**ABSTRACT**

The development of T-cell therapy for the treatment of human malignancy has been hindered, in large part, by a lack of identifiable tumor antigens. Studies to identify potential T-cell targets in humans have been difficult because of practical problems limiting the use of in viro immunization and a lack of reproducible in viro priming methods. Oncogenic proteins are involved in malignant transformation and maintenance of the transformed phenotype and theoretically are potential targets to T-cell therapy. HER-2/neu protein is a protooncogene product overexpressed in a variety of human malignancies and is associated with malignant transformation and aggressive disease in human breast cancer. Previous studies have shown that some patients with breast cancer have existent helper/inducer T-cell immunity to p185\(^{HER-2/neu}\) protein and peptides. The current study represents initial attempts to identify candidate cytokotoxic T-lymphocyte (CTL) epitopes. Synthetic peptides were constructed identical to HER-2/neu protein segments with amino acid motifs similar to the published motif for HLA-A2.1-binding peptides. Four peptides were synthesized and two were shown to be avid binders to HLA-A2.1. Two of the four peptides could be shown to elicit peptide-specific CTL by primary in viro immunization in a culture system using peripheral blood lymphocytes from a normal individual homozygous for HLA-A2. p185\(^{HER-2/neu}\) protooncogene protein contains immunogenic epitopes capable of generating human CD\(^{8+}\) CTL. The identification of candidate CTL epitopes will allow studies to determine whether some cancer patients have existent CTL immunity to HER-2/neu protein. The demonstrated ability to generate human peptide-specific CTL in vitro allows screening of other oncogenic proteins to identify candidate T-cell epitopes potentially useful for future immunotherapy studies.

**INTRODUCTION**

In animal models, established tumors can be eradicated by the adoptive transfer of T-cells that are specifically immune to the malignant cells (1–6). Techniques of adoptive T-cell therapy have recently been applied to the treatment of human viral disease (7), but the application of similar T-cell therapy for human malignancy has been hindered by the lack of well defined tumor antigens recognizable by autogenous T-cells. A common strategy in the search for tumor antigens is to isolate T-cells from peripheral blood or from tumor infiltrating lymphocytes and attempt to identify the antigens recognized by the T-cells (8, 9). Another ploy is to determine whether an immune response can be generated to proteins that are known to be involved in malignant transformation (10–12). The current studies represent initial experiments to identify candidate T-cell epitopes on p185\(^{HER-2/neu}\) protein, appropriate for evaluating cytotoxic T-cell responses. The HER-2/neu-specific T-cells identified, to date, have been helper/inducer T-cells (13).

The HER-2/neu protooncogene encodes a tyrosine kinase with homology to epidermal growth factor receptor (14) with a relative molecular mass of 185 kDa. HER-2/neu protein is a receptor-like transmembrane protein that consists of a large cysteine-rich extracellular domain which probably functions in ligand binding, a short transmembrane domain, and a small cytoplasmic domain (14). HER-2/neu is amplified and overexpressed in many human cancers, largely adenocarcinomas of breast, ovary, colon, and lung. In breast cancer, HER-2/neu overexpression is associated with aggressive disease and is an independent predictor of poor prognosis (15, 16). HER-2/neu amplification may also be related to cancer formation, with overexpression being detectable in 50–60% of in situ ductal breast carcinomas (17).

HER-2/neu protein has been examined as a possible target for T-cell-mediated immunotherapy (10), and is an appealing candidate target for several reasons: (a) the protein is large (1255 amino acids) and therefore should contain epitopes appropriate for binding to most, if not all, MHC\(^{3}\) molecules and thus be potentially recognizable by all individuals; (b) HER-2/neu is greatly overexpressed on malignant cells and thus T-cell therapy may be selective with minimal toxicity. In viral systems the ability of T-cells to recognize infected cells can be limited by the availability of endogenously processed and presented antigen (18). Overexpression of HER-2/neu by malignant cells (19, 20) may allow selective lysis of malignant cells, whereas lower concentrations in normal cells may disallow destruction of normal tissues (21, 22); (c) the oncogenic protein is intimately associated with the malignant phenotype and with the aggressiveness of the malignancy, especially in breast and ovarian carcinomas (15, 16, 23, 24). If antigen-negative variants arise from treatment, the variant cells might be biologically less aggressive. Thus, treatment might provide benefit even without cure.

