryomatrix™ (Thermo Electron Corporation, Anatomical Pathology USA Clinical Diagnostics, Pittsburg, PA) and stored in liquid nitrogen. 20 µm thick cryosections were cut using a SHANDON AS-620E Cryotome (Thermo Electron Corporation, Erlangen, Germany) on silanized slides. Samples were fixed in 4% formaldehyde for 30 minutes, washed twice in PBS with 1% bovine serum albumin (BSA) (pH 7.4) for 20 minutes at room temperature. Slides were labeled with a saturating concentration (10-20 µg/ml) of dye-conjugated antibody in 100 µl PBS containing 1% BSA overnight on ice, washed twice with PBS, and covered with 15 µl Mowiol (Merck, Budapest, Hungary) to avoid bleaching of fluorescent dyes.

Image analysis: Confocal microscopic images were analyzed with the DipImage toolbox (Delft University of Technology, Delft, The Netherlands) under Matlab (Mathworks Inc., Natick, MA). The cell membrane was identified by a manually-seeded watershed algorithm (29) using a custom-written interactive algorithm implemented in DipImage/Matlab. The cell membrane was used as a mask and the fluorescence intensity was evaluated only in pixels under the mask.

***In vitro* ADCC assay.** ADCC assays were performed as described previously (17, 30), with modifications. Peripheral blood mononuclear cells (PBMCs) were separated from the blood of healthy donors by Ficoll density gradient centrifugation (Histopaque-1077, Sigma-Aldrich, Irvine,

UK). The fraction containing PBMCs was resuspended in DMEM containing 10 % FCS. JIMT-1, JIMT X-, JIMT X+ and SKBR-3 tumor cells were harvested, washed once in PBS containing 1 % bovine serum albumin (BSA), and labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) (Molecular Probes, Inc, Eugene, OR) at a concentration of 10 μM at 37 ℃ for 10 minutes. Then, tumor cells were washed 3 times with DMEM containing 10 % FCS and 1 % BSA to remove unreacted and unbound CFDA, SE. Between the washing steps, cells were incubated in the same medium at 37 ℃ for 5 minutes. Finally, the labeled target cells were resuspended in DMEM containing 10 % FCS and mixed with PBMCs at effector : target ratios (E:T) of 2:1, 6:1, 15:1, 30:1 and 60:1. Trastuzumab, rituximab or trastuzumab-F(ab´)$_2$ were added to the mixed suspensions at a concentration 100 μg/ml. The samples were incubated at 37 ℃ for 8 hours followed by staining of dead cells with 50 μg/ml propidium-iodide. Cells were analyzed on a Becton-Dickinson FACScan flow cytometer (Mountain View, CA, USA). The negative control sample was prepared identically, but did not contain PBMCs. Tumor cells killed by 4% formaldehyde served as the positive control. The percentage of cells killed was calculated according the following formula = (% of living cells in the negative control – % of living cells in the sample)/ % of living cells in the negative control.

Statistical analysis. Data are expressed as the mean ± SE. The statistical significance of the differences between means were determined using Student's t test for two samples after verifying that data pass the normality test and the groups compared have equal variance. Differences were statistically significant at $P < 0.05$.

RESULTS

Effect of trastuzumab on established JIMT-1 xenograft tumors. We have shown previously that established JIMT-1 xenograft tumors (tumor volume 200-500 mm^3) are insensitive to trastuzumab treatment in immunodeficient nu/nmri mice (26). First, we tested the effects of trastuzumab on smaller xenograft tumors of 100-200 mm^3 volume in SCID mice that had been inoculated subcutaneously with 5×10^6 JIMT-1 cells in suspension only 9 days before treatment. Weekly treatment with trastuzumab or saline was continued until the end of the experiment. Tumor growth was partially but significantly inhibited by trastuzumab from day 16 to 44 ($P < 0.05$) when compared to the control group.

Effect of trastuzumab on non-established JIMT-1 tumors in *nude* or *SCID mice*. Since the growth of small JIMT-1 xenograft tumors was partially inhibited by trastuzumab, we next studied the effect of trastuzumab treatment started at the time when 5×10^6 JIMT-1 cells were inoculated subcutaneously into nude or SCID mice.

