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HER-2/neu-Mediated Regulation of Components of the MHC Class I Antigen-Processing Pathway

Felix Herrmann,[1] Hans-Anton Lehr,[2] Ingo Drexler,[4] Gerd Sutter,[4,5] Jan Hengstler,[3] Ursula Wollscheid,[1] and Barbara Seliger[1]

Third Department of [1]Internal Medicine and [2]Pathology, Johannes Gutenberg-University, Mainz; [3]Institute of Toxicology, University of Mainz, Mainz; and [4]Gesellschaft für Umwelt und Gesundheit GmbH-Institute for Molecular Virology and [5]Institute of Virology, Technical University of Munich, Munich, Germany

ABSTRACT

Because of its amplification and/or overexpression in many human tumors, the *HER-2/neu* proto-oncogene represents an attractive target for T-cell-mediated vaccination strategies. However, overexpression of oncogenes is often associated with defective expression of components of the MHC class I antigen-processing machinery (APM), thereby resulting in an immune escape phenotype of oncogene-transformed cells. To determine whether HER-2/neu influences the MHC class I antigen-processing pathway, the expression pattern of different APM components was examined in murine *in vitro* models of constitutive and tetracycline-controlled HER-2/neu expression. In comparison with HER-2/neu$^-$ control cells, HER-2/neu$^+$ fibroblasts exhibit reduced levels of MHC class I surface antigens that were associated with impaired expression and/or function of the peptide transporter associated with antigen processing, the proteasome subunits low molecular weight protein 2 and low molecular weight protein 10, the proteasome activators PA28α and PA28β, and tapasin. These APM abnormalities resulted in reduced sensitivity to lysis by CTLs. The HER-2/neu-mediated immune escape phenotype could be corrected by IFN-γ treatment. The clinical relevance of this finding was supported by an inverse correlation between HER-2/neu and the peptide transporter associated with antigen-processing protein expression as determined by immunhistochemical analysis of a series of HER-2/neu$^-$ and HER-2/neu$^+$ breast cancer specimens. Thus, a functional link between deficient APM component expression and HER-2/neu overexpression is proposed that might influence the design of HER-2/neu-targeted T-cell-based immunotherapeutic strategies.

INTRODUCTION

Abnormalities in the MHC class I surface expression of tumors are often associated with reduced sensitivity to lysis by tumor-reactive CTLs (1–3). In addition, *in vitro* models of oncogenic transformation demonstrate an inverse correlation between MHC class I surface expression and malignant transformation induced by various oncogenes, including *myc*, *mos*, and *ras*. This is accompanied by reduced expression and function of genes involved in antigen processing, instability of MHC class I surface antigens, and impaired T-cell-based immune recognition (4–6). However IFN-γ treatment concomitantly corrects the expression of specific components of the MHC class I antigen-processing machinery (APM), such as the IFN-γ-inducible immunoproteasome subunits, the peptide transporter, and the chaperone tapasin, as well as MHC class I antigens (7, 8).

Because a reduced MHC class I surface expression of tumors is often associated with disease progression, it may be speculated that oncogene-mediated down-regulation of APM components is associated with enhanced tumor growth *in vivo*. Indeed, it has recently been reported in an *in vitro* model of ras transformation that ras overexpression causes loss of the transporter associated with antigen processing (TAP) expression and function (9), which is accompanied by immune escape of these ras transformants *in vivo*. This could be reverted by *TAP* gene transfer resulting in rejection of ras-induced tumors. Thus, impaired APM component expression directly affected tumor growth.

As demonstrated in a number of model systems, the different ras proteins act as signal transducers of receptor tyrosine kinases in the Ras-mitogen-activated protein kinase pathway (10–13). The activation of this pathway plays a key role in human cancers (14). This has been demonstrated for the proto-oncogene *HER-2/neu*, also known as *c-erbB2*. *HER-2/neu* encodes for a M_r 185,000 transmembrane glycoprotein with tyrosine kinase-specific activity and has extensive homology in structure and sequence to the epidermal growth factor receptor (15). HER-2/neu is expressed in a variety of normal tissues (16) but amplified and/or overexpressed in 20–30% of breast, ovarian, and renal cell carcinomas (17). Its up-regulated expression has been implicated in carcinogenesis and is often associated with rapid disease progression, chemoresistance, and poor prognosis of patients (18). Because of its oncogenic capacity, HER-2/neu has been used as a target for antitumor therapy, including treatment with the humanized monoclonal antibody (mAb) Herceptin (19–21) and T-cell-based immunotherapies in both animal models and humans (20, 22). However, a collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for complete eradication of HER-2/neu$^+$ tumors (23, 24).

Recently, Lollini *et al.* (25) demonstrated MHC class I down-regulation in rat c-neu transgenic mice, suggesting that activated c-neu may affect the expression and function of APM components. However, little information is available concerning the expression pattern of these components in HER-2/neu-overexpressing cells. In this study, *in vitro* models of HER-2/neu transformation were used (*a*) to compare the expression pattern of proteasome subunits, TAP, tapasin, and MHC class I antigens in HER-2/neu$^-$ and HER-2/neu$^+$ cells; (*b*) to analyze the effects of IFN-γ on APM component expression and function in these cells; and (*c*) to determine the role of APM component down-regulation for the immune escape. In addition, HER-2/neu$^-$ and HER-2/neu$^+$ breast carcinomas were analyzed for TAP protein expression to evaluate the clinical relevance of the data obtained in the *in vitro* system. The results obtained contribute to our understanding of the pathomechanisms of APM deficiencies and the immunobiology of HER-2/neu. They may also have consequences for the development of immunotherapeutic strategies for the treatment of patients with HER-2/neu-overexpressing tumors.

