[CANCER RESEARCH 60, 5228–5236, September 15, 2000]

Defining Promiscuous MHC Class II Helper T-Cell Epitopes for the HER2/*neu* Tumor Antigen[1]

Hiroya Kobayashi, Monique Wood, Yongsheng Song, Ettore Appella, and Esteban Celis[2]

Department of Immunology and Cancer Center, Mayo Clinic and Mayo Graduate School, Rochester, Minnesota 55905 [H. K., M. W., E. C.], and the National Cancer Institute, NIH, Bethesda, Maryland 20892 [Y. S., E. A.]

ABSTRACT

It is accepted that both helper and CTLs play a critical role in immune antitumor responses. Thus, the design of effective immune-based therapies for cancer relies in the identification of relevant tumor-associated antigens (TAAs) capable of eliciting strong helper and cytotoxic T-cell responses against tumor cells. The product of the HER2/*neu* oncogene is considered as a prototype TAA, because it is found overexpressed in a large variety of malignancies, whereas normal cells only produce low levels of this product. Several cytotoxic T-cell epitopes for HER2/*neu* have been identified that enable the design of peptide-based therapeutic vaccines for tumors expressing this TAA. Nevertheless, it is expected that inclusion of peptide epitopes capable of eliciting HER2/*neu*-specific T helper responses into these vaccines may enhance their effectiveness in the clinic. We describe here a strategy to identify helper T-cell epitopes for HER2/*neu* that focuses on peptides predicted to bind to numerous histocompatibility alleles (promiscuous epitopes), which would encourage their use in therapeutic vaccines for the general cancer patient population. Following this approach, we successfully identified several peptides that elicited T helper ($CD4^+$) proliferative responses to peptides derived from HER2/*neu*. Most of the T-cell responses appeared to reflect a low affinity for antigen, which could be the result of immune tolerance because HER2/*neu* is expressed in low levels in normal cells and possibly including lymphocytes and monocytes. Interestingly, one of these peptides, $HER2_{883}$, was recognized by T cells in the context of either HLA-DR1, HLA-DR4, HLA-DR52, and HLA-DR53, indicating a high degree of histocompatibility promiscuity. Furthermore, T cells that reacted with peptide $HER2_{883}$ could also recognize antigen-presenting cells that process HER2/*neu* recombinant protein. These results may be relevant for the design of more effective therapeutic vaccines for tumors expressing the HER2/*neu* oncogene product.

INTRODUCTION

$CD4^+$ HTL[3] responses play an essential role in immunologically mediated antiviral and antitumor cellular immunity (1). During the induction (afferent) phase of T cell-mediated immune responses, HTLs participate in the induction of antigen-specific CTLs, which are the main effector cells against virally infected or malignant cells. HTLs not only provide CTLs with growth-stimulating lymphokines, such as IL-2, but also prime/activate DCs to effectively present antigen to naive CTL precursors (2–4). In addition, HTLs appear to be important in the maintenance of long-lived CTL responses, which may be critical in the prevention of relapses and in the preservation of immune memory (5–7). For example, in a recent clinical study, the *in vivo* persistence of adoptively transferred antigen-specific $CD8^+$ CTLs was dependent on the endogenous response of $CD4^+$ HTLs (8, 9). Moreover, $CD4^+$ HTLs can exhibit effector function by directly recognizing and killing MHC class II^+ virally infected or tumor cells that present HTL epitopes on their surface (10–12).

The design and implementation of T cell-based immunotherapy (vaccines or adoptive cell therapy) for cancer rely heavily in the identification of TAAs that bear immunogenic T-cell epitopes expressed on tumor cells. The HER2/*neu* gene product, a homologue of the epidermal growth factor receptor, has been reported to be overexpressed in a large proportion of aggressive breast and ovarian tumors and in other cancers of epithelial origin (13, 14). The HER2/*neu* protein appears to be an ideal TAA for immunotherapy because CTL responses specific for MHC class I epitopes have been observed in some cancer patients (15–19). Furthermore, tumor-reactive CTL responses have been induced *in vitro* using various recently identified MHC class I-binding synthetic peptides derived from the HER2/*neu* sequence (20–23). Thus, the identification of CTL epitopes for HER2/*neu* opens the door to the possibility of using this TAA for immunotherapy against tumors overexpressing this molecule. However, there is concern that in the absence of antigen-specific HTL responses, the HER2/*neu*-reactive CTLs may not survive or expand sufficiently to be effective in producing antitumor therapeutic responses.

Our goal is to identify peptide sequences corresponding to MHC class II broadly restricted (promiscuous) HTL epitopes for HER2/*neu* to improve the design of CTL-based immunotherapy for tumors expressing this TAA. The use of promiscuous HTL epitopes should allow the use of these peptide sequences in patient populations expressing diverse MHC class II alleles. There is already some evidence of the existence of MHC class II T-cell responses to HER2/*neu*: (*a*) is the observation that some patients with HER2/*neu*-positive cancers (such as pancreatic or breast cancer) produce IgG antibodies against the HER2/*neu* protein, suggesting that this protein triggers $CD4^+$ helper T-cell responses that are necessary for IgG class switching (24); (*b*) there are reports that $CD4^+$ T cells from HER2/*neu*-positive cancer patients can proliferate and produce lymphokines as the result of stimulation with synthetic peptides or recombinant HER2/*neu* protein (24–29).

In the present study, we used a computer algorithm to select HER2/*neu* sequences with potential promiscuous HLA-DR binding characteristics (30). Synthetic peptides corresponding to potential HLA-DR promiscuous binding sequences were prepared and tested for their capacity to stimulate HER2/*neu*-specific $CD4^+$ T cells from healthy donors by primary *in vitro* immunization using DCs as APCs. Our results show that several of the predicted peptides were able to trigger HTL responses in individuals expressing diverse HLA-DR alleles. Furthermore, one of these peptides ($HER2_{883}$) induced T-cell responses restricted by HLA-DR1, HLA-DR4, HLA-DR52, and HLA-DR53, which were effective in recognizing naturally processed HER2/*neu* protein. We observed that in most instances, the HER2/*neu*-reactive HTLs had a low affinity for antigen (*i.e.*, required high quantities of peptide to become activated). The apparent absence of high-affinity HTLs for HER2/*neu* could be attributable to peripheral tolerance to this antigen because this protein is expressed in low

Received 3/20/00; accepted 7/19/00.

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 by Grant R01CA80782 from the NIH.

[2] To whom requests for reprints should be addressed, at Department of Immunology, GU421A, Mayo Clinic, Rochester, MN 55905. Phone: (505) 284-0124; Fax: (505) 266-5255; E-mail: celis.esteban@mayo.edu.

[3] The abbreviations used are: HTL, helper T lymphocyte; IL, interleukin; DC, dendritic cell; TAA, tumor-associated antigen; APC, antigen-presenting cell; ECD, extracellular domain; ICD, intracellular domain; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; GM-CSF, granulocyte/macrophage-colony stimulating factor; SI, stimulation index; RT-PCR, reverse transcription-PCR; ARB, average relative binding; TCL, T-cell line; HBsAg, hepatitis B surface antigen; RT, reverse transcriptase.

amounts by cells in normal tissues, including some blood mononuclear cells.

MATERIALS AND METHODS

Cell Lines. EBV-transformed lymphoblastoid cells (EBV-LCL) were produced from peripheral blood mononuclear cells of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, VA). Mouse fibroblast cell lines (L-cells), transfected and expressing individual human MHC class II molecules, were kindly provided by R. W. Karr (Park-Davis, Ann Arbor, MI).

Synthetic Peptides and Recombinant Protein. To predict potential HLA-DR promiscuous HER2/*neu* $CD4^+$ T-cell epitopes, the amino acid sequence of the HER2/*neu* protein was analyzed using a computer algorithm designed to look for promiscuous HLA-DR binding peptides. Peptides that have high algorithm scores for three HLA-DR alleles (*DRB1* 0101, DRB1*0401,* and *DRB1*0701*) were identified using the MHC class II binding prediction tables published by Southwood *et al.* (30). Peptides were synthesized according to standard solid phase methods using an Applied Biosystems synthesizer and purified by high-performance liquid chromatography. The purity (>95%) and identity of peptides were determined by mass spectrometry. Recombinant human HER2/*neu* ECD and ICD protein fragments were produced and provided by Corixa Corporation (Seattle, WA). The purity of these proteins was verified by SDS-PAGE.

***In Vitro* Generation of DCs.** PBMCs from $HLA\text{-}DR1^+$, $HLA\text{-}DR4^+$, or $HLA\text{-}DR7^+$ normal volunteers were isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) gradient centrifugation of leukopheresis products. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers. DCs were generated in tissue culture from $CD14^+$ monocyte precursors purified by positive immunoselection using an anti-CD14 mAb coupled onto magnetic microbeads (Miltenyi Biotech, Auburn, CA). The $CD14^+$ monocytes were cultured at 1×10^6 cells/ml in the presence of 50 ng/ml of GM-CSF and 1000 units/ml of IL-4 in RPMI 1640 supplemented with 10% human male AB serum (Gemini Bio-Products, Calabasas, CA), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. Cultures were fed on days 3 and 5 with fresh medium containing GM-CSF and IL-4. On day 7, the nonadherent cells were harvested and used as APCs, described below.