Tumors were formed in all of the 7 *nude mice* treated with rituximab (a negative control antibody directed to CD20, a transmembrane protein not expressed by JIMT-1 cells (data not shown)), while palpable tumors developed only in 2 of 7 trastuzumab treated animals. Trastuzumab administration was suspended on day 42 when the inhibitory effect of trastuzumab was obvious as compared to rituximab treatment. Trastuzumab had a significant inhibitory effect on tumor growth from day 21 to 56 ($P < 0.05$). After stopping trastuzumab administration tumors started to grow in the 2 nude mice whose tumors were palpable earlier.

The growth of tumors in all trastuzumab-treated *SCID mice* was minimal for up day 42. From the fifth week onwards, the tumors started to grow exponentially in all (8/8) trastuzumab treated mice. At this time point, trastuzumab treatment was suspended in half of the treated animals, and continued for another 6 weeks in the rest of the animals. There was no difference in the rate of tumor growth between these two sub-groups indicating complete resistance to trastuzumab. The effect of trastuzumab on tumor growth was significant from day 14 to 91 ($P < 0.05$).

Treatment of JIMT-1 tumors with trastuzumab-F(ab´)$_2$. Since JIMT-1 cells are intrinsically resistant to trastuzumab, we hypothesized that the effects on submacroscopic xenografts are owed to ADCC reaction. We have therefore generated trastuzumab-F(ab´)$_2$, which binds ErbB2 with high affinity and exhibits *in vitro* growth inhibition equivalent to that of intact trastuzumab IgG in both

SKBR-3 and BT-474 cell cultures (Fig. 2*A*). When using this trastuzumab-F(ab´)$_2$ for weekly treatment of SCID mice inoculated with 5×10^6 JIMT-1 cells, on a weekly basis, no effect on xenograft tumor growth was seen as compared to saline treatment. In the parallel positive control, intact trastuzumab IgG significantly inhibited the growth of JIMT-1 tumors (from day 14 to 42 ($P < 0.05$)) similarly to findings in the previous experiment.

Cell surface expression level of ErbB2 in JIMT-1 xenografts. In order to investigate whether trastuzumab IgG and trastuzumab-F(ab´)$_2$ were able to down-regulate ErbB2 *in vivo*, fast frozen tissue sections were labeled with a fluorescent anti-ErbB2 antibody erbB2-76.5. The ErbB2 expression level of trastuzumab-treated tumors was ~50% lower than that of saline-treated cells. Quantitative image analysis revealed that trastuzumab-F(ab´)$_2$ and trastuzumab IgG were equally effective in down-regulating ErbB2 expression. Six weeks after suspension of trastuzumab treatment the ErbB2 expression level returned to almost as high as in control mice.

***In vitro* ADCC activity against trastuzumab-sensitive and resistant breast cancer cell lines.** The fact that trastuzumab-F(ab´)$_2$, unlike trastuzumab IgG, was ineffective against JIMT-1 xenografts *in vivo* suggested that the immune effector cells of SCID mice are responsible for growth retardation, irrespective of intrinsic cellular resistance. We next performed *in vitro* ADCC assays using human peripheral leukocytes (PBMCs) as effector cells. The baseline killing of tumor target cells by PBMCs in the presence of rituximab (IgG control) was low; there was no significant difference in target cell killing between assays performed with rituximab or with trastuzumab-F(ab´)$_2$. In contrast, the presence of trastuzumab IgG significantly increased killing of all breast cancer cell lines ($P < 0.05$). The ADCC activity of PBMCs evoked by trastuzumab was equally strong against trastuzumab-sensitive (SKBR-3) or resistant (JIMT-1) breast cancer cells, with dose-dependent cell death reaching ~50-60% killing at an effector:target ratio of 60:1. In addition to the native JIMT-1 cells, two sublines, JIMT-1 X- recovered from xenografted mice treated with saline, and JIMT-1 X+, a "superresistant" subline recovered after continuous treatment with trastuzumab were also equally sensitive to ADCC as SKBR-3.