In “classic” immune responses CD\(^{4+}\) helper/inducer T-cells secrete cytokines in response to soluble antigen presented in the class II antigen processing pathway and CD\(^{8+}\) CTL respond to and kill cells presenting antigenic proteins in the class I antigen processing pathway. Methods for eliciting and detecting CD\(^{4+}\) helper/inducer and CD\(^{8+}\) CTL are markedly different given that, in general, CTL recognize only proteins synthesized by the target cell to the exclusion of exogenous proteins. Studies to elicit class I MHC-restricted CD\(^{8+}\) CTL have been greatly facilitated by the determination that CD\(^{8+}\) CTL recognize short peptides bound in the groove of class I MHC molecules and that peptides binding to particular class I MHC molecules share discernible amino acid sequence motifs (25). The knowledge that CTL recognize peptide fragments binding in the groove of class I MHC molecules has been used extensively to define epitopes of proteins recognized by immune T-cells. Priming of CTL has classically required in vivo immunization with either peptides or with stimulator cells synthesizing the nominated target protein and capable of processing and presenting that protein in the class I MHC pathway (26–28). However, in mice it has recently been shown to be possible to prime in vivo and elicit CTL by using peptides with the appropriate class I MHC-binding motif and thereby circumvent the need to immunize with a viable cell synthesizing the nominated antigen. In a few instances in mice it has been possible to elicit CTL by priming to...
similar peptides in vitro. In humans, the conditions for in vivo and in vitro priming to peptides have not yet been determined. Studies to elicit murine CTL by in vitro priming have in general used spleen cells or dendritic cells purified from the spleen as stimulator cells. To our awareness no published studies have demonstrated that peptide-specific CTL can be similarly elicited by in vitro priming using human peripheral blood leukocytes as responders and stimulators.

The experiments described here show it is possible to generate peptide-specific CTL to oncogenic proteins in humans. The appropriateness of an oncogenic protein as a target for CTL in a human system depends on several factors; whether that protein has the proper configuration to bind class I, whether peptide/MHC complexes are present at the cell surface in high enough concentration for recognition by the T-cell, and whether the peptide/MHC complex is within the T-cell repertoire of a given individual. The development of a reproducible system to prime human T-cells in vitro will allow determination of which binding peptides are immunogenic and, once generated, which peptide-specific CTL can lyse cancer cells. The current identification of two candidate T-cell epitopes in the HER-2/neu oncogene protein gives credence to the approach and increases the likelihood that CTL cell reagents appropriate for eventual testing in therapy can be generated.

MATERIALS AND METHODS

Synthetic Peptides. Four peptides were constructed, derived from the amino acid sequence of HER-2/neu protein, to correspond to the reported HLA-A2.1 binding motif (25). The peptides used in the study were synthesized and purified by Dr. P. S. H. Chou (University of Washington, Seattle, WA) or M. K. Doljevi (Fred Hutchinson Cancer Research Center). The peptides were dissolved in sterile water or phosphate-buffered saline (pH 7.4), to give 2 mg/ml stock solutions. Prior to aliquoting they were sterile filtered and then stored at −70°C.

HLA-A2 Lymphocytes. A normal individual was identified as a source of homologous A2 lymphocytes: HLA typing, A2, B13, B8, BW4, BW6, C6. After signed consent the individual underwent leukapheresis. The PBMC were isolated by Ficoll/Hypaque density gradient centrifugation. Cells were washed and resuspended in RPMI 1640 (Gibco, Grand Island, NY), with 2.5 × 10^5 M2-2E, 200 units/ml penicillin, 200 units/ml streptomycin, 10 mM L-glutamine, and 10% FCS; 10% dimethyl sulfoxide was added. They were then cryopreserved in aliquots of 2 × 10^7 PBMC. All cultures were initiated from stocks of previously frozen PBMC.