Antigen-specific $CD4^+$ T-Cell Stimulation Using Synthetic Peptides and DCs. The cytokine-generated DCs were pulsed for 2 h at 37°C with 10 μg/ml synthetic peptide in a 5% CO_2 incubator. The peptide-pulsed DCs (1×10^4) were then irradiated (4200 rads) and mixed with 3×10^4 autologous $CD4^+$ T cells (purified by positive selection with anti-CD4 mAb coupled to magnetic microbeads from Miltenyi Biotech) in 200 μl of culture medium in each well of a 96-well, round-bottomed culture plate. Culture medium consisted of RPMI 1640 supplemented with 5% human male AB serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. Seven days later, half of the medium was removed from each culture well, and irradiated autologous PBMCs (1×10^5/well) pulsed for 2 h with peptide (at 10 μg/ml) in 100 μl of medium were added to each of the culture wells. Two days after the second stimulation with peptide, human recombinant IL-2 was added to each well at a final concentration of 10 IU/ml. One week later, the microcultures were tested for their proliferative responses to peptide-pulsed and irradiated (4200 rads) HLA-DR-transfected L-cells (used as APCs, see below). Those microwells showing a proliferative response to peptide (at least 2.5-fold over background) were transferred to 24- or 48-well plates and restimulated at weekly intervals with irradiated autologous PBMCs (1×10^6/well) pulsed with peptides (3 μg/ml) in medium containing 25 IU/ml IL-2. In some instances, TCLs were cloned by limiting dilution for further studies.

Antigen-specific Proliferative Response of T Cells. T cells (3×10^4/well) were mixed with irradiated autologous PBMCs (1×10^5/well), DCs (5×10^3/well), or HLA-DR-expressing L-cells (3×10^4/well) in the presence of various concentrations of antigen (peptides, recombinant HER2/*neu* protein) in 96-well culture plates. The cultures were incubated at 37°C in a 5% CO_2 incubator for 72 h, and during the last 16 h, each well was pulsed with 0.5 μCi/well of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter after harvesting the cell cultures onto glass fiber filters. To determine MHC restriction molecules involved in antigen presentation, blocking of the antigen-induced proliferative response was investigated by adding anti-HLA-DR mAb L243 or anti-HLA-DQ mAb SPVL3 (10 μg/ml) throughout the 72-h assay. All assessments of proliferative responses were carried out at least in triplicate, and results corresponded to the means. The SI was calculated by dividing the mean radioactivity (cpm) obtained in the presence of antigen by the mean radioactivity (cpm) obtained in the absence of antigen but in the presence of APCs.

Detection of HER2/*neu* Expression in Blood Mononuclear Cells. PBMCs from two normal volunteers were separated into $CD4^+$, $CD8^+$, and $CD14^+$ populations by positive selection using magnetic beads coated with specific antibodies (Miltenyi). Cytofluorometric analysis revealed that these cells were >98% pure for each of these populations (data not shown) The presence of HER2/*neu* transcripts in positively selected $CD4^+$, $CD8^+$, and $CD14^+$ cells was assessed by RT-PCR. A set of specific primers for HER2/*neu* was selected that spans several intron sequences, allowing the distinction of DNA from RNA. In addition, all RT-PCR reactions that were performed in the absence of reverse transcriptase (Taq Gold; Perkin-Elmer) did not yield any product indicating the absence of DNA in the RNA samples, which were prepared using RNeasy mini kits (Qiagen, Valencia, CA). The HER2/*neu* primers used were 5′-GTC TAC AAG GGC ATC TGG AT-3′ (5′ primer) and 5′-CCC CAA AAG TCA TCA GC-3′ (3′ primer), which yielded a 559-base product using the cDNAs that were derived from the purified RNA samples.

RESULTS

Prediction and Selection of Potential HTL Epitopes for HER2/*neu*. Because our goal was to identify promiscuous MHC class II HTL epitopes from HER2/*neu*, we examined the amino acid sequence of this protein for the presence of peptide sequences containing binding motifs for HLA-*DR*0101, DR*0401,* and *DR*0701* using the algorithm tables published by Southwood *et al.* (30). These algorithms take into account the potential (predicted) binding interactions of primary and secondary anchors of a 9-amino acid "core region" with each MHC allele. An estimated ARB for each possible core region

Table 1 *Peptides predicted to be promiscuous HTL epitopes*

Sequence (9-mer core)	DR1		DR4		DR7		Rank sum[a]	Peptide name	Peptide sequence[b] (15–18)mer
	ARB	Rank	ARB	Rank	ARB	Rank			
YVAPLTCSP	85.03	6	66.35	1	27.51	3	10	HER_{1124}	TDGYVAPLTCSPQPE
YVMAGVGSP	24.69	17	29.64	4	19.52	11	32	HER_{765}	KEILDEAYVMAGVGSPYVS
WCMQIAKGM	35.48	13	9.56	15	22.72	6	34	HER_{822}	LLNWCMQIAKGMSYL
WMALESILR	13.07	30	20.24	6	45.23	2	38	HER_{883}	KVPIKWMALESILRRRF
LDEAYVMAG	15.18	28	5.42	29	19.77	10	67	HER_{765}	KEILDEAYVMAGVGSPYVS
IKWMALESI	5.07	51	7.33	21	57.56	1	73	HER_{883}	KVPIKWMALESILRRRF
LSYMPIWKF	16.41	24	3.54	41	20.01	9	74	HER_{605}	KPDLSYMPIWKFPDE
LPTNASLSF	2.44	66	8.17	17	10.65	18	101	HER_{62}	LTYLPTNASLSFLQD
LTSIISAVV	1.78	77	7.02	23	9.57	19	119	HER_{648}	ASPLTSIISAVVGIL

[a] Rank sum = DR1 rank + DR4 rank + DR7 rank.
[b] These peptides were selected for synthesis for T-cell studies. The core region is shown underlined.

Table 2 *Proliferative responses to HER2/neu peptides*

Donor[a] peptides	T-cell responses (SI)[b]											
	DR1/11;DR52;DQ5/7			DR1/13;DR52;DQ5/7			DR4/15;DR51/53;DQ6/7			DR7/17;DR52/53;DQ2		
	TCL[c]	PBMC	L-DR1	TCL	PBMC	L-DR1	TCL	PBMC	L-DR4	TCL	PBMC	L-DR7
HER_{1124}	12F	1.1	NTd	8F	1.0	NT	10B	1.3	NT	7G	1.2	NT
HER_{822}	11C	31.3	NT				3B	207.1	NT	4E	117.7	NT
							7B	9.6	NT			
HER_{765}	3B	1.1	NT				8A	14.7	26.9	9B	252.7	NT
							7D	23.5	423.3			
HER_{883}	11A	334.3	NT	8F	92.2	271.7	6D	143.5	80.4	1D	22.8	1.5
				2A	82.3	43.2	7C	67.6	1.9	1E	115.7	3.5
										12F	181.3	NT
HER_{605}	12H	1.9	385.4				12B	12.2	18.3	4A	61.0	NT
	1H	200.3	NT				6B	207.4	NT			
HER_{62}	3H	0.9	NT				6F	114.9	484.3	4E	10.3	241.5
							11F	8.7	624.0	5D	12.7	183.2
HER_{648}	1H	1.2	1.0				NT			8E	75.7	590.9

[a] Four normal donor volunteers were tested in these experiments (their respective HLA-DR and -DQ alleles are shown).
[b] T-cell proliferative responses are expressed as SI, calculated as described in "Materials and Methods."
[c] Several TCLs were analyzed for their proliferative responses to peptide presented by either autologous PBMCs or L-cells transfected with individual DR molecules.
[d] NT, not tested.

peptide was estimated using the algorithm values. A total of 1247 9-amino acid peptide sequences for HER2/*neu* (1255 residues) were then ranked separately according to their algorithm values for each of the three HLA-DR alleles studied, *e.g., DR*0101* rank of 1 represents the peptide sequence with the highest probability of binding to this allele (highest ARB value). The three rank values for *DR*0101, DR*0401,* and *DR*0701* were added for each 9-residue sequence (rank sum), and the 10 sequences exhibiting the lowest rank sum values were identified and selected for peptide synthesis (Table 1). Although nine sequences were selected for peptide synthesis, only seven peptides were prepared because some of these sequences overlapped (*e.g.*, $HER2_{765}$/$HER2_{769}$ and $HER2_{883}$/$HER2_{885}$); therefore, a single peptide could be prepared that contained both core regions. Because in general HTLs prefer to recognize peptides of ~15 residues, these sequences were extended at least three amino acids at both amino and carboxyl ends for peptide synthesis (Table 1).

