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Characterization of Cytotoxic T Lymphocyte Epitopes of a Self-Protein, p53, and a Non-Self-Protein, Influenza Matrix: Relationship Between Major Histocompatibility Complex Peptide Binding Affinity and Immune Responsiveness to Peptides

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Summary: We previously described a motif prediction of major histocompatibility complex allele-specific peptides and an in vitro assay for actual measurement of peptide binding to human leukocyte antigen HLA-A2.1 molecules. Using this method we have identified candidate cytotoxic T lymphocyte (CTL) epitopes derived from a non-self-protein (influenza matrix) and self-protein (p53). We now show that results of binding assays performed over a range of peptide concentrations indicate that distinct differences in HLA-A2.1 peptide binding affinities exist between the influenza matrix and p53 protein. The results for the influenza matrix protein indicate that the peptide that shows the highest binding affinity to HLA-A2.1 is identical to the known immunodominant peptide recognized by influenza virus–specific CTLs. The results for p53 indicate that one of the peptides with a low binding affinity is capable of inducing specific CTL responses, but CTLs recognizing the highest affinity binding peptides were not obtained. These findings are discussed in terms of the distinct implications for induction of cellular immune responses directed against peptides with different binding affinities for HLA-A2.1 of proteins that constitute attractive targets for tumor immunotherapy. **Key Words:** p53—Influenza virus—Cytotoxic T lymphocyte—Peptide major histocompatibility complex affinity—Immuno(sub)dominance.

In addition to surgery, chemotherapy, and irradiation, alternative therapy is needed for those cancer patients whose prognoses have not been markedly improved using standard treatment. A potential nontoxic therapeutic method is the treatment of patients with tumor-specific T cells and/or vaccination with tumor-specific peptides capable of inducing T-cell responses (1–3). A critical step toward this goal is the identification of tumor-specific T-cell epitopes (4). In the majority of human malignancies, the p53 tumor suppressor gene product is overexpressed and/or mutated (5). Processing of overexpressed/mutated p53 in tumor cells may give rise to cytotoxic T lymphocyte (CTL) epitopes that differ in quantity or quality from the p53 epitopes found in

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normal cells. Therefore p53 might yield useful tumor-specific target epitopes. Loss of function of the p53 gene, an important step in carcinogenesis, is mostly due to a missense mutation of one allele, leading to stabilization and overexpression of p53 (6). Overexpression also occurs after binding of p53 to a cellular or viral protein, such as the SV-40 large T antigen, adenovirus type 5 E1B, heat shock protein members, or MDM2 (6).

CTLs recognize peptides presented by major histocompatibility complex (MHC) class I molecules at the cell surface (7,8). Binding of peptides to a specific MHC molecule is dependent on so-called allele-specific peptide motifs (9–12). The human cell line 174CEM.T2 (T2) is unable to present endogenously synthesized peptides to CTLs because of a homozygous deletion of the MHC class II region located on chromosome 6 (13,14). The HLA-A2.1 molecules are the only human leukocyte antigen molecules present at the cell surface of the T2 cell line, and these molecules are empty or occupied by peptides derived from the signal peptide domains of normal cellular proteins (11,15,16). The level of stable HLA-A2.1 cell surface molecules can be increased by exogenously adding an HLA-A2.1 binding peptide. We used the T2 cell line to identify peptides of the influenza matrix protein (12) and the p53 protein (17) that bind to HLA-A2.1 by measuring HLA-A2.1 cell-surface expression.

This study reports an evaluation of the assumption that peptides of non-self-proteins binding with the highest affinity to an MHC molecule are the peptides of choice to yield immunodominant CTL epitopes. On the other hand, it is likely that T cells, bearing receptors capable of recognizing the best binding peptides derived from self-proteins, are subject to negative selection in the thymus. It would therefore be important to determine the immunogenicity of peptides of a self-protein, such as p53, in comparison with a non-self-protein, such as influenza matrix protein, in relation to MHC class I binding affinity (18,19). Influenza matrix could be used as a model protein in our study because HLA-A2.1–restricted CTLs recognizing an immunodominant peptide (influenza matrix 58–66) of the influenza matrix have been described (20,21). In this study the 15 HLA-A2.1–binding influenza matrix peptides and four wild-type p53 peptides were tested with respect to their binding affinities for the HLA-A2.1 molecule and their published (influenza matrix) or tested (p53) CTL response-inducing potential.

