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*J Immunol* 2003; 170:4273-4280; doi: 10.4049/jimmunol.170.8.4273

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Identification and Characterization of the Immunodominant Rat HER-2/neu MHC Class I Epitope Presented by Spontaneous Mammary Tumors from HER-2/neu-Transgenic Mice

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The HER-2/neu (neu-N)-transgenic mice are a clinically relevant model of breast cancer. They are derived from the parental FVB/N mouse strain and are transgenic for the rat form of the proto-oncogene HER-2/neu (neu). In this study, we report the identification of a MHC class I peptide in the neu protein that is recognized by CD8+ T cells derived from vaccinated FVB/N mice. This 10-mer was recognized by all tumor-specific FVB/N T cells generated regardless of the TCR Vβ region expressed by the T cell or the method of vaccination used, establishing it as the immunodominant MHC class I epitope in neu. T cells specific for this epitope were able to cure FVB/N mice of transplanted neu-expressing tumor cells, demonstrating that this is a naturally processed peptide. Altered peptide analogs of the epitope were analyzed for immunogenicity. Vaccination with dendritic cells pulsed with a heteroclitic peptide provided FVB/N and neu-N mice with increased protection against tumor challenge as compared with mice immunized with dendritic cells loaded with either wild-type or irrelevant peptide. Discovery of this epitope allows for better characterization of the CD8+ T cell responses in the neu-N mouse model in which neu-specific tolerance must be overcome to produce effective antitumor immunity. The Journal of Immunology, 2003, 170: 4273–4280.

A n important role for Ag-specific CD8+ CTLs in antitumor immunity has been demonstrated in both murine tumor models and cancer patients. In mice, immunization with model and naturally expressed tumor Ags or their MHC class I (MHC-I) epitopes induces antitumor CTL that mediate in vivo tumor rejection (1–12). Patients with established disease have shown some response when given adoptively transferred tumor-specific CTL (13–15). Similarly, clinical trials testing the efficacy of immunizing with MHC-I tumor epitopes from the human papilloma virus Ag E7 have resulted in T cell responses in some patients with cervical cancer (16–18). Several trials undertaken with peptides derived from melanoma-associated Ags have resulted in the induction and expansion of Ag-specific CTL. In some cases, immunization was also associated with clinical responses (19–22). However, in the majority of patients, immunization with MHC-I peptides induces T cell responses that are weak and ineffective in inducing significant tumor regression. As with all cancer therapies, the application of spontaneously arising mouse cancer models is central to the development of enhanced immunotherapies for human cancer (23).

We previously described neu-N-transgenic mice as a model of breast cancer that closely mimics immune tolerance described in some patients with cancer (24). These mice, derived from the parental FVB/N strain, express the wild-type rat HER-2/neu (neu) cDNA under the control of a mouse mammary tumor virus promoter (25). Female mice spontaneously and stochastically develop mammary tumors beginning at 4 mo of age. Mammary glands that have become tumorigenic overexpress the neu transgene relative to neu expression in normal glands in the same mice. Because the neu tumor Ag is endogenous to the host, this allows for the development of tolerance to the Ag, as evidenced by the poor ability of these mice to develop neu-specific antitumor immunity following vaccination as compared with the parental FVB/N mice. In the parental strain, depletion of CD8+ T cells before neu-specific vaccination inhibits their ability to reject a subsequent tumor challenge (24). Similarly, depletion of CD8+ T cells in transgenic mice before vaccination accelerates tumor outgrowth (26–29). However, unlike the parental mice, the transgenic mice are rarely cured of neu-expressing tumors. These data suggest that neu-specific CTL in neu-N mice are either weak lytic agents or are actively tolerated, either through deletion or anergy induction among high-avidity T cells.

The current study was designed to identify epitopes expressed by the rat neu and recognized by FVB/N (H-2b)-derived CTL.
Identification and characterization of these epitopes are critical to understanding the difference in neu-reactive T cell responses between the transgenic and parental mice. Numerous tolerance studies have shown that T cells reactive with endogenous Ags expressed in the thymus are deleted (30–33), although some T cells can escape and exist in the periphery in a functionally unresponsive state (34, 35). Similarly, T cells reactive with endogenous Ags not expressed in the thymus may be deleted or rendered nonresponsive in the periphery (36–38). Knowledge of the MHC-I epitopes in neu will allow us to study the fate of T cell responses directed at these epitopes in the neu-N-transgenic tolerant mice. In addition, it may be possible to alter these epitopes to identify heteroclitic epitopes with improved immunogenicity in the transgenic mice.

