Overexpression of HER-2/Neu in Uterine Serous Papillary Cancer[1]

Alessandro D. Santin,[2] Stefania Bellone, Murat Gokden, Michela Palmieri, Donna Dunn, Jamshed Agha, Juan J. Roman, Laura Hutchins, Sergio Pecorelli, Timothy O'Brien, Martin J. Cannon, and Groesbeck P. Parham

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology [A. S., S. B., M. P., D. D., T. O., G. P.], Departments of Pathology [M. G.], Medicine [J. A., L. H.], and Microbiology and Immunology [M. J. C.], University of Arkansas, Little Rock, Arkansas 72205-7199, and Division of Gynecologic Oncology, University of Brescia, 25123 Brescia, Italy [A. S., S. B., M. P., S. P.]

ABSTRACT

Purpose: **Uterine serous papillary carcinoma (USPC) is a highly aggressive variant of endometrial cancer and histologically similar to high-grade ovarian cancer. HER-2/neu, the transmembrane receptor encoded by the c-*erbB2* gene, is overexpressed by immunohistology in ~25% of ovarian cancers. In this study, we have evaluated the expression of HER-2/neu in several fresh, established, paraffin-embedded, fixed USPCs. In addition, we have tested the sensitivity of USPC cells to Herceptin treatment.**

Experimental Design: **Ten consecutive USPC specimens were assessed by immunohistochemistry for the intensity of expression of HER-2/neu. In addition, three USPC cell lines were analyzed for expression of HER-2/neu by flow cytometry as well as for sensitivity to Herceptin-mediated, complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), and inhibition of cell proliferation.**

Results: **Eight of 10 (80%) of the USPCs assessed immunohistochemically for the intensity of expression of HER-2/neu stained heavily for HER-2/neu (2+ to 3+). Fresh and established primary USPC cell lines were found to express significantly more HER-2/neu receptor by flow cytometry (on the average, 10-fold greater) when compared with HER-2/neu-positive primary or established breast and ovarian cancer cell lines ($P < 0.001$). Importantly, although these USPC cell lines were resistant to chemotherapy *in vivo* and to natural killer- and complement-mediated cytotoxicity *in vitro*, they were found to be highly sensitive to Herceptin-mediated ADCC. USPC cell proliferation was also inhibited by Herceptin. A significant enhancement of ADCC was demonstrated when effector cells were exposed to low doses of IL-2 *in vitro*. Physiological concentrations of human serum IgG did not inhibit Herceptin-mediated ADCC against USPC.**

Conclusions: **On the basis of these findings and previous reports showing a positive *in vivo* correlation between efficacy of Herceptin therapy and the level of HER-2/neu overexpression by tumor cells, we propose that Herceptin might be a novel and attractive therapeutic strategy in patients harboring chemotherapy-resistant, recurrent, or metastatic USPC.**

INTRODUCTION

Cancer of the uterine corpus represents the most common gynecological malignancy, with ~37,400 new cases diagnosed in the United States in 1999 (1). USPC[3] (2) is a histological subtype of endometrial cancer constituting up to 10% of all endometrial cancers. Histologically similar to high-grade ovarian cancer (2, 3), USPC has a propensity for early intraabdominal and lymphatic spread even at presentation (4, 5) and is characterized by highly aggressive biological behavior (1–4). In contrast to ovarian cancer, however, it is a chemoresistant disease at its onset with responses to combined cisplatin-based chemotherapy ~20% and of short duration (6, 7). The survival rate is dismal, even when USPC is only a minor component of the histologically more common endometrioid adenocarcinoma (3, 5). The overall 5-year survival is 30 ± 9% for all stages, and the recurrence rate after surgery is extremely high (50–80%). Novel therapeutic strategies effective in the treatment of residual and/or metastatic USPC are urgently needed.

Proto-oncogenes are a group of normal genes that play important roles in the regulation of cell proliferation. Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and maintenance of the malignant phenotype. The human HER-2/neu (c-erbB2) gene product, similar to the epidermal growth factor receptor, is a transmembrane receptor protein that includes a cysteine-rich extracellular ligand-binding domain, a hydrophobic membrane spanning region, and an intracellular tyrosine kinase domain (8). With no direct ligand identified to date, HER-2/neu functions as a preferred partner for heterodimerization with other members of the epidermal growth factor receptor family (*i.e.*, HER-1 or ErbB1, HER-3 or ErbB3, and HER-4 or ErbB4) and thus plays an important role in coordinating the complex ErbB signaling network that is responsible for regulating cell growth and differentiation (9, 10). Initially identified as the proto-oncogene

Received 11/12/01; revised 2/12/02; accepted 2/22/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by grants from the Angelo Nocivelli and the Camillo Golgi Foundations, Brescia, Italy.
[2] To whom requests for reprints should be addressed, at University of Arkansas for Medical Sciences Medical Center, Division of Gynecologic Oncology, University of Arkansas, 4301 West Markham, Little Rock, AR 72205-7199. Phone: (501) 686-7162; Fax: (501) 686-8091.

[3] The abbreviations used are: USPC, uterine serous papillary carcinoma; MAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; IL, interleukin; PBL, peripheral blood lymphocyte; UAMS, University of Arkansas for Medical Sciences; FACS, fluorescence-activated cell sorter; NK, natural killer.

associated with the development of neuroblastomas in rats exposed to ethylnitrosourea *in utero* (11), HER-2/neu has subsequently been shown to be overexpressed in approximately one-third of primary ovarian carcinomas and breast carcinomas as well as other human tumors including colon, lung, prostate, and cervical cancers (12). In breast and ovarian cancer, several but not all studies have reported that the amplification of this gene is associated with resistance to treatment and poor survival, suggesting that cells overexpressing HER-2/neu may manifest a more aggressive biological behavior and may have a selective growth advantage over HER-2/neu-negative tumor cells (11, 13–16).

Recently, a humanized MAb to HER-2/neu, Herceptin, has been reported to have significant therapeutic effects in patients with strongly (*i.e.*, scoring 2+ and 3+) HER-2/neu-positive breast carcinomas, particularly when combined with chemotherapeutic drugs (17, 18). In ovarian, lung, and prostate cancers, clinical studies are currently investigating the efficacy of Herceptin in patients whose tumors exhibit strong plasmalemmal immunoreactivity for this protein (19). In contrast to ovarian cancer, however, very little is known about HER-2/neu expression by the histologically similar but biologically more aggressive USPC. In this study, we report for the first time that this variant of uterine cancer commonly overexpresses HER-2/neu (*i.e.*, scoring 2+ or more in 80% of samples tested), and that the levels of protein expression on primary USPC cell lines recorded by flow cytometry are on average 10-fold higher when compared with fresh or established breast and ovarian HER-2/neu-positive cancer cell lines. Importantly, we show that although USPC cell lines are resistant to NK-dependent cytotoxicity *in vitro*, they retain high sensitivity to anti-HER-2/neu ADCC, and that their *in vitro* proliferation is significantly inhibited by anti-HER-2/neu MAb (Herceptin). Furthermore, a significant enhancement of ADCC was demonstrated when peripheral blood effector cells were incubated with USPC cells in the presence of low doses of IL-2. We propose that Herceptin therapy may be a novel and attractive therapeutic strategy in patients harboring this biologically aggressive and chemotherapy- and radiotherapy-resistant variant of endometrial cancer.

