

A Polyepitope DNA Vaccine Targeted to Her-2/ErbB-2 Elicits a Broad Range of Human and Murine CTL Effectors to Protect against Tumor Challenge

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Abstract

A cDNA vaccine (pVax1/pet-neu) was designed to encode 12 different Her-2/ErbB-2-derived, HLA-A*0201-restricted dominant and high-affinity heteroclitic cryptic epitopes. Vaccination with pVax1/pet-neu triggered multiple and ErbB-2-specific CTL responses in HLA-A*0201 transgenic HHD mice and in HLA-A*0201 healthy donors *in vitro*. Human and murine CTL specific for each one of the 12 ErbB-2 peptides recognized *in vitro* both human and murine tumor cells overexpressing endogenous ErbB-2. Furthermore, vaccination of HHD mice with pVax1/pet-neu significantly delayed the *in vivo* growth of challenged ErbB-2-expressing tumor (EL4/HHD/neu murine thymoma) more actively when compared with vaccination with the empty vector (pVax1) or vehicle alone. These data indicate that the pVax1/pet-neu cDNA vaccine coding for a poly-ErbB-2 epitope is able to generate simultaneous ErbB-2-specific antitumor responses against dominant and cryptic multiple epitopes. [Cancer Res 2007;67(14):7028–36]

Introduction

The human *erbB-2* proto-oncogene codifies for a transmembrane protein (Her-2/ErbB-2) member of the epidermal growth factor receptor family with tyrosine kinase activity whose ligand is still unknown. It is commonly overexpressed in several human tumors, including breast, ovarian, and lung carcinomas (1–3). Overexpression of ErbB-2 is associated with aggressive disease and poor prognosis (4, 5). ErbB-2-specific antibodies and tumor-infiltrating CTL occur naturally in breast, renal, and ovarian cancer patients, confirming the *in vivo* immunogenicity and ability of ErbB-2 to break self-tolerance (6–10). To date, multiple ErbB-2 epitopes presented by HLA-A*0201 or HLA-A*0301 have been identified and used as targets of specific CTL responses (9–18).

Antitumor vaccination strategies have been taking many forms, including free peptides, dendritic cells loaded with peptides or tumor lysates, and DNA-based vaccines. Although the strategies of

vaccination with peptides are very attractive over other forms for the feasibility, many studies using native unmodified peptides resulted in low-level responses showing a high variability from one patient to another (9–23). Active immunotherapy strategies aimed to generate specific T-cell responses are currently being investigated in animal models or in clinical trials (24, 25). To date, increasingly powerful methods to stimulate antitumor immune responses have been developed into rat Her-2/Neu transgenic mice, including vaccination with electroporated DNA coding for the same transgene product (rat-Her-2/neu) or with “foreign” antigens through plasmid DNA xeno-immunization expressing both extracellular and transmembrane domains of the human *erbB-2* oncogene product (26, 27). Yet, the stringent role of humoral or cell-mediated immune response in *in vivo* tumor inhibition was not completely clarified; however, depletion of CD8⁺ T cells in ErbB-2 transgenic mice before vaccination accelerated tumor outgrowth (28, 29). The major limits of such models remain the presence of the *erbB-2* transgene during ontogenesis for immune tolerance shaping and the fact that DNA vaccines coding whole proteins from extracellular and transmembrane domains of the receptor might produce unpredictable autoimmune side effects in humans (30).

To overcome epitope variability among peptide presentation of tumors and to reduce the risk of tolerance toward the ErbB-2 high-affinity epitope peptides, we explored the possibility to mobilize *in vivo* a large ErbB-2-derived T-cell epitope repertoire in HLA-A*0201 transgenic mice and in an *in vitro* human model, wherein human peripheral blood mononuclear cells (PBMC) were used to trigger a multiple antitumor CTL response aimed to target simultaneously the HLA-A*0201-restricted dominant and cryptic epitopes of the human ErbB-2 protein.

Here, we present a cDNA vaccine (pVax1/pet-neu) encoding 12 ErbB-2-derived, HLA-A*0201-restricted dominant and cryptic epitopes. The pVax1/pet-neu vaccine was able to trigger a polyspecific CTL response in HLA-A*0201 transgenic HHD mice and induce an ErbB-2-specific antitumor immunity *in vivo*. Moreover, pVax1/pet-neu stimulated a marked polyspecific CTL response when used to sensitize human PBMC *in vitro*.

Materials and Methods

Mice. The HLA-A*0201 transgenic HHD mice were previously described (31). All *in vivo* experiments were done in accordance with the Institut Gustave Roussy ethical guidelines.

Note: A. Scardino and M. Alimandi contributed equally to this work.

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Cell culture. Breast carcinoma cell line MCF-7-T103 (HLA-A2.1⁺/ErbB-2⁺) was purchased from the American Type Culture Collection. C1R is a human lymphoblastic cell line which is defective in the synthesis of class I HLA molecules. C1R-A2, conversely, is a derivative subline stably transfected with the HLA-A(*02:01) gene, which expresses large amounts of empty HLA-A(*02:01) on the cell membranes and does not express other class I HLA molecules (32). These cells were provided by Dr. Jeffrey Schlom (Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD). RMA5/HHD and EL4/HHD cells (mouse thymoma^{-/-} for murine MHCs and transduced with HHD construction) were previously described (33). EL4/HHD/ErbB-2 cells were obtained by transfection of EL4/HHD cells with a long terminal repeat-2/*erbB-2*-gpt expression retroviral vector encoding for the human ErbB-2. The ErbB-2 expression was verified by immunoblot and fluorescence-activated cell sorting analysis using the ErbB-2-specific C-18 monoclonal antibody (mAb; Calbiochem). EL4/HHD/Tel-AML transfectants [Tel/Aml is a t(12;21) translocation critical for B-cell leukemogenesis; ref. 34] were a kind gift of Dr. P. Langlade-Demoyen (Institut Pasteur, Paris, France). Tumor cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (all purchased from Life Technologies, Inc.).

Peptides. Peptides were synthesized by Syntem (Nimes, France). They were dissolved in PBS, 3% DMSO and stored at -20°C. Sequences of the human ErbB-2 peptides are illustrated in Fig. 1A.