T-Cell Responses to Peptides from HER2/*neu*. The peptides selected from the promiscuous algorithm analysis were tested for their capacity to stimulate $CD4^+$ T cells isolated from four healthy, MHC-typed individuals (HLA-DR1/11, DR1/13, DR4/15, and DR7/17) in primary *in vitro* cultures using peptide-pulsed autologous DCs. After the primary screening assay (which was done only in single-well determinations because of low numbers of cells; see "Materials and Methods" for details), the T-cell cultures that displayed a SI of ≥2.5 were expanded and retested (this time in triplicates) for their capacity to react with peptide and recombinant HER2/*neu* protein in the presence of APC (PBMCs, DCs, or L-cells). The results in Table 2 show examples of the types of responses that we observed with several TCLs. In some cases, the SIs were close to a value of 1 (*e.g.*, $HER2_{1124}$ for all alleles), indicating that these T cells did not proliferate specifically to the peptides. It should be noted that a SI value of ~1 was the result of either: (*a*) the T cells did not proliferate at all in the presence or absence of peptide; or (*b*) because of high proliferative responses observed both with and without the addition of peptide, indicating that these cells were autoreactive. Nevertheless, a large number of antigen-specific T-cell responses, which were evident by SIs ≥2.5 (our arbitrary cutoff value) were observed with several of the peptides that were tested (Table 2). Most importantly, four of the peptides ($HER2_{822}$, $HER2_{765}$, $HER2_{605}$, and $HER2_{62}$) were capable of inducing HTL responses to more than one MHC class II allele, indicating some degree of promiscuity.

Interestingly, the results presented in Table 2 indicate that one of TCLs (TCL-12H from a DR1 donor) recognized peptide $HER2_{605}$ in an antigen-specific manner only when DR1-transfected L-cells were

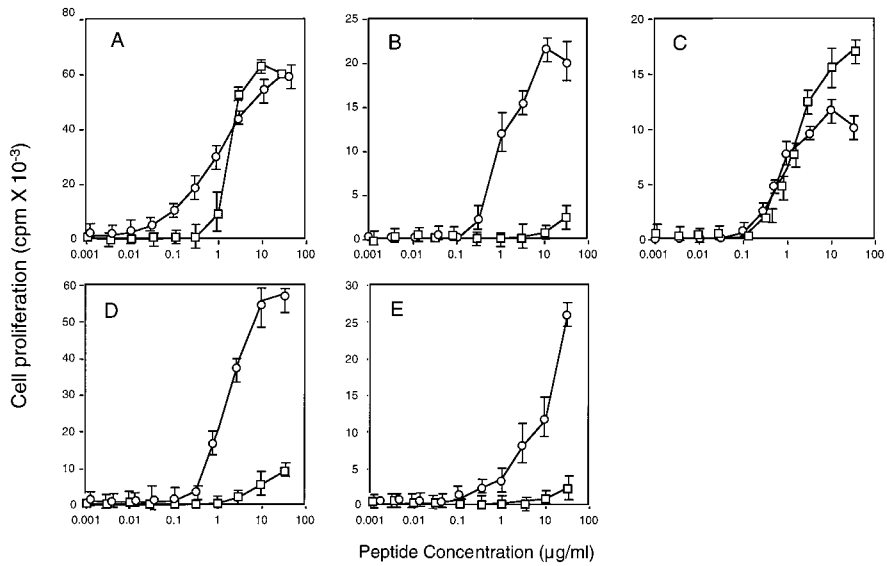

Fig. 1. Proliferative responses of $HER2_{883}$-reactive TCLs to various concentrations of peptide. $CD4^+$ T cells (3×10^4) were tested in triplicates for their ability to proliferate in response to several peptide concentrations in the presence of irradiated autologous PBMCs (○) or DR-transfected L-cells (□). The following TCLs were studied: *A*, TCL-8F (from the DR1/15, DR52 donor); *B*, TCL-7C (DR4/15, DR51/53); *C*, TCL-6D (DR4/15, DR51/53); *D*, TCL-1E (DR7/17, DR52/53); and *E*, TCL-1D (DR7/17, DR52/53). Transfected L-cells used as APCs were: L-DR1 (*A*); L-DR4 (*B* and *C*); and L-DR7 (*D* and *E*). Values shown are the means of duplicate determinations; *bars,* SD.

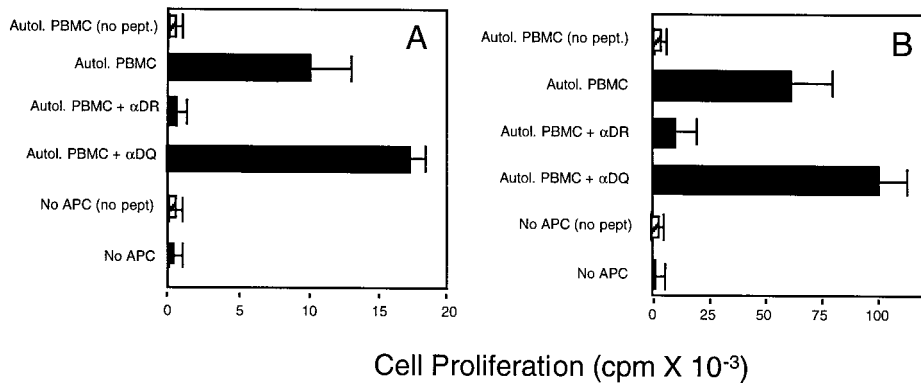

Fig. 2. MHC restriction analysis of $CD4^+$ $HER2_{883}$-reactive TCLs. mAbs specific for HLA-DR (L243) or HLA-DQ (SPVL3) were tested at 10 μg/ml for their capacity to inhibit the proliferation of TCL-8F (*A*) and TCL-6D (*B*) to peptide $HER2_{883}$ (used at 2.5 μg/ml; ■) in the presence of irradiated autologous APCs. For comparison, proliferative responses observed in the absence of peptide are shown (▨). Values shown are the means of triplicate determinations; *bars*, SD.

used as APCs ([^{3}H]thymidine incorporation was 54,342 cpm in the presence of peptide *versus* 141 cpm in the absence of peptide; SI, 385.4) but not when autologous PBMCs were used. The apparent lack to reactivity to peptide with PBMCs was attributable to the high [^{3}H]thymidine incorporation obtained in the absence of peptide (91,842 cpm with peptide *versus* 50,885 cpm without peptide; SI, 1.8). Similar results were obtained with several T-cell clones derived from this TCL (data not shown). The high levels of proliferation in the absence of peptide suggest the possibility that APCs in the PBMCs (but not in the L-cells) may be expressing and presenting a peptide of a similar sequence as $HER2_{605}$.

MHC Restriction Analysis. In some of the results presented above (Table 2), it is evident that in certain cases the antigen-induced proliferative responses were high only when autologous APCs (PBMCs or DCs) were used and not when HLA-DR-transfected cells were used to present peptide to the T cells (*e.g.*, TCL-7C from the DR4 donor and TCL-1D from the DR7 donor). The differences in the capacity of PBMC *versus* DR-transfected L-cells to serve as APCs are further illustrated in the peptide titration curves presented in Fig. 1. These results show that some TCLs responded well to various concentrations of peptide, regardless of whether PBMCs or DR-transfected cells were used as APCs, suggesting that these TCLs are restricted by the DR molecules expressed by the L-cells (Fig. 1, *A* and *C*). The antibody blocking experiments presented in Fig. 2 further corroborate that the two TCLs are MHC class II restricted and recognize peptide in the context of HLA-DR molecules (and not HLA-DQ).

Some of the $HER2_{883}$-reactive TCLs proliferated well to antigen only when autologous PBMCs were used as APCs, whereas the DR-transfected L-cells were inefficient in presenting the peptide (Fig. 1, *B*, *D*, and *E*). One possible explanation for these results could be that the DR-transfected L-cells are poor APCs because they do not express sufficient MHC molecules or because they may lack the appropriate adhesion/costimulatory molecules. However, this effect should be less evident at high concentrations of antigen but was still observed, even when 30 μg/ml of peptide was used (Fig. 1, *B*, *D*, and *E*). Moreover, the high SIs obtained with other TCLs when using the same L-cells as APCs (TCL-6F and TCL-11F in the DR4 donor and TCL-4E, TCL-5D, and TCL-8E in the DR7 donor in Table 2 and Fig. 1, *A* and *C*) indicate that these cells can function well as APCs when peptide is used as the source of antigen.