MATERIALS AND METHODS

Immunostaining of Formalin-fixed Tumor Tissues. Formalin-fixed, paraffin-embedded tissue blocks from 10 USPC cases were retrieved from the surgical pathology files of the UAMS. Study blocks were selected after histopathological review by a surgical pathologist from patients who underwent primary surgical therapy for invasive USPC at UAMS between 1998 and 2001. Only specimens showing pure histopathological characteristics of USPC were evaluated in this study. Tumors were staged according to the International Federation of Gynecologists and Obstetricians operative staging system. Patient characteristics are described in Table 1. Total abdominal hysterectomy and regional lymph node sampling for invasive USPC were performed in all cases. In addition, in an attempt to establish primary USPC tumor cell lines from these patients, four USPC fresh tumor biopsies were obtained at the time of surgery through the Gynecological Oncology Division and the Pathology Department of UAMS, under approval of the Institutional Review Board.

Table 1 Characteristics of the patients

Patients	Age	Race	Year of diagnosis	Stage	HER-2/neu positivity
USPC-1	62	Afro-American	1998	IVa	3+
USPC-2	63	Afro-American	1998	IVb	3+
USPC-3	59	Caucasian	2001	IVb	3+
USPC-4	73	Caucasian	2000	Ib	1+
USPC-5	73	Caucasian	1999	IIb	3+
USPC-6	62	Afro-American	2000	Ia	3+
USPC-7	58	Afro-American	1998	Ib	2+
USPC-8	63	Afro-American	2000	IIIc	3+
USPC-9	63	Caucasian	1999	IIIa	2+
USPC-10	64	Afro-American	2000	IVa	1+

The level of expression of HER-2/neu was evaluated by standard immunohistochemical staining by an external independent laboratory (PhenoPath Laboratories, Seattle, WA). The most representative H&E-stained block sections were used for each specimen. When available, both primary and metastatic sites were evaluated for HER-2/neu expression. Briefly, immunohistochemical stains were performed on 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue. After pretreatment with 10 mM citrate buffer at pH 6.0 using a steamer, they were incubated with anti-HER-2/neu MAb (Dako, Glostrup, Denmark) at a 1:2000 dilution. Slides were subsequently labeled with streptavidin-biotin (LSAB; Dako), stained with diaminobenzidine and counterstained with hematoxylin. The intensity of staining was graded as 0 (staining not greater than negative control), 1+ (light staining), 2+ (moderate staining), or 3+ (heavy staining).

Establishment of USPC Cell Lines. Three primary USPC cell lines (USPC-1, USPC-2, and USPC-3) were established after sterile processing of the tumor samples from surgical biopsies as described previously for ovarian carcinoma specimens (20). All three tumor samples were derived from USPC patients who experienced rapid tumor progression during adjuvant chemotherapy after primary surgical debulking. Primary USPC cell lines were analyzed by flow cytometry for HER-2/neu expression immediately after tumor processing and after *in vitro* culture from 1 week to 3 years (USPC-1 and USPC-2). Similarly, two primary serous papillary ovarian carcinoma cell lines established in our laboratory during the same study period from advanced-stage ovarian cancer patients (OVA-4 and OVA-5) as well as two established and previously characterized serous ovarian cancer (UCI-101 and UCI-107; kindly provided by Dr. Alberto Manetta, University of California, Irvine, CA) and breast cancer (B7–474 and SK-BR-3; American Type Culture Collection) cell lines, the latter shown previously to highly overexpress HER-2/neu (21), were analyzed as positive controls by flow cytometry for HER-2/neu expression (see below).

Flow Cytometry. The clinically marketed anti-HER-2/neu MAb Herceptin (Genentech, San Francisco, CA) was used for most of our study. For comparison, we also used an unconjugated anti-HER-2/neu (mouse IgG1) MAb obtained from Oncogene Science (Uniondale, NY). Herceptin is an IgG1κ that contains human framework regions with the complementary-

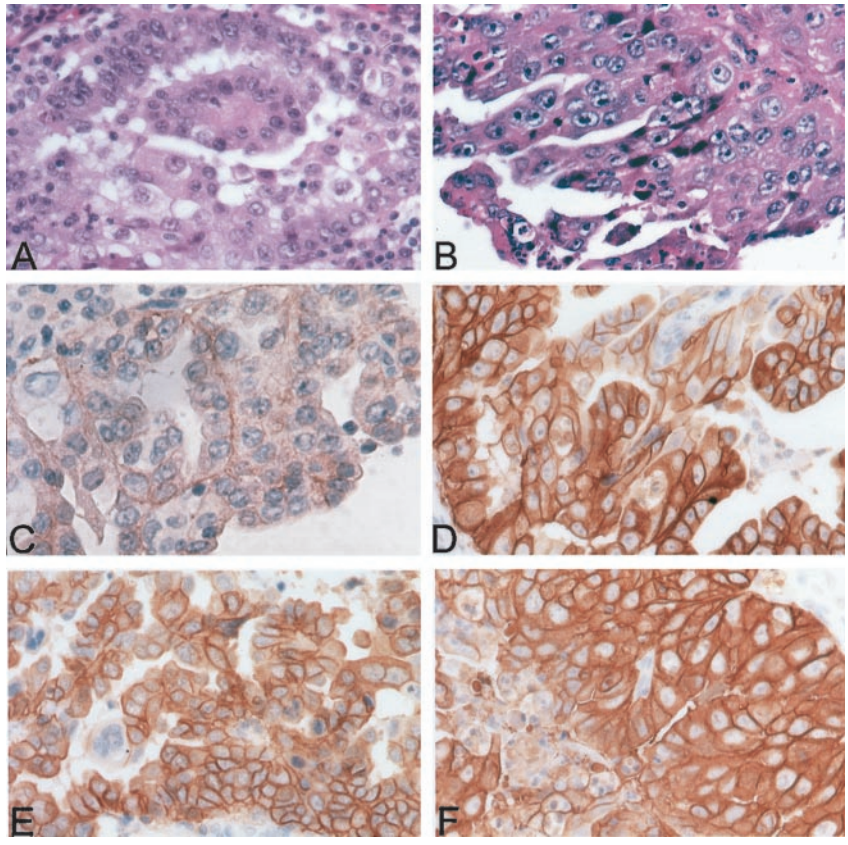

Fig. 1 *A* and *B*, representative H&E sections of USPC-4 and USPC-1 that stained light (1+) for HER-2/neu (*A*) and heavy (3+) for HER-2/neu (*B*), respectively. *C–F*, immunohistochemical staining for HER-2/neu expression on paraffin-embedded USPC specimens. *C*, USPC-4 with light (1+) staining for HER-2/neu. *D–F*, USPC-1, USPC-2, and USPC-3, respectively, from which heavy (3+) staining for HER-2/neu was detected. ×400.

determining regions of a murine MAb that binds to the M_r 185,000 extracellular determinant of HER-2/neu. For staining by Herceptin, a FITC-conjugated goat antihuman $F(ab1)_2$ immunoglobulin was used as a secondary reagent (BioSource International, Camarillo, CA). For staining by unconjugated anti-HER-2/neu (mouse IgG1), a goat antimurine FITC-labeled mouse IgG1 (Beckman-Coulter, Miami, FL) was used. Analysis was conducted with a FACScan, using cell Quest software (Becton Dickinson).