Detection of circulating tumor cells (CTCs) in the blood of JIMT-1 xenografted SCID mice using immunohistochemistry. In order to investigate whether we are able to detect CTCs in our SCID mouse model system, we inoculated 3 SCID mice with 5×10^6 JIMT-1 cells. When tumors reached 1000 mm^3 in size we sacrificed the mice, the whole blood was taken, and cells collected from the buffy coat were labeled with the Fab fragments of monoclonal antibody against human

MHC-I (W6/32-Fab) and with the mouse pan-CD45-specific mAb to distinguish cells of human and mouse origin. Fab fragments of the anti-human MHC-I were used to ensure they bind exclusively to human JIMT-1 cells, and not to Fc-receptor bearing immune cells of the mouse. The pan-anti-CD45 antibody was used to visualize mouse white blood cells since CD45 is expressed in all haematopoietic lineages at all stages of development and CD45 has been frequently used as a negative marker in multiparametric identification of CTCs. The anti-CD45 antibody was used as whole IgG, so that immune-competent cells of the mouse can bind it also via their Fc-receptors. We could clearly distinguish the MHC-I expressing human CTCs from the CD45 positive mouse cells.

Detection of CTCs in the blood of JIMT-1 xenografted SCID mice using FISH. We looked for an alternative way to detect human CTCs in mouse blood. Peripheral white blood cells from JIMT-1 xenografted mice isolated as described in the previous paragraph were fixed in methanol:acetic acid and used for double target FISH. We could distinguish circulating JIMT-1 cells identified by the SpectrumGreen-labeled human X chromosome probe from mouse cells detected by the SpectrumRed-labeled mouse DNA probe.

The effect of trastuzumab and rituximab on non-established JIMT-1 tumors in SCID mice. We previously showed that although JIMT-1 human breast cancer cells were resistant to trastuzumab *in vitro*, trastuzumab retarded the outgrowth of macroscopically detectable JIMT-1 xenografts for up to 5-7 weeks *in vivo*. The effect was likely to be mediated via ADCC, since trastuzumab-F(ab')$_2$ was ineffective in this model. 5×10^6 JIMT-1 cells were inoculated subcutaneously into SCID mice treated with saline, trastuzumab or rituximab by weekly i.p. injection. Therapy was started at the time of JIMT-1 inoculation and continued until the end of the experiment. The growth inhibitory effect of trastuzumab on JIMT-1 xenografts was similar to what we have previously reported. The size of tumors in all trastuzumab treated mice (8/8) decreased until day 21. From the fourth week onwards, the tumors started to grow exponentially in all trastuzumab treated animals. Trastuzumab had significant inhibitory effect on tumor growth from day 14 to 42 ($P < 0.05$). Interestingly, rituximab, an antibody directed to CD20, a transmembrane protein not expressed by JIMT-1 cells, which was used as a negative control, tended to enhance tumor growth, although this effect was not significant compared to the saline treated group.

Detection of circulating tumor cells (CTC) in the blood, and of disseminated tumor cells (DTC) in the bone marrow of JIMT-1 xenografted SCID mice. Mice were euthanized when their tumor size reached ~800 mm^3 which happened on day 42 in saline and rituximab treated

animals, and on day 56 in trastuzumab treated ones. The whole blood and bone marrow from the femurs were taken, and cells collected from the buffy coat were labeled with the Fab fragments of monoclonal antibodies against human ErbB1 (528-Fab), ErbB2 (2C4-Fab) and MHC-I (W6/32-Fab).

We were able to detect CTCs in the blood and DTCs in the bone marrow of saline, rituximab and trastuzumab treated mice. All detected tumor cells were positive for MHC-I, ErbB2 and ErbB1. Tumor cells shed surface antigens making detection of CTCs and DTCs potentially difficult. However, it is unlikely that each of the tested antigens (ErbB1, ErbB2, MHC-I) is completely lost from the surface of a single cell making our method robust. The blood of all saline and rituximab treated mice contained CTCs. Trastuzumab treatment significantly reduced the mean value of CTCs and the proportion of mice with detectable CTCs compared to the control ($P < 0.05$). Trastuzumab significantly decreased the number of DTCs in the bone marrow, and the fraction of mice with detectable DTCs compared to the control ($P < 0.05$).