MATERIALS AND METHODS

Cell Lines and IFN-γ Treatment. The parental murine HER-/neu$^-$ fibroblast cell line NIH3T3 was obtained from the American Type Culture Collection. NIH3T3 cells constitutively overexpressing HER-2/neu [cytomegalovirus (CMV)-HER-2/neu$^+$] were kindly provided by H. Bernhard (University Hospital of the Technical University, Munich, Germany). Conditional HER-2

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Requests for reprints: Barbara Seliger, III. Department of Internal Medicine, Johannes Gutenberg University, Langenbeckstrasse 1, 55131 Mainz, Germany. Phone: 0049-6131-176760; Fax: 0049-6131-176678; E-mail: B.Seliger@3-med.klinik.uni-mainz.de.

expression was achieved using the TET-OFF system originally established by Gossen and Bujard (26). Briefly, NIH3T3 cells were cotransfected with three vectors (pUHD15-1, pTBC1 Hygro, and pTBC HER2/SEAP) as described by Baasner *et al.* (27), resulting in a cell line termed tetracycline-HER-2/neu$^+$ (tet-HER-2/neu$^+$). These cells exhibit a tetracycline-controlled HER-2/neu expression, and the addition of anhydrotetracycline hydrochloride led to a complete HER-2/neu down-regulation (tet-HER-2/neu$^-$; Ref. 27). Selection for stable transfections and their expression was achieved in the presence of 125 μg/ml hygromycin B (Sigma-Aldrich, Schnelldorf, Germany). All experiments including exposure to anhydrotetracycline hydrochloride were done in the absence of hygromycin B.

Wild-type NIH3T3 and the HER-2/neu-transfected cells were routinely maintained in DMEM (PAN Systems, Aidenbach, Germany) supplemented with 10% (v/v) FCS or fetal bovine serum (tetracycline tested; Clontech, Palo Alto, CA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2 humidified air. For IFN-γ stimulation, cells were incubated in the presence of 100 units/ml murine recombinant IFN-γ (Roche Diagnostics, Mannheim, Germany) for 24 h at 37°C.

Flow Cytometry. The mAbs used in this study were the anti-H-2L^{d}L^q, anti-H-2K^q, anti-H2D^q (Cedarlane Laboratories Ltd., Hornby, Canada), anti-HLA-A2.1 (28), anti-HER-2/neu (DAKO, Hamburg, Germany), and, as secondary antibody, FITC-labeled goat antimouse immunoglobulin (FITC-GAM; Beckman/Coulter, Krefeld, Germany). For inhibition of HER-2/neu expression, tet-HER-2/neu$^+$ cells were incubated with 10 mg/ml anhydrotetracycline hydrochloride (Acros Chimica) for 0, 1, 3, and 7 days before flow cytometry was performed. For all four incubation periods, cells were cultured for 8 days after plating and harvested simultaneously.

Indirect immunofluorescence analysis of MHC class I antigens and HER-2/neu was performed as described previously (5) using a flow cytometer (Coulter Epics XL MCL; Beckman/Coulter).

Reverse Transcription-PCR (RT-PCR) Analysis. Total cellular RNA was isolated using standard methods. The primers and the conditions used for one-step RT-PCR analysis have been recently described in detail (29).

Western Blot Analysis. For Western blot analysis, 30 μg protein/lane obtained from total protein extracts were size fractionated by 10% or 15% SDS-PAGE. Proteins were visualized by Ponceau S staining, transferred onto nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) as described previously (30), and subsequently identified using the anti-TAP1, anti-TAP2, anti-low molecular weight protein (LMP) 2, and anti-LMP7 mAbs [all kindly provided by K. Früh (Howard Johnsson, La Jolla, CA)] as well as the anti-LMP10, anti-PA28α, and anti-PA28β mAbs [purchased from Affinity Research Products (Exeter, United Kingdom)]. Western blots were developed with peroxidase-coupled goat antirabbit immunoglobulin (DAKO) and enhanced chemiluminescence (Pharmacia, Freiburg, Germany).

For the detection of tapasin protein expression, membrane-associated proteins were separated from cytosolic proteins as described by Bordier *et al.* (30) and then subjected to Western blot analysis as described above. The antitapasin mAb was kindly provided by Dr. B. Ortmann.[6]

Peptide Translocation Assay. Peptide translocation assay was performed with 2.5×10^6 cells/reaction using the radio-iodinated model peptides RYWANATRSI and TNKTRIDGQY, respectively, which were kindly provided by F. Momburg (German Cancer Research Center, Deutsches Krebsforschungszentrum, Heidelberg, Germany) as described previously (31).

Cytotoxicity Assay of HLA-A2-Expressing Cells. Parental HER-2/neu$^-$ NIH3T3 cells and the CMV-HER-2/neu$^+$ transformants were stably transfected with the HLA-A*0201 expression vector as described recently (32). Stable neomycin-resistant HLA-A2-expressing transfectants were selected in a culture medium supplemented with 500 μg/ml G418. Three independent HLA-A2$^+$ HER-2/neu$^-$ and HLA-A2$^+$ CMV-HER-2/neu$^+$ clones expressing comparable amounts of HLA-A2 mRNA but distinct levels of HLA-A2 surface antigens were used as targets for HLA-A2-restricted tyrosinase-specific CTLs using the standard chromium release assay. Cells were infected with 10 units/cell vaccinia tyrosinase virus and incubated for 3 h at 37°C before targets were labeled with ^{51}Cr. Twelve h after infection, cytotoxicity assays were performed as described by Drexler *et al.* (33). ^{51}Cr-labeled targets were mixed with different concentrations of effectors (E:T ratio) in triplicates and incubated for 4 h at 37°C. Supernatants were then harvested, and radioactivity was counted in a gamma counter. The percentage of specific lysis was calculated as 100 $\times$ (experimental release $-$ spontaneous release)/(maximal release $-$ spontaneous release). Three independent experiments were performed using three HLA-A2$^+$ HER-2/neu$^-$ and HLA-A2$^+$ CMV-HER-2/neu$^+$ cells.

