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Site-directed mutagenesis of an *HLA-A3* gene identifies amino acid 152 as crucial for major histocompatibility complex-restricted and alloreactive cytotoxic T-lymphocyte recognition

(major histocompatibility antigen/class I molecule/antigenic determinant)

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ABSTRACT **Major histocompatibility complex-restricted and alloreactive cytotoxic T lymphocytes (CTL) can discriminate between the HLA-A3.1 and HLA-A3.2 antigens. HLA-A3.1 and the rare variant HLA-A3.2 have been shown to differ by two amino acids in the α_2 domain at positions 152 (A3.1, glutamic acid; A3.2, valine) and 156 (A3.1, leucine; A3.2, glutamine). To determine the structural basis for the ability of CTL to differentiate A3.1 from A3.2, two site-directed mutants of the *HLA-A3.2* gene were produced, $152^{A3.1}$–$156^{A3.2}$ and $152^{A3.2}$–$156^{A3.1}$, that have the indicated codons for positions 152 and 156. These mutated *HLA-A3* genes, as well as the nonmutated *HLA-A3.1* and *HLA-A3.2* genes, were then transfected into the murine cell line P815-HTR and used as targets for human CTL. Influenza virus-specific HLA-A3.1-restricted CTL lysed virus-infected P815 cells transformed with the *HLA-A3.1* and $152^{A3.1}$–$156^{A3.2}$ genes, but not P815 cells transformed with the *HLA-A3.2* and $152^{A3.2}$–$156^{A3.1}$ genes. *HLA-A3.2*-allospecific CTL lysed the P815 cells transformed with the *HLA-A3.2* and $152^{A3.2}$–$156^{A3.1}$ genes but did not lyse P815 cells transformed with the *HLA-A3.1* or $152^{A3.1}$–$156^{A3.2}$ genes. Thus, a single amino acid change at position 152, substituting valine for glutamic acid and thereby introducing a charge difference, produces major structural changes in the epitopes recognized by major histocompatibility complex-restricted and alloreactive CTL.**

Class I major histocompatibility complex (MHC) molecules function as restriction elements for T cells that recognize foreign antigens and as targets for alloreactive T cells (1–3). The precise structural features of the class I molecules that are important for recognition by T cells are not clearly understood. Exon shuffling experiments have localized these determinants to the two amino-terminal domains of the class I heavy chain (α_1 and α_2 in the human) (4–9). Specific regions of these domains that may be important for T-cell recognition have been identified using murine class I mutants and human class I variants that are recognized differentially by cytotoxic T lymphocytes (CTL) (1–3). One region in particular that has repeatedly been observed to exhibit changes that are recognized by human CTL is in the α_2 domain between residues 146 and 160 (10–14). CTL-defined variants of HLA-A2 (10, 11), HLA-A3 (12, 13), and HLA-B27 (14) each have amino acid substitutions within residues 146–160. Additional supportive evidence for the importance of amino acids in positions 146–160 comes from studies with mutant mice (3). Of the bm series of H-2K^b murine mutants, the bm1 mutant is the most functionally divergent from wild type as assessed by virus-immune CTL recognition; H-2K^b-restricted CTL specific for each of seven different viruses do not recognize virus-infected bm1 targets (3). The bm1 mutant molecule differs from the parent H-2K^b molecule at positions 152, 155, and 156 (15, 16). Because there are multiple differences between these mutants (variants) and their respective parent molecules, it is not known whether all of these changes are required to produce the observed effects on T-cell recognition.