Tests for ADCC. A standard 5-h chromium (^{51}Cr) release assay was performed to measure the cytotoxic reactivity of Ficoll-Hypaque-separated PBLs from several healthy donors and one USPC patient in combination with Herceptin against tumor target cell lines. The release of ^{51}Cr from the target cells was measured as described (22) as evidence of tumor cell lysis after exposure of tumor cells to various concentrations of Herceptin (ranging from 1 to 5 μg/ml). Controls included the incubation of target cells alone or with PBLs or MAb separately. The chimeric anti-CD20 MAb Rituximab (Rituxan; Genentech) was used as control for Herceptin in all bioassays. ADCC was calculated as the percentage of killing of target cells observed with MAb plus effector cells as compared with ^{51}Cr release from target cells incubated alone.

Test for Complement-mediated Target Cell Lysis and γ-Globulin Inhibition. A standard 5-h chromium (^{51}Cr) release assay identical to those used for ADCC assays, except that human serum (as a source of complement) diluted 1:2 to 1:4 was added in place of the effector cells, was used to test for complement-mediated target cell lysis. To test for the possible inhibition of ADCC against USPC cell lines by physiological human plasma concentrations of γ-globulin, heat-inactivated (56°C for 30 min) human serum was diluted 1:2 to 1:4 before being added in the presence or absence of effector PBLs. In some experiments, non-heat-inactivated human serum (diluted 1:2 or 1:4) was added in the presence of effector PBLs. Controls included the incubation of target cells alone or with either lymphocytes or MAb separately. Rituxan was used as control MAb.

IL-2 Enhancement of ADCC. To investigate the effect of IL-2 on Herceptin-mediated ADCC, effector PBLs were incubated at 37°C at a final concentration of IL-2 (Aldesleukin; Chiron Therapeutics, Emeryville, CA) ranging from 50 to 100 IU/ml in 96-well microtiter plates. In some experiments, effector PBLs were incubated with IL-2 only during the standard 5-h chromium (^{51}Cr) release assay, whereas in other experiments effector PBLs were preincubated for up to 72 h with IL-2 before ADCC assay. Target cells were primary USPC cell lines exposed to Herceptin (concentrations ranging from 1 to 5 μg/ml), whereas controls included the incubation of target cells alone or with PBLs in the presence or absence of IL-2 or MAb, respectively. Rituxan was used as a control MAb. ADCC was calculated as the percentage of killing of target cells observed with MAb plus effector PBLs, as compared with target cells incubated alone. Each experiment was performed with at least two

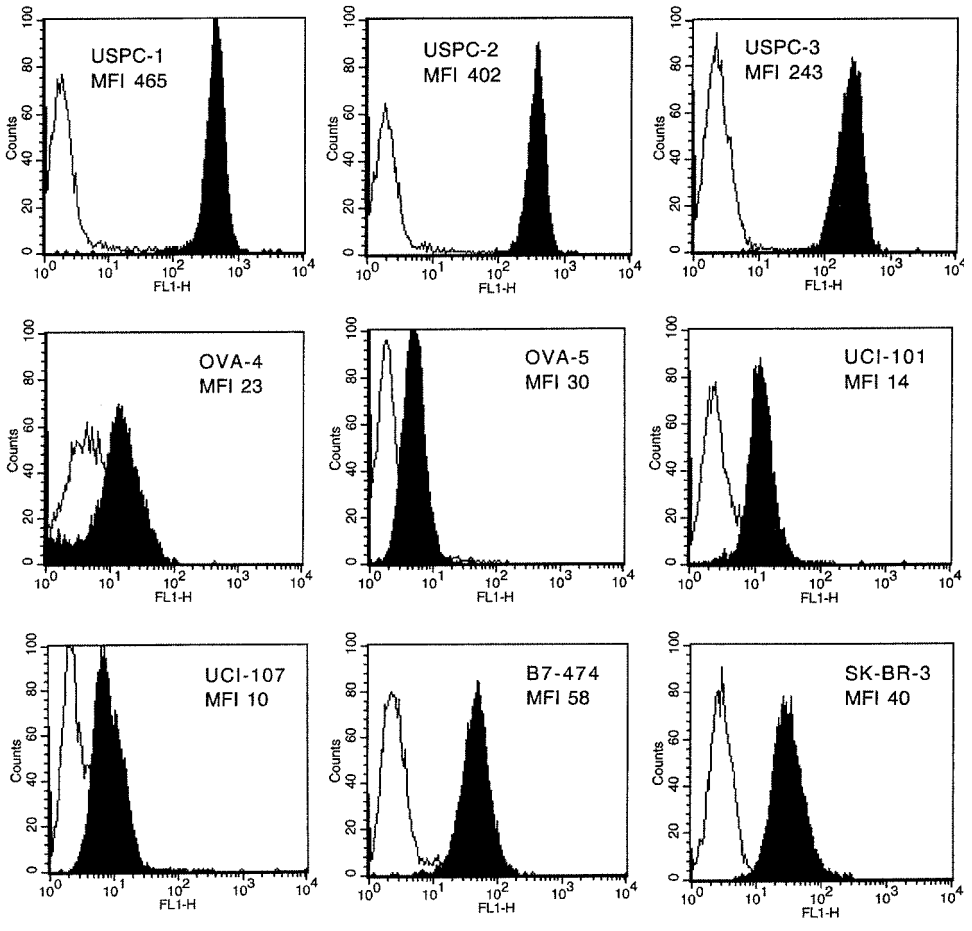

Fig. 2 Representative FACS analysis of Herceptin staining of primary USPC cells and primary and established ovarian and breast cancer cell lines. Data with Herceptin are shown in *solid black*; isotype control MAb profiles are shown in *white*. Similar results were obtained with FITC-labeled anti-HER-2/neu MAb (Oncogene Science)-stained tumor cell lines (data not shown). HER-2/neu expression was significantly higher on USPC cell lines compared with fresh and established ovarian cancer cell lines and established breast cancer cell lines ($P < 0.001$, Student's *t* test).

normal donors, with results from a representative donor presented.

Cell Proliferation Assay. Primary USPC cell lines from patients USPC-1 and USPC-2 were plated at 2500 cells/well in U-bottomed 96-well plates in the presence or absence of various concentrations of Herceptin on day 0, using Rituxan as a control. On day 4, cells were pulsed with [H^3]thymidine (1 μCi/well) for 6 h and then placed in a −20°C freezer for 1 h. After thawing at room temperature, cells were harvested using a Packard Filtermate Harvester Unifilter-96, and incorporated radioactivity was measured as described (23).