MATERIAL AND METHODS

T2 Binding Assay

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) using Fmoc chemistry. The purity of the peptides was determined by reverse-phase high performance liquid chromatography. Peptides were dissolved in dimethyl sulfoxide (DMSO) (final DMSO concentration 0.25%), diluted in 0.9% NaCl to a peptide concentration of 2 mg/ml, and stored at −20°C. The T2 cell line, a gift from Dr. P. Cresswell (Yale University, New Haven, CT, U.S.A.), was cultured in Iscove's modified Dulbecco's medium (IMDM) (Biochrom KG; Seromed, Berlin, Germany) with 2 m*M* glutamine, 100 IU/ml penicillin, 100 μg/ml kanamycin and 10% fetal calf serum (FCS) (Hyclone Laboratories Inc., Logan, UT, U.S.A.).

The T2 binding assay was performed as previously described (12). In short, washed T2 cells were incubated overnight with peptide or 0.9% NaCl. Peptides binding to the HLA-A2.1 molecule will stabilize this molecule at the cell surface of the T2 cell line and therefore increase HLA-A2.1 cell-surface expression. Cells were stained by indirect immunofluorescence, with the anti-HLA-A2.1 monoclonal antibody BB7.2 as a first antibody and goat-anti-mouse (GAM) fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ fragments as a second antibody, and measured on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). The fluorescence ratio (FR) was calculated by the formula mean fluorescence experimental sample/mean fluorescence background.

Induction of CTL Responses

The induction of CTL responses has been previously described (17). In short, peptide-loaded and mitomycin-C treated T2 cells were used as antigen-presenting cells with HLA-A2.1-positive peripheral blood lymphocytes of a healthy donor as responder cells. Cells were cultured in RPMI medium (Gibco, Paisley) containing glutamine, antibiotics, 15% pooled human serum, and 40 μg/ml peptide. Responding cells were restimulated weekly with feeder cells, Epstein-Barr virus–transformed B cells, and peptide. From week 3 on, 1% leucoagglutinin (Pharmacia, Uppsala, Sweden) and human recombinant interleukin-2 (120 IU/ml; Eurocetus, Amsterdam, The Netherlands) were added.

Cytotoxic specificity of responding cells against peptide-sensitized target cells was tested in standard 4-h ^{51}Cr-release assays.

RESULTS

T2 Binding Assay

Fifteen influenza matrix (IM) peptides and four wild-type p53 peptides were tested (Table 1) at different concentrations to determine their binding affinities for HLA-A2.1. Influenza matrix peptides IM (2–11), IM (2–12), IM (3–11), IM (58–66), and IM (59–68) had the highest affinity for the HLA-A2.1 molecule (Table 2). Peptide IM (58–66), the known immunodominant epitope of the influenza matrix, appeared to be the peptide with highest affinity for HLA-A2.1 (Table 2). Two p53 peptides, p53 (187–197) and p53 (65–73), had a similar binding affinity to the HLA-A2.1 molecule (Table 2). The p53 (264–271) peptide is the third-best binding peptide (Table 2).

Induction of CTL Responses

We have already been successful in generating stable, peptide-specific CTL clones against the p53 (264–271) peptide (17). Using the same protocol, we induced p53 (264–271) peptide-specific HLA-A2.1–restricted CTL lines. Against the other wild-type p53 peptides, p53 (25–33), p53 (65–73), and p53 (187–197), only a weak specific response at week 7 of culture was observed (Table 3). FACScan analysis performed during week 11 showed that in three of four CTL lines, the majority of responding cells were $CD4^+$ (data not shown). Only the CTL line against the p53 (264–271) peptide appeared to be $CD8^+$ (>97% $CD8^+$, data not shown).

TABLE 1. *Influenza matrix peptides and p53 peptides identified as peptides binding to HLA-A2.1*

Seq. no.	Peptide
Influenza matrix (IM) peptides	
IM (2–11)	SLLTEVETYV
IM (2–12)	SLLTEVETYVL
IM (3–11)	LLTEVETYV
IM (3–12)	LLTEVETYVL
IM (41–51)	VLMEWLKTRPI
IM (50–59)	PILSPLTKGI
IM (51–59)	ILSPLTKGI
IM (51–60)	ILSPLTKGIL
IM (58–66)	GILGFVFTL
IM (58–68)	GILGFVFTLTV
IM (59–68)	ILGFVFTLTV
IM (134–142)	RMGAVTTEV
IM (145–155)	GLVCATCEQIA
IM (164–172)	QMVTTTNPL
IM (164–173)	QMVTTTNPLI
p53 peptides	
p53 (25–33)	LLPENNVLS
p53 (65–73)	RMPEAAPPV
p53 (187–197)	GLAPPQHLIRV
p53 (264–271)	LLGRNSFEV

The sequence numbers (seq. no.) of the first and last amino acids are shown. The peptides are ranked according to first seq. no. Peptides were selected using sequence analysis and in vitro assay for identifying peptides capable of binding to HLA-A2.1 (12,17).