We have identified the immunodominant MHC-I epitope in the rat neu protein. This peptide is recognized by all neu-specific T cell lines and clones we derived from the splenocytes of vaccinated rat neu protein. This peptide is protective in vivo. Furthermore, immunization with a heteroclitic version of this peptide is also protective against challenge with mammary tumors expressing the naturally processed epitope.

Materials and Methods

Peptides and primers

A panel of 135 peptides from fragment 4 (see Fig. 2) used in the initial screening were synthesized by Chiron (Emeryville, CA). All other peptides (>95% purity) were from the Johns Hopkins Biosynthesis and Sequence Facility (Department of Biochemistry, Johns Hopkins School of Medicine, Baltimore, MD). The PCR primers used to create the neu fragments (see Fig. 1) are as follows: fragment 1 (bp 1–508), 5′-CCGGCGCAATTCCGAATGTCCTGTTCTGAGGTTTCCACCGCAGGTAACGTTCTCCTGACACTCATGAGTCCCGGGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAGGACATGAAGTTGCGGCTCCCTGATGGACATTAATGCTCTGTTT; fragment 2 (bp 458–886), 5′-GACATGAAGTTGCGGCTCCCTGATGATCCCGGAACTCTGCTAGTCTCACTGAGGATCTGAGAAGCCTCTCTACCGCGTATCGTGATCCTTACCCGGTACCCTAGCAGATTGGAGGCTGAGGTAG; fragment 3 (bp 887–1294), 5′-GACATGAAGTTGCGGCTCCCTTCCACAGTACAGCTGGTGACACATCCTCTGGTGTTCTCCACCTCTCAACTGCTGACACTCATGAGTCCCGGGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAGGACATGAAGTTGCGGCTCCCTGATGGACATTAATGCTCTGTTT; fragment 5 (bp 1607–2077), 5′-GACATGAAGTTGCGGCTCCCTATCACAGGTTACCTGTAACCCGGGCTCCCTGATGATCCCGGAACTCTGCTAGTCTCACTGAGGATCTGAGAAGCCTCTCTACCGCGTATCGTGATCCTTACCCGGTACCCTAGCAGATTGGAGGCTGAGGTAG; fragment 7 (bp 2405–2872), 5′-GACATGAAGTTGCGGCTCCCTTCCACAGTACAGCTGGTGACACATCCTCTGGTGTTCTCCACCTCTCAACTGCTGACACTCATGAGTCCCGGGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAGGACATGAAGTTGCGGCTCCCTGATGGACATTAATGCTCTGTTT; fragment 6 (bp 2004–2476), 5′-GACATGAAGTTGCGGCTCCCTTCCACAGTACAGCTGGTGACACATCCTCTGGTGTTCTCCACCTCTCAACTGCTGACACTCATGAGTCCCGGGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAGGACATGAAGTTGCGGCTCCCTGATGGACATTAATGCTCTGTTT; fragment 8 (bp 2801–3271), 5′-GACATGAAGTTGCGGCTCCCTTCCACAGTACAGCTGGTGACACATCCTCTGGTGTTCTCCACCTCTCAACTGCTGACACTCATGAGTCCCGGGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAGGACATGAAGTTGCGGCTCCCTGATGGACATTAATGCTCTGTTT.

Lin and medium

The IT22 cell line derives from a spontaneously transformed mouse fibroblast line (ATCC CRL-1658; American Type Culture Collection (ATCC), Manassas, VA). Rat neu cDNA was retrovirally inserted into rat neu-expressing recombinant vaccinia virus as described previously (44). T cells were maintained at 37°C, 5% CO2 in RPMI 1640 medium supplemented with 10% FBS, 1% l-glutamine, and 0.5% penicillin/streptomycin.

