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

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**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 vivo immunization and a lack of reproducible in vitro 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 existed helper/inducer T-cell immunity to p185HER-2/neu protein and peptides. The current study represents initial attempts to identify candidate cytotoxic 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 vitro immunization in a culture system using peripheral blood lymphocytes from a normal individual homozygous for HLA-A2. p185HER-2/neu protooncogene protein contains immunogenic epitopes capable of generating human CD8+ 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 autologous 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 p185HER-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, MHC3 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 CD4+ helper/inducer T-cells secrete cytokines in response to soluble antigen presented in the class II antigen processing pathway and CD8+ CTL respond to and kill cells presenting antigenic proteins in the class I antigen processing pathway. Methods for eliciting and detecting CD4+ helper/inducer and CD8+ 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 CD8+ CTL have been greatly facilitated by the determination that CD8+ 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 oncoergic proteins in humans. The appropriateness of an oncoergic 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 culture 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/nu oncoergic 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/nu 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. Dolejsi (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, B18, 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-ME, 200 units/ml penicillin, 200 units/ml streptomycin, 10 mM c-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 × 10^7) 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% CO₂/95% air. HLA-A2 binding was determined by indirect immunofluorescence. The cells were stained on ice for surface HLA-A2 with a mouse monoclonal anti-HLA-A2 antibody at a 3% solution in phosphate-buffered saline (Atlantic Antibodies, Stillwater, MN), followed by a second layer antibody, fluorescein isothiocyanate-F(ab')2 rabbit anti-mouse IgG (H+L) (Zymed Laboratories, San Francisco, CA) was also used in a 3% solution. A control stain of T2 incubated similarly with no exogenous peptide added was then cryopreserved and used as a negative control. 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 (4-6 × 10^7) were thawed, washed, and resuspended in 25-cm², 100-ml tissue culture flasks (Corning, Corning, NY) containing 10 ml of media with equal parts of EHAA 120 (Biofluids) and RPMI 1640 (Gibco) with 10% FCS, penicillin/streptomycin, 2-mercaptoethanol, and 10% AB serum (human AB CELLeCt; ICN Flow, Costa Mesa, CA). Appropriate peptide in a concentration of 10 μg/ml was added directly to the bulk cultures. To provide T-cell help, low concentrations of tetanus toxoid (2.5 μg/ml) were added to each culture in the initial in vitro sensitization along with peptide. So as not to overwhelm or dominate the culture with the tetanus toxoid response, titrations of tetanus toxoid had previously been assessed in a standard proliferation assay with the donor’s lymphocytes and the concentration of tetanus toxoid that provided the lowest detectable stimulation index (stimulation index, 1.85) was used. The cells were incubated for 7 days at 37°C in 5% CO₂/95% air, at the end of which they were harvested and washed, and 2 × 10^7 cells were resuspended in 10 ml media with 10% AB serum in 25-cm² upright flasks. Thawed autologous PBMC, 2 × 10^7, were incubated for 2 h with 10 μg/ml of the appropriate peptide, irradiated to 3000 rads, and added directly to culture.

IL-2 (human recombinant IL-2; Hoffmann La Roche Inc., Nutley, NJ) at a maximum concentration of 5 units/ml was added directly to culture on day +2 and again on day +4 after this second in vitro stimulation. Most experiments used 2 units/ml. The restimulation schedule followed a 7-day cycle with IL-2 added as described on day 2 and 4 of each cycle. At the third stimulation, the cells were split to 24-well flat-bottom plates (Costar, Cambridge, MA), at a concentration of 2 × 10^6 cells/ml of media in each well with antigen-presenting cells still consisting of 1 × 10^6 irradiated PBMC with added peptide. Routine cytotoxic assay was performed after the 4th IVS and again after the 10th IVS.

Cytotoxicity Assay. At the time of acquisition of donor PBMC, autologous (LCL) were generated and maintained in culture with RPMI 1640/10% FCS. LCLs were generated by the incubation of 5 × 10^7 PBMC in 2 ml of RPMI/10% FCS with 2 ml of Epstein-Barr virus-conditioned supernatant. Cyclosporin was added at a concentration of 400 ng/ml. Media were changed and cyclosporin was added every 7 days for 1 month until stable transformed B-cell lines were obtained. These LCLs were used as targets. After the fourth IVS, 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 51Cr-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 3% solution. The stained cells were then preserved in 1% paraformaldehyde and stored at 4°C. Flow cytometry was performed.

RESULTS

HER-2/nu 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/nu 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 absence of anchor residues L and V. The peptide motifs were then assigned to the appropriate HLA-A2 alleles based on the amino acid sequence motif shown in Table 1. The data were then compared with the results of experiments performed on the four motifs shown in Figure 1. It is clear that the peptide motifs shown in Table 1 have the ability to bind to HLA-A2.1.

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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).
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 despite low levels of already occupied class I MHC. T2 without exogenous peptide 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 empiric 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 primed CD4+ T-cell response; (c) IL-2 added late to culture in low concentration; and (d) multiple restimulations.

Table 3 Peptides with a motif theoretically appropriate for binding to HLA-A2.1 can be shown to actually bind to HLA-A2 in a class I MHC molecule stabilization assay.

