

Featured Article

Pilot Study of the Mechanism of Action of Preoperative Trastuzumab in Patients with Primary Operable Breast Tumors Overexpressing HER2

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ABSTRACT

Purpose: To elucidate the mechanism by which trastuzumab, a humanized monoclonal antibody against HER2 with proven survival benefit in women with HER2-positive metastatic breast cancer, mediates its antitumor activity.

Experimental Design: A pilot study including 11 patients with HER2-positive tumors treated in a neo-adjuvant setting with trastuzumab was performed. Trastuzumab was administered i.v. at a dose of 4 mg/kg followed by three weekly i.v. doses of 2 mg/kg. The primary tumor was surgically removed 7 days after the last treatment. Surgical samples, tumor biopsies, and lymphocytes from these patients were collected for biological studies.

Result: Clinical data indicated one complete pathological remission and four partial remissions using RECIST (Response Evaluation Criteria in Solid Tumors). Trastuzumab was well tolerated and neither serious adverse events

nor changes in cardiac function were observed during this short-term treatment and after surgery. The biological data showed that, independent of response, (a) all patients showed high levels of circulating trastuzumab; (b) saturating level of trastuzumab was present in all of the tumors; (c) no down-modulation of HER2 was observed in any tumors; (d) no changes in vessel diameter was observed in any tumors; (e) no changes in proliferation was observed in any tumors; and (f) a strong infiltration by lymphoid cells was observed in all cases. Patients with complete remission or partial remission were found to have a higher *in situ* infiltration of leukocytes and a higher capability to mediate *in vitro* antibody-dependent cellular cytotoxicity activity.

Conclusions: The results of this pilot study argue against trastuzumab activity in patients through down-modulation of HER2 but in favor of antibody-dependent cellular cytotoxicity guiding efforts to optimize the use of trastuzumab in breast cancer patients.

INTRODUCTION

Trastuzumab is a high-affinity humanized monoclonal antibody that has been shown to produce response rates of 15–40% when used as single agent in patients with metastatic HER2-overexpressing breast cancer (1) and to prolong survival when combined with chemotherapy in the first-line therapy of such patients (2). A recent study has also shown that preoperative trastuzumab in combination with other chemotherapeutic agents was active against HER2-overexpressing early-stage breast cancer (3). Although trastuzumab has become a standard of care for women with HER2-overexpressing metastatic breast cancer, the *in vivo* mechanism of action of this new drug is not completely understood. Efforts addressing the mechanism(s) mediating trastuzumab activity would allow for optimal use of this humanized antibody for all patients with HER2-overexpressing tumors. *In vitro* treatment of HER2-overexpressing breast carcinoma cell lines with trastuzumab resulted in down-modulation of the receptor and inhibition of tumor growth (4). In contrast, the activity of trastuzumab examined in animal models was found to depend on the engagement of Fc-receptor expressing lymphocytes (5), indicating antibody-dependent cellular cytotoxicity (ADCC) as the major mechanism of antibody action. A recent study in a preclinical model consisting of immunodeficient severe combined immunodeficiency mice transplanted with human breast carcinoma cells overexpressing HER2 showed that trastuzumab treatment induced a 30% reduction of tumor volume attributable to a reduction in vessel volume, introducing a new mechanism of action for this antibody (6).

To establish the role of trastuzumab therapy in patients overexpressing HER2, this study investigated the efficacy and

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Note: R. Gennari and S. Menard contributed equally to this work. The study was conducted in accordance with the ethical principles stated in the most recent version of the Declaration of Helsinki. The protocol was reviewed and approved by institutional review boards of all participating centers. All patients enrolled in the study signed an informed consent.

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safety of antibody administration as a single agent for early-stage breast cancer patients. Concomitantly, the availability of biological material from patients with HER2-positive breast carcinomas treated with trastuzumab as a single drug was unique to studying the mechanism of action of this innovative therapeutic tool. Indeed, the biomolecular analysis on tumor specimens before and after therapy has given insights into the putative trastuzumab mechanisms of action.