No micrometastasis was found in the bone marrow of saline- and trastuzumab-treated mice, however, we could detect micrometastasis in 5 out of 7 rituximab-treated mice. Four mice had only one micrometastasis (consisted of 3, 3, 3 and 6 cells) and one mouse had 3 micrometastases (consisted of 6, 7 and 16 cells). Furthermore, we detected a circulating multicellular tumor aggregate (CMTA) (consisting of 9 cells) in the blood of one rituximab treated mouse. Neither bone marrow micrometastasis nor CMTA was found in samples from saline or trastuzumab treated mice.

DISCUSSION

Trastuzumab is a recombinant antibody drug, which is widely used for the treatment of breast cancer. Despite encouraging clinical results some cancers are primarily resistant to trastuzumab, and a majority of those initially responding become resistant during prolonged treatment. The mechanisms of trastuzumab resistance have not been fully understood.

It is well documented that trastuzumab acts directly in cancer cell signaling, as well as indirectly via the immune system. We examined the possible mechanisms of trastuzumab resistance in detail using the breast cancer cell line JIMT-1 established from the pleural metastasis of a patient who was clinically resistant to trastuzumab. Despite ErbB2 gene amplification and receptor overexpression, JIMT-1 cells are resistant to trastuzumab *in vitro*, and also *in vivo*, if therapy is initiated 45 days after establishing xenografts.

Our main and unexpected finding was that trastuzumab caused a significant growth inhibition of the outgrowth of macroscopic JIMT-1 xenograft tumors in both nude and SCID mice. The effect was probably mediated via the Fc portion of trastuzumab IgG because the $F(ab')_2$ fragment of trastuzumab was ineffective in the SCID mouse model system, in spite of inhibiting proliferation of trastuzumab sensitive cells in vitro equally well as intact trastuzumab IgG. We attribute the Fc-mediated effects of trastuzumab to ADCC because both nude and SCID mice have functioning macrophages and natural killer cells capable of killing tumor cells by ADCC. These findings reflect the central role of the immune system in mediating the effects of trastuzumab in vivo. In a previous study, Clynes et al. inoculated trastuzumab-sensitive BT-474 cells into knock-out mice lacking activating FcRγIII receptors. In this model system, the antitumor activity of trastuzumab was reduced but not ablated: about 25% of the effect was retained. In the same manner, treatment of wild-type mice with the mutated form of 4D5, the parent antibody of trastuzumab, which was made unable to bind to Fc receptors, had a similar partial effect. These results indicate that in the case of BT-474 cells, trastuzumab probably triggers both the intrinsic growth-inhibitory and apoptotic regulatory pathways, as well as evokes ADCC. In the case of the intrinsicallyresistant JIMT-1 cells, the mechanism of action of trastuzumab seems to be exclusively ADCC.

The mutual independence of the intrinsic and immune-mediated effects was further evidenced by in vitro ADCC experiments using human peripheral leukocytes as effector cells. The capacity of these cells to kill JIMT-1 and SKBR-3 cells in the presence of trastuzumab by ADCC was the same

despite significant differences in the direct drug sensitivity assays. Moreover, we found that the downmodulation of ErbB2 receptor from the cell surface upon trastuzumab treatment, which has previously been postulated as the central phenomenon for direct growth inhibition, seems to be mechanistically unrelated to the action of trastuzumab in vivo.

Moreover, we found that the down-modulation of ErbB2 receptor from the cell surface upon trastuzumab treatment, which has previously been postulated as the central phenomenon for direct growth inhibition appears to be mechanistically unrelated to the action of trastuzumab *in vivo*.

The subline JIMT-X+, generated from a trastuzumab treated JIMT-1 xenograft, did not differ from the parental JIMT-1 cells in the *in vitro* ADCC assays. JIMT-1 X+ cells were able to form xenografts in SCID mice and the growth of the xenografts was reduced by trastuzumab administration, suggesting that JIMT-1 X+ cells were initially sensitive to trastuzumab also *in vivo*; although trastuzumab resistance has developed after 3 weeks.

Finally, we showed that trastuzumab therapy was able to reduce the number of both circulating tumor cells (CTC) in the blood and disseminated tumor cells (DTC) in the bone marrow, even when the antibody has already lost its tumor inhibitory effect on the primary tumor. To the best of our knowledge, this is the first study to investigate the effect of trastuzumab on circulating and disseminated tumor cells shed from a trastuzumab resistant primary tumor in a xenograft model. We suggest that ErbB2 expressing CTCs and DTCs may be sensitive to trastuzumab even if trastuzumab resistance of the primary tumor has already developed, especially if the mechanism of resistance is masking of ErbB2. So trastuzumab treatment might have benefit in the case of patients with already trastuzumab-resistant breast cancer.