An alternative and more plausible explanation for the differences in antigen presentation function observed between L-cells and PBMCs is that those TCLs could be restricted by an MHC class II allele or molecule (DQ or DP) different from the one expressed by the transfected L-cell. For example, it is possible that the TCL-7C (Fig. 1*B*), which was derived from the DR4/15;DR51/53;DQ6/7 individual may recognize peptide $HER2_{883}$ in the context of DR15, DR51, DR53, or a DQ or DP molecule and not DR4. If this were to be the case, it would signify that some of peptides that we have identified as promiscuous for DR1, DR4, and DR7 could be capable of binding to additional MHC class II molecules. To evaluate this possibility, we tested the capacity of several of the TCLs that reacted poorly to peptide when DR4- or DR7-transfected L-cells were used as APCs to proliferate to peptide presented by additional MHC-typed cell lines. In addition, we also determined whether monoclonal anti-DR or anti-DQ antibodies would inhibit the recognition of antigen presented by autologous PBMCs by the T cells. The results in Fig. 3 show that two TCLs, which were derived from different donors (Fig. 1, *B* and *E*), recognized peptide $HER2_{883}$ in the context of the DR53 molecule and that anti-DR, but not anti-DQ antibodies, inhibited the T-cell responses. Similarly, another TCL that was originally thought to be

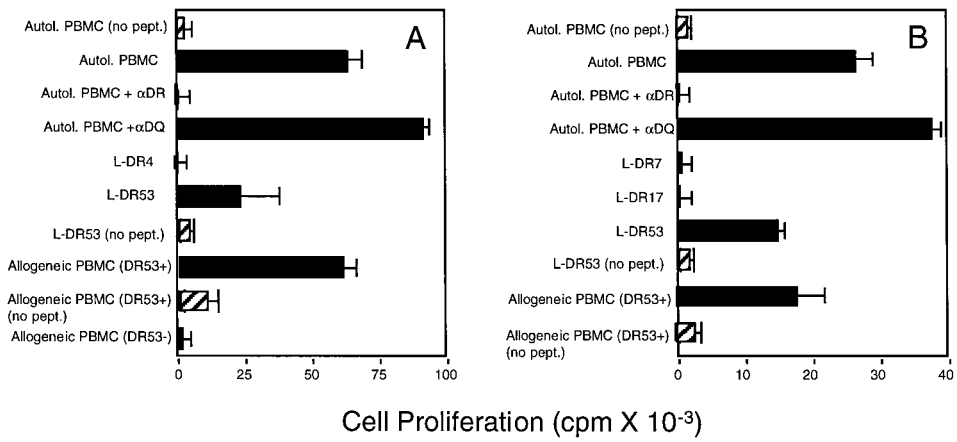

Fig. 3. HLA-DR53 can present peptide $HER2_{883}$ to HTLs. TCL-7C (*A*) and TCL-1E (*B*) were tested for their ability to proliferate to peptide $HER2_{883}$ presented by various MHC-typed APCs (as indicated in the figure). The allogeneic PBMCs used in these experiments shared only HLA-DR53 (and not other DR alleles) with the MHC type of the TCL. mAbs specific for HLA-DR (L243) and HLA-DQ (SPVL3) were tested for their capacity to inhibit the peptide-induced proliferation with autologous APCs. Values shown are the means of triplicate samples; *bars*, SD.

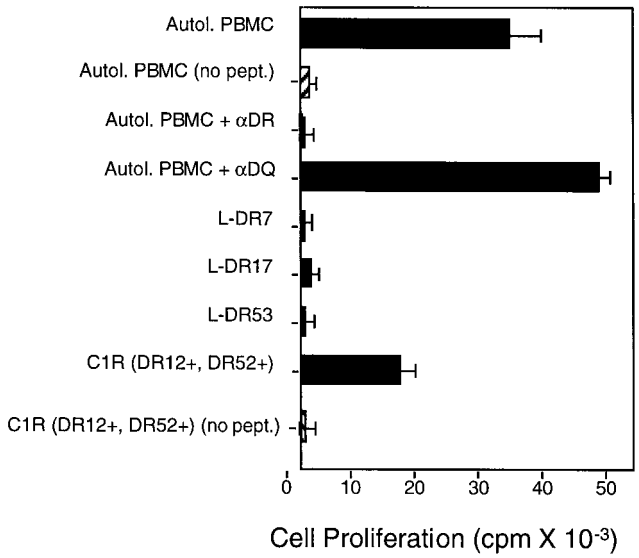

Fig. 4. HLA-DR52 can also present peptide $HER2_{883}$ to HTLs. A T-cell clone derived from TCL-1D (Fig. 1*E*) was tested for its capacity to proliferate to peptide $HER2_{883}$ presented by various MHC-typed APCs (as indicated in the figure). The C1R cells used in this experiment shares only HLA-DR52 (and not other DR molecules) with the MHC type of the TCL. Proliferative responses in the absence of peptide were in all cases <1000 cpm (▨ and data not shown). mAbs specific for HLA-DR (L243) and HLA-DQ (SPVL3) were tested for their capacity to inhibit the peptide-induced proliferation with autologous APCs. Values shown are the means of triplicate determinations; *bars*, SD.

restricted to DR7, but responded poorly to peptide presented by L-DR7 cells (Fig. 1*E*), reacted with peptide in the context of DR52 (Fig. 4). These results indicate that peptide $HER2_{883}$, which was predicted to bind to DR1, DR4, and DR7, is also capable of stimulating T-cell responses in the context of DR52 and DR53 and, thus, has a higher degree of promiscuity than originally thought.

Recognition of Processed Antigen by Peptide-reactive HTL. The results presented thus far indicate that several of the peptides from HER2/*neu* that were predicted to serve as promiscuous MHC class II T-cell epitopes were indeed capable of inducing $CD4^+$ T-cell responses in the context of more than one HLA-DR allele. However, it is necessary to determine whether these peptides represent true T-cell epitopes that would be relevant for the development of tumor immunotherapy. Thus, it becomes important to determine whether APCs that naturally capture and process the protein antigen that bears the putative T-cell epitope (in this case, the HER2/*neu* protein) are capable of stimulating the peptide-reactive T cells. We consequently proceeded to test the capacity of T cells that had shown reactivity to HER2/*neu* peptides ($HER2_{883}$, $HER2_{822}$, $HER2_{765}$, $HER2_{605}$, and $HER2_{62}$) to recognize naturally processed antigen in the form of recombinant HER2/*neu* protein. For these experiments, we used autologous PBMCs or DCs as APCs and recombinant DNA derived ICD or ECD protein fragments of HER2/*neu* as a source of antigen.

The data presented in Fig. 5 show that four $HER2_{883}$-reactive TCLs proliferated well to HER2/*neu* ICD (which encompasses the $HER2_{883}$ peptide) but not to HER2/*neu* ECD (which lacks the $HER2_{883}$ sequence; data not shown) when DCs were used as the source of APCs. Similar results were observed when the $HER2_{883}$-reactive TCLs were tested for their capacity to produce lymphokines (measured by a commercial ELISA kit) as a result of stimulation for 24 h, using DCs pulsed with either peptide or HER2/*neu* ICD. For example, TCL-7C (2×10^4 cells) produced 850 pg/ml when stimulated with DCs (5×10^3) pulsed with $HER2_{883}$ peptide and 400 pg/ml of this lymphokine when the DCs were pulsed with HER2/*neu* ICD, whereas DCs alone failed to induce the production of GM-CSF (<10 pg/ml).