MATERIALS AND METHODS

Patients. From May to December 2002, 47 patients with primary operable breast cancer (cT_{1-3} , N_{0-2}) underwent tumor biopsy for selection and tumor biological characterization. Eleven patients classified as HER2-positive tumors (herceptest/3+ or herceptest/2+ and fluorescence in situ hybridization-positive) were enrolled in the study to receive preoperative trastuzumab as a single loading dose of 4 mg/kg followed by 3 weekly treatments at 2 mg/kg. The short treatment protocol, corresponding to the time interval between diagnosis and planned surgery in our institution, was chosen for ethical reasons, to avoid delay until efficacy of treatment was proven. Surgery was performed 1 week after the last dose. Patients underwent either quadrantectomy with sentinel node biopsy and/or complete auxiliary dissection or modified radical mastectomy. Left ventricular ejection fraction was determined at baseline and before surgical treatment. After surgery, patients received adjuvant treatments in accordance to standard practice guidelines.

Antibodies. Biological parameters were analyzed by immunohistochemical staining and the following markers were tested: expression of the MHC of class I (MHC) with W6/32 monoclonal antibody (MAb; DAKO, Glostrup, Denmark) directed against a monomorphic epitope on the M_1 , 45 000 polypeptide products of the *HLA-A*, *-B*, and *-C loci*; the cell-adhesion molecule e-cadherin using the MAb clone NCH38 (DAKO); tumor proliferation using MIB-1 MAb (Immunotech, Marseille, France) directed against Ki67 proliferation antigen expressed during the active phases of the cell cycle but absent in resting cells; infiltrating leukocytes using anti-CD45RO MAb (clone UCHL1, DAKO) labeling mature activated T cells, granulocytes and monocytes, anti-CD68 MAb (clone Ki-M6, DAKO) directed against a marker expressed by monocytes and macrophages, and anti CD-56 MAb (clone 123C3, ZYMED Laboratories Inc. South San Francisco CA) staining natural killer (NK) cells; and tumor angiogenesis after vessel staining with anti-CD31 MAb (DAKO).

Evaluation of Trastuzumab in Treated Patients. To investigate whether injected trastuzumab was localized at tumor level, frozen sections of tumors were tested with biotinylated trastuzumab at a concentration ranging from 50 to 10 μ g/ml followed by incubation with streptavidin-conjugated horseradish peroxidase (DAKO). To verify if the lack of trastuzumab staining was attributable to down-modulation of the HER2 receptor after treatment, the presence of oncoprotein was evaluated by streptavidin-biotin-peroxidase technique incubating a slide for each sample with CB11 MAb (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom) directed against a

synthetic peptide corresponding to a site on the internal domain of receptor (1:30) and not cross-reacting with trastuzumab.

The amount of trastuzumab present in patient sera displacing biotinylated trastuzumab from cell membrane of HER2-positive cell line (SKBR3) was measured by competition assay using biotinylated trastuzumab as probe tested on an HER2-positive cell line (SKBR3) in cytofluorimetric analysis and calculated based on a calibration curve using different quantities of non-biotinylated trastuzumab starting from a concentration 25-fold higher than that of labeled reagent.

Cell-Dependent Cytotoxicity. Two distinct aliquots of peripheral blood, with and without anticoagulant, were drawn from patients before and after therapy with trastuzumab. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech, Uppsala, Sweden) of peripheral blood collected in anticoagulant. After washings with medium containing 10% FCS, cells were resuspended in 90% FCS + 10% DMSO solution on ice at cell concentration $<10 \times 10^6$ /ml and finally frozen in liquid nitrogen after intermediate cooling at -80°C in a methanol-containing device. After thawing, viability of PBMCs was consistently higher than 85%, as evaluated with flow cytometry by standard propidium iodide incorporation. Concomitantly, peripheral blood collected at the same time, but without anticoagulant, was used for separation of autologous serum that was further incubated 30 minutes at 56°C to inactivate complement, and serum was finally frozen at -80°C until use.