Previous studies have shown that a variant HLA-A3 molecule (A3.2) differs from the predominant type (A3.1) by two amino acids, at positions 152 and 156 (12, 13). These differences produce the following significant changes in T-cell recognition: (*i*) HLA-A3.1-restricted virus-immune CTL do not recognize infected HLA-A3.2-positive targets (17); and (*ii*) HLA-A3.2-positive cells induce a strong A3.2-specific CTL response from HLA-A3.1-positive responder cells (18). Thus, these differences affect self-restriction elements as well as alloantigenic determinants. It is unclear whether both amino acid differences contribute equally to the formation of these antigenic determinants, or whether a single residue difference can affect the formation of either type of epitope. To address these issues, we have used the technique of site-directed mutagenesis to create HLA-A3 molecules that differ from one another at position 152 or 156 only and have analyzed their ability to be recognized by virus-immune and alloimmune CTL. The results indicate that the difference of glutamic acid (A3.1) and valine (A3.2) at position 152 is of primary importance in forming determinants recognized by both self-restricted and allospecific T cells.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear leukocytes (PBL) were obtained by batch leukapheresis of normal adult volunteers and were cryopreserved (17). HLA serotyping of PBL was kindly performed by the Human Leukocyte Antigen Typing Laboratory (Department of Transfusion Medicine, National Institutes of Health) and the Blood Center of Southeastern Wisconsin (Milwaukee). P815-HTR1-TK$^-$ is a highly transfectable thymidine kinase negative variant of the murine mastocytoma P815 (19) and was generously provided by T. Boon (Ludwig Institute for Cancer Research, Brussels).

Antibodies. The monoclonal antibodies used in the present study are GAP.A3 (20) (anti-HLA-A3, IgG ascites), B9.12 (21) (anti-HLA class I monomorphic; IgG culture fluid; a kind gift of F. Koning, National Institutes of Health), and 34-2-12 (22) (anti-H-2D^d; IgG ascites). All antibodies used for immunofluorescence analyses were used at saturating concentrations as determined by titration on a fluorescence-activat-

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Abbreviations: CTL, cytotoxic T lymphocyte(s); MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte(s); PHA, phytohemagglutinin.

ed cell sorter. Fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG were obtained from Cappel Laboratories (Cochranville, PA).

Synthetic Oligonucleotides. The two oligonucleotides used to prime site-directed mutagenesis, 5′ CGGCCCATGAGGCGGAGC 3′ (to generate the $152^{A3.1}$–$156^{A3.2}$ mutant) and 5′ CGGAGCAGCTGAGAGCCT 3′ (to generate the $152^{A3.2}$–$156^{A3.1}$ mutant), and the HLA-A3 exon 3 sequencing primer 5′ GACTGGGCTGACCGC 3′ (used to confirm the presence of mutations and the integrity of sequences) were obtained in acrylamide gel-purified form from OCS Labs (Denton, TX). The mutagenic oligonucleotides were 5′ phosphorylated using T4 polynucleotide kinase (Bethesda Research Laboratories).

Site-Directed Mutagenesis of HLA-A3.2. A 212-base-pair (bp) *Kpn* I fragment of the *HLA-A3.2* gene, containing all but 68 5′-nucleotides and 1 3′-nucleotide of exon 3, was subcloned into the *Kpn* I site of the single-stranded phage vector M13mp18. A subclone containing the noncoding strand of the exon 3 fragment on the positive strand of the vector was used as a template for oligonucleotide-primed site-directed mutagenesis, using methods described (23–25). Briefly, 20 pmol each of mutagenic primer and M13 universal sequencing primer (Bethesda Research Laboratories) and 2 pmol of M13 hybridization primer (New England Biolabs) were hybridized to 1 pmol of the single-stranded template. The product was then extended and ligated using the Klenow fragment of DNA polymerase (Bethesda Research Laboratories) and T4 ligase (New England Biolabs) in a vol of 30 μl for 24 hr at 14°C. Part of the resulting mixture was used to transform competent JM103 cells. Selected plaques were amplified in 2-ml cultures and were screened by dot blot hybridization using the 5′-end-labeled mutant oligonucleotide (23). Clones that were positive following washing of the filter at 70°C (for $152^{A3.1}$–$156^{A3.2}$) or 68°C (for $152^{A3.2}$–$156^{A3.1}$) were analyzed by DNA sequencing, using the chain-termination method (26), to confirm the presence of the mutation.

The mutagenized *Kpn* I fragment was excised from the replicative form of the M13 clone and ligated into an M13mp11 clone of HLA-A3.2 that lacked the 212-bp *Kpn* I fragment. The correct orientation of the inserted fragment was confirmed by DNA sequencing. The resulting mutant *HLA-A3.2* genes were then subcloned into the plasmid vector pUC8 (27), which was prepared by standard procedures (28) and purified twice on cesium chloride gradients.