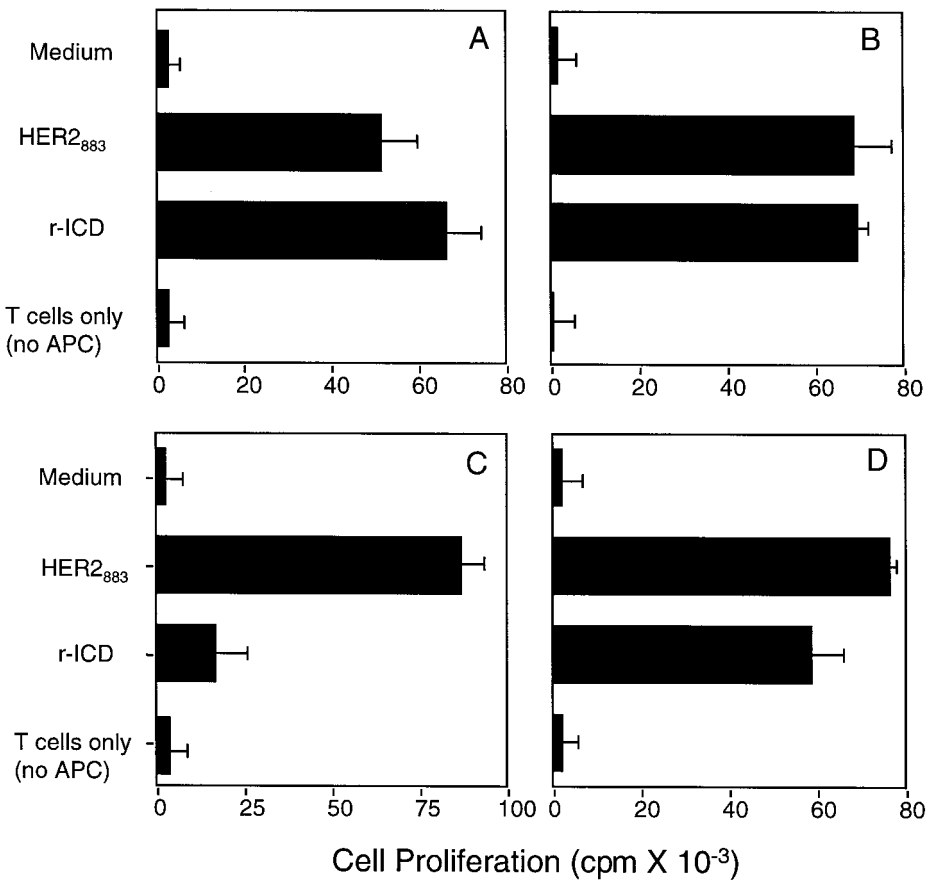

Fig. 5. $HER2_{883}$-specific $CD4^+$ T cells can recognize recombinant HER2/*neu* intracellular domain (*r-ICD*) protein presented by autologous DCs. The $HER2_{883}$-reactive HTLs, TCL-7C (*A*), TCL-6D (*B*), a clone of TCL-1D (*C*), and TCL-1E (*D*), were tested for their capacity to proliferate to autologous DCs in the presence of $HER2_{883}$ peptide (2.5 μg/ml) or recombinant HER2/*neu* recombinant ICD protein (10 μg/ml). No significant proliferative response was observed against HER2/*neu* ECD protein (data not shown). Values shown are the means of triplicate determinations; *bars*, SD.

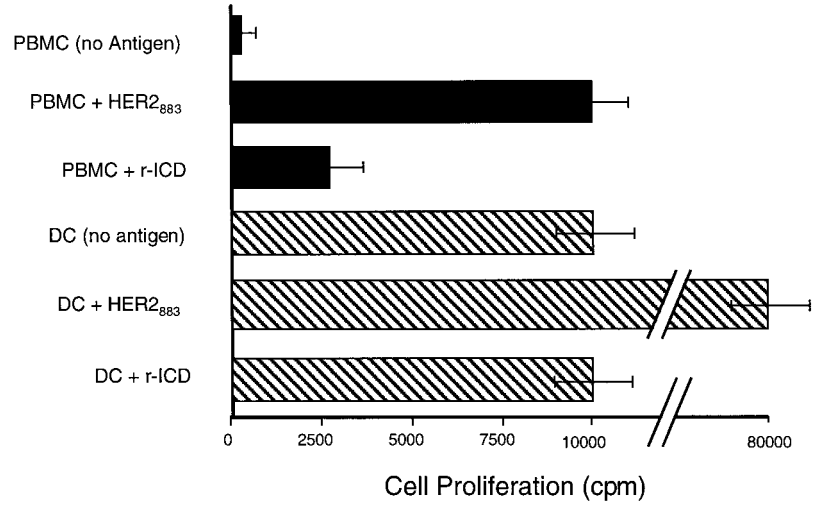

Fig. 6. HER2/*neu* recombinant ICD (*r-ICD*) protein presented by PBMCs can trigger specific $CD4^+$ HTL responses. The $HER2_{883}$-reactive TCL-8F was tested for its ability to proliferate to $HER2_{883}$ peptide (2.5 μg/ml) and recombinant HER2/*neu* ICD protein (10 μg/ml) presented by autologous PBMCs and DCs. Results show high response to peptide when either PBMCs or DCs were used as APCs, but significant proliferation to HER2/*neu* ICD protein was evident only with PBMCs, attributable to high background observed with DCs alone. Values shown are the means of triplicate determinations; *bars*, SD.

It was interesting to observe that the four $HER2_{883}$-reactive TCLs failed to proliferate or produce lymphokines to HER2/*neu* ICD when PBMCs were used as APCs (not shown). These results suggest that either the HER2/*neu* ICD is processed differently by monocytes and DCs or that the affinity of the TCLs for antigen is low, and they require large numbers of peptide/MHC complexes and/or adhesion molecules (provided by the DCs) to achieve their signaling threshold.

A fifth TCL (TCL-8F), also reactive with peptide $HER2_{883}$ (restricted by HLA-DR1; Fig. 1*A*) was capable of recognizing HER2/*neu* ICD with autologous APCs (Fig. 6). However, in this case, PBMCs (presumably monocytes) functioned well as APCs, whereas DCs appeared to induce a nonspecific proliferative response (high background). These results raise the possibility that DCs may express an endogenous peptide/MHC complex that cross-reacts with $HER2_{883}$, which is capable of stimulating the reactivity of the 8F TCL.

The capacity to recognize processed HER2/*neu* protein was also tested in those TCLs specific for peptides $HER2_{822}$, $HER2_{765}$, $HER2_{605}$, and $HER2_{62}$, but in neither case were we able to detect significant proliferative responses when either PBMCs or DCs were used as APCs (data not shown). These results could be interpreted by the possibility that these epitopes may not be produced by APCs that process the protein antigen. Alternatively, these results could be explained on the basis of the low affinity of the T cells for antigen, which would necessitate a higher number of peptide/MHC complexes than those expressed on the APCs to trigger the proliferative responses. In support of the latter possibility, the example presented in Fig. 7 illustrates the large differences in affinities observed between T cells that respond to processed antigen and those that do not. The results show that the $HER2_{883}$-reactive TCL 8F, which recognizes protein presented by PBMCs (Fig. 6), can respond to peptide concentrations as low as 10 ng/ml, whereas a TCL specific for peptide $HER2_{765}$, which is unable to respond to protein presented by either PBMCs or DCs (data not shown) requires >1 μg/ml of peptide to proliferate. For comparison, we present the peptide titration response curve of a $CD4^+$ TCL specific for a peptide from HBsAg, which proliferates well to the HBsAg protein (31) and is capable of reacting with <1 ng/ml of peptide (Fig. 7). This high level of affinity for antigen is typical of what we have observed with T helper cell lines and clones reactive viral antigens from hepatitis B and other viruses (31–33), which reflects the lack of immunological tolerance to these epitopes.

Expression of HER2/*neu* in PBMCs. As mentioned above, some of our results showing high proliferative responses in the absence of antigen when autologous APCs were used suggest that a cross-reactive peptide epitope or the HER2/*neu* product may be expressed in a subpopulation of PBMCs. Although HER2/*neu* is considered to be an epithelial cell marker, there are reports that this molecule is expressed by some hematopoietic cells and hematological malignancies. To explore the possibility that HER2/*neu* may be present in blood mononuclear cells, we examined the expression of this gene in purified $CD4^+$, $CD8^+$, and $CD14^+$ cells by RT-PCR (Fig. 8). These results clearly indicate that all three populations of cells express message for a transcript corresponding to HER2/*neu*. The bands corresponding to 559 bp are derived from mRNA because the primer PCR set used for these experiments crosses four introns, and no bands were observed in the absence of RT. Similar results have been obtained using PBMCs from five different individuals (data not shown). The presence of cell surface HER2/*neu* protein on blood mononuclear cells could not be detected by conventional cytofluorometric analysis using several monoclonal antibodies specific for this antigen (data not shown). Thus, the results shown in Fig. 8 indicate that HER2/*neu* product may be expressed to some extent in T lymphocytes and monocytes. However, the amount of cell surface product may be very low, or alternatively, the protein may be degraded before it reaches the cell membrane.

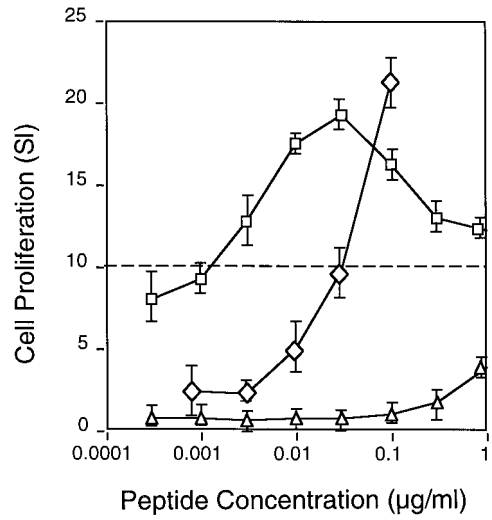

Fig. 7. Comparison of peptide titration response of TCLs specific for HBsAg and HER2/*neu*. TCLs were cultured in triplicate with peptide-pulsed autologous PBMCs in the presence of various concentrations of the corresponding synthetic peptide. ◇, TCL-8F with $HER2_{883}$; △, TCL-7D with peptide $HER2_{765}$; □, TCL-HBsAg with peptide S1d from HBsAg. Values shown are the means of duplicate determinations; *bars*, SD.