To obtain a reliable comparison between cytotoxic activity before and after therapy, PBMCs isolated from the same patient, but at a different time, were thawed and tested in parallel in the same experiment. After thawing, cells were washed in FCS-containing medium, and finally suspended in RPMI 1640 containing 20% of autologous human serum that had been collected at the same time of PBMCs with the ultimate goal to restore *in vitro* conditions overlapping to those found *in vivo*.

Cell cytotoxicity was measured in a 4-hour ^{51}Cr release assay using the MDA-MB361 human breast cancer cell line as a target because of its high HER2 expression. Five-thousandths ^{51}Cr -labeled MDA-MB361 cells were added to each well in which various concentrations of PBMCs were suspended in 200 μ L of RPMI 1640 buffered with HEPES and containing 20% of autologous serum. After a 4-hour incubation at 37°C , the release of ^{51}Cr into supernatants was determined in triplicate at E:T ratios of 12.5:1, 25:1 and 50:1. Twenty-five μ L of supernatant were collected from each well, seeded in Lumaplate 96 scintillation plates, and counted by a TopCount gamma counter (Packard Instruments Company, Meriden, CT). Maximum and spontaneous ^{51}Cr release values were obtained by adding either 1% NP40 detergent (BDH Biochemicals, Poole, United Kingdom) or complete medium, respectively, to microtiter wells containing 5×10^3 labeled target cells. The percentage of lysis was calculated according the following formula = $(\text{cpm experimental} - \text{cpm spontaneous release}) / (\text{cpm maximum release} - \text{cpm spontaneous release}) \times 100$.

To estimate a correlation between ADCC activity from various patients and their own responses observed after trastuzumab therapy, cell cytotoxicity data were also expressed in terms of lytic units 20 (LU20) per 10^7 mononuclear cells,

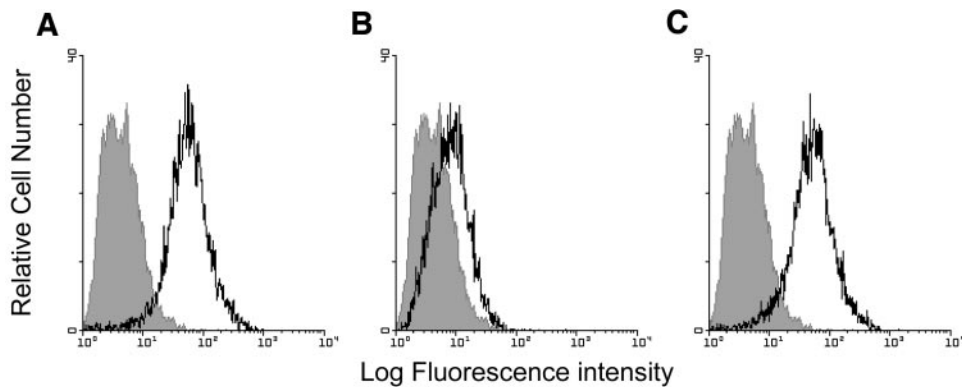


Fig. 1 Analysis of the presence of trastuzumab in sera of patient. Fluorescence-activated cell sorter analysis of the reactivity of biotinylated trastuzumab on HER2-positive cells alone (A) or in competition with sera of patient after (B) or before (C) trastuzumab treatment. Representative data obtained with samples from one patient are expressed. The *solid area* shows cytofluorimetric profile obtained with only fluorescein isothiocyanate streptavidin (negative control).

calculated according to Pross *et al.* (7), on the basis of the dose-response curve. One LU20 corresponds to the number of effector cells necessary to lyse 20% of the targets.