Measurement of peptide/HLA-A*0201 relative affinity and stability. A protocol used to measure relative affinity has previously been described (35). Briefly, T2 cells were incubated with various concentrations of peptides (0.1–100 µmol/L) for 16 h and then stained with the BB7.2 mAb to quantify the expression of HLA-A*0201. For each peptide concentration, the HLA-A*0201-specific staining was calculated as the percentage of the staining obtained with 100 µmol/L of the reference peptide HIVpol₅₈₉ (IVGAETFYV). Relative affinity was determined as concentration of each peptide / concentration of the reference peptide that was able to induce 20% of HLA-A*0201 expression. For peptide/HLA-A*0201 complex stability measure, T2 cells were incubated overnight with 100 µmol/L of each peptide at 37°C. Cells were then treated with brefeldin A for 1 h; washed; incubated at 37°C for 0, 2, 4, and 6 h; and then stained with the BB7.2 mAb. DC₅₀ was defined as the time required for the loss of 50% of the HLA-A*0201, as previously described (35).

Design and construction of the pVax1/pet-neu polyepitope. A polyepitope cDNA (pet-neu) was designed using optimized codons from the human *erbB-2* sequence and incorporating the following elements from the 5' end: a *Bam*HI restriction site, a Kozak consensus start sequence, 12 consecutive epitopes, a tag epitope recognized by the SV5-pk (pk) mAb, an *Xba*I restriction site to facilitate future modification, a translational stop codon, and an *Eco*RI restriction site at the 3' end. To construct pet-neu (404 bp), six oligonucleotides were synthesized (Imperial Cancer Research Fund Oligonucleotide Synthesis Service, Leeds, United Kingdom) that overlapped by 20 bp and together covered the polyepitope sequences. Polyepitope cDNAs were assembled by PCR. Adjacent primers were dimerized (1 µg of each) in separate PCR reactions containing 1 unit of Deep Vent DNA polymerase, 1× thermopol buffer, and 200 µmol/L deoxynucleotide triphosphates (New England Biolabs, Inc.) in a final volume of 100 µL overlaid with 40 µL of mineral oil (Sigma). A hot start PCR reaction was carried out at 94°C, during which time primers and DVP were added. The cycling conditions were 94°C for 20 s, 42°C for 30 s, 72°C for 30 s, five cycles. Reactions were held at 72°C and 20 µL of adjacent primer dimer reactions were combined before a further five cycles of amplification. The third primer dimer reaction (20 µL) was then added to the second primer dimer combination for the final five cycles. PCR products from the oligonucleotide splicing by overlap extension steps were used as templates for a final PCR reaction using terminal primers complementary to the polyepitope. The final PCR reaction product was purified by electrophoresis, cloned into pVAX1 (Invitrogen), and recombinant plasmids examined for mutation by DNA sequencing.

CTL generation in vaccinated HHD mice. For peptide *in vivo* priming, HHD mice were injected s.c. with 100 µg of peptide emulsified in

incomplete Freund's adjuvant in the presence of 140 µg of the IA^b-restricted HBVcore₁₂₈ T-helper (Th) epitope. After 11 days, spleen cells (5 × 10⁷ in 10 mL) were then stimulated *in vitro* with cognate peptide (10 µmol/L). On day 6 of culture, the bulk responder populations were tested for specific cytotoxicity. For pVax1/pet-neu *in vivo* priming, HHD mice were injected i.m. with 10 µmol/L cardiotoxin (Latoxan) in 50-µL PBS. Twenty-five days later, mice were injected i.m. with 100 µg of pVax1/pet-neu. Spleen cells were collected 1 week after the second vaccination and stimulated with lipopolysaccharide (LPS) blasts loaded with the cognate peptide (splenocytes/LPS blasts ratio, 3:1) in the presence of 10% T-cell growth factor-enriched supernatant. Effector CTL were tested after four cycles of *in vitro* stimulation. Positive responses were considered when peptide-specific lysis was at least 10% above the background level (RMA5/HHD targets pulsed with the irrelevant HIVgag₇₆ peptide).

ELISPOT assay. Peptide-specific T cells from vaccinated mice were counted by IFN-γ ELISpot (36). IFN-γ-secreting cells were counted using the automated image analysis system ELISpot Reader (AID). The Wilcoxon two-tail rank test was done to determine whether there was a statistically significant difference between the numbers of IFN-γ-secreting cells in the wells stimulated with the ErbB-2- or the HIVgag₇₆-derived peptides.

Generation of human CTL. Blood samples were obtained from normal healthy donors. Dendritic cells were obtained by stimulation of adherent cells from PBMC with 50 ng/mL granulocyte macrophage colony-stimulating factor and 0.5 ng/mL IL-4 (both purchased from R&D Systems) for 7 days. The PBMC for CTL primary cultures were washed thrice in Dulbecco's PBS and then resuspended in AIM-V medium (Life Technologies), supplemented with 5% pooled human AB serum (Valley Biomedical), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). Cells (2 × 10⁵) in a 100-µL volume of complete medium were added to each well of a 96-well flat-bottomed assay plate (Corning, Costar Corp.). One hundred thousand dendritic cells were grown in six-well microplates at 37°C and transfected with 1 µg of pVax1/pet-neu using the Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Transfected dendritic cells were irradiated and added to the lymphocyte cultures at a final dendritic cell/PBMC ratio of 1:5. The cocultures were then incubated for 5 days at 37°C in a humidified atmosphere containing 5% CO₂. The cultures were fed with 20 IU/mL human interleukin-2 (IL-2; Cetus Corp.) for 10 days and IL-2-containing medium was replenished every 3 days. Incubation for 5 days with pVax1/pet-neu-pulsed dendritic cells plus 10 days with IL-2 constitutes one *in vitro* stimulation cycle. Cytotoxicity was tested after four *in vitro* stimulation cycles.

Cytotoxicity assay. Targets were labeled with 150 µCi of ⁵¹Cr, plated in 96-well round-bottomed plates (10⁴ per well in 100 µL of RPMI 1640 + 3% FCS), and, when necessary, loaded with the peptide (1 µmol/L) for 90 min. Effectors were added to targets at different ratios and plates were incubated at 37°C for 6 h. After incubation, supernatants were collected and radioactivity was measured in a gamma counter. Percentage of specific lysis was calculated as (experimental spontaneous / maximal spontaneous ⁵¹Cr release) × 100.