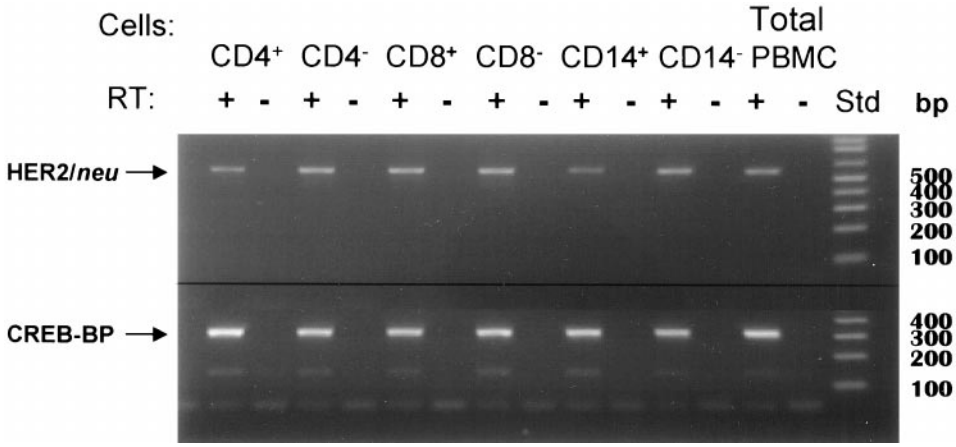

Fig. 8. Expression of HER2/*neu* in PBMCs. HER2/*neu* transcripts were detected by RT-PCR in cDNAs prepared from purified mRNAs from $CD4^+$, $CD8^+$, and $CD14^+$ cells as described in "Materials and Methods." Total PBMCs, positively selected ($CD4^+$, $CD8^+$, and $CD14^+$) and negatively selected (CD4−, CD8−, and CD14−) cells were used to prepare cDNA. RT-PCR was performed both in the presence (RT+) and absence (RT−) of RT to ensure that the PCR products were derived from RNA and not from contaminating DNA. The *bottom part* of the figure presents RT-PCR for transcripts corresponding to the cyclic AMP-responsive element binding protein (*CREB-BP*), a "housekeeping gene" that we use to assess the quality of the RNA and cDNA of the samples.

DISCUSSION

To date, the research pertaining to T-cell immunity against tumors has focused mainly on $CD8^+$ HLA class I-restricted CTL responses. Numerous MHC class I CTL epitopes have been identified for a large variety of TAAs, some expressed in melanoma (34–43) and others found in solid tumors (20–23, 44, 45). We have reported recently the identification of several CTL epitopes for HER2/*neu*, some restricted by HLA-A2 (20, 21) and others by HLA-A3 (22), which are commonly found MHC alleles. Although antitumor CTLs have been the main trend for the development of cancer immunotherapy, the importance of tumor-reactive HTLs in the development of effective immunotherapy has been stressed by several investigators (1). There is some agreement that tumor antigen-reactive HTLs are important not only for the induction of CTLs but also that these regulatory T cells may be critical for the maintenance of CTL responses (8, 9), which will be necessary for the establishment of long-lasting immune responses capable of preventing tumor relapses. In view of this, several groups have begun to search for HTL epitopes contained in known TAAs that could be used to enhance immune responses to peptide vaccination aimed toward inducing tumor-reactive CTLs. Recently, several HTL epitopes have been identified for TAAs, such as tyrosinase, MART-1, NY-ESO, and MAGE-3, which are expressed preferentially in melanomas and for p53 and MUC-1, which are overexpressed in a variety of malignancies (46–53). Herein we describe several peptides from HER2/*neu* that were capable of stimulating MHC class II-restricted HTL responses, which could be used to potentiate CTL responses to HER2/*neu* peptide-based antitumor vaccines.

A major drawback of T-cell epitope-based immunotherapy for both CTLs and HTLs is the constraint imposed by MHC restriction, which limits the use of these epitopes to the patient population that expresses the particular MHC alleles. For MHC class I CTL epitopes, the proposed strategy to broaden the population coverage by peptide epitopes has been to target those alleles that are most predominantly found in humans, such as HLA-A1, HLA-A2, HLA-A3, HLA-A24, and HLA-B7. Furthermore, this strategy has been refined by selecting peptide epitopes that can bind to more than one allele (*i.e.*, promiscuous), which usually occurs in MHC molecules that have a high degree of homology in their residues that participate in peptide-binding function. As the result, MHC molecules capable of binding peptides bearing similar anchor residues have been grouped into MHC "supertypes," which include the A2, A3/A11, and B7 supertype families (54–57). In view of these, MHC supertype-binding peptides become the choice for developing CTL epitope-based vaccines, because they offer broad population coverage.

In the case of MHC class II molecules, peptides capable of binding to more than one allele have also been described. Peptide sequences derived from natural antigens and some designer synthetic constructs have been shown to bind to numerous MHC class II molecules and to elicit HTL responses (58–62). Thus, the existence of peptide-binding promiscuity in the MHC class II system points to the possibility of identifying broadly DR-reactive HTL epitopes from TAAs, which would be advantageous for increasing population coverage and enhancing the effectiveness of T-cell epitope-based vaccines. As the result of a comprehensive study of peptides binding to purified MHC class II molecules of various alleles, Southwood *et al.* (30) reported a predictive algorithm to identify promiscuous HLA-DR binding peptides. This algorithm takes into account the role of primary MHC binding anchors at positions 1 and 6 and of secondary anchors at positions 2–5 and 7–9 for peptides of nine residues to predict their binding to the *DRB1*0101*, *DRB1*0401,* and *DRB1*0701* allelic products. A large number of peptide sequences with high algorithm values for all three alleles were shown to bind well, not only to the three above-mentioned alleles but also to other MHC class II molecules, including *DRB1*1501*, *DRB1*0901*, *DRB1*1302,* and *DRB5*0101*. Here, we have applied the Southwood algorithm (30) to predict peptide sequences from HER2/*neu* that may constitute cross-reactive (degenerate) MHC class II binders with the goal of identifying promiscuous HTL epitopes. Our results demonstrate that this approach is effective for selecting peptide sequences from TAAs, such as HER2/*neu*, that are capable of triggering HTL responses restricted to two to four different HLA-DR alleles. Furthermore, at least one of these peptides ($HER2_{883}$) was shown to trigger HTL responses restricted by two additional MHC class II molecules (DR52 and DR53), which were not included in the studies by Southwood *et al.* (30). These results suggest that the predictive algorithm may function for a larger number of alleles than thought previously.

Our studies show that the majority of the peptides that were predicted to bind to MHC class II molecules were capable of inducing primary *in vitro* HTL responses using lymphocytes from normal volunteers. The induction of HTLs required the use of DCs, which were generated from monocyte precursors with GM-CSF and IL-4, to present the peptide to $CD4^+$ T-cell precursors. Various attempts to induce HTL responses to HER2/*neu* peptides using unfractionated PBMCs (in the absence of DCs) failed, indicating that the naive precursors may require the strong costimulatory activity and high MHC/peptide complex density provided by DCs to become activated (data not shown). In contrast, it has been reported that HTLs from breast/ovarian cancer patients can respond to HER2/*neu* peptides

presented by PBMCs (presumably monocytes), suggesting that the HER2/*neu*-reactive HTLs have been primed *in vivo* (24–29).

The capacity of peptide-reactive HTLs to recognize naturally processed antigen presented by APCs has been considered as a prerequisite that the corresponding peptide represents a therapeutically relevant T-cell epitope. The lack of reactivity of peptide-reactive HTLs with APCs that process and present the protein antigen could indicate that the peptide epitope is not correctly processed and cannot be efficiently presented by class II molecules to the T cells (*i.e.*, cryptic epitopes). Alternatively, it is also possible that HTLs with low affinity for peptide may not respond to APCs presenting naturally processed antigen because of their requirement for a high number of surface peptide/MHC complexes, which can only be attained by the addition of a high concentration of exogenous peptide. Although six of the seven peptides that we tested were able to elicit peptide-reactive HTL responses, in our hands only peptide $HER2_{883}$ stimulated HTLs that reacted with protein antigen (recombinant HER2/*neu*) that was processed and presented by autologous APCs. The low affinity of the HTLs induced with peptides $HER2_{822}$, $HER2_{765}$, $HER2_{605}$, and $HER2_{62}$, which was evident by the requirement for a high peptide concentration to induce proliferation (example presented in Fig. 7), suggests that this may be the reason why these cells failed to respond to recombinant HER2/*neu* protein, even when DCs were used as APCs (data not shown). In addition to cell proliferation assays, we were not able to detect T-cell reactivity to HER2/*neu* protein presented by autologous APCs (DCs or PBMCs) using ELISA and ELISPOT assays (data not shown). From the present results, we cannot exclude the possibility that the epitopes represented by peptides $HER2_{822}$, $HER2_{765}$, $HER2_{605}$, and $HER2_{62}$ are not produced by APCs that process exogenous captured antigen. In addition, we cannot eliminate the possibility that some of the peptide-reactive TCLs that do not recognize processed HER2/*neu* protein could be T cells that recognize immunogenic contaminants that may be present at undetectable concentrations (by HPLC) in our peptide preparations.