RESULTS

Response to trastuzumab therapy was evaluated based on clinical, pathological, and radiologic examination of the tumor before and after treatment. Clinical and pathological data revealed one complete pathological remission, four partial remissions (>30% reduction of the major tumor diameter), and six patients with no responses using RECIST (Response Evaluation Criteria in Solid Tumors; ref. 8). No serious adverse events were observed, except for an infusion-associated flu-like syndrome in 3 of 11 (27%) patients. No cardiac dysfunction was recorded.

Sera from all 11 patients collected immediately before

surgery (Fig. 1B) completely inhibited the binding of biotinylated trastuzumab on HER2-overexpressing cells (Fig. 1A), contrary to samples obtained from the same patients before trastuzumab administration (Fig. 1C). This indicates the presence of circulating trastuzumab after antibody treatment. Levels of humanized antibody calculated based on a calibration curve using different quantities of non-biotinylated trastuzumab were estimated around 60 $\mu\text{g/ml}$ and were similar in all patients.

No change in HER2-positive staining was observed at any dilution of the anti-HER2 monoclonal antibody non-cross-reacting with trastuzumab in the 10 tumors found at surgery (Fig. 2A) in comparison to staining on the pretreatment biopsies (Fig. 2B), indicating that the tumor after treatment with the trastuzumab continued to express the same amount of oncoprotein on the membrane.

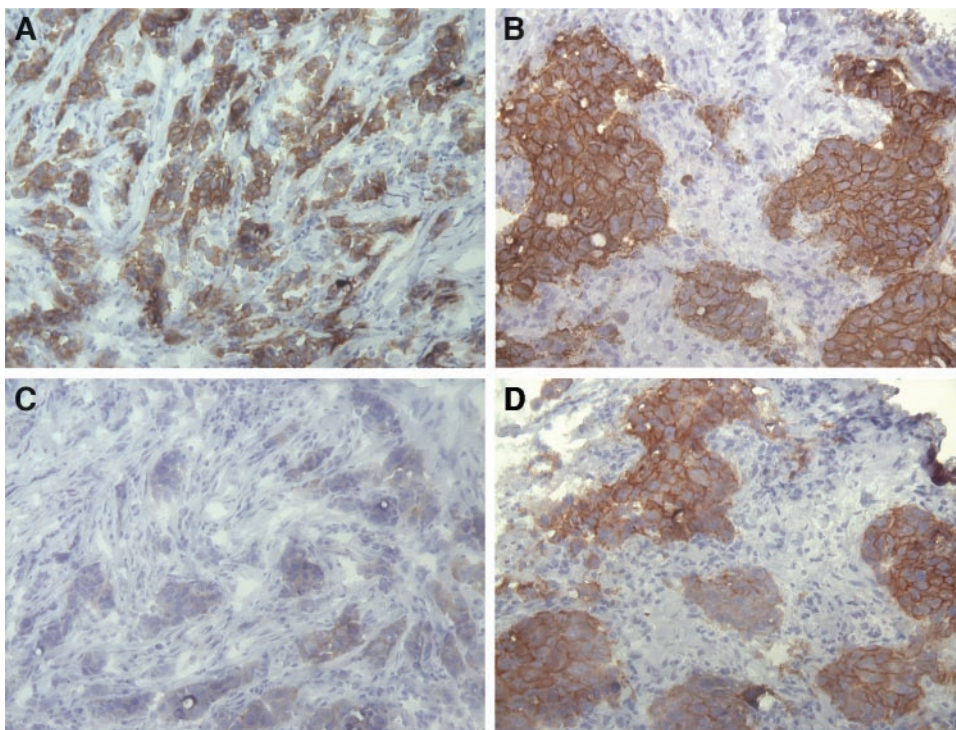


Fig. 2 Analysis of tumor specimens. Immunohistochemical analysis of frozen sections from surgical specimen (A and C) and core biopsy (B and D) using MAb CB11 (A and B) or biotinylated trastuzumab (C and D). Representative data obtained with samples from one patient are shown.