RESULTS

HER-2/neu Expression by Immunohistology on USPC. Immunohistochemically detectable HER-2/neu protein (*i.e.*, score from 1+ to 3+) was noted in 100% of the USPC samples evaluated (*i.e.*, 10 of 10 samples), with 8 of 10 of the USPC samples showing moderate (2+, 2 samples) to heavy (3+, 6 samples) stain for HER-2/neu (Table 1). In four cases in which peritoneal metastases were present, HER-2/neu expression was evaluated in both the primary tumor and one metastatic site. In all cases, the intensity of staining was the same when the two sites were compared (data not shown). This included one case in which low (1+) HER-2/neu expression was seen and three cases in which high (3+) HER-2/neu expression was seen. All three primary USPC cell lines established during the study period were from specimens derived from patients harboring USPCs with a score 3+ for HER-2/neu by immunochemistry, *i.e.*, USPC-1, USPC-2, and USPC-3 (Fig. 1). We were unable to establish a primary USPC cell line from patient USPC-4, whose tumor scored 1+ for HER-2/neu by immunohistochemistry. The three primary USPC cell lines were further studied by flow cytometry as well as in biological assays evaluating the *in vitro* efficacy of anti-HER-2/neu MAb therapy (see below).

HER-2/neu Expression by Flow Cytometry on USPC, Serous Papillary Ovarian, and Breast Cancer Cell Lines. HER-2/neu expression was evaluated by FACS analysis on the primary USPC cell lines, two primary serous papillary ovarian carcinoma cell lines, and two ovarian (*i.e.*, UCI-101 and UCI-107) and two breast cancer (*i.e.*, BT-474 and SK-BR-3) cell lines, the latter being reported previously to highly overexpress HER-2/neu (21, 24). In addition, as negative controls, several B-cell lines (EBV-transformed lymphoblastoid B-cell lines) established from the same USPC and ovarian cancer patients from which the tumor cell lines had been established were also studied. Extremely high reactivity against HER-2/neu receptor was found on all three primary USPC cell lines (100% positive cells for all three USPCs), with mean fluorescence intensity

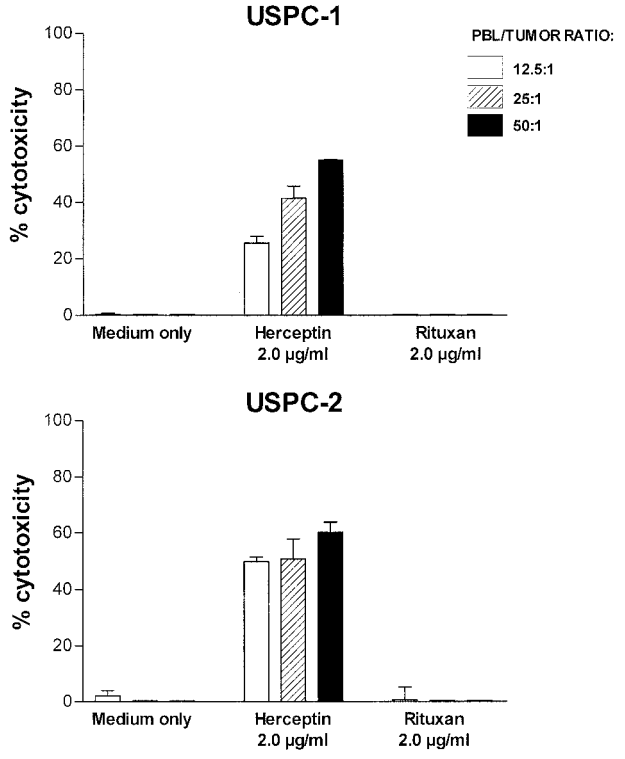

Fig. 3 ADCC mediated by Herceptin (2 μg/ml) against ^{51}Cr-labeled USPC-1 (*upper panel*) and USPC-2 (*lower panel*) cells (10,000 cells/sample), as measured in combination with effector PBLs from a representative healthy, heterologous donor in a 5-h assay. The percentage of target cell lysis is shown; *bars*, ±SD. Effector cells plus Rituxan (2 μg/ml) were used as controls. Similar ADCC results were obtained with the use of Herceptin at 1 or 5 μg/ml (data not shown). No killing was detected in the presence of Herceptin but in the absence of effector PBLs (data not shown).

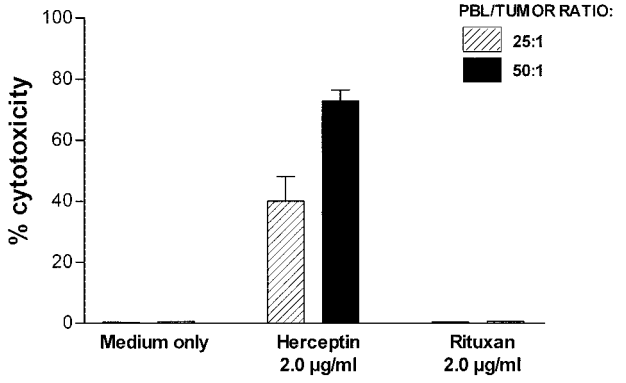

Fig. 4 ADCC mediated by Herceptin (2 μg/ml) against ^{51}Cr-labeled USPC-3 cells (10,000 cells/sample), as measured in combination with autologous effector PBLs in a 5-h assay. The percentage of target cell lysis is shown; *bars*, ±SD. PBLs plus medium only or Rituxan (2 μg/ml) were used as controls. Similar ADCC results were obtained with the use of Herceptin at 5 μg/ml (data not shown).

ranging from 200 to 450 (USPC-1), 150 to 250 (USPC-2), and 170 to 230 (USPC-3), respectively (Fig. 2). Similarly, all primary and established ovarian cancer and breast cancer cell lines were also found to overexpress HER-2/neu by FACS (Fig. 2). Primary and established ovarian and breast cancer cell lines, however, were found to express significantly lower levels of HER-2/neu (average mean fluorescence intensity was 10-fold lower) than that expressed by USPC cells ($P < 0.001$). This finding was particularly remarkable, because the breast cancer cell lines have been shown previously to highly overexpress HER-2/neu and are commonly used as positive controls in several assays evaluating HER-2/neu overexpression (21, 24). All autologous B-cell lines tested were consistently negative for HER-2/neu expression (data not shown).

USPCs Are Resistant to NK Activity but Sensitive to Herceptin-mediated ADCC. Primary USPC cell lines were tested for their sensitivity to NK cytotoxicity when challenged with PBLs collected from several healthy donors in a standard 5-h ^{51}Cr release assay. As shown in Fig. 3, USPC cell lines were consistently found to be resistant to NK-mediated killing when combined with PBLs at E:T ratios varying from 12.5:1 to 50:1 (range of killing from 0 to 3% with all E:T ratios). Similarly, USPC cell lines incubated with Rituxan control antibody were not significantly killed (range of killing from 0 to 3% with all E:T ratios; Fig. 3). In strong contrast, USPC cell lines were found to be highly sensitive to PBLs from heterologous donors combined with Herceptin to mediate ADCC (range of killing, from 25 to 60%, from 12.5:1 to 50:1 E:T ratio; Fig. 3). This experiment was repeated five times with similar results.