Immunohistochemistry (IHC). Nineteen HER-2/neu$^-$ and 19 HER-2/neu$^+$ cases of invasive breast cancer cases were selected from the routine diagnostic service. For IHC, 4-μm-thick sections were cut from paraffin blocks of formalin-fixed tumor tissues onto positively charged slides, and sections were deparaffinized and rehydrated in graded alcohols. The slides were subjected to heat-induced epitope retrieval and then incubated with an anti-HER-2/neu polyclonal antibody (1:1,000 dilution; Dako Corp., Carpinteria, CA) for 30 min at room temperature. Negative controls were incubated with PBS instead of the primary antibody. Antibody was localized using peroxidase, counterstained with hematoxylin. The TAP1 and MHC class I studies were similarly performed using anti-TAP1-specific mAb 148.3 (34), anti-TAP2 mAb 429.3 (35), and anti-HC10 mAb recognizing the MHC class I heavy chain in a dilution of 1:8.

Interpretation of IHC was performed by one investigator (H-A. L.) in a blinded fashion. For HER-2/neu, the extent of membranous immunostaining intensity (in multiple representative $\times$20 fields/case) was assessed, and cases were termed positive if $>$15% of the slide showed clear cut, complete, membranous immunostaining, corresponding to a score of +++ on the currently applied DAKO scoring system. For each case, infiltrating carcinoma and adjacent nonneoplastic breast tissue were scored separately, and a final subtracted score of tumor minus nonneoplastic tissue was used to correct for variability in background staining of nonneoplastic HER-2/neu$^-$ tissue as approved by the United States Food and Drug Administration. The extent of TAP and MHC class I immunostaining was scored on a four-tier scoring system: 0, negative; 1, faint immunostaining intensity; 2, moderate immunostaining intensity; and 3, strong immunostaining intensity. The scoring was performed by the same pathologist who was blinded to the results of the HER-2/neu test.

RESULTS

HER-2/neu Overexpression Results in Down-Regulation of MHC Class I Surface Expression. An inverse correlation between overexpression of particular oncogenes, including the ras proteins, and MHC class I abnormalities has been demonstrated, a correlation that is attributable to APM component deficiencies (4, 6). Because ras proteins can activate a signal transduction pathway downstream of receptor tyrosine kinases, it can be speculated that HER-2/neu overexpression also modulates APM component expression and, consequently, MHC class I surface expression. To prove this hypothesis, murine *in vitro* models of fibroblasts expressing constitutive or tet-controlled HER-2/neu were analyzed by flow cytometry using anti-H-2 mAbs. As shown representatively in Fig. 1*A* for H-2L^q, HER-2/neu overexpression in both the constitutive and tet-regulated systems was associated with a significant down-regulation of H-2 surface expression. In addition, all H-2 loci were down-regulated in CMV-HER-2/neu$^+$ cells when compared with parental HER-2/neu$^-$ fibroblasts (Fig. 1*B*). Interestingly, this effect could be reversed by IFN-γ stimulation of CMV-HER-2/neu$^+$ cells, resulting in H-2 surface expression levels comparable with that of untreated wild-type NIH3T3 cells (Fig. 1, *B* and *C*), although IFN-γ treatment does not influence HER-2/neu expression. These data suggest that impaired H-2 surface antigen expression is not caused by genetic alterations occurring during the selection of stable transfectants. These data were further confirmed by the tet-controlled HER-2/neu system. Untreated tet-HER-2/neu$^+$ cells demonstrated impaired H-2 surface expression in the presence of high HER-2/neu surface levels (Fig. 1*A*). Forty-eight-h anhydrotetracycline hydrochloride treatment of tet-HER-2/neu$^+$ cells caused a 1.8-fold induction of H-2 surface expression, which

[6] B. Ortmann, personal communication.