ny-specific T cell lines and clones and hemagglutinin (HA)-specific T cell line

Some CD8+ T cell lines were derived from FVB/N mice that were s.c. vaccinated with 1×106 irradiated 3T3 neu GM at each of three sites (two forelimbs and one hindlimb). Mice were sacrificed, and spleens were excised 2 wk later. Splenocyte cultures were initially stimulated every 5 days with irradiated, IFN-γ-treated NT5B7-1 cells and then every 9 days by the addition of irradiated 3T3 neu B7-1 cells as stimulators and FVB/N-derived clones as feeders. Clones were developed from this line by limiting dilution. Other CD8+ T cell lines were produced from the splenocytes of mice vaccinated with a neu-expressing recombinant vaccinia or neu plasmid DNA as described previously (24). A T cell line specific for the irrelevant Ag HA was derived from mice vaccinated with HA recombinant vaccinia virus as described previously (44). T cells were maintained at 37°C, 5% CO2 in RPMI 1640 supplemented with 10% FBS, 1% l-glutamine, 0.1% 2-ME (Sigma-Aldrich), and 0.5% penicillin/streptomycin supplemented with 10 cts U/ml murine IL-2 (supernatant from B16 IL-2 line (45)).

Chromium release assays

Lysis assays were performed in triplicate in 96-well V-bottom plates as previously described (24). Briefly, target cells were resuspended in 100 μl of CTL medium and labeled with 0.2 μCi of 51Cr/107 cells at 37°C and 5% CO2 for 1 h. Cells were washed in CTL medium and resuspended at 6×104 cells/ml in RPMI 1640. To pulse peptide onto targets, 100 μl of peptide in RPMI 1640 was added to 50-μl targets for 1 h at room temperature in each well. After the removal of 100 μl of supernatant, 150 μl of CTL medium was added for the indicated E:T ratio. After a 4-h incubation, 100 μl of supernatant was assayed for 51Cr release and percent specific lysis was determined by the formula: (experimental Cr release − spontaneous Cr release target alone)/(maximum Cr release target alone − spontaneous Cr release target alone)×100. For the Ab blocking assays, the H-2Dq Ab 30-5-78 (ATCC HB-31) was added for 30 min at 37°C to 100 μl of target cells resuspended in CTL medium at a final concentration of 50 μM before addition of effector T cells.

Development and transfection of neu fragments

Nine overlapping fragments of the neu cDNA were created using specific primers and PCR amplification (Fig. 1A) and then ligated into pcDNA3.1. The fragment constructs were then transfected into NIH 3T3 cells using electroporation (20 μg/107 cells) or Lipofectamine (1.5 mg/5×106 cells; Life Technologies). Limiting dilution produced several clones of each construct. RT-PCR was performed using primers described above to determine which clones expressed fragment mRNA.

Mice and dendritic cell immunizations

FVB/N mice were purchased at 6–8 wk of age from the National Cancer Institute (Bethesda, MD). FVB/N mice (25) were bred to homozygosity as verified by Southern blot analysis. Splenic dendritic cells were generated from FVB/N mice as previously described (46). On the day of immunization, cells were collected, resuspended at 4×107/ml in AIM-V medium (Life Technologies), and pulse labeled with 300 μg/ml peptide for 3 h. Cells were then washed three times in HBSS (pH 7.4) and resuspended at 2×106 cells/ml. Mice were given 0.1 ml s.c. injections in each hindlimb on days 0 and 7 and challenged with NT2 mammary tumor cells in the right hindlimb on day 14.

GM-CSF release ELISA

Peptides were pulsed into NIH 3T3 cells (which were grown overnight at 26°C to increase the number of empty MHC on the cell surface) in RPMI 1640 at 26°C for 1 h at a final concentration of 0.1 μM. Targets were then

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Cloning and transfection of H-2Dq

Murine H-2Dq was cloned from the L-Dq cell line (5′ primer, ATGGCTCGCGCCACGCTGCT; 3′ primer, TCACGCTTTACAATCTCGGGA) and ligated into pcDNA3.1. The T2 cell line is a B lymphoblast/T lymphoblast hybrid cell line deficient in the MHC-I TAP transporter molecule (ATCC CRL-1992). These cells were transfected with Dq by electroporation (20 μg/107 cells) to create the T2Dq cell line. T2Dq cells were maintained at 37°C and 5% CO2 in RPMI 1640 (Life Technologies) supplemented with 10% FBS (HyClone), 1% l-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 0.5% penicillin/streptomycin.

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washed twice in CTL medium, and $1 \times 10^5$ T cells were added for a 1:1 E:T ratio. Plates were incubated at 37°C and 5% CO$_2$ for 24 h, and supernatants were harvested for the ELISA (Endogen, Woburn, MA). For the screening of neu fragments, $1 \times 10^5$ T cells and $3 \times 10^5$ targets were plated in CTL medium for 24 h and an ELISA was performed.