Herceptin-mediated ADCC by Autologous PBLs. Because in experimental models and human beings (25, 26) alteration in number and function of NK cells has been associated with tumor progression, we investigated the ability of autologous PBLs from patients harboring USPCs to kill tumor cells in the presence or absence of Herceptin. The USPC-3 cell line was challenged with PBLs collected from patients in a standard 5-h ^{51}Cr release assay. Similarly to the results obtained using healthy donor PBLs against USPC-1 and USPC-2 cell lines (Fig. 3), USPC-3 was found to be highly resistant to autologous NK-mediated killing at all of the E:T ratios tested (*i.e.*, from 25:1 to 50:1; range of killing, from 0 to 1% with all E:T ratios; Fig. 4). The USPC-3 cell line incubated with Rituxan (anti-CD20) control antibody was not significantly killed (range of killing, from 0 to 1% with all E:T ratios; Fig. 4). In contrast, USPC-3 was found to be highly sensitive to Herceptin when combined with autologous PBLs to mediate ADCC (range of killing, from 35 to 75%, from 25:1 to 50:1 E:T ratio; Fig. 4). This experiment was repeated twice with similar results.

Effect of Complement and Physiological Concentrations of IgG on Herceptin-mediated ADCC against USPC. To evaluate primary USPC cell lines for their sensitivity to complement-mediated cytotoxicity and to evaluate possible inhibition of ADCC by physiological concentrations of IgG, USPC cell lines were challenged by adding human serum diluted 1:2 to 1:4 (with or without heat inactivation) in the presence or absence of the effector cells and Herceptin to standard 5-h ^{51}Cr release assays. As shown in Fig. 5, addition of untreated serum with or without Herceptin or Rituxan was not able to induce significant cytotoxicity against USPC-1 (Fig. 5, *A*, *C*, and *E*) and USPC-2 (Fig. 5, *B*, *D*, and *F*) cell lines. These data illustrate the lack of significant cytotoxicity mediated by

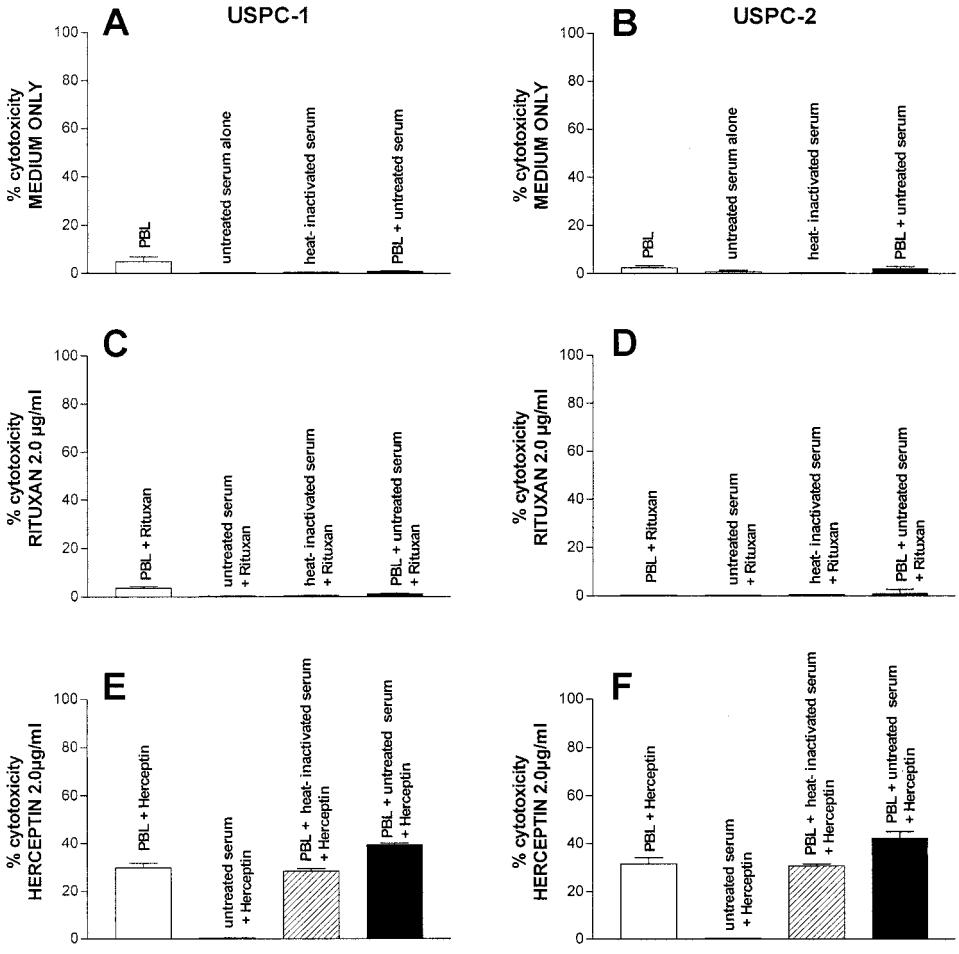

Fig. 5 Effect of complement and serum immunoglobulin (dilution 1:2) on cytotoxicity mediated by Herceptin (2 μg/ml) against ^{51}Cr-labeled USPC-1 (*A*, *C*, and *E*) and USPC-2 (*B*, *D*, and *F*) cells (10,000 cells/sample), measured in the presence or absence of effector PBLs from a representative heterologous healthy donor in a 5-h assay. The percentage of target cell lysis is shown at E:T ratios of 25:1; *bars*, SD. Effector PBLs with medium alone or with Rituxan (2 μg/ml) plus or minus serum were used as controls. Herceptin-mediated ADCC in the presence of heat-inactivated human serum and effector PBLs was not significantly different from the results obtained in the absence of serum. Herceptin-mediated ADCC in the presence of untreated human serum and effector PBLs were significantly increased compared with the results obtained in the absence of serum ($P < 0.03$).

complement proteins in the absence of effector cells. Addition of physiological concentrations of IgG (*i.e.*, heat-inactivated serum diluted 1:2 to 1:4) to PBLs in the presence of Herceptin did not significantly alter the degree of ADCC achieved in the presence of Herceptin (Fig. 5). In contrast, addition of untreated serum (diluted 1:2 to 1:4) to PBLs in the presence of Herceptin consistently increased Herceptin-mediated cytotoxicity against USPCs ($P < 0.03$; Fig. 5).