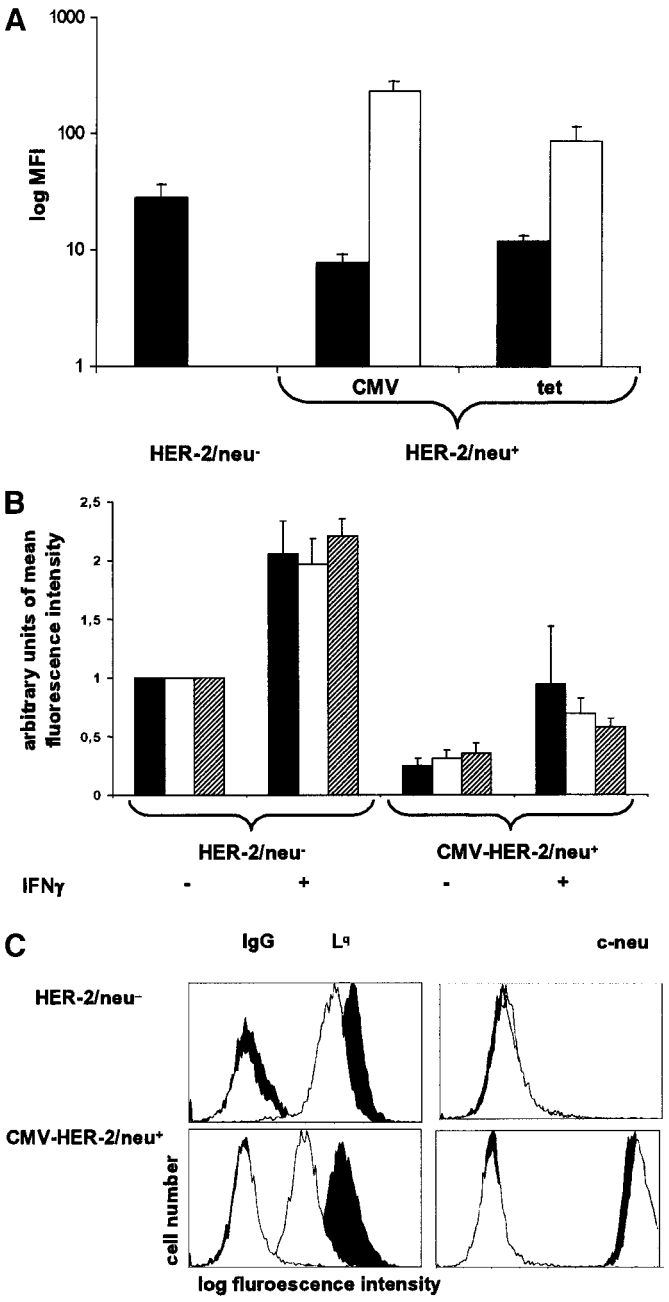

Fig. 1. MHC class I surface expression on HER-2/neu$^-$ and HER-2/neu$^+$ cells. The cells were grown in the presence or absence of 100 units/ml IFN-γ for 24 h before flow cytometry was performed as described in "Materials and Methods." *A*, down-regulation of the H-2 surface expression in CMV-HER-2/neu$^+$ and tet-regulated HER-2/neu$^+$ cells. The results are expressed as log mean specific fluorescence. *White bars* represent HER-2/neu expression, and *black bars* represent H-2 expression. *MFI*, mean fluorescence intensity. *B*, the down-regulation of MHC class I surface expression is represented by impaired expression of all three haplotypes (■, L^q; □, K^q; ▨, D^q; parental NIH3T3 set 1), which could be induced by IFN-γ. The results are expressed as mean specific fluorescence, which was set as 1 for HER-2/neu$^-$ cells. *C*, the flow histograms demonstrate a down-regulation of H-2L^q expression (*white histogram*) in CMV-HER-2/neu-expressing cells (*bottom left panel*) when compared with parental cells (*top left panel*), which could be up-regulated in both cell lines by IFN-γ treatment (*black histograms, left panels*). CMV-HER-2/neu transfectants demonstrate high HER-2/neu surface expression levels (*white histogram, bottom right panel*), and HER-2/neu expression was not altered after IFN-γ stimulation (*bottom right panel, black histogram*). The results are expressed as log fluorescence intensity and represent one of three independent experiments.

was accompanied by a significant down-regulation of HER-2/neu (tet-HER-2/neu$^-$; data not shown) However, a kinetics with extended anhydrotetracycline hydrochloride treatment combined with determination of H-2 surface antigen expression was not possible because of apoptotic signals caused by this treatment, which might negatively interfere with the MHC class I surface expression.

Down-Regulation of MHC Class I Surface Expression Is Associated with APM Deficiencies. The reduced levels of H-2 surface antigens in HER-2/neu$^+$ fibroblasts raised the possibility of impaired generation and/or processing of antigenic peptides attributable to deficiencies in various components of the MHC class I processing pathway. Therefore, the mRNA and protein expression pattern of MHC class I APM components were analyzed in CMV-HER-2/neu$^+$ fibroblasts by RT-PCR using β-actin as control and by Western blot analysis, respectively. Heterogeneous but significantly lower TAP1, TAP2, PA28, LMP2, LMP10, and tapasin mRNA and/or protein levels were detected in CMV-HER-2/neu$^+$ cells when compared with HER-2/neu$^-$ parental cells (Fig. 2, *A* and *B*). In contrast, the transcription of the constitutive proteasome subunits; the chaperones calnexin, calreticulin, and ER60; the protein disulfide isomerase; and the MHC class I heavy and light chain was not influenced by HER-2/neu transformation (Fig. 2*A*; data not shown). The unaltered transcription of MHC class I heavy chain molecules in CMV-HER-2/neu$^+$ cells when compared with HER-2/neu$^-$ fibroblasts suggests that the down-regulation of MHC class I surface expression in CMV-HER-2/neu$^+$ cells is posttranscriptionally controlled and may be attributable to a reduced generation or supply of antigenic peptides in the endoplasmic reticulum. Thus, impaired TAP expression and function might be crucial for efficient antigen processing. Indeed, the low TAP expression levels in CMV-HER-2/neu$^+$ cells were accompanied by impaired peptide transporter function as demonstrated in peptide translocation assays using streptolysin *O*-permeabilized HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells and two radioiodinated reporter peptides. Although the peptide transport efficiency is generally low in murine fibroblasts (5), HER-2/neu$^-$ NIH3T3 cells exhibit a statistically higher transport rate of both peptides when compared with CMV-HER-2/neu$^+$ cells (Wilcoxon test, $P < 0.05$; Fig. 3).

Impaired APM Expression Is Associated with Reduced Immune Response. To investigate whether the HER-2/neu-associated reduced APM component expression affects the T-cell response, vaccinia tyrosinase-infected HLA-A2$^+$ HER-2/neu$^-$ and HLA-A2$^+$ CMV-HER-2/neu$^+$ cells were used as targets in cytotoxicity assays. Before the chromium release experiments, both HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells were stably transfected with HLA-A2 cDNA. Subsequently, three independent HLA-A2$^+$ HER-2/neu$^-$ and HLA-A2$^+$ CMV-HER-2/neu$^+$ clones were analyzed for HLA-A2 surface expression. As expected, all three HLA-A2$^+$ HER-2/neu$^-$ clones expressed higher HLA-A2 surface levels than the HLA-A2$^+$ CMV-HER-2/neu$^+$ cells. IFN-γ treatment of the different transfectants enhanced HLA-A2 surface expression of both HER-2/neu$^-$ and CMV-HER-2/neu$^+$ clones, suggesting that the human HLA-A2 molecule could be loaded with peptides generated in the murine cell system. These transfectants were then used as targets in T-cell assays. Three independent experiments were performed demonstrating that HLA-A2$^+$ parental HER-2/neu$^-$ cells were specifically recognized by HLA-A2-restricted tyrosinase-specific CTLs, whereas the CTL-mediated lysis of HLA-A2$^+$ CMV-HER-2/neu$^+$ cells was strongly inhibited (Fig. 4). Although the overall lysis rates were low, the difference in lysis between HER-/neu$^-$ and CMV-HER-2/neu$^+$ clones is statistically significant (Wilcoxon test, $P < 0.05$). In addition, IFN-γ treatment, which enhances the expression of the major APM components but not the expression of the exogenously introduced HLA-A2, leads to enhanced lysis rates of CMV-HER-2/neu$^+$ clones (Fig. 4). Thus, reduced sensitivity to CTL recognition is attributable to an impaired peptide generation, processing, and pres-