Flow cytometry (FACS)

Cells were stained by washing them two times in FACS buffer (1 × HBSS (pH 7.4), 2% FBS, 1% HEPES (Life Technologies), and 0.1% NaN$_3$ (Sigma-Aldrich)) and incubating with Ab for 20 min at 4°C. Staining of TCR V$\beta$4, V$\beta$14, and V$\beta$17 was done using supernatants from hybridomas KT4-10, 14-2, and KJ23, respectively (47). Abs to V$\beta$2, -6, and -7, and fluorescence-conjugated secondary Abs were purchased from BD PharMingen (La Jolla, CA). Ab to CD8 was purified from the 2.43 hybridoma (ATCC HB-27). MHC staining was done using supernatant collected from hybridomas 30-5-7S, 113, and 28-14-8S, which are specific for H-2D$^b$ (42). The secondary Ab for all three Abs was FITC-conjugated goat anti-mouse IgG (BD PharMingen).

Intracellular cytokine staining (ICS)

ICS was performed as directed using a BD PharMingen kit for detection of murine IFN-$\gamma$. Briefly, $1 \times 10^6$ splenocytes (nylon wool purified to deplete B cells and macrophages) were incubated 12–16 h with an equal ratio of indicated targets in the presence of GolgiStop. Cells were washed in FACS buffer and stained with FITC-conjugated Ab to CD8, fixed and permeabilized, and stained with PE-conjugated Ab to IFN-$\gamma$.

Results

Generation of neu-specific CD8$^+$ T cells from vaccinated FVB/N mice

Three T cell lines were generated to characterize the T cell populations that result from different vaccination experiments as well as from different neu targeted vaccine approaches shown to be potent in other tumor models (48). A T cell line was therefore generated from mice vaccinated with the 3T3neuGM whole cell vaccine, and 11 neu-specific T cell clones were derived from this line. In addition, lines were created from mice vaccinated with the vaccinia vector expressing the entire neu protein and from mice vaccinated with neu plasmid DNA. All lines and clones were shown to lyse the full-length neu protein in a $^{51}$Cr release assay using 3T3neu vs 3T3 wild-type targets (not shown). Analysis of the V$\beta$ usage of these T cell lines and clones suggests that the neu CD8$^+$ T cell response is oligoclonal. Specifically, TCRs utilizing six V$\beta$ regions (TCR V$\beta$2, -4, -6, -7, -14, and -17) were identified (data not shown).

Identification of RNEU$_{420-429}$, a peptide epitope contained in the extracellular domain of the rat neu protein, as the T cell target of all FVB/N-derived T cell lines and clones

As an initial approach to roughly map the position of neu epitopes, the neu cDNA was divided into nine approximately equal fragments overlapping by 15–25 aa (Fig. 1A). NIH 3T3 cells were

![FIGURE 1. Extracellular fragment 4 of neu contains a neu MHC-I epitope recognized by the FVB/N-derived T cell clones. A. Nine overlapping fragments of neu cDNA were created using specific primers and PCR amplification. The numbers under each fragment refer to the starting and ending base pair within the entire neu cDNA. Each fragment was sequenced and confirmed to be the original rat neu sequence before ligation into the vector pcDNA3.1. The fragments were then transfected into 3T3 cells for use as targets in T cell assays. T cells ($1 \times 10^5$) and $3 \times 10^5$ targets were plated in duplicate in 96-well plates and incubated at 37°C for 24 h. Supernatants were collected and tested for levels of GM-CSF by ELISA. Data are shown as degree of color change (OD). B. Effectors were FVB/N V$\beta$4A clone (TCR V$\beta$4) (B) and FVB/N V$\beta$6A clone (TCR V$\beta$6) and FVB/N V$\beta$14A clone (TCR V$\beta$14) (C). T cells show recognition of full-length neu (■) and of fragment 4 (□).]
transfected with these constructs and used as targets in GM-CSF release assays to identify which fragment contained the peptide(s) recognized by the clones. Fig. 1B shows recognition of 3T3 cells expressing fragment 4 (aa 410–553) by the FVB/N-derived T cell clone FVB/N Vβ4A (expressing TCR Vβ4A). Recognition of fragment 4 was also seen with FVB/N Vβ6A clone (TCR Vβ6) and FVB/N Vβ14A clone (Vβ14) (Fig. 1C). This fragment is located in the extracellular domain of neu near the transmembrane region.