DNA-Mediated Gene Transfer. Plasmid clones containing the mutant *HLA-A3.2* genes were introduced into P815-HTR cells by cotransformation with pSV2gpt, which permits growth in the presence of mycophenolic acid. Transformation of the cells was done according to the calcium phosphate method described by Maryanski *et al.* (29), using 20 μg of class I plasmid and 10 μg of pSV2gpt. Selection took place in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD), supplemented with 10% fetal calf serum (Hazelton Dutchland, Denver, PA)/2 mM glutamine/gentamicin (50 μg/ml) (GIBCO) and containing mycophenolic acid (6 μg/ml) (GIBCO), xanthine (0.25 mg/ml) (Sigma), and hypoxanthine (15 μg/ml) (Sigma) (selective medium).

Transformed cells were analyzed for expression of *HLA-A3* gene products by indirect immunofluorescence using a FACS-IV cell sorter (Becton Dickinson) and the anti-HLA-A3 antibody GAP.A3 as described (30). The brightest 10% of the positive cells were selected by sorting on the cell sorter and expanded in selective medium (see above). Transformed cells were retested by indirect immunofluorescence approximately every 2 weeks to ensure stability of expression of the transfected gene.

Generation and Assay of CTL. Generation of the HLA-A3.1 plus A/JAP influenza virus-specific CTL line 1D3 was performed as follows: PBL from donor 49610 (HLA-A3,11, -B14,35, -DR1,6) were stimulated *in vitro* with A/JAP/305/57 (H2N2) influenza virus as described (17); 10 days later, the primed responder cells were stimulated in secondary culture with A/JAP-infected 2000-R-irradiated (1 R = 2.58×10^{-4} C/kg) PBL (17) from donor 45019 (HLA-A3,29, -B7,44, -DR2,7) for 5 days and in tertiary culture with irradiated A/JAP-infected PBL from donor 44034 (HLA-A1,3, -B8,39, -DR2,7). After 4 days of tertiary stimulation, responder cells were restimulated in limiting dilution culture at 10 cells per well with irradiated A/JAP-infected autologous PBL as stimulator cells (17). The specificity of the 1D3 line for HLA-A3.1 plus A/JAP was established by assaying the line on a panel of A/JAP-infected and uninfected HLA-typed targets.

The HLA-A3.2 allospecific CTL line M3 anti-E1 was generated as described (18). The HLA-A2 allospecific CTL line A5 was generated by priming responder cells from donor 43597 (HLA-A1,25, -B8,35, -DR3) with stimulator cells from donor W5 (HLA-A2, -B8, -DR3) and repeatedly stimulating the primed responder cells with $HLA\text{-}A2^+$ stimulator PBL (18). The A5 line was derived from these responder cells by limiting dilution at 10 responder cells per well. Specificity for HLA-A2 was established by assaying the A5 line on a panel of HLA-typed target cells.

CTL activity was assayed in a 4-hr ^{51}Cr-release cytotoxicity assay as described (17). Target cells were phytohemagglutinin (PHA)-stimulated PBL (17) or P815 cells; influenza virus-infected targets were prepared as described (17). Results are expressed as the mean percentage specific lysis of triplicate determinations (17).

RESULTS

Generation of Site-Directed Mutants. HLA-A3.1 and HLA-A3.2 molecules differ from each other at amino acid positions 152 and 156. The goal of these experiments was to generate HLA-A3 molecules that differed from the HLA-A3.1 molecule at position 152 or 156 alone. As shown in Fig. 1, this could be readily accomplished by means of single base changes in HLA-A3.2. Changing thymine to adenine in the second position of codon 152 of HLA-A3.2 results in the substitution of glutamic acid for valine ($152^{A3.1}$–$156^{A3.2}$). Changing adenine to thymine in the second position of codon 156 of HLA-A3.2 results in the substitution of leucine for glutamine ($152^{A3.2}$–$156^{A3.1}$). The technique of oligonucleotide-primed site-directed mutagenesis was used to generate these molecules (24–26). Rather than perform the mutagenesis on the entire *HLA-A3.2* gene, a 212-bp *Kpn* I fragment that contained 75% of HLA-A3.2 exon 3 (encoding the α_2 domain) was subcloned. A fragment of this size allowed us to easily monitor mutagenized clones by DNA sequencing to ensure that no bases other than those intended were changed. The mutagenized fragment was then inserted into an HLA-A3.2 clone that lacked the 212-bp *Kpn* I fragment.