The percentage of increase of class I and if the surface of the cell is measured by increased fluorescent intensity of cells incubated with peptide compared to cells incubated in medium alone. Values are results of 3 separate analyses along with the mean. Peptides p48-56 and p789-797 are particularly avid binders.

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
A Fourth in vitro stimulation

\[ p48-56 \]

B Tenth in vitro stimulation

\[ p48-56 \]

Fig. 1. CTL specific for HER-2/neu peptides can be generated by in vitro immunizations. PBMC (4–6 × 10^6) from a homozygous HLA-A2 normal donor were incubated with peptide at a concentration of 10 μg/ml. The lymphocytes were tested for lytic activity after 4 and then 10 in vitro sensitizations. A, data after the fourth IVS. Target cells consisted of 51Cr-labeled autologous Epstein-Barr virus-transformed B-lymphocytes incubated without peptide (C), with stimulated peptide (△), or irrelevant peptide (○) for 2 h prior to use. In this assay irrelevant peptide for p48-56 was p109-117, a peptide derived from the rat HER-2/neu sequence and for p789-797 it was p851-859. A 4-h chromium release assay was performed. The results represent the percentage of specific lysis at the indicated effector:target ratio. Activity against K562 and Daudi at this time was less than 10% lysis. These targets were included to evaluate natural killer cell and lymphokine-activated killer cell activity. B, data for the same CTL lines after the 10th IVS. The execution of the chromium release assay is as described except the irrelevant peptide for p48-56 was p789-797, and for p789-797 it was p48-56. In all of these assays after the 10th IVS the lytic activity against K562 and Daudi were less than 5% at all effector:target ratios.

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^{ras} protein and the joining region segment of p210^{abl-erbB} 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
experimentation but are consistent with the current paradigm de
veloped by others. Conditions include: (a) large numbers of T-cells
to compensate for a presumably low precursor frequency (52); (b) a
currently stimulated primed CD4+ T-cell response to provide re
quisite T-cell help. Many studies have emphasized the role of CD4+
t-cells in the generation of CTL (53, 54), including the demonstra
tion 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 detect-
able 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) mul
tiple restimulations. Although peptide-specific CTL could be de
tected 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-cells. No T-cell repertoire and that peptide-specific CTL can be
generated to multiple epitopes. The same 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 expe
rience (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 concentra
tion for T-cell recognition. If the currently identified candidate peptide-
specific CTL prove unable to specifically lyse HER-2/neu-positive
HER-2/neu-positive cancer cells, the same methods can be used to identi
fy a greater spectrum of candidate peptides. In addition, there are
many alternative strategies for priming in vivo that may favor the
development of CTL that will lyse tumor. First, peptide-specific CTL
capable of lysing targets synthesizing the target protein can often be
elicited from peptide-specific populations by repeated stimulation of
the peptide-specific CTL by the target cells (52, 56). The presumption is
that the peptide-specific CTL line contains the progeny of many
different clones, each with slightly different specificity and/or af
finity for peptide and/or ability to recognize different configurations of
the MHC/peptide complex. Only a small subset of elicited peptide-
specific CTL present in the line are able to recognize “naturally”
processed peptides. Multiple restimulations in vitro induce selective
expansion of the appropriate effector clone(s). Second, methods to
increase the number of class I molecules/stimulator cells may poten
tiate priming. One factor limiting in vitro priming may be low concen
trations of peptide bound to MHC molecules. In murine studies,
priming to appropriately constructed peptides has been shown to elicit
CTL in circumstances in which priming to parental cells synthesizing
the protein has been impossible (52). Apparently, priming with exogenic
peptide complexes may result in the expression of a greater number of
MHC/peptide complexes per cell (52), and, a minimum number of MHC/peptide complexes are required for T-cell recognition. The cur
rent study used PBMC from an individual homozygous for HLA-A2 on
the presumption that a homozygous cell would express 2-fold the
number of MHC/peptide complexes per cell. Whether similar results
could be obtained with cells from heterozygous HLA-A2 individuals
has not yet been examined. Third, unfractonated PBMC were used as
antigen-presenting cells. Enriched dendritic cells might be more ef
fective. In vitro culture systems may be relatively deficient in den
drritic cells which are particularly effective antigen-presenting cells.
Increased efficacy of priming in vitro may lead to increased ability of
peptide-specific CTL to lyse tumor. Culture conditions requiring mul
tiple restimulations will most likely result in the expansion and over
growth of only one or two T-cell clones and might therefore randomly
select against T-cell clones capable of lysing tumor. More efficient
stimulation of CT responses would most ensure that all or most of
the potential CT precursors would be activated which would increase the
likelihood that all precursors capable of lysing tumor would respond.

In humans, the majority of published studies examining CD8+ CTL
responses to class I MHC-binding peptides have studied in vivo
primed responses to viral proteins. In vitro generation of peptide-
specific CTL with viral peptides is possible, but again, lymphocytes
were derived from subjects who were previously infected. However,
studies of response to peptide segments of self-proteins have received
less attention and studies to identify candidate CTL epitopes in cir
cumstances in which in vivo priming has not occurred have been
hampered 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
heu 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.

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