Peptides 10 aa in length were synthesized, and these peptides spanned the entire sequence of fragment 4 (excluding the overlapping regions with fragments 3 and 5) and overlapped by nine residues each. Of the 135 peptides made, one (RNEU420–429) was consistently recognized by clones in GM-CSF release ELISA (data not shown). This peptide (PDSLRDLSVF), along with its 8- and 9-aa derivatives, was synthesized and tested for recognition by neu-specific T cells in Cr51 release lysis assays. As shown in Fig. 2, the 10-mer was recognized more efficiently than the shorter peptides regardless of the TCR Vβ region expressed by the T cell. This peptide was subsequently found to be recognized by all T cell lines and clones derived, suggesting that RNEU420–429 is the immunodominant MHC-I neu epitope recognized by FVB/N-derived T cells.

To further analyze the CD8+ T cell response to neu in FVB/N mice, frequency analysis was performed on the spleens of five individual mice given a s.c. 3T3neuGM vaccine. Fourteen days after vaccination, spleens were excised and cultured with 3T3neuB7-1 cells for 7 days before performing ICS to determine the response to full-length neu and to RNEU420–429. As shown in Table I, all vaccinated FVB/N mice developed CD8+ T cells that were specific for both full-length neu and for RNEU420–429. This further supports the immunodominance of RNEU420–429 in FVB/N mice.

Table I. T cell specificity of vaccinated FVB/N and neu-N mice

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>% neu Specific</th>
<th>% RNEU420–429 Specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/N 1</td>
<td>1.83</td>
<td>2.48</td>
</tr>
<tr>
<td>FVB/N 2</td>
<td>0.88</td>
<td>1.92</td>
</tr>
<tr>
<td>FVB/N 3</td>
<td>1.47</td>
<td>2.75</td>
</tr>
<tr>
<td>FVB/N 4</td>
<td>0.06</td>
<td>0.48</td>
</tr>
<tr>
<td>FVB/N 5</td>
<td>0.28</td>
<td>1.16</td>
</tr>
<tr>
<td>neu-N 1</td>
<td>0.00</td>
<td>0.32</td>
</tr>
<tr>
<td>neu-N 2</td>
<td>0.13</td>
<td>0.22</td>
</tr>
<tr>
<td>neu-N 3</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td>neu-N 4</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>neu-N 5</td>
<td>0.02</td>
<td>0.26</td>
</tr>
<tr>
<td>neu-N 6</td>
<td>0.00</td>
<td>0.07</td>
</tr>
<tr>
<td>neu-N 7</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td>neu-N 8</td>
<td>0.16</td>
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</tr>
<tr>
<td>neu-N 9</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>neu-N 10</td>
<td>0.14</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Specificity determined by the percentage of CD8+ splenocytes staining positive for IFN-γ in response to target in ICS assay.

** RNEU420–429 specificity determined by the percentage responding to IT22neu minus the percentage responding to IT22.

The original identified 10-mer RNEU420–429 is the optimal epitope recognized by neu-specific T cell clones. Full-length and truncated versions of the epitope (as well as the irrelevant lymphocytic choriomeningitis virus nucleoprotein peptide NP118–126) were pulsed onto 3 x 10^3 chromium-labeled IT22 cells for 1 h at 37°C. T cells were added before a 4-h incubation at 37°C. E:T ratios ranged from 15:1 to 25:1. Effectors were FVB/N Vβ2A clone (Vβ2) (A), FVB/N Vβ4A clone (Vβ4) (B), FVB/N Vβ6A clone (Vβ6) (C), and FVB/N Vβ14A clone (Vβ14) (D). Inserts. Surface staining of T cell clones. Effectors were stained with Ab to CD8 (thin line), Ab to appropriate TCR Vβ region (thick line), or the irrelevant Ab rat IgG (dotted line).
FVB/N mice. The same analysis was done on the spleens of 10 individual neu-N mice given the same vaccine. As shown in Table I, the tolerized neu-N mice do not demonstrate appreciable T cell activity for the immunodominant epitope.