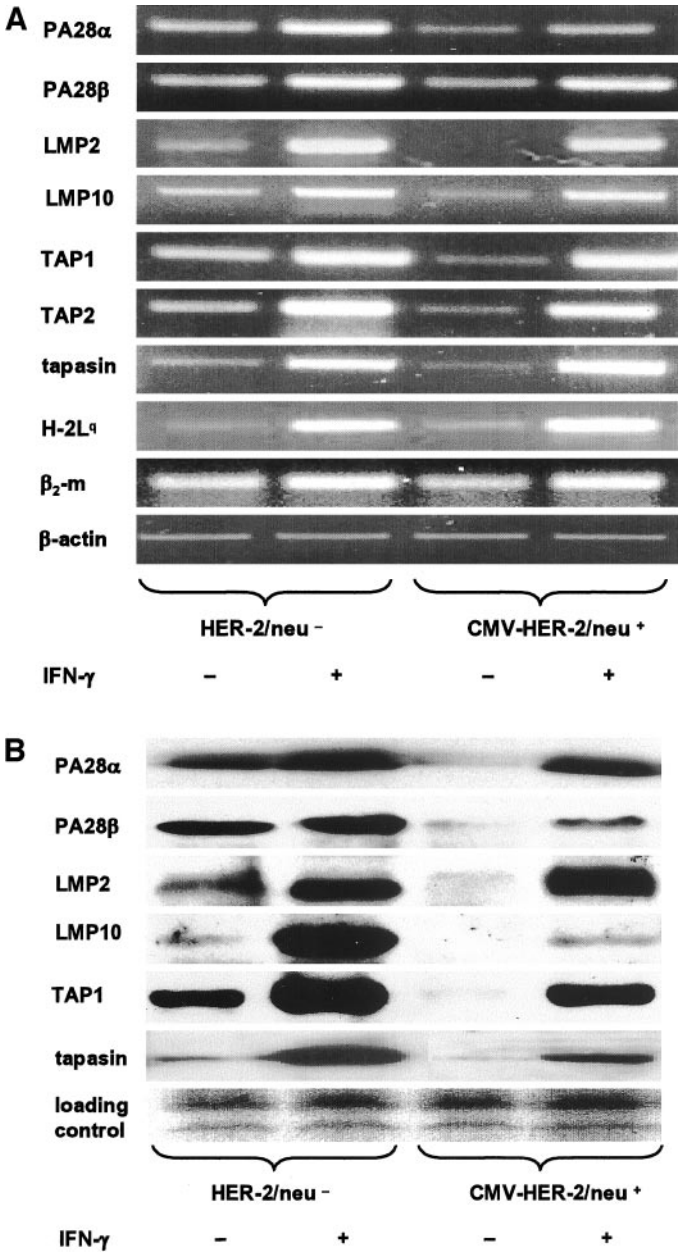

Fig. 2. Impaired mRNA and protein expression of MHC class I APM components in HER-2/neu-transformed cells. *A*, total RNA from HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells either left untreated or treated with 100 units/ml IFN-γ for 24 h was extracted and subjected to RT-PCR analysis as described previously. Amplification of the β-actin cDNA served as internal control. β_2-*m*, β_2-microglobulin. *B*, proteins from HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells either left untreated or treated with IFN-γ for 24 h were extracted, and 30 μg protein/lane were subjected to Western blot analysis using anti-APM-specific antibodies. Detailed information on the antibodies is given in "Materials and Methods."

entation that is in accordance with the impaired expression and function of MHC class I APM components, including TAP (Fig. 3).

IFN-γ Treatment Restores Impaired APM Component Expression in HER-2/neu$^+$ Cells. IFN-γ treatment of HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells resulted in a significant increase in the steady-state mRNA levels of PA28α and β, LMP2, LMP10, both TAP subunits, tapasin, and MHC class I molecules, which was more pronounced in CMV-HER-2/neu$^+$ cells than in HER-2/neu$^-$ fibroblasts (Fig. 2). In contrast, the expression of calnexin, calreticulin, ER60, protein disulfide isomerase, and HER-2/neu was not effected by this cytokine treatment. The IFN-γ-mediated induction of PA28, LMP, and TAP transcription was accompanied by increased levels of protein expression, enhanced peptide transport rate, up-regulation of MHC class I surface antigens, and increased CTL-mediated lysis (Figs. 1, 2*B*, 3, and 4).

Down-Regulation of APM Components Can Also Be Found *in Vivo* in HER-2/neu$^+$ Tumor Specimens. The results of the *in vitro* models of HER-2/neu transformation postulate that HER-2/neu overexpression in human cancers might also be associated with APM deficiencies. Therefore, HER-2/neu$^-$ and HER-2/neu$^+$ breast carci-

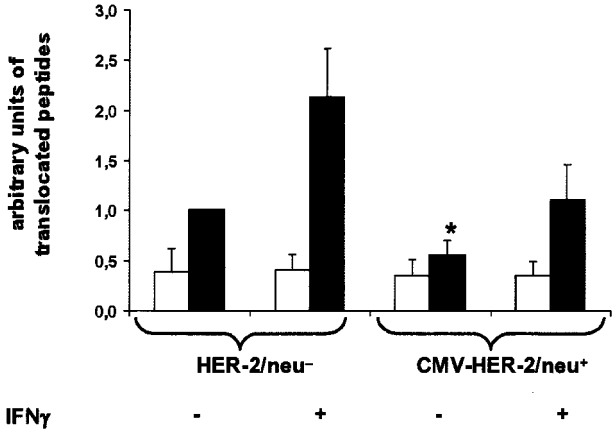

Fig. 3. TAP-dependent peptide transport of model peptides. The iodinated model peptides RYWANATRSI and TNKTRIDGQY, respectively, were translocated in the presence (■) and absence (□) of ATP using streptolysin *O*-permeabilized HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells. The translocated peptides were isolated and quantified as described previously, and results are expressed as a percentage of input peptides. The data represent the mean of four independent experiments, expressed as arbitrary units, and compared with peptide transport in untreated HER-2/neu$^-$ cells in the presence of ATP (set as 1).