Vaccination and analysis of antitumor activity *in vivo*. HHD mice were vaccinated twice either with pVax1/pet-neu or with the empty pVax1 at 2-week intervals or with the vehicle PBS. Ten days after the second vaccination, mice were challenged with 2 × 10⁴ EL4/HHD/Tel-Aml or EL4/HHD/ErbB-2 cells. For each immunogen, groups of nine HHD mice were vaccinated. The number of injected tumor cells was previously defined as 2-fold the minimal dose that gives 100% of tumors in HHD mice. Tumor volumes were measured by a caliper in two dimensions, and the volumes were calculated using the formula (width² × length) / 2. Animals were monitored twice a week for the development of palpable tumors. Mice were sacrificed when they showed any symptom of very sickness, hard to breathe or rare movement, or when their tumors grew larger than 1,000 mm³ to avoid unnecessary suffering. Survival was recorded until 65 days. All *in vivo* experiments were done in accordance with ethical guidelines of the Institut Gustave Roussy.

Statistical analysis. Statistical significance of median survival was determined by survival data by Mantel-Haenszel log-rank test. Differences

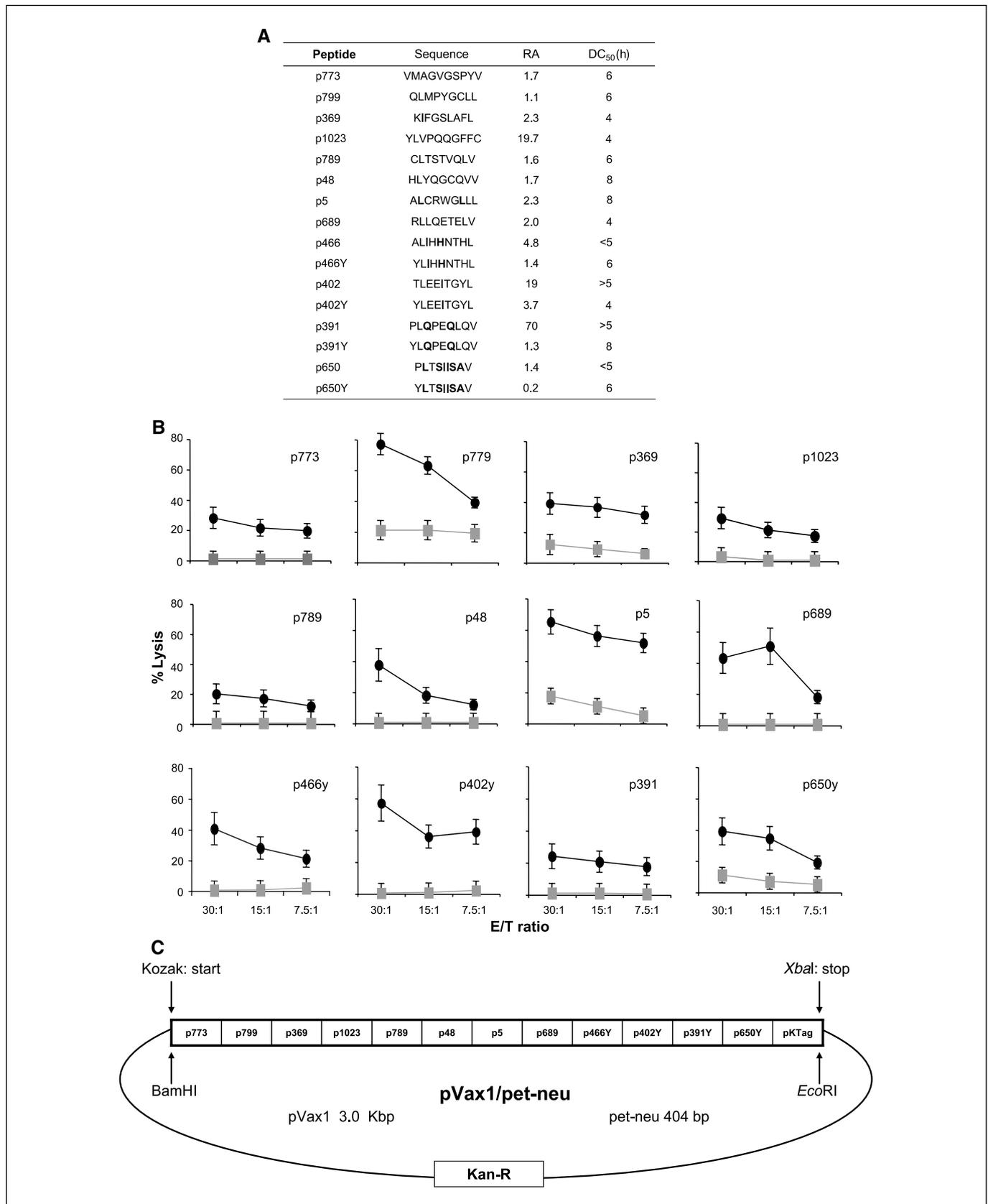


Figure 1. A, affinity for HLA-A*0201 of ErbB-2 peptides (RA, relative affinity; DC₅₀, 50% of stably bound peptide to MHC molecules at given time). In boldface are amino acids differing from Her-2 murine sequence. B, generation of specific CTL peptide-vaccinated HHD mice. Spleen cells from peptide-primed HHD mice were *in vitro* stimulated as described in Materials and Methods. CTL activity was tested against RMAS/HHD cells loaded with the HIVgag₇₆ (■) or the cognate ErbB-2 native (●) peptides. E/T ratio, effector/target ratio. Bars, SD. C, design of the pVax1/pet-neu polyepitope.

Table 1. Polyspecific anti-ErbB-2 response generated in HHD mice vaccinated with pVax1/pet-neu

Peptide	pVax1/pet-neu											pVax1			
	Mouse no.											Mouse no.			
	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4
p369	49	65	112	73	51	24	68	14	46	8	12	5	6	26	9
p5	60	56	113	166	25	48	8	45	68	23	9	11	9	19	10
p48	21	79	121	76	14	36	17	27	26	20	68	8	10	21	9
p1023	20	116	111	97	16	19	16	25	59	46	15	9	5	14	7
p773	7	39	119	57	6	6	4	58	7	19	38	10	7	15	11
p689	8	110	121	28	27	52	55	55	9	53	45	8	11	20	9
p789	0	80	42	0	46	9	23	9	33	24	28	9	12	19	6
p799	29	39	110	131	13	19	27	15	10	31	17	7	8	14	9
p650Y	1	40	99	39	27	40	35	36	22	40	40	5	10	15	7
p402Y	29	88	144	144	11	37	23	6	36	24	80	13	8	14	6
p466Y	30	57	100	43	7	4	27	5	24	61	75	10	10	13	8
p391Y	37	96	119	42	16	51	39	37	19	20	67	9	7	18	6
HIVgag76	6	31	75	4	4	4	3	4	3	5	4	7	9	15	7

NOTE: Results represent the mean of IFN- γ -producing cells/ 10^5 CD8 cells. Means in boldface are statistically significant compared with HIVgag76 ($P < 0.05$, Wilcoxon two-tail rank test).

in average tumor volume were evaluated by a two-tailed t test, whereas statistical differences in tumor incidence at defined time points were evaluated using the Fisher exact test. The Wilcoxon two-tail rank test was done to determine whether there was a statistically significant difference between the numbers of IFN- γ -secreting cells in the wells stimulated with the ErbB-2- or the HIVgag₇₆-derived peptides.