		152				156	
A3.2	CAT	VAL GTG	GCG	GAG	CAG	GLN CAG	AGA
A3.1	---	-A- GLU	---	---	---	TT- LEU	---
$152^{A3.1}$–$156^{A3.2}$	---	GLU -A-	---	---	---	GLN ---	---
$152^{A3.2}$–$156^{A3.1}$	---	--- VAL	---	---	---	-T- LEU	---

FIG. 1. Sequence comparison of HLA-A3.1, HLA-A3.2, and the site-directed mutants $152^{A3.1}$–$156^{A3.2}$ and $152^{A3.2}$–$156^{A3.1}$. Amino acids and their corresponding codons derived from HLA-A3.1 are indicated by a black background. A dash (–) indicates homology to the HLA-A3.2 nucleotide sequence.

Expression of HLA-A3 Genes in P815-HTR Cells Following DNA-Mediated Gene Transfer. Plasmids containing the A3.1, A3.2, $152^{A3.1}$–$156^{A3.2}$, and $152^{A3.2}$–$156^{A3.1}$ genes were introduced into P815-HTR cells and, after selection (see *Materials and Methods*), were assayed by indirect immunofluorescence for cell-surface expression of molecules that reacted with the anti-HLA class I monomorphic antibody B9.12 (21). Fig. 2 shows the results of such an assay. The level of expression of HLA molecules detected by this anti-monomorphic antibody on $P152^{A3.1}$–$156^{A3.2}$ was almost indistinguishable from the levels expressed by the PA3.1 and PA3.2 transformants (Fig. 2*A*). The cell-surface expression of HLA antigens on transformant $P152^{A3.2}$–$156^{A3.1}$ was found to be significantly (5- to 10-fold) higher than on the other transformants. As a control for levels of expression of endogenous class I molecules, the transformants were assayed for the ability to bind an anti-H-$2D^d$ antibody. The results (Fig. 2*B*) demonstrate that all of the transformants expressed similar levels of this mouse antigen.

MHC-Restricted Recognition of P815-HTR Cells Transformed with *HLA-A3* Genes. Virus-specific MHC-restricted CTL can discriminate between HLA-A3.1 and HLA-A3.2 (17). Thus, influenza virus-specific CTL populations from $A3.1^+$ donors fail to lyse influenza-infected targets that express the A3.2 molecule. Experiments shown in Fig. 3*A* examine the effects of the single amino acid substitutions in $152^{A3.1}$–$156^{A3.2}$ and $152^{A3.2}$–$156^{A3.1}$ on target-cell recognition by virus-specific A3.1-restricted CTL. The results demonstrate that the A/JAP influenza virus-specific A3.1-restricted CTL line 1D3 lyses the A/JAP-infected PA3.1 and $P152^{A3.1}$–$156^{A3.2}$ transformants but does not lyse the A/JAP-infected PA3.2 and $P152^{A3.2}$–$156^{A3.1}$ transformants or any uninfected transformant (data points shown only for uninfected PA3.1). This same pattern of reactivity has been observed with three other independently derived influenza-specific CTL lines obtained from $A3.1^+$ donors (data not shown). These observations are apparently unrelated to class I molecule density on the target cells. Infected $P152^{A3.2}$–$156^{A3.1}$ transformants are not lysed despite the 5- to 10-fold higher expression of the human class I molecules (Fig. 2*A*). These results indicate that not only do virus plus A3.1-specific CTL discriminate between A3.1 and A3.2 as restriction elements on the virus-infected transformants, but also that the single substitution of the A3.2-encoded valine for glutamic acid at position 152 in the A3.1 molecule completely destroys the A3.1 restriction elements. In contrast, retention of glutamic acid at position 152 and substitution of leucine for glutamine at position 156 did not have a detectable effect on A3.1-restricted recognition [$P152^{A3.1}$–$156^{A3.2}$ was lysed by A3.1-restricted A/JAP-specific CTL to a similar extent as PA3.1 (Fig. 3*A*)].