H-2D\textsuperscript{\alpha} is the restriction element for RNEU\textsubscript{420–429}

Initial CTL blocking studies using an Ab specific for H-2D\textsuperscript{\alpha} and -L\textsuperscript{\alpha} (30-5-7S) indicated that lysis of neu was restricted to one of these two molecules. MHC restriction was further determined by pulsing RNEU\textsubscript{420–429} onto mouse L cells that were transfected with either H-2D\textsuperscript{\alpha} or -L\textsuperscript{\alpha}. FVB/N V\textsubscript{β}2A clone (V\textsubscript{β}2) (A), FVB/N V\textsubscript{β}4A clone (V\textsubscript{β}4) (B), FVB/N V\textsubscript{β}6A clone (V\textsubscript{β}6) (C), and FVB/N V\textsubscript{β}14A clone (V\textsubscript{β}14) (D). T cell clone FVB/N V\textsubscript{β}4A demonstrates enhanced lysis of the RNEU\textsubscript{420–429} peptide with an alanine substituted at position 2 (RNEU\textsubscript{420–429} A2). E, T2\textsuperscript{D\textsuperscript{\alpha}} cells were incubated with Ab to D\textsuperscript{\alpha} (28-14-8S) followed by FITC-conjugated Ab to mouse IgG (thick line) to confirm surface expression of the MHC molecule (thin line, secondary Ab alone).

Adoptive transfer of RNEU\textsubscript{420–429} specific T cells into tumor-bearing FVB/N mice confirms that this peptide is a tumor rejection epitope naturally expressed by mammary tumor cells in vivo

FVB/N mice were given a s.c. leg injection of neu-expressing tumor cells (NT2) followed 1 day later by i.v. transfer of an FVB/N T cell line (derived from mice given neu plasmid vaccine) specific for RNEU\textsubscript{420–429}. This line was chosen because it demonstrated the highest lysis of NT2 cells in vitro. As shown in Fig. 4, mice receiving these T cells showed protection from tumor outgrowth as compared with mice receiving a control T cell line specific for the irrelevant Ag HA (p < 0.0008). This experiment was repeated using several RNEU\textsubscript{420–429} specific T cell clones and lines with similar results.
Identification of a heteroclitic peptide of RNEU_{420-429} that can immunize mice in vivo against outgrowth by the mammary tumor

Identification of the immunodominant neu peptide allowed us to search for altered peptide analogs with potentially enhanced immunogenicity. Altered forms of RNEU_{420-429} were created by substituting alanine at each of the 10 positions. This type of approach has been successful in identifying heteroclitic T cell peptides in both rodent and human settings (6, 49–54). In the majority of cases, substitutions did not enhance recognition. However, when alanine was substituted for glutamate at position 2, this peptide (designated RNEU_{420-429A2}) demonstrated markedly improved recognition by the T cell clone FVB/N V/H9252 in a lysis assay as compared with wild-type peptide (Fig. 3). Interestingly, the heteroclitic peptide was found to have a lower binding affinity than the wild-type RNEU_{420-429} peptide in a T2D^p MHC stabilization assay (data not shown), suggesting that its improved stimulatory capacity was instead due to the enhanced stability of the TCR/MHC/peptide complex.

To determine whether this heteroclitic peptide can immunize mice against mammary tumor expressing the natural RNEU_{420-429} epitope, both wild-type RNEU_{420-429} and the heteroclitic variant RNEU_{420-429A2} were used to vaccinate mice. Dendritic cells derived from FVB/N mice were pulsed in vitro with either of these peptides (or with an irrelevant peptide) and then injected s.c. into FVB/N and neu-N mice followed by a s.c. NT2 tumor challenge. As shown in Fig. 5, mice immunized with dendritic cells pulsed with the wild-type peptide developed tumor at about the same rate as mice immunized with an irrelevant peptide (p < 0.15 for FVB/N mice; p < 0.39 for neu-N mice). However, FVB/N mice immunized with the heteroclitic peptide showed a lag in tumor growth as compared with FVB/N mice immunized with the irrelevant peptide (p < 0.012). Although not statistically significant, neu-N mice demonstrated a promising trend toward protection when vaccinated with the heteroclitic peptide (p < 0.21).