Results

Affinity for HLA-A*0201 and immunogenicity of ErbB-2 peptides. Twelve HLA-A*0201-restricted ErbB-2 epitopes were included in the polyepitopic construct (pet-neu; Fig. 1A). Eight of them (p5, p48, p369, p689, p773, p789, p799, and p1023) were described to be targets of tumor-infiltrating lymphocytes in breast, ovarian, and gastric cancers and renal cell carcinoma (8–11, 15). They exhibited high affinity for HLA-A*0201 (relative affinity <5 ; DC₅₀ >4 h) except p1023, which is considered an intermediate binder (relative affinity >5 ; DC₅₀ >4 h). All epitopes show full homology with murine Her-2, with the exception of ErbB peptide epitope p5, chosen as internal positive control for tolerance. The remaining four epitopes (p391, p402, p466, and p650) were recently described to be cryptic ErbB-2 epitopes (17). These epitopes had a DC₅₀ <2 but they varied in their relative affinity (p391 and p402 had a relative affinity <5 whereas p466 and p650 had a relative affinity >5). Moreover, they were nonimmunogenic in both humans and HHD mice (17, 35). They were therefore used as high-affinity heteroclitic variants having a tyrosine at the first position (PIY variants) as previously described (ref. 17; Fig. 1A). The immunogenicity of the dominant and the heteroclitic PIY variants was verified in HHD mice. Spleen cells from peptide-vaccinated mice were *in vitro* restimulated with peptide and then tested for their ability to kill RMAS/HHD cells coated with the native ErbB-2 peptides or the negative control HIVgag₇₆ peptide. Results show no evidence of tolerance toward dominant epitopes per se when injected as ErbB-2 minimal essential epitopes. Specific CTL response was triggered for all ErbB-2 epitopes

injected, and CTL generated by the heteroclitic variants recognized the corresponding native epitopes (Fig. 1B). Thus, such peptide epitope sequences have been included in the pVax1-pet-neu construction design (Fig. 1C).

Induction of CTL in HHD mice vaccinated with pVax1/pet-neu. The pet-neu DNA was synthesized and cloned into the DNA vaccine vector pVax1. The SV5-pk tag was included at the 3' end of the polyepitope to enable study of the expression of the pet-neu protein. In fact, COS-7 cells transfected with pVax1/pet-neu expressed the pet-neu as assessed by confocal microscopy (data not shown). To determine whether the polyepitopic construct could prime ErbB-2-specific CTL *in vivo*, we vaccinated HHD mice either with pVax1/pet-neu (eleven mice) or with the empty pVax1 (four mice). CTL responses against each one of the 12 ErbB-2 epitopes were evaluated by IFN- γ ELISpot assay done *ex vivo* for each vaccinated mouse (Table 1). Peptide-specific, IFN- γ -producing cells were not detected in pVax1-vaccinated mice. Conversely, multiepitope responses were induced in pVax1/pet-neu-primed mice. These responses were different from one mouse to another and were simultaneously directed against 8 to 11 epitopes. Specific responses to peptides p48, p1023, and p391 were observed in all the tested mice, whereas other peptides were recognized only by variable percentages of mice. In a second set of experiments, spleen cells from three pVax1/pet-neu-vaccinated mice were *in vitro* stimulated with LPS-treated blasts loaded with each peptide and CTL were tested against peptide pulsed RMAS/HHD target cells. Positive responses were considered when peptide-specific lysis was at least 10% above the background level (RMAS/HHD targets pulsed with the irrelevant HIVgag₇₆ peptide). In agreement with the ELISpot results, each mouse generated CTL against the majority of peptides but the response varied from one mouse to another (Fig. 2). Taken together, these results show that vaccination with pVax1/pet-neu triggers a polyspecific CTL response *in vivo*.

pVax1/pet-neu-induced CTL recognize the naturally processed epitopes on ErbB-2-expressing tumors. We next wanted

to evaluate the *in vitro* antitumor ability of the multiple CTL response induced in pVax1/pet-neu-primed mice using the syngeneic murine tumor cell line EL4/HHD expressing human ErbB-2 (EL4/HHD/ErbB-2) or Tel-Aml (EL4/HHD/Tel-Aml) as target cells. Figure 3A shows the CTL activity from 2 representative HHD mice of the 11 vaccinated with pVax1/pet-neu; results indicate that EL4/HHD/ErbB-2 cells were killed by CTL specific for ErbB-2 peptides, whereas EL4/HHD/Tel-Aml cells were not, showing specific CTL being able to recognize the corresponding natural epitopes. To show that generation of ErbB-2-specific CTL was due to the *in vivo* priming with pVax1/pet-neu and not to the repetitive *in vitro* stimulation with peptide-loaded LPS-activated spleen cells, we vaccinated mice with either pVax1/pet-neu or the empty pVax1 and stimulated pooled spleen cells from each group