To verify if the lack of HER2 down-modulation was attributable to a failure of trastuzumab to reach the tumor mass, immunostaining with biotinylated trastuzumab was performed. In all cases the staining with the biotinylated antibody, which was intense on the core biopsies (Fig. 2D), was almost completely abrogated in the surgical specimen (Fig. 2C), indicating the presence of trastuzumab bound to HER2 on the tumor cell membrane.

Several variables, including tumor grade, hormone receptor expression, and age of the patients were found to be independent of response (Table 1). Tumor cell proliferation based on the frequency of MIB1-positive cells in the core biopsies *versus* the surgical specimens did not change during therapy nor did the vessel diameter evaluated based on staining for CD31 antigen. Only the diameter of vessels present in the core biopsies was found to weakly correlate with the therapeutic response ($r = 0.63$, $P = 0.04$), with largest vessels associated with response.

Because HER2-positive tumors were known to be often but not always infiltrated by leukocytes, the presence of a lymphoid infiltrate, evaluated as the number of CD68-, CD45RO-, and CD56-positive cells in the tumors before trastuzumab therapy was analyzed. The frequency of cases with a strong infiltrate was higher in the responding group compared with the non-responsive one (four of five *versus* one of six, respectively; Table 1), and a weak association with response evaluated as a percentage of reduction of major tumor diameter was observed ($r = 0.70$, $P = 0.02$). The number of NK cells, although quite low, were proportional to the total number of infiltrating cells and increased in the surgical specimens compared with pretreatment biopsies as well as other leukocytes.

HLA class I expression, known to inhibit ADCC when expressed on the target cells (9), was investigated in the 10 tumor surgical specimens. As expected, about half of the tumors were HLA class I-negative, but no association with response to trastuzumab therapy was observed. Also levels of e-cadherin, a homophilic adhesion molecule with inhibition that resulted in enhanced ADCC activity in a preclinical model (10), were found to be heterogeneous in the tumor specimens but were not associated with response.

Cell-mediated cytotoxic activity tested on ^{51}Cr -labeled HER2-positive target cells was found to be significantly increased, although at different levels at the end of the trastuzumab treatment in 8 of the 10 patients tested (Fig. 3A). These results were obtained *ex vivo* by directly comparing for each patient the activity of PBMCs in the presence of autologous serum. Thus, although the specific phenotype of effector cells and their homing potential for non-lymphoid peripheral tissue (such as breast) were not determined in this preliminary phase of the study, we could demonstrate that short-term therapy with trastuzumab induced significant anti-HER2 PBMC-dependent cytotoxic activity in most treated patients. We also tested the dose-response relationship at different E:T ratios to express data from single patients as arbitrary lytic units corresponding to the number of effector cells necessary to lyse 20% of the targets (LU20; Fig. 3B). By using this parameter, patients showing a clinical response were found to have a significantly higher cytotoxic activity at the end of trastuzumab treatment (180 ± 50 LU20), as compared with those not responding (47 ± 15 LU20; $P < 0.01$). In addition, considering response as a continuous variable using the reduction of tumor diameter percentage, a strong correlation was found with cell cytotoxic activity ($r = 0.89$, $P = 0.0005$; Fig. 4). After excluding the patient who underwent a complete remission and had a particularly high level of lytic unit, the relationship between response and ADCC was still statistically significant, although at a lower level ($P = 0.03$). Interestingly, it may be worth to note that, shortly before diagnosis of breast carcinoma, the patient who underwent a complete pathological remission experienced a herpes virus infection that might have increased NK activity, as reported recently (11). In conclusion, these preliminary data indicate that cell-dependent cytotoxicity, among various biological effects having therapeutic potential that we tested, was the most likely mechanism mediating trastuzumab activity in this short-term protocol.