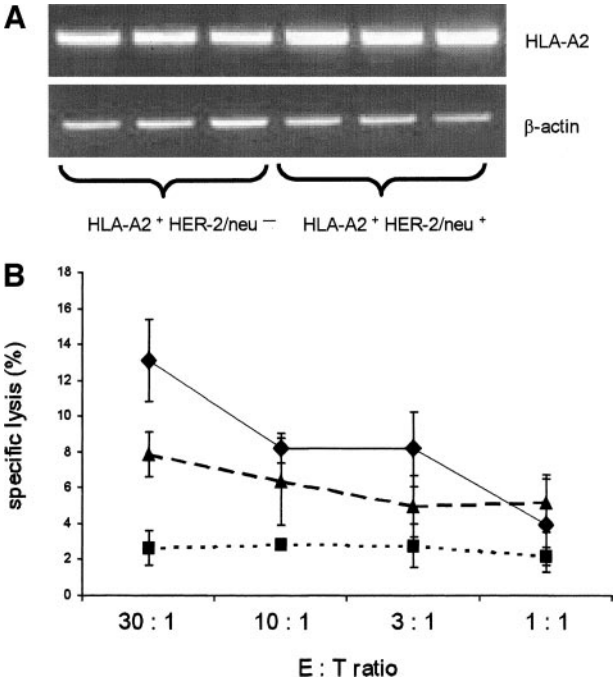

Fig. 4. Reduced CTL-mediated lysis in CMV-HER-2/neu cells. Three HLA-A2-transfected clones of HER-2/neu$^-$ and CMV-HER-2/neu$^+$ cells, respectively, were infected with 10 units/cell vaccinia tyrosinase virus and used in chromium release assays with HLA-A2-specific CTLs as described in "Materials and Methods." *A*, RT-PCR analysis of β-actin and HLA-A2 confirmed equal expression of HLA-A2 in all clones. *B*, specific lysis of a HLA-A2 $^+$ HER-2/neu$^-$ (◆), a representative untreated HLA-A2$^+$ CMV-HER-2/neu$^+$ (■), and an IFN-γ-treated HLA-A2$^+$ CMV-HER-2/neu$^+$ (▲) clone are shown using E:T ratios ranging from 30:1 to 1:1.

nomas obtained from the routine pathology diagnostic service were analyzed for the putative association between HER-2/neu overexpression and APM down-regulation. Immunohistochemical staining of 19 consecutive HER-2/neu-overexpressing and 19 HER-2/neu$^-$ breast cancer cases was performed using the anti-TAP1 and anti-TAP2 mAbs as well as anti-HC10 mAb recognizing the MHC class I heavy chain. As shown representatively for TAP1, IHC revealed a statistically significant ($P < 0.05$) lower TAP1 expression in HER-2/neu$^+$-overexpressing breast carcinomas (Fig. 5). In addition, TAP2 down-regulation was often found in both TAP1$^+$ and TAP1$^-$ HER2/neu$^+$ cells (Table 1). Because TAP is only functional as a heterodimer, the frequency of functional TAP deficiencies is even higher than that observed for TAP1 alone. In contrast, the MHC class I heavy chain expression is not effected by HER-2/neu overexpression in breast carcinomas, which is in accordance with the *in vitro* expression studies (data not shown). Thus, the HER-2/neu-mediated transformation seems to be associated with low levels of specific APM components in *in vitro* models of oncogenic transformation as well as in tumor specimens *in situ*.

Table 1 *Inverse association of HER-2/neu and TAP subunit expression in breast cancer*

TAP1 and TAP2 IHC scores ranging from 0 to 3 as described in "Materials and Methods" of 19 HER-2/neu$^-$ and 19 HER-2/neu$^+$ lesions are shown. The results are expressed as the number of tumor lesions corresponding to the TAP1/TAP2 IHC score. Note the inverse correlation of HER-2/neu with TAP expression, which was statistically significant for TAP1 ($P < 0.05$, Wilcoxon test).

HER-2/neu	No. of cases analyzed	No. of tumor lesions corresponding to the TAP1/TAP2 IHC score			
		O	1	2	3
+	19	6/6	9/10	3/3	1/0
−	19	0/5	7/3	7/9	5/2

DISCUSSION

Tumor-specific immune responses have been detected in patients with cancer, including those with overexpression of the *HER-2/neu* oncogene (20, 22, 29). However, many patients fail to generate an immune response that could protect them from progressive tumor growth. Central to any immune response is the proper processing and presentation of such tumor-associated antigens in the context of MHC class I molecules. In breast cancer, both HER-2/neu overexpression (17) and MHC class I loss (2, 3) have frequently been described. This was further confirmed by the work of Lollini *et al.* (25), who reported a partial or complete loss of MHC class I expression in breast carcinomas of c-neu transgenic mice. However, a correlation between these two phenotypes has not yet been analyzed.

In the present study, HER-2/neu overexpression in surgically removed lesions of human breast carcinomas and/or in murine *in vitro* models of oncogenic transformation is associated with a down-regulation of MHC class I surface expression, which may be attributable to impaired expression and/or function of various APM components (Figs. 1–3 and 5). These abnormalities were accompanied by strongly reduced CTL-mediated lysis (Fig. 4), which might influence the outcome of T-cell-based immunotherapies. Indeed, the inhibition of HER-2/neu expression in ovarian cancer cells results in an up-regulation of MHC class I surface antigens and an increased sensitivity of the tumor cells for CTL lysis (36). The IFN-γ-mediated effects on APM component expression in HER-2/neu$^+$ cells and wild-type NIH3T3 fibroblasts highlight the importance of additional use of immunostimulatory molecules in T-cell-based immunotherapies. It is noteworthy that MHC class I surface levels and the efficacy of CTL-mediated lysis of IFN-γ-treated CMV-HER-2/neu$^+$ cells could not reach the levels of IFN-γ-treated HER-2/neu$^-$ cells (Figs. 1 and 4). This still raises the question of sufficient immunogenicity of HER-2/neu$^+$ cells even in the presence of cytokines.