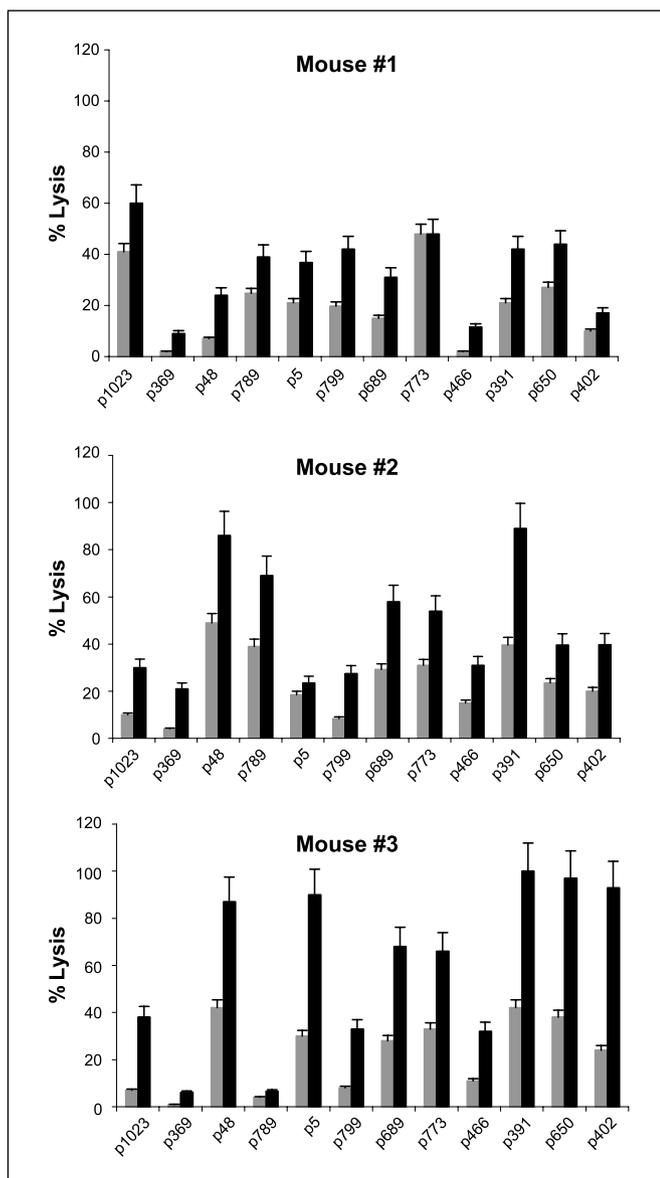


Figure 2. Generation of peptide-specific CTL in pVax1/pet-neu-vaccinated HHD mice. Spleen cells from pVax1/pet-neu-vaccinated mice were *in vitro* stimulated with peptide-loaded LPS blasts; CTL effectors were tested after the fourth restimulation against RMA/SH2 targets pulsed with the irrelevant HIVgag₇₆ (gray columns) or the cognate native ErbB-2 peptides (black columns) at an effector/target ratio of 40:1. Bars, SD.

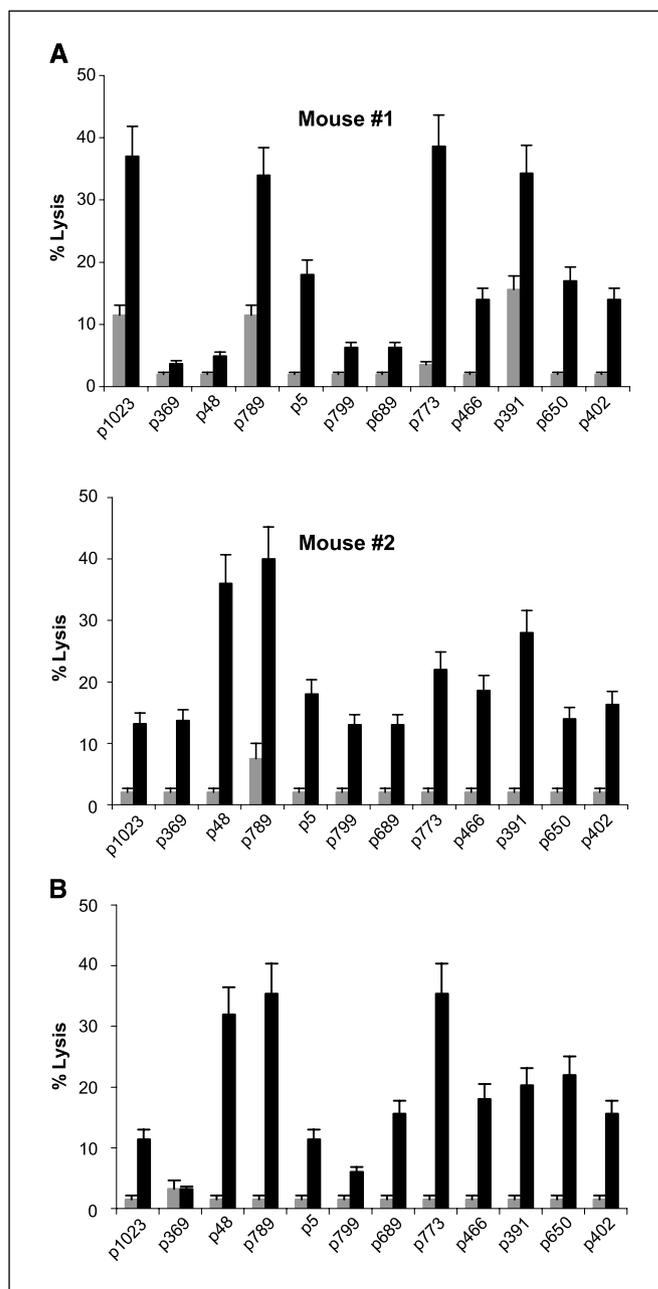


Figure 3. Recognition of endogenous ErbB-2 by CTL induced in pVax1/pet-neu-vaccinated mice. Peptide-specific CTL generated in pVax1/pet-neu-vaccinated mice were tested against EL4/HHD/neu (black columns) and EL4/HHD/Tel-Aml (gray columns) targets at an E/T ratio of 40:1. Bars, SD. Spleen cells from HHD mice vaccinated with pVax1/pet-neu (black columns) or empty pVax1 (gray columns) were *in vitro* restimulated with peptide-loaded LPS blasts and tested for cytotoxicity against EL4/HHD/neu and EL4/HHD/Tel-Aml at an E/T ratio of 40:1. Results represent the ErbB-2-specific lysis (lysis of EL4/HHD/ErbB-2 cells – lysis of EL4/HHD/Tel-Aml cells). Bars, SD.

with LPS-generated blasts pulsed with peptide in the same conditions. Results in Fig. 3B show that *in vitro* stimulated spleen cells from pVax1/pet-neu mice developed ErbB-2-specific CTL whereas *in vitro* stimulated spleen cells from mice vaccinated with pVax1 vector alone did not.