TABLE 2. *Influenza matrix and p53 peptides binding to HLA-A2.1*

	Concentration (μg/ml)						
Peptide	100	50	25	12.5	6.3	3.1	1.5
IM (58–66)	2.32	2.43	2.25	1.81	1.59	1.49	1.17
IM (2–11)	2.50	2.33	2.07	1.57	1.44	1.22	1.11
IM (3–11)	2.67	2.69	2.44	1.85	1.43	1.22	1.09
IM (2–12)	3.16	2.67	2.10	1.60	1.30	1.15	—
IM (59–68)	2.35	2.34	1.90	1.38	1.21	1.10	—
IM (164–172)	2.48	2.28	1.63	1.57	1.19	—	—
IM (41–51)	2.04	2.06	1.82	1.55	1.19	—	—
IM (51–59)	2.43	2.21	1.77	1.44	1.12	—	—
IM (3–12)	2.41	2.20	1.69	1.27	1.06	—	—
IM (134–142)	1.95	1.69	1.37	1.11	—	—	—
IM (58–68)	1.76	1.69	1.66	1.11	—	—	—
IM (50–59)	1.58	1.47	1.25	1.11	—	—	—
IM (164–173)	1.73	1.43	1.19	—	—	—	—
IM (145–155)	1.43	1.34	1.17	—	—	—	—
IM (51–60)	1.41	1.20	1.04	—	—	—	—
p53 (187–197)	2.63	2.39	1.99	1.61	1.32	1.16	1.06
p53 (65–73)	2.59	2.11	1.80	1.51	1.28	1.11	1.09
p53 (264–271)	2.69	2.28	1.68	1.22	1.08	—	—
p53 (25–33)	1.77	1.52	1.16	—	—	—	—

Binding of influenza matrix and p53 peptides at concentrations of 100, 50, 25, 12.5, 6.25, 3.1, and 1.6 μg/ml (final concentration in the test). Dashes indicate HLA-A2.1 at the cell surface was not up-regulated. The peptides are ranked in order of binding affinity to HLA-A2.1.

DISCUSSION

In recent reviews the potential therapeutic value of T-cell-mediated immune responses has been appraised (1–3). In addition to identifying new tumor-specific target antigens of choice for T-cell therapy, it is also important to identify the immunogenic epitopes within antigens. Although p53 is a self-protein subject to the laws of immunological tolerance, overexpressed p53 might serve as a tumor-specific antigen. Targeting overexpressed p53 could lead to therapy of the many types of cancer in which p53 is involved.

Processing of p53 could be altered in cancer cells compared with normal cells, stemming from, for example, a much longer half-life of p53 (22). Tumor cells may then end up displaying other p53 peptides

TABLE 3. *Lytic activity of CTL lines against four p53 epitopes*

No. of weeks in culture	Anti-p53 (264–271) line (sp–asp)	Anti-p53 (25–33) line (sp–asp)	Anti-p53 (65–73) line (sp–asp)	Anti-p53 (187–197) line (sp–asp)
Week 7	80–2	35–20	35–26	17–6
Week 9				
E/T 40:1	88–0	23–36	13–13	3–13
E/T 20:1	76–0	25–28	11–13	8–28
Week 11				
E/T 40:1	78–29	13–22	38–37	0–5
E/T 20:1	79–21	6–19	19–16	2–5

T2 was used as the target cell line in the ^{51}Cr release assays. sp refers to the percentage of specific lysis in the ^{51}Cr release assay (T2 loaded with the specific peptide). asp refers to the percentage of aspecific lysis in the ^{51}Cr release assay (T2 loaded with an aspecific HLA-A2.1–binding peptide). E/T is the effector/target ratio. On week 7 a split-well assay was performed with an unknown E/T ratio.

or different quantities of the same p53 peptides compared with normal cells, allowing selective presentation at the cell surface and recognition by CTLs. For presentation by MHC class II, mutation in the staphylococcal nuclease protein enhanced the processing and/or presentation of T-cell epitopes (23), even though it is not directly involved in T-cell epitope recognition. A similar mechanism of enhanced processing might operate for mutant p53 in the case of MHC class I.