Numerous attempts in our laboratory to isolate higher affinity HER2/*neu*-reactive HTLs (*e.g.*, by using lower concentrations of peptide for priming and restimulation) thus far have failed. By comparison, using similar conditions as the ones used here, our laboratory has been successful on numerous occasions in isolating high-affinity HTLs for foreign antigens such as HBsAg, tetanus toxoid, and rabies viral proteins, which can recognize naturally processed antigen (31–33). Thus, this raises the possibility that the high-affinity HTLs for the HER2/*neu*-derived peptide epitopes that we studied here have been rendered tolerant because this protein is expressed in low amounts by normal epithelial cells. Moreover, results presented here suggest that $CD4^+$ and $CD8^+$ T lymphocytes and monocytes ($CD14^+$ mononuclear cells) may all express some level of HER2/*neu* product, increasing the likelihood of peripheral tolerance to this antigen. Alternatively, the possibility exists that peptide-pulsed DCs are able to elicit high-affinity HER2/*neu*-reactive HTLs, but these T cells may not be readily identified because of their nonspecific (autoreactive) proliferative responses, because the autologous APCs themselves may be expressing the antigen. The high background responses (in the absence of peptide) obtained with many of the TCLs and clones support this possibility.

Whether high-, intermediate-, or low-affinity HER2/*neu*-reactive HTLs will exhibit biological (antitumor) activity *in vivo* remains to be determined. Various factors such as the amounts of HER2/*neu* protein expressed/produced by tumor cells and the effectiveness of APCs to capture and process antigen (in the form of apoptotic, necrotic tumor cells) are likely to contribute to the generation of cell surface MHC/peptide complexes, the density of which will determine whether the HTLs can respond or not to the antigenic stimulus. Lastly, the possibility that high-affinity HTL responses to HER2/*neu* *in vivo* could trigger detrimental autoimmunity must be considered, because these cells will react with APCs presenting normal levels of HER2/*neu*.

In summary, we report here the identification of several promiscuous HTL epitopes for HER2/*neu*. Because HTLs play such an important role in tumor immunity, we believe that the use of these epitopes should be considered for the design of therapeutic vaccines against tumors that overexpress the HER2/*neu*.

REFERENCES

1. Topalian, S. L. MHC class II restricted tumor antigens and the role of CD4+ T cells in cancer immunotherapy. Curr. Opin. Immunol., *6:* 741–745, 1994.
2. Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature (Lond.), *393:* 480–483, 1998.
3. Ridge, J. P., Di Rosa, F., and Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T- helper and a T-killer cell. Nature (Lond.), *393:* 474–478, 1998.
4. Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature (Lond.), *393:* 478–480, 1998.
5. Riddell, S. R., Watanabe, K. S., Goodrich, J. M., Li, C. R., Agha, M. E., and Greenberg, P. D. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science (Washington DC), *257:* 238–241, 1992.
6. Riddell, S. R., and Greenberg, P. D. Principles for adoptive T cell therapy of human viral diseases. Annu. Rev. Immunol., *13:* 545–586, 1995.
7. Heslop, H. E., Ng, C. Y., Li, C., Smith, C. A., Loftin, S. K., Krance, R. A., Brenner, M. K., and Rooney, C. M. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nat. Med., *2:* 551–555, 1996.
8. Reusser, P., Riddell, S. R., Meyers, J. D., and Greenberg, P. D. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood, *78:* 1373–1380, 1991.
9. Li, C. R., Greenberg, P. D., Gilbert, M. J., Goodrich, J. M., and Riddell, S. R. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood, *83:* 1971–1979, 1994.
10. Krensky, A. M., Clayberger, C., Reiss, C. S., Strominger, J. L., and Burakoff, S. J. Specificity of OKT4+ cytotoxic T lymphocyte clones. J. Immunol., *129:* 2001–2003, 1982.
11. Yasukawa, M., and Zarling, J. M. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. I. Lysis restricted by HLA class II MB and DR antigens. J. Immunol., *133:* 422–427, 1984.
12. Tite, J. P., and Janeway, C. A., Jr. Cloned helper T cells can kill B lymphoma cells in the presence of specific antigen: Ia restriction and cognate *vs.* noncognate interactions in cytolysis. Eur. J. Immunol., *14:* 878–886, 1984.
13. 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 (Washington DC), *244:* 707–712, 1989.
14. Yokota, J., Yamamoto, T., Toyoshima, K., Terada, M., Sugimura, T., Battifora, H., and Cline, M. J. Amplification of the c-erbB-2 oncogene in human adenocarcinomas *in vivo*. Lancet, *1:* 765–767, 1986.
15. Lustgarten, J., Theobald, M., Labadie, C., LaFace, D., Peterson, P., Disis, M. L., Cheever, M. A., and Sherman, L. A. Identification of Her-2/Neu CTL epitopes using double transgenic mice expressing HLA-A2.1 and human CD8. Hum. Immunol., *52:* 109–118, 1997.
16. Yoshino, I., Goedegebuure, P. S., Peoples, G. E., Parikh, A. S., DiMaio, J. M., Lyerly, H. K., Gazdar, A. F., and Eberlin, T. J. HER2/neu-derived peptides are shared antigens among human non-small lung cancer and ovarian cancer. Cancer Res., *54:* 3387–3390, 1994.
17. Disis, M. L., Smith, J. W., Murphy, A. E., Chen, W., and Cheever, M. A. *In vitro* generation of human cytolytic T-cells specific for peptides derived from the HER-2/*neu* protooncogene protein. Cancer Res., *54:* 1071–1076, 1994.
18. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc. Natl. Acad. Sci. USA, *92:* 432–436, 1995.
19. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/*neu* protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med., *181:* 2109–2117, 1995.
20. Kawashima, I., Hudson, S., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A., and Celis, E. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. Hum. Immunol., *59:* 1–14, 1998.
21. Kono, K., Rongcun, Y., Charo, J., Ichihara, F., Celis, E., Sette, A., Appella, E., Sekikawa, T., Matsumoto, Y., and Kiessling, R. Identification of HER2/neu-derived peptide epitopes recognized by gastric cancer-specific cytotoxic T lymphocytes. Int. J. Cancer, *78:* 202–208, 1998.
22. Kawashima, I., Tsai, V., Southwood, S., Takesako, K., Sette, A., and Celis, E. Identification of HLA-A3-restricted cytotoxic T lymphocyte epitopes from carcino-