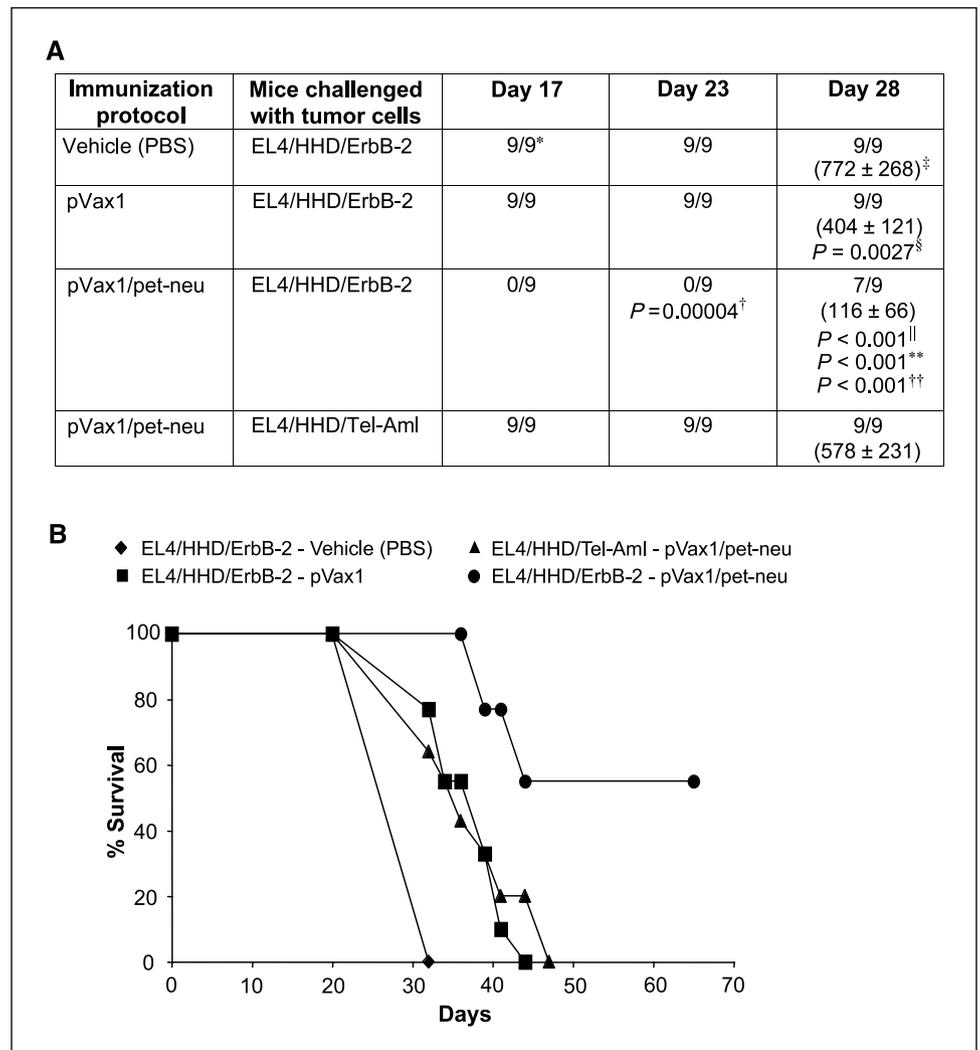
Antitumor protective immunity in pVax1/pet-neu immunized mice. Next, we determined whether the administration of the pVax1/pet-neu polyepitope vaccine could prevent the

establishment of ErbB-2-expressing tumor transplants. Ten days after the second vaccination with the pVax1/pet-neu or pVax1 plasmid, HDD mice were challenged with 2×10^4 EL4/HHD/ErbB-2 cells. To test the specificity of the antitumor immune response, a group of pVax1/pet-neu-vaccinated mice challenged with 2×10^4 EL-4/HHD/Tel-Aml cells was added. Tumor growth was monitored twice a week until day 32 when all the unvaccinated mice died or were sacrificed. Mice of the remaining groups were followed for time survival. Results of tumor growth are shown in Fig. 4A. By day 17, tumor overexpressing ErbB-2 had developed in all mice vaccinated with the control vector or vehicle alone. By contrast, none of the mice immunized with the pVax1/pet-neu vaccine exhibited tumor growth. In this group of vaccinated mice, tumor protection continued through day 23 in all animals ($P = 0.00004$). Conversely, tumor growth of EL-4/HHD/Tel-Aml cells was not affected by the pVax1/pet-neu vaccination. These results indicated the appearance of an anti-ErbB-2-specific immune interference with tumor growth elicited by the pVax1/pet-neu immunization. At day 28, tumors developed also in seven of the nine pVax1/pet-neu-vaccinated mice. However, the tumors developed in these animals (116 ± 66) were smaller than those observed in mice immunized with vehicle only (772 ± 268 ; $P < 0.001$) or with vector alone (404 ± 121 ; $P < 0.001$). It is worth noting that at this stage of

tumor development, vaccination with pVax1 vector alone affected tumor growth with respect to that with vehicle alone ($P = 0.0027$). The *in vivo* antitumor protective immunity elicited by the pVax1/pet-neu immunization was specific for ErbB-2 because vaccinated mice were not protected against tumor cells lacking ErbB-2 (EL4/HHD/Tel-Aml tumor cell line). All these mice developed tumors by day 17 and the average of tumor volume measured at day 28 was 578 ± 231 ($P < 0.001$). Furthermore, pVax1/pet-neu vaccination increased the survival of tumor-bearing mice. Results are presented in Fig. 4B. Whereas unvaccinated mice died at day 32, the mean survival for the pVax1-vaccinated mice was 37.3 ± 4.4 days ($P = 0.04$, compared with unvaccinated mice). The time of survival was significantly improved in the group of pVax1/pet-neu-vaccinated mice; five of nine mice were still alive at day 65 ($P = 0.02$ and $P = 0.0007$, compared with pVax1 and PBS immunized mice, respectively). Overall, these results showed that interference with tumor growth of cells overexpressing ErbB-2 was obtained by active immunization with pVax1/pet-neu.