A POTENTIAL EXPLANATION OF TRASTUZUMAB-RESISTANCE OF JIMT-1 CELLS

In our *in vivo* experiments, trastuzumab had inhibitory effect on JIMT-1 xenografts in SCID mice despite the *in vitro* resistance of these cells to trastuzumab, but only when its administration was started at small xenograft size, or at the time of tumor cell inoculation. The growth inhibitory effect was mediated via ADCC, but resistance developed after 5-7 weeks. A subline, JIMT-X+, generated from a trastuzumab-resistant xenograft, did not differ from the parental JIMT-1 cells in *in vitro* ADCC assays and was equally sensitive to trastuzumab when re-grafted to mice. Furthermore, trastuzumab significantly reduced the number of circulating and disseminated tumor cells (CTCs and DTCs, which have a central role in metastasis development) at a time when the primary tumor was already unresponsive to trastuzumab, suggesting that ErbB2 positive CTCs and DTCs could be sensitive to trastuzumab-mediated ADCC even if when the primary tumor is already non-responsive. Taken together, it is likely that in this model single tumor cells or their smaller clusters are sensitive to trastuzumab-mediated ADCC, but lose their sensitivity to ADCC when cell-cell and cell-matrix junctions are formed and the cells develop into a 3 dimensional tumor tissue. It was previously suggested that trastuzumab resistance is caused by masking of ErbB2 by MUC4 or hyaluronan. These molecules may not only block the binding of trastuzumab, but could also prevent the recruitment of Fc receptor bearing immune effector cells. When single tumor cells shed into the bloodstream they certainly leave connective tissue glycosaminoglycans behind and probably lose some of their cell surface molecules responsible for masking. These events unmask ErbB2 and could render CTCs and DTCs sensitive to trastuzumab. It is probably also of importance that single tumor cells are better tackled by the immune system than larger tumor masses.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

1. **Barok M.**, Isola J., Pályi-Krekk Zs., Nagy P., Juhász I., Vereb Gy., Kauraniemi P., Kapanen A., Tanner T., Vereb Gy., Szöllősi J.: Trastuzumab causes ADCC-mediated growth-inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Molecular Cancer Therapeutics, 6 (2007): 2065-2072. **IF: 5,131**

2. **Barok M.**, Balázs M., Nagy P, Rákosy Zs., Treszl A., Tóth E., Juhász I., Park J.W., Isola J., Vereb Gy., Szöllősi J.: Trastuzumab decreases the number of circulating and disseminated tumor cells despite trastuzumab resistance of the primary tumor. Cancer Letters, *accepted for publication* **IF: 3.277**

OTHER PUBLICATIONS:

1. Pályi-Krekk Zs, **Barok M.**, Isola J., Tammi M., Szöllősi J., Nagy P.: Hyaluronan-induced masking of ErbB2 and CD44-enhanced trastuzumab internalization in trastuzumab resistant breast cancer. European Journal of Cancer, 2007 Sep 30. **IF: 4,167**

2. Rákosy Zs., Vizkeleti L., Ecsedi S., Voko Z., Bégány A, **Barok M.**, Krekk Zs., Gallai M., Szentirmay Z., Ádány R., Balázs M.: EGFR gene copy number alterations in primary cutaneous malignant melanomas are associated with poor prognosis. International Journal of Cancer 2007 Oct 15;121(8):1729-37. **IF: 4,693**

3. Pályi-Krekk Zs, **Barok M.**, Kovács T., Saya H., Nagano O., Szöllősi J., Nagy P.: EGFR and ErbB2 are functionally coupled to CD44 and regulate CD44 shedding. *under review*

4. **Barok M.**, Balázs M., Lázár V., Rákosy Zs., Tóth E., Treszl A., Park J.W., Vereb Gy., Szöllősi J.: Characterization of a trastuzumab resistant novel breast cancer cell line by CGH and FISH. *manuscript*