DISCUSSION

Our study indicates that trastuzumab is safe, well tolerated, and can induce therapeutic responses in patients with primary

Table 1 Analysis of different parameters according to response to trastuzumab

	No. cases/total	
	Responders	Not responders
No. patients	5 (45%)	6 (55%)
HER2-pos 3+	5/5	5/6
ER-neg	2/5	4/6
PgR-neg	2/5	4/6
G III	3/4*	5/6
Down-modulation of HER2	0/5	0/6
Presence of trastuzumab in the tumor	5/5	6/6
Decrease Ki67-positive cells	0/5	1/6
Decrease of vessel diameter	0/5	0/6
Strong lymphoid infiltration before therapy	4/5	1/6
Strong lymphoid infiltration after therapy	5/5	4/5
	Mean \pm SE (range)	Mean \pm SE (range)
Age of patients	55.8 \pm 9.0(44–69)	47.8 \pm 5.2(26–64)
ADCC in lytic units	180 \pm 50	47 \pm 15

* Tumor grading was not determined for case with complete response.

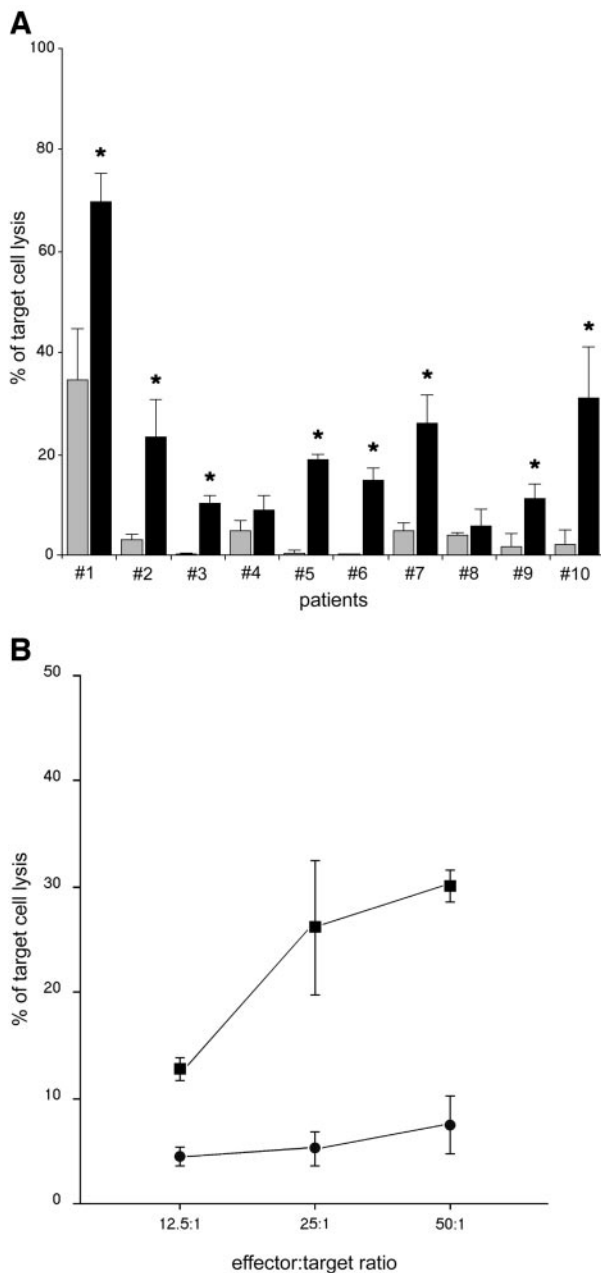


Fig. 3 Cytotoxic activity of patients against HER2. **A**, cytotoxic activity against HER2-overexpressing target cells of the PBMC of patients collected before (gray bars) or at the end of therapy with trastuzumab (black bars). Data are expressed as percentage of target cell lysis obtained from short-term chromium release assays. Data obtained at the 25:1 E:T ratio are shown. Comparison was made by Student's *t* test for each patient and $P < 0.05$ (*) was considered significant. **B**, a representative experiment with increasing E:T ratios is shown (patient 10) using PBMC collected before (●) or at the end of therapy with trastuzumab.