Human ErbB-2-specific CTL response to pVax1/pet-neu. The pVax1/pet-neu vaccine capacity to mobilize a human T-lymphocyte repertoire specific for the epitopes encoded in the construction was investigated by CTL priming with pVax1/pet-neu-transduced dendritic cells. CTL lines were established

Figure 4. A, effects of immunization with pVax1/pet-neu on the growth of EL4/HHD cells transduced with the cDNA encoding for ErbB-2. *, number of mice with tumors/total number of mice; †, pVax1/pet-neu-vaccinated mice versus the other groups of immunized mice (Fisher's exact test, two-tailed analysis); ‡, average tumor volume (mm^3) \pm SD; §, average tumor volume of pVax1-vaccinated mice versus vehicle (Student's *t* test); ||, average tumor volume of pVax1/pet-neu-vaccinated mice versus vehicle (Student's *t* test); **, average tumor volume of pVax1/pet-neu versus pVax1-vaccinated mice (Student's *t* test); ††, average tumor volume of pVax1/pet-neu-vaccinated mice challenged with EL4/HHD/ErbB-2 versus pVax1/pet-neu-vaccinated mice challenged with EL4/HHD/Tel-Aml (Student's *t* test). B, effects of immunization with pVax1/pet-neu on time survival of mice challenged with EL4/HHD cells transduced with the cDNA encoding for ErbB-2. Nine HDD mice in each group were vaccinated with vehicle (PBS), empty pVax1 vector, or pVax1/pet-neu encoding the polyepitope ErbB-2 vaccine. Ten days after the second vaccination, mice immunized with pVax1/pet-neu were challenged with 2×10^4 EL4/HHD/ErbB-2 (●) or EL4/HHD/Tel-Aml (▲) tumor cells, whereas mice vaccinated with vehicle alone (◆) or pVax1 vector (■) were challenged with 2×10^4 EL4/HHD/ErbB-2 cells. The time of survival determined until day 65 was significantly improved in the pVax1/pet-neu-vaccinated mice group compared with the other groups of immunized mice ($P = 0.02$ and $P = 0.0007$, pVax1/pet-neu versus pVax1 or PBS, respectively).



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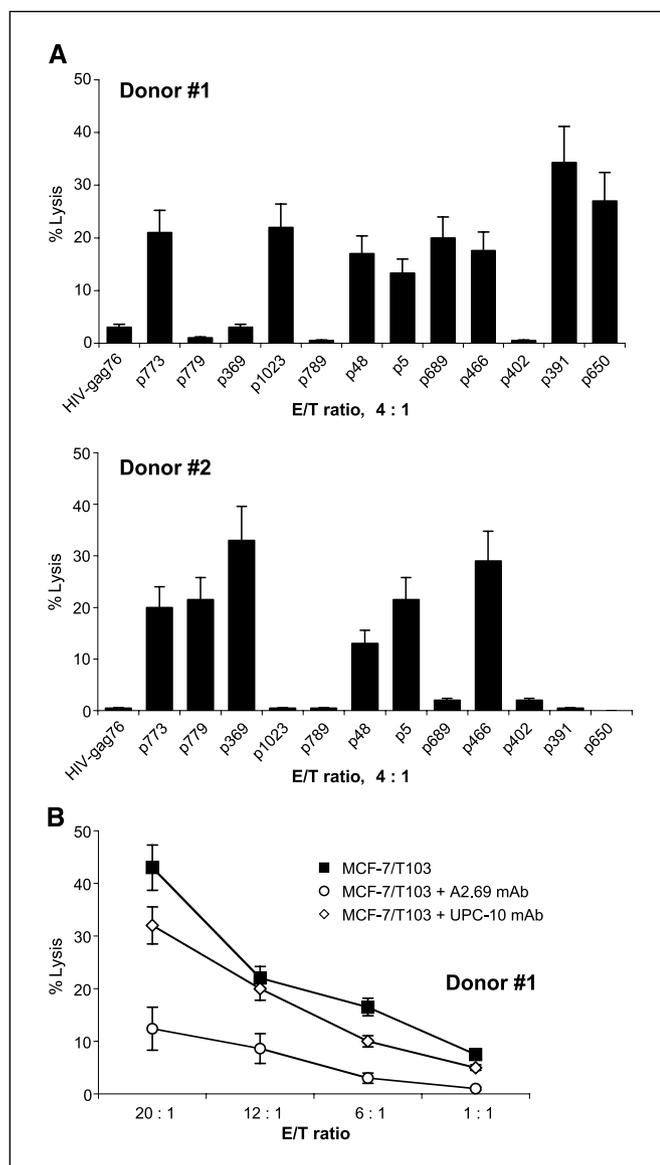


Figure 5. *In vitro* stimulation of ErbB-2 polyspecific human CTL by pVax1/pet-neu. PBMC from HLA-A*0201 healthy donors were stimulated with autologous dendritic cells transduced with pVax1/pet-neu. CTL were tested after the fourth *in vitro* stimulation against CIR.A2 cells loaded with ErbB-2 peptides (A) or ErbB-2⁺ MCF-7/T103 in the presence of the HLA-A*0201 specific A2.69 and the irrelevant UPC-10 mAbs (B). Bars, SD.

by *in vitro* priming and cyclic restimulations with autologous dendritic cells prepulsed with pVax1/Pet-neu in the presence of IL-2. After the fourth stimulation, lymphocyte cultures were tested for ErbB-2-specific cytolytic activity against CIR-A2 (HLA-A*0201⁺) target cells previously pulsed with each one of the eight dominant peptides included in the pVax1/pet-neu construct as well as the native cryptic epitope sequences of the four heteroclitic variants. CTL against the ErbB-2 peptides included in pVax1/pet-neu were generated in two of three healthy donors tested. As documented in Fig. 5A, donor #1 developed CTL against seven peptides (p773, p1023, p48, p5, p689, p466, p391, and p650) whereas donor #2 developed CTL against six peptides (p773, p779, p369, p48, p5, and p466). The multispecific CTL population was also able to kill the HLA-A*0201⁺, ErbB-2⁺ MCF-7-T103 carcinoma cell line *in vitro* (Fig. 5B). The HLA-A*0201

specific A2.69 mAb abrogated the MCF-7-T103 lysis whereas the irrelevant UPC-10 did not, thus showing the HLA-A*0201 restriction of the MCF-7-T103 recognition.