Peptide-binding Assay. The mutant cell line, T2, was used as a measure of in vitro binding. T2 cells (1 × 10^5) were incubated in RPMI 1640/10% FCS with individual peptides at a concentration of 25 μg/ml for 18 h at 37°C in 5% CO_2/95% air. HLA-A2 binding was determined by indirect immunofluorescence. The cells were stained on ice for surface HLA-A2 with a mouse anti-human antibodies, fluorescein isothiocyanate-F(ab')/2 rabbit anti-mouse IgG (H+L) (Zymed Laboratories, South San Francisco, CA) was also used in a 3% solution. The stained cells were then preserved in 1% paraformaldehyde and stored at 4°C. Flow cytometry was performed.

In Vitro Induction of CTL. Previously frozen homologous HLA-A2 PBMC (4 × 10^7) were thawed, washed, and resuspended in 25×10^7/cm³ tissue culture flasks (Corning, Corning, NY) containing 10 ml of media with equal parts of EEHAA 120 (Biofluids) and RPMI 1640 (Gibco) with 10% FCS. Media were changed and fresh media were added as described on day 2 and 4 of each cycle. The cells were tested for cytotoxic activity against designated target cells. The targets, LCLs, were labeled overnight with 51Cr, then incubated for 2 h with no peptide, stimulating peptide, or an irrelevant peptide. These targets, along with 3C5I-labeled K562 and Daudi cell lines, were then used in a 4-h chromium release assay. The procedure was repeated after the 10th IVS. Class I blocking studies were performed at the 10th IVS by using mouse monoclonal anti-HLA-A2 antibody to designated experimental wells at a 5% solution in phosphate-buffered saline.

Cell Surface Antigen Characterization. Characterization of the cell lines was performed after the 10th IVS by using mouse anti-human antibodies directed against CD4 and CD8 (Becton Dickinson, San Jose, CA). Cells (1 × 10^6) were stained on ice for surface markers with the appropriate antibody at a 1:50 dilution. The stained cells were then preserved in 1% paraformaldehyde and stored at 4°C. Flow cytometry was performed.

RESULTS

HER-2/neu Protein Contains Multiple Segments with an Amino Acid Sequence Motif Appropriate for Binding the Class I MHC Molecule HLA-A2.1. Recently, it has been determined that peptides binding to particular MHC molecules share discernible sequence motifs (25). A peptide motif for binding in the groove of HLA-A2.1 identifies a typical or average peptide as being 9 aa long with dominant anchor residues occurring at positions 2(L) and 9(V) (Table 1). We evaluated the 1255 aa structure of HER2/neu protein and determined 10 peptide segments of 9 aa in length that contained both the dominant anchor residues. Emphasis was placed on the presence or

Table 1 Peptides were given a score based on the defined HLA-A2.1 binding motif. Data represent the HLA-A2.1-restricted binding motif described by Falk et al. (25) along with an arbitrary scoring system, adapted from Nijman et al. (30).

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absence of dominant anchor residues as they appear to be of prime importance for peptide binding to MHC (29). The peptides were given a total score based on the similarity to the current published motif (30). The arbitrary scoring system awarded a +3 for a dominant anchor residue, +2 for a strong binding residue, and +1 for a weak binding residue. Four of 10 peptides were synthesized, 2 with relatively low homology to epidermal growth factor receptor, 33%, and 2 with high homology but not identical to epidermal growth factor receptor, 78 and 89% (Table 2). One is located in the extracellular domain of the protein and three are located in the intracellular domain (14).

The Four Peptides with a Motif Theoretically Appropriate for Binding to HLA-A2.1 Can Be Shown to Bind to HLA-A2 in a Class I MHC Molecule Stabilization Assay. Having identified and synthesized peptides with the theoretical likelihood of binding to HLA-A2.1, the next series of experiments evaluated whether the constructed peptides in fact could bind the *sine qua non* of CTL generation. Peptides were tested for the capacity to bind to HLA-A2 in an assay utilizing the mutant cell line T2. T2 is a human T- and B-cell hybrid that has a large homozygous deletion of the MHC class II region (31-33). This deletion includes TAP1 and TAP2 genes encoding for the transporters associated with antigen processing. HLA-A2.1 molecules are present at low density on the cell surface and are, at least partially, occupied with peptides. These peptides are derived from signal domains of normal cellular proteins (34, 35). T2 cells can present exogenously added peptides if they are strong binders of HLA-A2.1 even at low levels of already occupied class I MHC. T2 could not use exogenous peptide that had a low expression of HLA-A2 (30-50%). When incubated with peptides able to bind HLA-A2, class I MHC molecules stabilize on the cell surface and increases can be measured. The T2 transport deletion mutant may be used as an indicator of peptide ability to bind class I MHC (30, 36, 37). The binding of signal sequence peptides, however, may decrease the sensitivity of the assay. The data in Table 3 represent the mean from 3 separate fluorescence-activated cell sorter analyses. Percentage of stabilization could vary 5-7% between each assay.