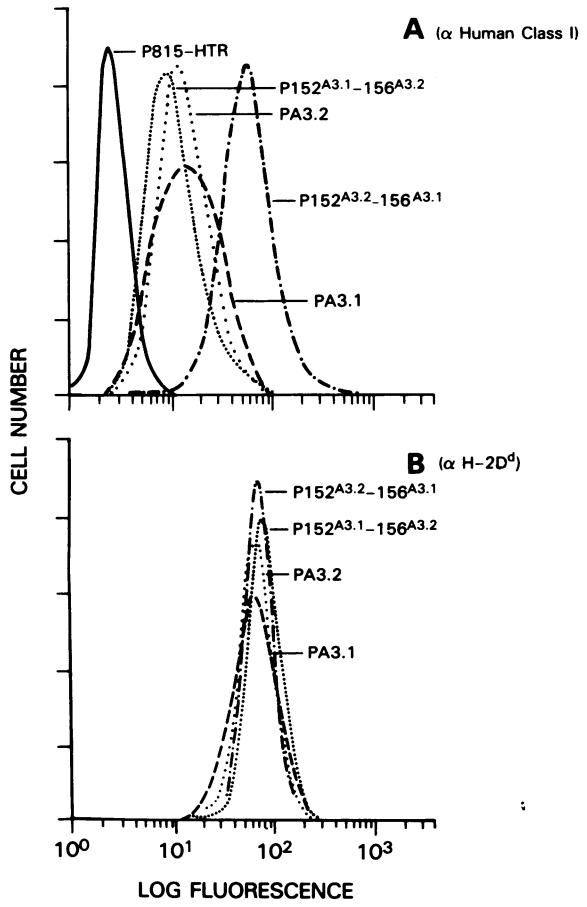

FIG. 2. Cell-surface expression of HLA molecules on P815-HTR transfectants. The indicated cells were incubated with the anti-HLA class I monomorphic antibody B9.12 (*A*) or the H-$2D^d$-specific antibody 34-2-12 (*B*). After reaction with fluorescein-conjugated anti-mouse antiserum, cells were washed and analyzed on the fluorescence-activated cell sorter.

Allorecognition of P815-HTR Cells Transformed with *HLA-A3* Genes. Previous studies have demonstrated that the A3.2 molecule could be distinguished from the A3.1 molecule by alloreactive CTL, and that the A3.2-specific alloreactive CTL line M3 anti-E1 could recognize the product of the A3.2 gene after it was transfected and expressed on murine L cells (18). P815-HTR transformants expressing the mutant or parent molecules were assessed for their abilities to be lysed by the M3 anti-E1 CTL line (Fig. 3*B*). The A3.2-specific CTL line lysed the PA3.2 but not the PA3.1 transformant. The level of lysis of the PA3.2 mouse cell transformant was ≈1/2 that observed on the human PHA blast target E1 that carries the A3.2 molecule. The $P152^{A3.2}$–$156^{A3.1}$ transformant was lysed as well as the PA3.2 transformant, whereas no lysis of $P152^{A3.1}$–$156^{A3.2}$ or P815-HTR was observed [similar results were obtained with three sublines derived from this CTL line (data not shown)]. None of the transformants was lysed by the HLA-A2-specific CTL line A5 (Fig. 3*C*). The A5 line did lyse the E1 PHA blasts, which also express the A2 antigen. These results indicate that the alloantigenic determinant(s) that distinguish A3.2 from A3.1 are critically dependent on the presence of valine at position 152 of the A3.2 molecule, and that substitution of glutamic acid for valine destroys the determinant(s). In contrast, the substitution of leucine for glutamine at position 156 had no detectable effect on the recognition of A3.2 by A3.2-specific alloreactive CTL.