IL-2 Enhancement of ADCC against USPC. To investigate the effect of low doses of IL-2 in combination with Herceptin (2 μg/ml) on ADCC against USPC cell lines, PBLs from healthy donors were incubated for 5–72 h in the presence of 50–100 IU/ml of IL-2. As representatively shown in Fig. 6, Herceptin-mediated ADCC was significantly increased in the presence of low doses of IL-2. Administration of 100 IU/ml of IL-2 to the effector PBLs at the start of the assay increased the cytotoxic activity against USPC cell lines compared with the use of Herceptin alone, whereas no significant increase in cytotoxicity was detected after 5-h IL-2 treatment in the absence of Herceptin or in the presence of Rituxan control MAb (Fig. 6). Longer periods of preincubation (72 h) of effector PBLs with IL-2 showed a similar increase in ADCC in the presence of Herceptin. However, a small but significant increase in cytotoxicity was also detectable in the absence of Herceptin and in presence of Rituxan against the USPC cell line tested, possibly related to lymphokine-activated killer activity ($P < 0.05$; Fig. 6).

Growth of HER-2/neu-positive USPC Can Be Inhibited by Herceptin *in Vitro*. Experiments were performed to investigate whether the proliferation of two different HER-2/neu-positive USPC cell lines (USPC-1 and USPC-2) can be inhibited by Herceptin, as compared with Rituxan, which was used as a control. Data presented in Table 2 show this to be the case. The proliferation of both cell lines was significantly inhibited in the presence of Herceptin, with the percentage of inhibition varying from 30 to 62% for USPC-1 and from 22 to 52% for USPC-2 ($P > 0.05$; Table 2).

DISCUSSION

In the last few years, several clinical studies have shown that HER-2/*neu* gene amplification and/or protein overexpression represents the prototype of a stable molecular abnormality endowed with well-characterized functional consequences that are detectable in several of the most common human solid tumors including breast, ovarian, colon, non-small cell lung cancer, and prostate and cervical cancer (reviewed in Refs. 8, 11, and 19). In the clinical setting, high levels of HER-2/neu in

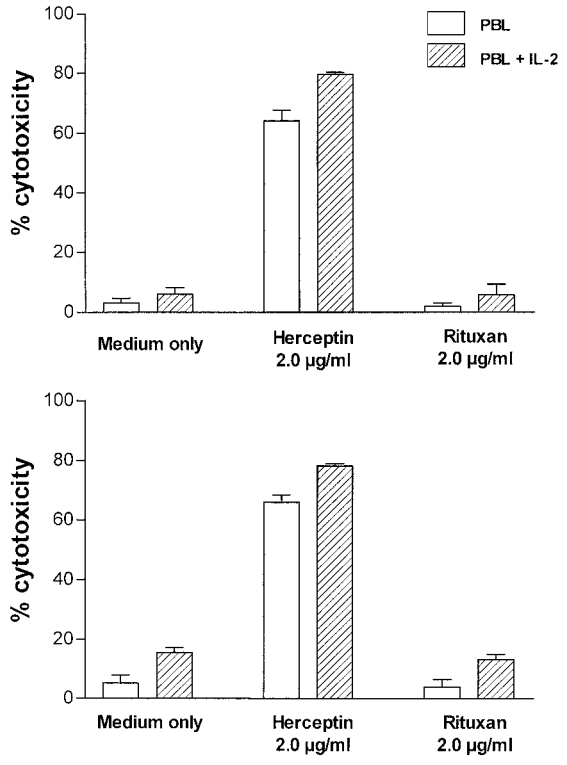

Fig. 6 Enhancement of ADCC mediated by Herceptin (2 μg/ml) against ^{51}Cr-labeled USPC-2 cells (10,000 cells/sample) in the presence of 100 IU/ml of IL-2 for 5 h (*upper panel*) or after preincubation of effector PBLs with 100 IU/ml of IL-2 for 72 h (*lower panel*) as measured in a 5-h assay. The percentage of target cell lysis is shown at E:T ratios of 50:1; *bars*, SD. Effector PBLs with medium only or in the presence of Rituxan (2 μg/ml) were used as controls. Herceptin-mediated ADCC was significantly enhanced ($P < 0.01$, Student's *t* test). A small but significant increase in cytotoxic activity was seen at 72 h of IL-2 exposure in the absence of Herceptin and in the presence of Rituxan ($P < 0.05$). Similar results were obtained after incubation of effector PBLs with 50 IU/ml of IL-2.

tumor tissue have been associated with shorter patient survival (13–15), resistance to antiestrogens (27) and chemotherapeutic drugs (13–15), and resistance to tumor necrosis factor-α, activated macrophages, and lymphokine-activated killer cells (28).

In this study, we report for the first time that USPC, a histological variant of endometrial cancer characterized by an early intraabdominal and lymphatic spread, an extreme inborn resistance to radiotherapy, chemotherapy, and hormonal therapy, and a highly aggressive biological behavior (2–7), commonly overexpresses HER-2/neu. Eight of 10 (80%) of the USPCs tested in our series by immunohistology on paraffin-embedded tissue stained moderately (score 2+) or strongly (score 3+) positive for HER-2/neu surface expression. Patients from whom the primary USPC cell lines described in this study were established experienced rapid disease progression during chemotherapy (regimen including Taxol, carboplatin, and doxorubicin) and radiation treatment (data not shown). These findings are therefore consistent with the published evidence regarding the extremely aggressive biological behavior of this subset of uterine tumors (2–7) and further support the notion that

Table 2 Inhibition of [^{3}H]thymidine uptake by USPC cells grown in the presence of Herceptin[a]

Experiment no.	Target cells	Herceptin dose (μg/ml)	% inhibition
1	USPC-1	1	58% ($P < 0.01$)
		2	61% ($P < 0.01$)
		5	62% ($P < 0.01$)
2	USPC-2	1	41% ($P < 0.01$)
		5	52% ($P < 0.01$)
3	USPC-1	1	30% ($P < 0.03$)
		5	34% ($P < 0.03$)
4	USPC-2	1	22% ($P < 0.05$)
		2	26% ($P < 0.05$)

[a] There were six to eight replicates/group. Rituxan was used as control MAb and gave no inhibition when compared with culture medium alone.

HER-2/neu overexpression may be a major prognostic factor in endometrial cancer (29, 30).

All primary USPC cell lines established in our laboratory and tested in this study were derived from specimens that stained strongly positive for HER-2/neu by immunohistochemistry. In agreement with these results, flow cytometric analysis of all USPC cell lines demonstrated a striking overexpression of HER-2/neu receptor that was significantly higher than several positive control primary and well-established ovarian cancer cell lines ($P < 0.001$). More surprisingly, however, two breast cancer cell lines (BT-474 and SK-BR-3) known to highly overexpress HER-2/neu and commonly used in many laboratories as positive controls for HER-2/neu receptor expression (21, 24) were found to have significantly lower levels of HER-2/neu receptor when compared with primary USPC cell lines ($P < 0.001$). These data further suggest a correlation between the extremely aggressive biological behavior of USPC, their common resistance to standard cytotoxic treatments *in vivo*, and their remarkable overexpression of the HER-2/neu receptor. Nevertheless, this constitutive and striking expression of HER-2/neu in USPC might turn out to be a useful adjunctive tool to help to differentiate primary USPCs with spread to the ovaries and/or abdominal cavity, which is often the case in the clinic, with the similar but histologically indistinguishable serous papillary ovarian tumors. This differential diagnosis might have important clinical and therapeutic implications.