operable breast cancer, even when used as a single agent and for a very short time period in the preoperative setting. Interestingly, our data also indicates that short-term therapy with trastuzumab is sufficient to induce significant anti-HER2 cell-dependent cytotox-

icity in most patients with early-stage disease and still fully functional immune systems. Furthermore, we found a correlation between cytotoxic activity and clinical responses, thus suggesting that ADCC plays a substantial role in the therapeutic efficacy of trastuzumab. As expected (12), the number of NK was found to be quite low in the tumor infiltrates, suggesting macrophages and granulocytes as principal mediators of ADCC. By contrast, we failed to observe other biological effects bearing therapeutic implication, including down-modulation of the receptor or activity on vessels. All together, these preliminary data are strongly consistent with earlier observation from *in vivo* preclinical models indicating that the engagement of the Fc of trastuzumab with FcR γ is required for the majority of trastuzumab activity in the BT474 xenograft model (5) but do not support the role of other mechanisms supposed to be involved in trastuzumab activity.

Our failure to detect HER2 down-modulation during trastuzumab therapy may constitute an unexpected finding. However, previously reported results of preoperative trial of association of trastuzumab with paclitaxel also showed that the majority of the cases maintained the HER2 expression after therapy (3). Our series is probably too small to observe the low frequency of cases suggested to become HER2-negative after therapy in Burstein's study. Alternately, the observed down-modulation might be attributable to the longer treatment (11 weeks) or to a role of paclitaxel in selecting HER2-negative cells.

This information is relevant for optimization of trastuzumab treatment for several reasons. First, the use of trastuzumab in combination with other drugs should take into consideration the immunosuppressive effect of most chemotherapeutic drugs, and the still undetermined optimal timing of rescue of cells mediating antibody-dependent cell-mediated cytotoxicity. Note that taxane treatment, which synergizes with trastuzumab, induces immunosuppression of adaptive immunity, but selectively increases NK activity (13). Second, specific cytokines might be used to stimulate NK activity. Although this approach has been attempted using interleukin 2 without success, that particular study was

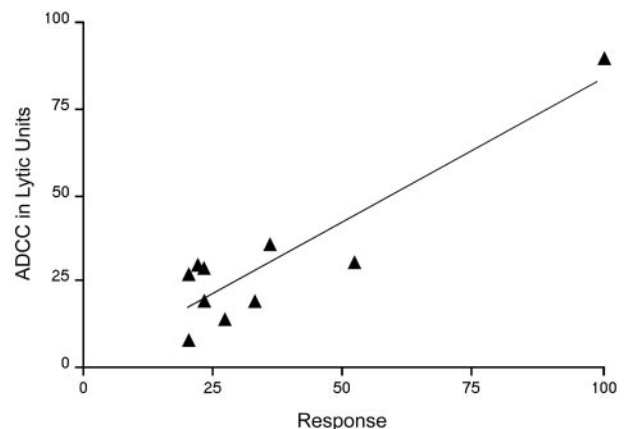


Fig. 4 ADCC activity and trastuzumab efficacy. Correlation between response to trastuzumab evaluated as a reduction of tumor diameter after treatment and ADCC expressed as lytic units. Triangles represent patients.

performed in patients with metastatic disease who were pre-treated with intensive chemotherapy so that induction of NK activity was poor and interleukin 2 toxic (14). Finally, the experimental *in vitro* data on synergy between trastuzumab and other drugs may have no relevance at least for *in vivo* short-term treatment because the activity of trastuzumab *in vitro* differs from that *in vivo* (e.g., trastuzumab down-modulates HER2 *in vitro* but not *in vivo*). One possibility to explain this discrepancy may rest in the lack of bivalent binding of the antibody, required to induce internalization of the complex receptor/antibody, attributable to a different fluidity of the membrane when cells are in a three-dimensional context *in vivo*.

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