DISCUSSION

The present study examines the structural basis for epitopes that are recognized by CTL that discriminate between two subtypes of HLA-A3. These A3 subtypes, A3.1 and A3.2, differ from each other by only two amino acids, at positions 152 and 156 in the α_2 domain (12, 13). It is apparent from this study that it is the change at position 152 (from a glutamic acid in HLA-A3.1 to a valine in HLA-A3.2) that results in the formation of the allospecific and influenza virus-specific HLA-A3-restricted CTL-defined differences between HLA-A3.1 and HLA-A3.2. The conclusion that the substitution at position 152 is crucial for T-cell recognition of the HLA-A3 molecule is based on results obtained with A3.2-allospecific and type A influenza virus-specific A3.1-restricted CTL lines. Although it is conceivable that CTL clones exist whose recognition of HLA-A3 is not affected by the change at position 152, the fact that A3 recognition by uncloned CTL populations is so dramatically affected by a change at this position indicates that this amino acid is crucial for recognition by a substantial proportion of T-cell clones. It is not

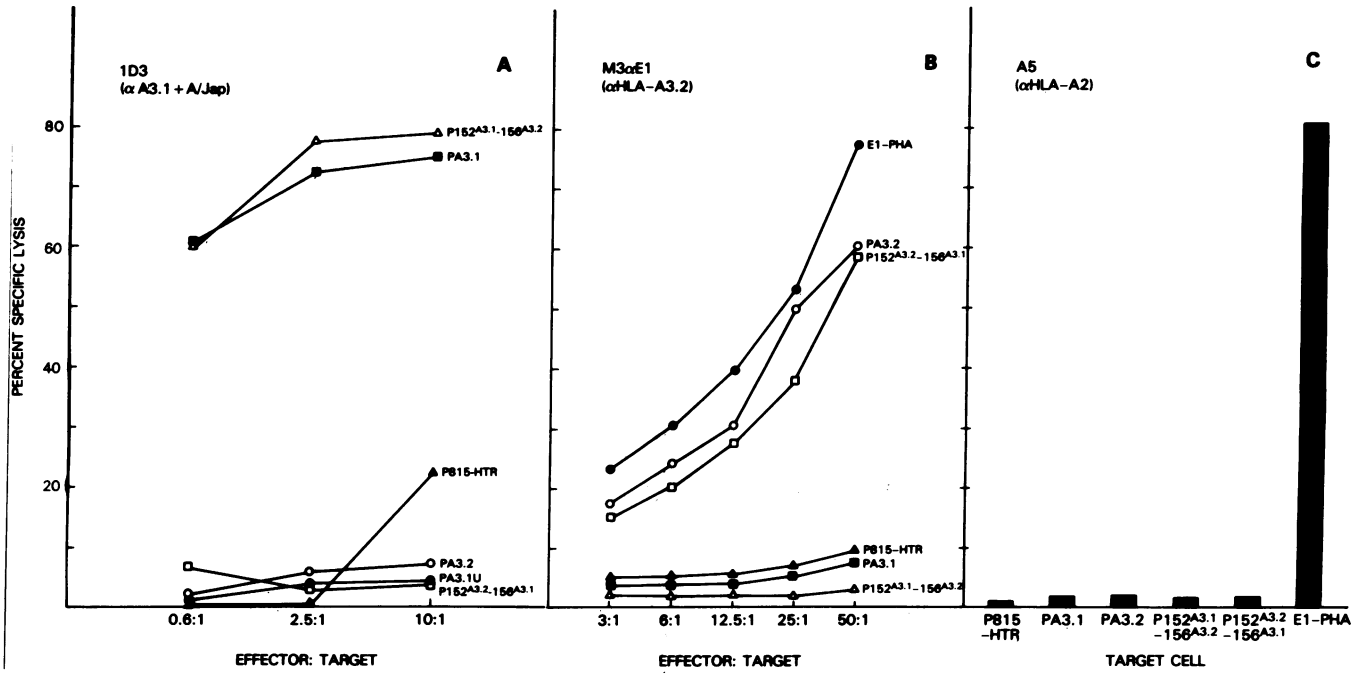

FIG. 3. MHC-restricted and allospecific recognition of HLA-A3-transfected P815-HTR cells by human CTL. (*A*) A/JAP-infected targets and uninfected PA3.1 targets (PA3.1U, ●) were assayed for their ability to be lysed by the HLA-A3.1 plus A/JAP-specific CTL line 1D3. Infected targets were PA3.1 (■), PA3.2 (○), $P152^{A3.1}$–$156^{A3.2}$ (△), $P152^{A3.2}$–$156^{A3.1}$ (□), and P815-HTR (▲). (*B*) Targets were assayed for their ability to be lysed by the HLA-A3.2-specific CTL line M3 anti-E1. The targets used were E1-PHA blasts (●), PA3.1 (■), PA3.2 (○), $P152^{A3.1}$–$156^{A3.2}$ (△), $P152^{A3.2}$–$156^{A3.1}$ (□), and P815-HTR (▲). (*C*) The same targets were assayed for their ability to be lysed by the HLA-A2-specific CTL line A5.

known whether the difference in amino acids at position 152 alters recognition by virus-immune CTL as a result of altering epitopes recognized by the antigen-specific T-cell receptor or whether it alters the ability of the molecule to interact with viral antigens.

Results of the present study of human T-cell recognition of *HLA-A3* gene products have utilized target cells that express only one human gene, *HLA-A3*. Thus, differences in recognition of this gene product by human CTL cannot be attributed to effects of other cell-surface molecules. We and others (31) have readily demonstrated HLA-A3- and HLA-A2-restricted influenza-immune CTL recognition of P815 transformants, indicating that these murine targets are able to serve as appropriate targets for these human CTL responses. However, it has been difficult to demonstrate strong lysis of the P815-A3.1 transformant by human alloreactive CTL specific for HLA-A3.1 (data not shown). The reason for this poor reactivity is unclear. A similar situation may exist for recognition of HLA-A2 because, in general, human A2-specific alloreactive CTL are unable to lyse mouse L cells transfected with HLA-A2 (32–34). An examination of A3.2-restricted influenza-immune CTL recognition was precluded by the finding that A3.2 is apparently a low-responder allele for influenza-specific T-cell responses (data not shown).