Discussion

We evaluated the *in vivo* efficacy of a polyepitope vaccine (pVax1/pet-neu) composed of 12 ErbB-2-derived, HLA-A*0201-restricted epitopes. We showed that HLA-A*0201 transgenic HHD mice vaccinated with pVax1/pet-neu developed simultaneously CTL against the majority of these epitopes. These CTL recognize the endogenous ErbB-2 epitopes and provide partial protection of HHD mice from the growth of the murine tumor EL4, engineered to express human ErbB-2. Moreover, pVax1/pet-neu stimulates *in vitro* from HLA-A*0201 human PBMC a multispecific CTL response capable to lyse tumor cells.

Several variables could influence the vaccine efficacy of a polyepitope, such as the presence of a Th epitope, the optimal cleavage of the different epitopes by the proteasome which depends on the epitope rearrangement, the addition of spacers between the epitopes, the creation of new junctional epitopes, and, finally, the role of affinity of the peptide epitopes included in the construction for immunodominance and skewing of the multiple specific CTL response *in vivo*. However, data in the literature do not allow for the establishment of a consensus on the role of each one of these variables on polyepitope efficacy. For instance, results of Mateo et al. (37) suggest that there is a correlation between affinity and immunogenicity of peptides included in a melanoma polyepitope. Furthermore, whereas Ishioka et al. (38) showed that, using an HIV polyepitope, the affinity of the epitopes does not interfere with their capacity to induce a CTL response. Palmowski et al. (39) showed the role of affinity diversity among peptide epitopes included within a polyepitope construction as crucial for skewing of immune response and CTL immunodominance in HLA-A*0201 transgenic mice. Concerning the need for a Th epitope, whereas Velders et al. (40) did not find any difference in antitumor immunity between normal and CD4^{-/-} alleles in mice vaccinated with a polyepitope containing the TT Th epitope. A consensus, however, seems to be established for the role of the polyepitope organization (epitope rearrangement, addition of spacers) that should allow for the appropriate cleavage of all the epitopes and should prevent the creation of new junctional peptides with high affinity for the HLA molecule (38, 39). Therefore, to design the pVax1/pet-neu polyepitope, we took care that the arrangement of the 12 epitopes (a) allows the processing of each one at its COOH-terminal position; (b) does not create new junctional peptides with high affinity for HLA-A*0201; and (c) includes native or high-affinity heteroclitic PIY variants of peptide epitopes showing similar strong binding affinity *in vitro* for the MHC-I molecule and high immunogenicity *in vivo* into mouse models. The processing at the COOH-terminal position was evaluated by two predictive models of proteasome cleavage (41, 42). Among the 10 virtual arrangements screened, we have chosen the pet-neu construct that fit better with the two criteria. p733, p369, p1023, p5, p466Y, p402Y, p391Y, and p650Y were predicted to be processed and only five new junctional peptides with predicted high affinity for HLA-A*0201 could be generated (p67₉, p94₉, p17₉, p91₁₀, and p63₁₀) but only one (p91₁₀) was predicted to be processed. Although we have not quantified the processing of the ErbB-2 and the junctional

epitopes, our results showing that HHD mice vaccinated with pVax1/pet-neu develop CTL against all the ErbB-2 epitopes show that all these epitopes are processed.

The major point addressed in our work is the possibility to include heteroclitic cryptic tumor epitopes in polyepitopic vaccines. The *ex vivo* ELISpot assay done after mice vaccination with pVax1/pet-neu did not show any immunodominance among native or heteroclitic peptide epitopes included within the construction, suggesting the possibility to increase *in vivo* a wide response directed simultaneously against multiple epitopes independently of their nature. Furthermore, results on CTL specific for ErbB-2 showed variability of response among different mice. Interpretation of such results might also involve sequence differences between some ErbB-2-derived epitopes (p5, p466Y, p391Y, and p650Y) and the murine Her-2 sequence, but the possibility to increase a multiple and diverse CTL response having antitumor activity was strongly indicated. Furthermore, results on human PBMC showed a CTL response variability among two individuals showing the possibility to mobilize a human T-cell repertoire specific for multiple ErbB-2 epitopes including cryptic epitopes. The multiple CTL response was capable to kill MCF-7 tumor cells and no correlation with the ErbB-2 specificity of CTL response evoked into mice was observed. Thus, the major evidence of the study is that pVax1/pet-neu generates a multiple CTL response and that response was addressed concomitantly to dominant and cryptic epitopes with antitumor effects *in vitro* and *in vivo*.

The interest of cryptic tumor epitopes in tumor immunotherapy is suggested in our previous work (17, 35) and is mainly based on the fact that the specific CTL repertoire is not submitted to the thymic or peripheral negative selection and must, therefore, be large and avid. The mechanisms of tolerance to self are particularly important for the design of new cDNA vaccines for the prevention of ErbB-2-expressing malignancies. Previous data from vaccines tested into mice models transgenic for rat-p185 cannot be directly translated to humans because the mechanisms of tolerance to human self-p185neu could be different from those of rat p185neu expressed in transgenic mice and the escape of human preneoplastic lesions may be more difficult to overcome in patients. Yet,

the group of Jafee showed that vaccination of Her-2/ErbB-2 transgenic mice with immunodominant rat Her-2/Neu MHC class I epitope presented by spontaneous tumors (RNEU₄₂₀₋₄₂₉) did not indicate antitumor immunity probably because of tolerance. On the other hand, dendritic cells pulsed with a heteroclitic variant of such epitope (RNEU₄₂₀₋₄₂₉^{A2}) induced improved protection against tumors that expressed the natural epitope, suggesting the mobilization of a more avid T-cell repertoire capable of recognizing the natural peptide onto tumor cells (43). In this study, the presence of immunodominant and heteroclitic peptide epitopes in the pVax1/pet-neu construction modulates a multiple response generating simultaneously different human self T-cell repertoires. This could circumvent the variability of peptide presentation by tumor cells and the different repertoire avidity of T-cell receptors specific for self peptides *in vivo*. In fact, the cell-mediated immune control on preneoplastic lesions or small foci of tumor recurrences should be more effective *in vivo* compared with the inhibition of the growth of fast-growing transplanted tumors, like the aggressive murine thymoma (EL4/HHD/neu) used in this work as a theoretical model.

In summary, we describe a polyepitopic ErbB-2-based recombinant vaccine that stimulates *in vitro* in humans and *in vivo* in humanized HLA-A*0201 transgenic mice a polyspecific CTL response and induces a partial antitumor protective immunity. This vaccine could be used for immunotherapy of ErbB-2-expressing tumors and as a model for the design of multiepitope cDNAs including native or heteroclitic peptide epitopes for future vaccine strategies.

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