embryonic antigen and HER-2/neu by primary *in vitro* immunization with peptide-pulsed dendritic cells. Cancer Res., *59:* 431–435, 1999.
23. Rongcun, Y., Salazar-Onfray, F., Charo, J., Malmberg, K. J., Evrin, K., Maes, H., Kono, K., Hising, C., Petersson, M., Larsson, O., Lan, L., Appella, E., Sette, A., Celis, E., and Kiessling, R. Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J. Immunol., *163:* 1037–1044, 1999.
24. Disis, M. L., Calenoff, E., McLaughlin, G., Murphy, A. E., Chen, W., Groner, B., Jeschke, M., Lyndon, N., McGlynn, E., Livingston, R. B., Moe, R., and Cheever, M. A. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res., *54:* 16–20, 1994.
25. Disis, M. L., Gralow, J. R., Bernhard, H., Hand, S. L., Rubin, W. D., and Cheever, M. A. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, an oncogenic self-protein. J. Immunol., *156:* 3151–3158, 1996.
26. Cheever, M. A., Disis, M. L., Bernhard, H., Gralow, J. R., Hand, S. L., Huseby, E. S., Qin, H. L., Takahashi, M., and Chen, W. Immunity to oncogenic proteins. Immunol. Rev., *145:* 33–59, 1995.
27. Disis, M. L., and Cheever, M. A. HER-2/neu protein: a target for antigen-specific immunotherapy of human cancer. Adv. Cancer Res., *71:* 343–371, 1997.
28. Tuttle, T. M., Anderson, B. W., Thompson, W. E., Lee, J. E., Sahin, A., Smith, T. L., Grabstein, K. H., Wharton, J. T., Ioannides, C. G., and Murray, J. L. Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. Clin. Cancer Res., *4:* 2015–2024, 1998.
29. Fisk, B., Hudson, J. M., Kavanagh, J., Wharton, J. T., Murray, J. L., Ioannides, C. G., and Kudelka, A. P. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res., *17:* 45–53, 1997.
30. Southwood, S., Sidney, J., Kondo, A., del Guercio, M. F., Appella, E., Hoffman, S., Kubo, R. T., Chesnut, R. W., Grey, H. M., and Sette, A. Several common HLA-DR types share largely overlapping peptide binding repertoires. J. Immunol., *160:* 3363–3373, 1998.
31. Celis, E., Ou, D., and Otvos, L., Jr. Recognition of hepatitis B surface antigen by human T lymphocytes. Proliferative and cytotoxic responses to a major antigenic determinant defined by synthetic peptides. J. Immunol., *140:* 1808–1815, 1988.
32. Celis, E., Kung, P. C., and Chang, T. W. Hepatitis B virus-reactive human T lymphocyte clones: antigen specificity and helper function for antibody synthesis. J. Immunol., *132:* 1511–1516, 1984.
33. Celis, E., Ou, D., Dietzschold, B., and Koprowski, H. Recognition of rabies and rabies-related viruses by T cells derived from human vaccine recipients. J. Virol., *62:* 3128–3134, 1988.
34. Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., Van Pel, A., De Plaen, E., Amar-Costesec, A., and Boon, T. A nonapeptide encoded by human gene *MAGE-1* is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J. Exp. Med., *176:* 1453–1457, 1992.
35. Wolfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer, Z. B. 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.
36. Coulie, P. G., Brichard, V., Van Pel, A., Wàlfel, T., Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurkin, C., Szikora, J. P., Renauld, J. C., and Boon, T. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med., *180:* 35–42, 1994.
37. Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P. F., Rivoltini, L., Yannelli, J. R., Appella, E., and Rosenberg, S. A. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J. Exp. Med., *180:* 347–352, 1994.
38. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T., and Rosenberg, S. A. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. Proc. Natl. Acad. Sci. USA, *91:* 6458–6462, 1994.
39. Cox, A. L., Skipper, J., Chen, Y., Henderson, R. A., Darrow, T. L., Shabanowitz, J., Engelhard, V. H., Hunt, D. F., and Slingluff, C. L., Jr. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science (Washington DC), *264:* 716–719, 1994.
40. Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P. A., Chesnut, R. W., Grey, H. M., Sette, A., and Serra, H. M. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc. Natl. Acad. Sci. USA, *91:* 2105–2109, 1994.
41. Tsai, V., Southwood, S., Sidney, J., Sakaguchi, K., Kawakami, Y., Appella, E., Sette, A., and Celis, E. Identification of subdominant CTL epitopes of the gp100 melanoma-associated tumor antigen by primary *in vitro* immunization with peptide-pulsed dendritic cells. J. Immunol., *158:* 1796–1802, 1997.
42. Kawashima, I., Tsai, V., Southwood, S., Takesako, K., Celis, E., and Sette, A. Identification of gp100-derived, melanoma-specific cytotoxic T-lymphocyte epitopes restricted by HLA-A3 supertype molecules by primary *in vitro* immunization with peptide-pulsed dendritic cells. Int. J. Cancer, *78:* 518–524, 1998.
43. Tahara, K., Takesako, K., Sette, A., Celis, E., Kitano, S., and Akiyoshi, T. Identification of a MAGE-2-encoded human leukocyte antigen-A24-binding synthetic peptide that induces specific antitumor cytotoxic T lymphocytes. Clin. Cancer Res., *5:* 2236–2241, 1999.
44. Nukaya, I., Yasumoto, M., Iwasaki, T., Ideno, M., Sette, A., Celis, E., Takesako, K., and Kato, I. Identification of HLA-A24 epitope peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. Int. J. Cancer, *80:* 92–97, 1999.
45. Alexander, R. B., Brady, F., Leffell, M. S., Tsai, V., and Celis, E. Specific T cell recognition of peptides derived from prostate-specific antigen in patients with prostate cancer. Urology, *51:* 150–157, 1998.
46. Manici, S., Sturniolo, T., Imro, M. A., Hammer, J., Sinigaglia, F., Noppen, C., Spagnoli, G., Mazzi, B., Bellone, M., Dellabona, P., and Protti, M. P. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. J. Exp. Med., *189:* 871–876, 1999.
47. Chaux, P., Vantomme, V., Stroobant, V., Thielemans, K., Corthals, J., Luiten, R., Eggermont, A. M., Boon, T., and van der Bruggen, P. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes. J. Exp. Med., *189:* 767–778, 1999.
48. Kobayashi, H., Kokubo, T., Sato, K., Kimura, S., Asano, K., Takahashi, H., Iizuka, H., Miyokawa, N., and Katagiri, M. $CD4^+$ T cells from peripheral blood of a melanoma patient recognize peptides derived from nonmutated tyrosinase. Cancer Res., *58:* 296–301, 1998.
49. Zarour, H. M., Kirkwood, J. M., Kierstead, L. S., Herr, W., Brusic, V., Slingluff, C. L., Jr., Sidney, J., Sette, A., and Storkus, W. J. Melan-A/MART-1(51–73) represents an immunogenic HLA-DR4-restricted epitope recognized by melanoma-reactive CD4(+) T cells. Proc. Natl. Acad. Sci. USA, *97:* 400–405, 2000.
50. Jager, E., Jager, D., Karbach, J., Chen, Y. T., Ritter, G., Nagata, Y., Gnjatic, S., Stockert, E., Arand, M., Old, L. J., and Knuth, A. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-*DRB4*0101–0103* and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. J. Exp. Med., *191:* 625–630, 2000.
51. Fujita, H., Senju, S., Yokomizo, H., Saya, H., Ogawa, M., Matsushita, S., and Nishimura, Y. Evidence that HLA class II-restricted human CD4+ T cells specific to p53 self peptides respond to p53 proteins of both wild and mutant forms. Eur. J. Immunol., *28:* 305–316, 1998.
52. Pieper, R., Christian, R. E., Gonzales, M. I., Nishimura, M. I., Gupta, G., Settlage, R. E., Shabanowitz, J., Rosenberg, S. A., Hunt, D. F., and Topalian, S. L. Biochemical identification of a mutated human melanoma antigen recognized by CD4(+) T cells. J. Exp. Med., *189:* 757–766, 1999.
53. Hiltbold, E. M., Ciborowski, P., and Finn, O. J. Naturally processed class II epitope from the tumor antigen MUC1 primes human $CD4^+$ T cells. Cancer Res., *58:* 5066–5070, 1998.
54. Kondo, A., Sidney, J., Southwood, S., del Guercio, M. F., Appella, E., Sakamoto, H., Grey, H. M., Celis, E., Chesnut, R. W., Kubo, R. T., and Sette, A. Two distinct HLA-*A*0101*-specific submotifs illustrate alternative peptide binding modes. Immunogenetics, *45:* 249–258, 1997.
55. Sidney, J., Grey, H. M., Southwood, S., Celis, E., Wentworth, P. A., del Guercio, M. F., Kubo, R. T., Chesnut, R. W., and Sette, A. Definition of an HLA-A3-like supermotif demonstrates the overlapping peptide-binding repertoires of common HLA molecules. Hum. Immunol., *45:* 79–93, 1996.
56. Sidney, J., Grey, H. M., Kubo, R. T., and Sette, A. Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs. Immunol. Today, *17:* 261–266, 1996.
57. Sidney, J., del Guercio, M. F., Southwood, S., Engelhard, V. H., Appella, E., Rammensee, H. G., Falk, K., Rotzschke, O., Takiguchi, M., Kubo, R. T., *et al.* Several HLA alleles share overlapping peptide specificities. J. Immunol., *154:* 247–259, 1995.
58. Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewal, A., Snoke, K., Serra, H. M., Kubo, R. T., Sette, A., *et al.* Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity, *1:* 751–761, 1994.
59. Sette, A., Adorini, L., Appella, E., Colon, S. M., Miles, C., Tanaka, S., Ehrhardt, C., Doria, G., Nagy, Z. A., Buus, S., *et al.* Structural requirements for the interaction between peptide antigens and I-Ed molecules. J. Immunol., *143:* 3289–3294, 1989.
60. Sinigaglia, F., Guttinger, M., Kilgus, J., Doran, D. M., Matile, H., Etlinger, H., Trzeciak, A., Gillessen, D., and Pink, J. R. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. Nature (Lond.), *336:* 778–780, 1988.
61. Busch, R., Strang, G., Howland, K., and Rothbard, J. B. Degenerate binding of immunogenic peptides to HLA-DR proteins on B cell surfaces. Int. Immunol., *2:* 443–451, 1990.
62. Hill, C. M., Liu, A., Marshall, K. W., Mayer, J., Jorgensen, B., Yuan, B., Cubbon, R. M., Nichols, E. A., Wicker, L. S., and Rothbard, J. B. Exploration of requirements for peptide binding to HLA DRB1*0101 and DRB1*0401. J. Immunol., *152:* 2890–2898, 1994.

Defining Promiscuous MHC Class II Helper T-Cell Epitopes for the HER2/ *neu* Tumor Antigen

Hiroya Kobayashi, Monique Wood, Yongsheng Song, et al.

Cancer Res 2000;60:5228-5236.