The data presented in these studies suggest that the amino acid at position 152, and not that at position 156, is crucial to the formation of HLA-A3 determinants recognized by CTL. This idea is supported by the observation that most CTL-distinguishable class I variants with known change(s) in the 146–160 segment possess changes at position 152 (11–16). However, it is important to consider the particular amino acid changes that result in alteration of epitopes recognized by CTL. Monos *et al.* (35) have postulated that amino acid substitutions that produce a charge variation will be the most significant type of change in forming antigenic structures recognized by CTL. The present observations are consistent with this hypothesis. Thus, the change from the negatively charged glutamic acid to the hydrophobic valine at position 152 is readily detected by CTL, whereas the change from the hydrophobic leucine to the uncharged hydrophilic glutamine at position 156 is not. The general importance of this hypothesis is suggested by the fact that the change(s) in the 146–160 segment of most CTL-distinguishable class I variants that have been sequenced to date results in a charge difference (11–16). Thus, it is possible that introducing a charge difference at position 156 could alter HLA-A3 CTL determinants as well.

It is striking that, among the human class I variants with changes in the 146–160 segment, only positions 152 and 156 vary. Furthermore, these same residues vary in the $H\text{-}2K^b$ murine class I mutant bm1 (in addition to the residue at position 155) (15, 16). In all of these cases, it is unclear whether a determinant including the residues at positions 152 and 156 is recognized directly or whether these residues influence the conformation of other parts of the molecule. Studies by Vega *et al.* (36) have predicted that residues 146–160 exist in the native molecule in the form of an α-helix, a structure that may be important for T-cell recognition (37). Residues 152 and 156, separated by three amino acids, would presumably be located on the same face of that α-helix on the outer surface of the class I molecule, forming an epitope that could be recognized by CTL. Site-directed mutagenesis of other class I variants coupled with data from x-ray crystallographic studies will be invaluable in better understanding the role that these residues play in epitope formation.

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