Discussion

We have identified a MHC-I epitope in rat HER-2/neu, RNEU_{420-429}, which is the dominant target of the CD8^+ T cell response in FVB/N mice. This peptide, which is contained in the extracellular region of neu, is recognized by all neu-specific CD8^+ T cell lines and clones derived from vaccinated FVB/N mice regardless of the neu-targeted vaccine approach used for immunization. This was true even though vaccination induced an oligoclonal neu-specific T cell response as determined by the panel of

T cell lines and clones tested expressing several different TCR Vβ types. This was unexpected because the neu gene is 4kB in size and encodes for a large protein. However, other studies have reported similar findings demonstrating that a single viral or tumor antigenic epitope is recognized by a panel of T cells derived from immunized mice (8, 55–57). In contrast, neu-specific T cells isolated from patients with neu-expressing breast and ovarian cancer typically recognize multiple epitopes in both the extracellular and intracellular domains of the protein (58–63). Therefore, our data suggest that the FVB/N non tolerized mice recognize neu as a foreign Ag.

FVB/N mice were protected against inoculation with a neu-expressing in vitro tumor line (derived from naturally arising mammary tumors in neu-N mice) when T cells specific for this epitope were adoptively transferred. This indicates that RNEU_{420-429} is the immunodominant MHC-I epitope in rat neu that serves as the naturally expressed tumor rejection target on spontaneously arising neu-expressing tumors. It is interesting to note that RNEU_{420-429} differs from the corresponding murine peptide in a 51Cr release lysis assay (data not shown). This probably explains its high degree of immunogenicity in the FVB/N mice.

Vaccination with the RNEU_{420-429} peptide pulsed onto dendritic cells did not demonstrate antitumor immunity. This is not surprising, because vaccinating mice or patients with solely MHC-I tumor epitopes has produced only modest results in most studies. For some Ags, altering the wild-type MHC-I epitope so that it binds more strongly to MHC and/or demonstrates greater recognition in vitro by Ag-specific T cells improves the immunization potential and the clinical outcome (54, 65–67). In this study, we show that vaccinating FVB/N mice with dendritic cells pulsed with a heteroclitic variant of the wild-type epitope also induces improved protection against tumors that express the natural RNEU_{420-429} epitope as compared with immunization with the RNEU_{420-429} epitope itself. neu-N mice show a promising trend toward protection when vaccinated with the heteroclitic peptide but not to the degree seen in FVB/N mice. This may reflect the neu-specific tolerance seen in neu-N mice. In any case, this regimen proved to be far less efficacious for either strain of mice than our whole-cell vaccine, further supporting the concept that vaccines that induce both CTL and Th cell responses may be more effective than vaccines that only enhance the CTL response (41, 48).
Few studies have been published dissecting the immune responses in neu-N mice, which are transgenic for rat neu and therefore express a tissue-specific tumor Ag (24, 26–29, 44). We have previously shown that these mice are tolerant to their transgene as compared with the FVB/N strain from which they were derived and that this tolerance is found both in the humoral and T cell arms of the immune response (24). However, we do not yet know the mechanism of this tolerance. It appears from our studies that neu-N mice given a whole-cell 3T3 neuGM vaccine do not develop T cells specific for the immunodominant peptide in neu, although mice vaccinated with the heteroclitic version of the peptide show a degree of protection from neu-expressing tumor challenge. This may indicate that, although the predominant response to neu in neu-N mice is to other, less immunogenic epitopes, T cells specific for the immunodominant peptide may be induced. However, neu-N mouse-derived T cells may recognize the immunodominant epitope with a lower avidity as compared with FVB/N-derived T cells. This tolerance may occur in the thymus, in the periphery, or in both compartments. The identification of RNEL_1420–429 as the immunodominant epitope contained in the rat neu protein will facilitate the further characterization of vaccine-induced immune responses in the rat neu-transgenic mouse model. To that end, an MHC/peptide tetramer can be made to identify T cells specific for the immunodominant epitope, because it is known that the restricting MHC molecule is H-2D^d. Identification of other epitopes in rat neu is also ongoing.

Acknowledgments

We thank David Woodland for providing TCR Abs, Ted Hansen for providing MHC Abs and L-D^d and L-L^d cells, Susan Ivie for dendritic cell culture, and Eric Lutz for assistance in cloning the H-2D^d cDNA. We also thank Drs. Drew Pardoll and Leisha Emens for their critiques.

References


42. Lee, D. R., R. J. Raboch, W. R. Lie, and T. H. Hansen. 1988. The murine MHC class I genes, H-2D\(^{a}\) and H-2L\(^{a}\), are strikingly homologous to each other, H-2L\(^{b}\), and two genes reported to encode tumor-specific antigens. J. Exp. Med. 168:1719.