CTL Specific for HER-2/neu Peptides Can Be Generated by Primary *In Vitro* Immunization. In initial studies (data not shown), conditions for detecting T-cell immunity to standard recall antigens were used, and no HER-2/neu peptide-specific CTL could be detected. We have derived a set of conditions which have allowed priming to the 2 peptides that are the strongest binders of HLA-A2 in the T2-binding assay. The conditions were derived by extensive empirical experimentation but are consistent with the current paradigm. Conditions include: (a) cultures with large numbers of responder T-cells to account for the low precursor frequency; (b) a concurrent stimulated responder T-cell population; (c) IL-2 added late to culture in low concentration; and (d) multiple restimulations.

Four of the peptides indicated in Table 2 were set up identically in culture conditions as outlined in “Materials and Methods.” After the fourth *in vitro* stimulation the lines were harvested and tested in a standard 4-h chromium release assay. Cells stimulated with p48-56 and p789-797, the strongest binders in the T2-binding assay, showed specific lysis of 18 and 6%, respectively (Fig. 1, *Column A*). These lines had no reactivity against autologous LCL alone or LCL lines that had been incubated with a closely related control peptide. Cytolytic reactivity against K562 and Daudi cell lines was low, mitigating against substantial natural killing or lymphokine-activated killing. The T-cell lines cultured with the remaining peptides showed either no difference in lytic potential between autologous LCL and peptide-coated LCL or no lytic activity.

All lines were retested after the 10th *in vitro* stimulation (Fig. 1, *Column B*). The 2 lines that showed specific lytic activity after stimulations showed greater lytic activity and peptide specificity after multiple additional restimulations. At the highest effector:target ratios p48-56 showed specific lysis of 66% and p789-797 lysed 58%. The percentage of specific lysis against K562 and Daudi cell lines in all assays after the 10th IVS was less than 5%. The two cytotoxic cell lines were of a mixed T-cell phenotype. The p48-56 line was 90% CD8+ and 10% CD4+, while the p789-797 line was 40% CD8+ and 60% CD4+. The majority of lysis was presumed to be mediated through CD8+ T-cells because lytic activity against peptide-coated target decreased dramatically in the presence of anti-human HLA-A2 antibody (data not shown). The remaining T-cell lines designated as p851-859 and p1172-1180 were nonspecific with little lytic activity. At the 10th IVS at an E:T ratio of 10:1 all lysis was below 10%.

**DISCUSSION**

CD8+ CTL recognize peptide bound in the groove of class I MHC molecules. Recent advances defining the character and nature of this interaction (25, 38) have led to the development of systems for generating and utilizing virus-specific CTL responding to peptide epitopes recognized in the context of class I MHC. It is well documented
that in vivo priming with peptide in murine models can elicit class I restricted CTL (26, 27, 39, 40). Although many investigators have studied the use of carrier systems to augment this response (41, 42), it is clear that CTL can be induced in vivo by the use of unmodified, carrier-free synthetic peptides (43–45). Studying peptide-specific CTL in humans is more difficult because of practical problems limiting the use of in vivo immunization and a lack of standard reproducible in vitro priming methods. Nevertheless, investigators have shown, in viral systems, that human peptide-specific CTL can be generated by immunization in vitro with synthetic peptides that correspond to class I binding motifs (46–48).