Thus far, limited information exists about APM component expression in HER-2/neu$^+$ tumors. Nijman *et al.* (37) demonstrated a down-regulation of MHC class I and β_2-microglobulin expression in only a minority of HER-2/neu$^+$ ovarian carcinoma patients, but the level of MHC class I antigens and HER-2/neu was not correlated. In contrast, our results argue for an impaired TAP activity in HER-2/neu$^+$ breast carcinoma because approximately 80% of these tumor lesions lack the expression of at least one TAP subunit, which therefore abrogates functional heterodimer formation (Fig. 5). However, additional studies that compare the level of HER-2/neu and APM expression as well as the impact of these expressions on immune response are urgently needed.

Recently, ras transformation has been shown to down-regulate MHC class I surface expression in a constitutive and an inducible system (5, 38), which may be at least attributable to suppression of TAP1 and LMP2 transcription (5). These results were in accordance with the data obtained in our HER-2/neu models and postulate a possible link of oncogene expression with deficient APM expression and function. Because HER-2/neu overexpression can activate several signal transduction pathways, including the ras/mitogen-activated protein kinase pathway, it can be speculated that this signal transduction cascade may be involved in the APM component down-regulation. However, the exact mechanisms by which HER-2/neu overexpression promotes APM deficiencies remain to be elucidated. Thus far, it is not known how HER-2/neu accomplishes such effects. However, the IFN-γ-mediated up-regulation of the mRNA and protein expression of the various APM components in HER-2/neu$^+$ cells (Fig. 2) suggests at least dysregulation rather than structural alterations of these molecules. The alternative explanation that these changes occurred during selection of transfectants could be excluded by the implementation of the tet-HER-2/neu model system (Fig. 1). The understanding of the strategies by which HER-2/neu$^+$ tumor cells circumvent proper APM expression, which appears to be a complex biological phenomenon, may provide essential information for the design of immunotherapeutic strategies to combat HER-2/neu-overexpressing cancers.

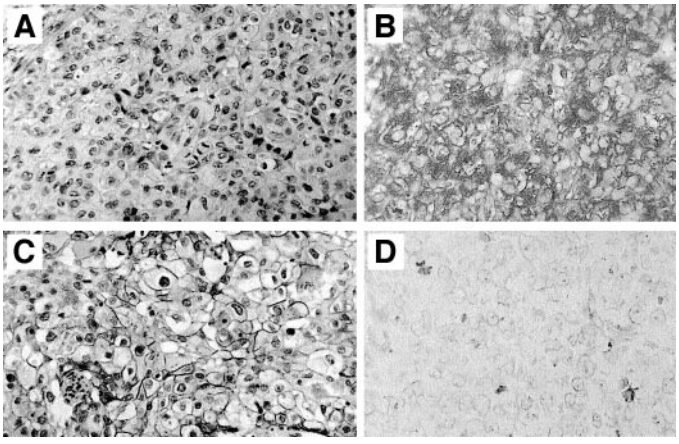

Fig. 5. Immunostaining intensity for TAP1 in HER-2/neu$^+$ and HER-2/neu$^-$ cases of invasive breast cancer. Representative immunohistochemical studies of two different breast cancer cases exhibiting strong TAP1 expression (*B*) in a HER-2/neu$^-$ (*A*) and absent TAP1 expression (*D*) in a HER-2/neu$^+$ case (*C*). Note that for HER-2/neu, only membranous immunostaining was considered positive.