Members of the heat shock protein (HSP) family selectively bind peptides and thereby influence antigen processing and presentation (24). This may occur in the case of p53 and MHC class I presentation, because p53 can form complexes with HSP (6). We suggest that processing of altered p53 complexes could give rise to new CTL epitopes. In the case of a self-protein such as p53, it is likely that tolerance will play a role in determining the frequency of specific CTLs recognizing (dominant) self-epitopes in the postthymic environment. The interactions involved in negative selection of developing T cells are probably the same as in clonal activation of mature T cells in the periphery; a threshold quantity of peptide/MHC complexes is recognized by a given T cell receptor (TCR) (25). CTL precursors capable of recognizing self-peptides that are not processed or presented at subthreshold levels are therefore likely to be spared negative selection.

An example of this process is found in a murine system in which CTLs were obtained against peptide determinants derived from several ubiquitous or tissue-restricted self-proteins, e.g., β_2-microglobulin, hemoglobin, and liver proteins (26). In the case of the cytochrome-c molecule, it was shown that not all possible MHC binding peptides are processed and presented at the cell surface (27). A so-called cryptic self-peptide was capable of breaking T-cell tolerance to other sites in the whole self-protein (27). The same is true in murine models of experimental allergic encephalomyelitis (EAE) (28). In response to both cytochrome-c and myelin basic protein in EAE, normally silent T cells are aroused. From these studies it can be inferred that these potentially self-reactive T cells are quiescent in vivo, possibly because the epitope recognized by these T cells is not processed at all or only at a very low level. Alternatively, the epitopes are presented in a nonphysiological manner, or the specific TCR-bearing T cells may be anergic (26–28). One of the possibilities explaining our finding of CTLs against an autologous peptide is that we may have overcome clonal anergy by high-concentration peptide presentation on the antigen-presenting T2 cells.

The p53 (264–271) peptide is not the peptide with highest affinity for HLA-A2.1 (Table 2). Yet the p53 (264–271) peptide so far is the only determinant against which strong CTLs have reproducibly been obtained. Comparison of the p53 peptide-binding results with the peptide binding data obtained with the influenza matrix peptides leads to the tentative conclusion that the p53 (264–271) peptide is a second-echelon peptide with respect to binding affinity. Stable CTLs capable of recognizing either of the two best binding peptides have not been obtained despite repeated efforts of the type that consistently yield p53- (264–271) specific CTLs. Our failure to obtain CTLs capable of recognizing the p53 peptide possessing the highest MHC binding affinity may reflect the greater chance of inducing tolerance against the immunodominant self-peptide. The fact that the same protocol consistently yielded specific CTLs when a p53 peptide of lesser binding

affinity was used also supports the view that binding affinity may influence immunodominance. It is important to know which of these p53 peptides are processed and presented at the cell surface in a complex with a HLA-A2.1 molecule.

Tolerance is thought to be induced against peptides with the optimal binding affinity present at threshold quantity on thymic or postthymic educator cells (19,25,29). By ignoring those peptides with a level of presentation below this threshold, the immune system can preserve the largest possible TCR repertoire. Hence, autoreactive CTLs against the most immunodominant self-peptides per MHC allele have a greater chance of inducing tolerance. According to Ohno (29), there seems to be a general need (e.g., by HLA-A2.1) to ignore every high-affinity self- or non-self-peptide, thus following the safe course by suspecting all high-affinity peptides as possible self-peptides. CTL epitopes of non-self-proteins, in this view, could be peptides that are endowed with suboptimal binding affinity to a given class I MHC molecule (29).

It is predicted on a theoretical basis that peptide IM (3–11) should be the best binder and therefore be ignored by HLA-A2.1–restricted CTLs (29). According to this theory, the peptide IM (58–66) recognized by the HLA-A2.1–restricted CTLs should be among the second-echelon MHC class I binding peptides in terms of binding affinity. However, our results show that the IM (58–66) peptide is the peptide with the highest affinity for HLA-A2.1 (Table 2). This finding supports our view that the best binding peptide of a non-self-protein is most likely the immunodominant CTL epitope. We have obtained similar data from various viral systems (adenovirus/Sendai virus) in mice (unpublished observations).

We are now testing whether CTL responses directed against influenza virus–infected targets can be induced against other HLA-A2.1–binding influenza matrix peptides and, if so, whether these CTLs can lyse virus-infected cells or cells expressing matrix protein. This testing would address the question of whether CTLs against the lower-affinity-binding peptides of matrix protein can be obtained and can lyse infected cells. The answer would be relevant for the response against a self-protein in which the best binding peptides might have induced tolerance and the second-echelon binding peptides are the most likely candidates to arouse T-cell responses against.

In conclusion, the best MHC-binding peptides of a self-protein possibly have induced tolerance. Lower-affinity MHC-binding peptides of self-proteins can induce CTLs, whereas the peptides with the highest binding affinity of non-self-proteins harbor the immunodominant CTL epitopes.

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