Our laboratory has been evaluating the potential of oncogenic proteins to serve as targets for T-cell therapy. Malignant transformation is increasingly being ascribed to the aberrant function of a series of definable cancer-related genes. Aberrant function is often the result of specific mutations or gene amplification. One approach to target oncogenic proteins for T-cell therapy is to target the cancer-specific mutated segment of the protein. Studies have demonstrated that the mutated segment of p21\textsuperscript{ras} protein and the joining region segment of p210\textsubscript{vpr-abl} chimeric protein can be immunogenic to T-cells (11, 12, 49) and thus, by definition, aberrant oncogenic proteins can serve as cancer-specific antigens.

Targeting aberrant oncogenic proteins offers the hope of cancer therapy with absolute specificity. However, one apparent problem for directing T-cell therapy against mutated segments of oncogenic proteins is that not every patient will be able to respond to a single epitope on a single protein. Overexpressed oncogenic proteins offer the advantage of providing many potential target epitopes and a chance for much broader reactivity, albeit without absolute specificity. The HER-2/neu protein was chosen for the current studies as a prototype-overexpressed oncogenic protein.

The generation of peptide-specific CTL to HER-2/neu-derived peptides in an HLA-A2 restricted fashion was aided by prior elucidation of the binding motifs (25). Others have shown that not only are the peptides that fit the binding motif extremely stable in the class I MHC cleft (50), but in addition there is a strong correlation between the ability to stabilize or up-regulate MHC class I in vitro and the generation of CTL responses in vivo (51). A similar correlation was observed in the current studies. The two peptides that stabilized HLA-A2 most effectively, i.e., the strongest binders in the T2-binding assay, were the peptides which could elicit CTL by in vitro priming.

In initial studies, standard T-cell culture conditions for detecting immunity to standard recall antigens were used and no peptide-specific CTL could be detected. The conditions developed to allow elici-
tation of peptide-specific CTL were derived by extensive empiric experiment but are consistent with the current paradigm developed by others. Conditions include: (a) large numbers of T-cells to compensate for a presumably low precursor frequency (52); (b) a concurrent stimulated primed CD4+ T-cell response to provide requisite T-cell help. Many studies have emphasized the role of CD4+ T-cells in the generation of CTL (53, 54), including the demonstration that in vivo depletion of CD4+ T-cells can completely inhibit the generation of CTL by peptide immunization (55). Tetanus toxoid was used at the lowest titrated concentration capable of providing detectable stimulation so as not to overwhelm the culture with CD4+ cells or to generate too much IL-2; (c) IL-2, but added late to culture (day +9 for primary IVS), and in very small amounts. IL-2 added early (day +2 or +4) or in greater concentration (5–10 units) resulted in the generation and expansion of nonspecific CTL and/or activated NK cells even after multiple restimulations (data not shown); and (d) multiple restimulations. Although peptide-specific CTL could be detected after 4 weeks in culture, responses became more marked after multiple restimulations.

The current experiments showed that peptides derived from the HER-2/neu oncogene protein sequence are within recognition of the human T-cell repertoire and that peptide-specific CTL can be generated to multiple epitopes. The next major question to be addressed will be whether the peptide-specific CTL can lyse HER-2/A2-positive, HER-2/neu-positive cancer cells. Extrapolating from our murine experience (49), our expectation is that some but not all peptide-specific CTL will be able to lyse cancer cells. Not every peptide will be appropriately processed and presented in high enough concentration for T-cell recognition. If the currently identified candidate peptide-specific CTL prove unable to specifically lyse HER-2/neu-positive A2A2-positive cancer cells, the same methods can be used to increase the number of class I molecules/stimulator cells may potentially by a lack of reproducible in vitro culture systems. The current study defines a reproducible in vitro culture system for the generation of human CTL. Using human responses to p185HER-2/neu protein in the context of HLA-A2 as a prototype model, the in vitro culture system allows rapid identification of immunogenic epitopes in the oncogenic protein.

ACKNOWLEDGMENTS

The authors wish to thank Anita Rogers and Kevin Whitham for their assistance in the preparation of the manuscript.

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CTL SPECIFIC FOR HER-2/neu PEPTIDES


In Vitro Generation of Human Cytolytic T-Cells Specific for Peptides Derived from the HER-2/neu Protooncogene Protein

Mary L. Disis, Joseph W. Smith, Ann E. Murphy, et al.