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REFERENCES

1. Goodenow, R., Vogel, J., and Linsk, R. Histocompatibility antigens on murine tumors. Science (Wash. DC), *230:* 777–783, 1985.
2. Garrido, F., Ruiz-Cabello, F., Cabrera, T., Perez-Villar, J. J., Lopez-Botet, M., Duggan-Keen, M., and Stern, P. L. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol. Today, *18:* 89–95, 1997.
3. Ferrone, S., and Marincola, F. M. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. Immunol. Today, *16:* 487–494, 1995.
4. Mausdley, D. J., and Pond, J. S. Modulation of MHC antigen expression by viruses and oncogenes. Immunol. Today, *12:* 429–431, 1991.
5. Seliger, B., Harders, C., Lohmann, S., Momburg, F., Urlinger, S., Tampé, R., and Huber, C. Down-regulation of the MHC class I antigen-processing machinery after oncogenic transformation of murine fibroblasts. Eur. J. Immunol., *28:* 122–133, 1998.
6. Seliger, B., Harders, C., Wollscheid, U., Staege, M. S., Reske-Kunz, A. B., and Huber, C. Suppression of MHC class I antigens in oncogenic transformants: association with decreased recognition by cytotoxic T lymphocytes. Exp. Hematol., *24:* 1275–1279, 1996.
7. Seliger, B., Maeurer, M. J., and Ferrone, S. Antigen-processing machinery breakdown and tumor growth. Immunol. Today, *21:* 455–464, 2000.
8. Seliger, B., Schreiber, K., Delp, K., Meissner, M., Hammers, S., Reichert, T., Pawlischko, K., Tampe, R., and Huber, C., Downregulation of the constitutive tapasin expression in human tumor cells of distinct origin and its transcriptional upregulation by cytokines. Tissue Antigens, *57:* 39–45, 2001.
9. Qin, Z., Harders, C., Cao, X., Huber, C., Blankenstein, T., and Seliger, B. Increased tumorigenicity, but unchanged immunogenicity, of transporter for antigen presentation 1-deficient tumors. Cancer Res., *62:* 2856–2860, 2002.
10. Hunter, T. Oncoprotein networks. Cell, *88:* 333–346, 1997.
11. Lowy, D. R., and Willumsen, B. M. Function and regulation of Ras. Annu. Rev. Biochem., *62:* 851–891, 1993.
12. Paumelle, R., Tulasne, D., Kherrouche, Z., Plaza, S., Leroy, C., Reveneau, S., Vandenbunder, B., Fafeur, V., Tulashe, D., and Reveneau, S. Hepatocyte growth factor/scatter factor activates the ETS1 transcription factor by a RAS-RAF-MEK-ERK signaling pathway. Oncogene, *21:* 2309–2319, 2002.
13. Norgaard, P., Law, B., Joseph, H., Page, D. L., Shyr, Y., Mays, D., Pietenpol, J. A., Kohl, N. E., Oliff, A., Coffey, R. J., Jr., Poulsen, H. S., and Moses, H. L., Treatment with farnesyl-protein transferase inhibitor induces regression of mammary tumors in transforming growth factor (TGF) α and TGF α/neu transgenic mice by inhibition of mitogenic activity and induction of apoptosis. Clin. Cancer Res., *5:* 35–42, 1999.
14. Sivaraman, V. S., Wang, H., Nuovo, G. J., and Malbon, C. C., Hyperexpression of mitogen-activated protein kinase in human breast cancer. J. Clin. Investig., *99:* 1478–1483, 1997.
15. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ulrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science (Wash. DC), *230:* 1132–1139, 1985.
16. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene, *5:* 953–962, 1990.
17. Slamon, D. J., Godolphin, W., Jones, L. A., Hilt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ulrich, A., and Press, M. F. Studies of the HER-2/neu protooncogene in human breast and ovarian cancer. Science (Wash. DC), *244:* 707–712, 1989.
18. Seshadri, R., Firgaira, F. A., Horsfall, D. J., McCaul, K., Setlur, V., and Kitchen, P. Clinical significance of HER-2/neu oncogene amplification in primary breast cancer. The South Australian Breast Cancer Study Group. J. Clin. Oncol., *11:* 1936–1942, 1993.
19. Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, 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 anti-p185HER2/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.
20. Murray, J. L., Przepiorka, D., and Ioannides, C. G. Clinical trials of HER-2/neu specific vaccines. Semin. Oncol., *27:* 71–75, 2000.
21. Baselga, J., Norton, L., Albanell, J., Kim, Y. M., and Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res., *58:* 2825–2831, 1998.
22. Disis, M., and Cheever, M. HER-2/neu protein: a target for antigen-specific immunotherapy of human cancer. Adv. Cancer Res., *71:* 343–371, 1997.
23. Reilly, R. T., Machiels, J. P., Emens, L. A., Ercolini, A. M., Okoye, F. I., Lei, R. Y., Weintraub, D., and Jaffee, E. M. The collaboration of both humoral and cellular HER-2/neu targeted immune responses is required for the complete eradication of HER-2/neu expressing tumors. Cancer Res., *61:* 880–883, 2001.
24. Knutson, K. L., Schiffman, K., and Disis, M. L. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T cell immunity in cancer patients. J. Clin. Investig., *107:* 477–484, 2001.
25. Lollini, P. L., Nicoletti, G., Landuzzi, L., De Giovanni, C., Rossi, I., Di Carlo, E., Musiani, P., and Nanni, P. Down regulation of major histocompatibility complex class I expression in mammary carcinoma of HER-2/neu transgenic mice. Int. J. Cancer, *77:* 937–941, 1998.
26. Gossen, M., and Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA, *89:* 5547–5551, 1992.
27. Baasner, S., von Melchner, H., Klenner, T., Hilgard, P., and Beckers, T. Reversible tumorigenesis in mice by conditional expression of the HER2/c-erbB2 receptor tyrosine kinase. Oncogene, *13:* 901–911, 1996.
28. Wölfel, T., van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer zum Büschenfelde, K. H., and Boon, T. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur. J. Immunol., *24:* 759–764, 1994.
29. Seliger, B., Wollscheid, U., Momburg, F., Blankenstein, T., and Huber, C. Coordinate downregulation of multiple MHC class I antigen processing genes in chemical-induced murine tumor cell lines of distinct origin. Tissue Antigens, *56:* 327–336, 2000.
30. Bordier, C. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biochem., *256:* 1604–1607, 1981.
31. Neefjes, J. J., Momburg, F., and Hämmerling, G. J. Selective and ATP-dependent translocation of peptides by MHC-encoded transporter. Science (Wash. DC), *261:* 769–771, 1993.
32. Wölfel, T., Schneider, J., Meyer Zum Buschenfelde, K. H., Rammensee, H. G., Rotzschke, O., and Falk, K. Isolation of naturally processed peptides recognized by cytolytic T lymphocytes (CTL) on human melanoma cells in association with HLA-A2.1. Int. J. Cancer, *57:* 413–418, 1994.
33. Drexler, I., Antunes, E., Schmitz, M., Wölfel, T., Huber, C., Erfle, V., Rieber, P., Theobald, M., and Sutter, G. Modified vaccinia virus Ankara for delivery of human tyrosinase as melanoma-associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen A*0201-restricted cytotoxic T cells *in vitro* and *in vivo*. Cancer Res., *59:* 4955–4963, 1999.
34. Meyer, T. H., van Endert, P. M., Uebel, S., Ehring, B., and Tampe, R. Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells. FEBS Lett., *351:* 443–447, 1994.
35. van Endert, P. T., Tampé, R., Meyer, T. H., Tisch, R., Bach, J. F., and McDewitt, H. O. A sequential model for peptide binding and transport by the transporters associated with antigen processing. Immunity, *1:* 491–500, 1994.
36. Castilleja, A., Ward, N. E., O'Brian, C. A., Swearingen, B., II, Swan, E., Gillogly, M. A., Murray, J. L., Kudelka, A. P., Gershenson, D. M., and Ionannides, C. G. Accelerated HER-2 degradation enhances ovarian tumor recognition by CTL: implications for tumor immunogenicity. Mol. Cell. Biochem., *217:* 21–33, 2001.
37. Nijman, H. W., van Diest, P. J., Poort-Keesom, K. J., von Mensdorff-Pouilly, S., Vertraeten, R. A., Kummer, A., Meijer, C. J., Melief, C. J., Hilgers, J., and Keenemans, P. T cell infiltration and MHC class I and class II expression in the presence of tumor antigens: an immunohistochemical study in patients with serous epithelial ovarian cancer. Eur. J. Obstet. Gynecol. Reprod. Biol., *94:* 114–120, 2001.
38. Lohmann, S., Wollscheid, U., Huber, C., and Seliger, B. Multiple levels of MHC class I downregulation by ras oncogenes. Scand. J. Immunol., *43:* 537–544, 1996.