Primary USPCs studied were found to be highly resistant to killing by NK cells and partially resistant to lymphokine-activated killer activity (*i.e.*, PBLs cultured for up to 72 h in 100 IU/ml of IL-2). Therefore, to our knowledge, these data showed for the first time that in addition to their high resistance to chemotherapy, radiation treatment, and hormonal therapy (2–7), USPC cells are also intrinsically highly resistant to NK activity. Furthermore, complement-mediated tumor cell lysis (in the absence of effector cells) was not observed, which may be attributable to the presence of membrane-associated complement regulatory proteins such as CD35 (complement receptor 1), CD55 (decay accelerating factor), or CD46 (membrane cofactor protein) on USPCs, as reported previously for other human tumors resistant to complement-dependent cytotoxicity (31). In strong contrast, however, all primary USPC cell lines tested were found to be highly susceptible to ADCC when incubated

with heterologous or autologous effector cells in the presence of Herceptin. These data, therefore, demonstrate that although these tumor cells are *per se* extremely resistant to any standard cytotoxic therapy in the clinic, they remain highly sensitive to the killing activity mediated by NK cells when triggered by HER-2/neu-specific antibody.

In vivo, ADCC applications are known to be dependent upon the availability of the effector cells to interact with the antibody at the target site in the presence of high concentrations of irrelevant human IgG. In this study, we show that ADCC against USPC was not significantly inhibited by high concentrations (up to 50%) of human serum. In fact, a consistent increase in cytotoxicity was detected in the presence of effector cells and non-heat-inactivated human serum. These data, therefore, suggest that in the presence of effector PBLs, human serum may augment Herceptin-mediated cytotoxicity against USPC. Moreover, these results indicate that the binding of Herceptin to the Fc receptor on mononuclear effector cells is of very high affinity and is likely to occur in the *in vivo* situation.

Treatment of cancer patients with combinations of MAbs and cytokines does not amount to a mere addition to the benefit of each treatment modality alone but has clearly been demonstrated to have synergistic potential (32, 33). Recently, low doses of recombinant IL-2 have been given by continuous infusion or s.c., with remarkable immunological results coupled with negligible toxicity (34, 35). This point is noteworthy because, both in experimental models and in human beings, modulation of both the number and function of NK cells has been associated previously with tumor progression (25, 26), and in addition, substantially suppressed ADCC responses have been reported in several cancer patients (36). Importantly, however, cytotoxicity levels in patients who demonstrate suppressed ADCC can be increased *in vitro* to levels similar to those of normal donors by prior exposure of effector cells to IL-2 (37). Consistent with this view, a significant increase in ADCC against USPC was detected after exposure of effector cells from healthy donors as well as one USPC patient (data not shown) to low doses of IL-2 *in vitro* for a brief time (*i.e.*, 5 h). Longer time periods of incubation (up to 3 days) with IL-2 under the same conditions showed similar results. These data, therefore, suggest that the administration of low (*i.e.*, nontoxic) doses of IL-2 *in vivo*, giving rise to a lytic effector cell that is markedly enhanced in its function by the addition of an antibody bridge, may significantly increase the efficacy of Herceptin therapy in USPC patients. Furthermore, on the basis of the high resistance of USPC to standard cytotoxic anticancer therapy, these combined therapies might be particularly important in the treatment of USPC patients.

Although the majority of previous reports investigating the antitumor effects of MAbs support the view that efficacy is primarily dependent on immune activation through the Fc receptor (38), others have shown that Herceptin retains ~40% of its antitumor activity in FcγRIII−/− mice compared with wild-type mice, indicating that some biological effects of MAbs can be independent of Fc receptor binding (39). Consistent with these data, in this study we were able to detect a significant inhibition in the proliferation of USPC cell lines by anti-HER-2/neu MAbs. These results demonstrated that USPCs behave similarly to ovarian cancer cell lines overexpressing HER-2/neu (16).

In conclusion, we show that HER-2/neu is highly expressed by USPCs, and we further demonstrate that USPC cells are exquisitely sensitive to Herceptin-mediated ADCC. On the basis of these findings and previous evidence showing a correlation between efficacy of Herceptin therapy in direct proportion to the HER-2/neu overexpression on tumor cells, we postulate that Herceptin might be a novel and attractive therapeutic strategy in USPC patients either for the prevention of recurrence after surgical treatment or for the treatment of metastatic disease. The future design and implementation of clinical trials in this regard will ultimately determine the validity of this approach.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics 1999. CA Cancer J. Clin., *49:* 8–31, 1999.

2. Sherman, M. E., Bitterman, P., Rosenshein, N. B., Delgado, G., and Kurman, R. J. Uterine serous carcinoma. A morphologically diverse neoplasm with unifying clinicopathological features. Am. J. Surg. Pathol., *16:* 600–610, 1992.

3. Carcangiu, M. L., and Chambers, J. T. Uterine papillary serous carcinoma: a study of 108 cases with emphasis on prognostic significance of associated endometrioid carcinoma, absence of invasion, and concomitant ovarian cancer. Gynecol. Oncol., *47:* 298–305, 1992.

4. Goff, B. A., Kato, D., Schmidt, R. A., Ek, M., Ferry, J. A., Muntz, H. G., Cain, J. M., Tamimi, H. K., Figge, D. C., and Greer, B. E. Uterine papillary serous carcinoma: pattern of metastatic spread. Gynecol. Oncol., *54:* 264–268, 1994.

5. Carcangiu, M. L., and Chambers, J. T. Early pathologic stage clear cell carcinoma and uterine papillary serous carcinoma of the endometrium, comparison of clinicopathological features and survival. Int. J. Gynecol. Pathol., *14:* 30–38, 1995.

6. Levenback, C., Burke, T. W., Silva, E., Morris, M., Gershenson, D. M. Kavanagh, J. J., and Wharton, J. T. Uterine papillary serous carcinoma (USPC) treated with cisplatin, doxorubicin, and cyclophosphamide (PAC). Gynecol. Oncol., *46:* 317–321, 1992.

7. Nicklin, J. L., and Copeland, L. J. Endometrial papillary serous carcinoma: pattern of spread and treatment. Clin. Obstet. Gynecol., *39:* 686–695, 1996.

8. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. Science (Wash. DC), *244:* 707–712, 1980.

9. Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Hynes, N. E., and Yarden, Y. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J., *15:* 254–264, 1996.

10. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. ErbB-2, the preferred heterodimerization partner of all erbB receptors, is a mediator of lateral signaling. EMBO J., *16:* 1647–1655, 1997.

11. Padhy, L. C., Shih, C., Cowing, D., Finkelstein, R., and Weinberg, R. A. Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas. Cell, *28:* 865–871, 1982.

12. Tal, M., Wetzler, M., Josefberg, Z., Deutch, A., Assaf, D., Kris, R., Shiloh, Y., Givol, D., and Schlessinger, J. Sporadic amplification of the *HER2/neu* protooncogene in adenocarcinomas of various tissues. Cancer Res., *48:* 1517–1520, 1988.

13. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of *HER-2/neu* oncogene. Science (Wash. DC), *235:* 177–182, 1987.

14. Wright, C., Angus, B., Nicholson, S., Sainsbury, R. C., Cairns, J., Gullick, W. C., Kelly, P., Harris, A. L., and Wilson Horne, C. H.

Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. Cancer Res., *49:* 2087–2090, 1989.

15. Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J. T., Dodge, R., Clarke-Pearson, D. L., Marks, P., McKenzie, S., Yin, S., and Bast, R. C. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res., *50:* 4087–4091, 1990.

16. Hellstrom, I., Goodman, G., Pullman, J., and Hellstrom, K. E. Overexpression of HER-2 in ovarian carcinomas. Cancer Res., *61:* 2420–2423, 2001.

17. Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Triphaty, D., Baly, D., Baughman, S. A., Twaddell, T., Glaspy, J. A., and Slamon, D. J. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized antip185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu overexpressing metastatic breast cancer refractory to chemotherapy treatment. J. Clin. Oncol., *16:* 2659–2671, 1998.

18. Slamon, D. L., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Pharm, D., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses Her2. N. Engl. J. Med., *344:* 783–792, 2001.

19. Agus, D. B., Bunn, P. A., Jr., Franklin, W., Garzia, M., and Ozols, R. F. HER-2/neu as therapeutic target in non-small cell lung cancer, prostate cancer, and ovarian cancer. Semin. Oncol., *27:* 53–63, 2000.

20. Santin, A. D., Rose, G. S., Hiserodt, J. C., Fruehauf, J., Eck, L. M., Garcia, R. I., Schranz, V., DiSaia, P. J., Pecorelli, S., and Granger, G. A. Effects of tumor necrosis factor-α plus interferon-γ combined with high dose γ irradiation on the expression of major histocompatibility complex molecules and intercellular adhesion molecule-1 in human ovarian cancers. Int. J. Cancer, *65:* 688–694, 1996.

21. Yip, Y. L., Smith, G., Koch, J., Dubel, S., and Ward, R. L. Identification of epitope regions recognized by tumor inhibitory and stimulatory anti-Erb-2 monoclonal antibodies: implications for vaccine design. J. Immunol., *166:* 5271–5278, 2001.

22. Santin, A. D., Hermonat, P. L., Ravaggi, A., Chiriva-Internati, M., Zhan, D., Pecorelli, S., Parham, G. P., and Cannon, M. Induction of human papillomavirus-specific CD4+ and CD8+ lymphocytes by E7 pulsed autologous dendritic cells in patients with HPV16 and 18 positive cervical cancer. J. Virol., *73:* 5402–5410, 1999.

23. Nazaruk, R. A., Rochford, R., Hobbs, M. V., and Cannon, M. J. Functional diversity of the CD8+ T cell response to Epstein-Barr virus (EBV): implications for the pathogenesis of EBV-associated lymphoproliferative disorders. Blood, *91:* 3875–3883, 1998.

24. Mass, R. The role of HER-2 expression in predicting response to therapy in breast cancer. Semin. Oncol., *27:* 46–52, 2000.

25. Introna, M., and Mantovani, A. Natural killer cells in human solid tumors. Cancer Metastasis Rev., *2:* 337–340, 1983.

26. Choe, B., Frost, P., Morrison, N., and Rose, N. Natural killer cell activity of prostatic cancer patients. Cancer Investig., *5:* 285–288, 1987.

27. Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. Epidermal growth factor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. J. Steroids Biochem. Mol. Biol., *37:* 811–814, 1990.

28. Ioannides, C. G., Ioannides, M. G., and O'Brian, C. A. T-cell recognition of oncogene products: a new strategy for immunotherapy. Mol. Carcinog., *6:* 77–82, 1992.

29. Berchuck, A., Rodriguez, G., Kinney, R. B., Soper, J. T., Dodge, R. K., Clarke-Pearson, D. L., and Bast, R. C., Jr. Overexpression of HER-2/neu in endometrial cancer is associated with advanced stage disease. Am. J. Obstet. Gynecol., *164:* 15–21, 1991.

30. Hetzel, D. J., Wilson, T. O., Keeney, G. L., Roche, P. C., Cha, S. S., and Podratz, K. C. HER-2/neu expression: a major prognostic factor in endometrial cancer. Gynecol. Oncol., *47:* 179–185, 1992.

31. Devine, D. The regulation of complement on cell surfaces. Trans. Med. Rev., *5:* 123–131, 1991.

32. Caron, P. C., Lai, L. T., and Scheinberg, D. A. Interleukin-2 enhancement of cytotoxicity by humanized monoclonal antibody M195 (anti-CD33) in myelogenous leukemia. Clin. Cancer Res., *1:* 63–70, 1995.

33. Hooijberg, E., Sein, J. J., van der Berk, P. C. M., Hart, A. A. M., van der Valk, M. A., Kast, W. M., Melief, C. J. M., and Hekman, A. Eradication of large human B cell tumors in nude mice with unconjugated CD20 monoclonal antibodies and interleukin 2. Cancer Res., *55:* 2627–2634, 1995.

34. Stein, R. C., Malkovska, V., Morgan, S., Galaazka, A., Aniszewski, C., Roy, S. E., Shearer, R. J., Marsden, R. A., Bevan, D., Gordon-Smith, E. C., and Coombes, R. C. The clinical effects of prolonged treatment of patients with advanced cancer with low-dose subcutaneous interleukin 2. Br. J. Cancer, *63:* 275–282, 1991.

35. Caligiuri, M. A., Murray, C., Robertson, M. J., Wang, E., Cochran, K., Cameron, C., Schow, P., Ross, M. E., Klumpp, T. R., Soiffer, R. J., Smith, K. A., and Ritz, J. Selective modulation of human natural killer cells *in vivo* following prolonged infusion of low-dose recombinant interleukin 2. J. Clin. Investig., *91:* 123–128, 1993.

36. Ortaldo, J. R., Woodhouse, C. S., Morgan, A. C., Jr., Herberman, R. B., Cheresh, D. A., and Reisfeld, R. A. Analysis of effector cells in human antibody-dependent cellular cytotoxicity with murine monoclonal antibodies. J. Immunol., *138:* 3566–3572, 1987.

37. Honsik, C. J., Jung, G., and Reisfeld, R. A. Lymphokine-activated killer cells targeted by monoclonal antibodies to the disialogangliosides GD2 and GD3 specifically lyse human tumor cells of neuroectodermal origin. Proc. Natl. Acad. Sci. USA, *83:* 7893–7897, 1986.

38. Clynes, R., Takechi, Y., Moroi, Y., Houghton, A., and Ravetch, J. V. Fc receptors are required in passive and active immunity to melanoma. Proc. Natl. Acad. Sci. USA, *95:* 652–656, 1998.

39. Clynes, R., Towers, T. L., Persta, L. G., and Ravetch, J. V. Fc receptors modulate *in vivo* cytotoxicity against tumor targets. Nat. Med., *6:* 443–446, 2000.