RECOGNITION OF INFLUENZA A MATRIX PROTEIN BY HLA-A2-RESTRICTED CYTOTOXIC T LYMPHOCYTES

Use of Analogues to Orientate the Matrix Peptide in the HLA-A2 Binding Site

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Since the original implication of class I molecules of the MHC in CTL recognition of virus-infected cells (1), significant advances have been made in our understanding of such recognition of viral antigens. CTL recognition originally was assumed to involve recognition of surface viral glycoproteins on infected cells. However while anti-MHC mAbs inhibited recognition by CTL, anti-virus glycoprotein antibodies did not (2). Also, CTL for a polymorphic virus such as influenza A usually reacted with all influenza virus strains that have differing glycoproteins on the surface (3, 4).

For rare murine T cell clones that distinguished between influenza virus strains, it was possible to map specificity to virus polymerase or nucleoprotein (NP)† (5, 6). The latter was confirmed by transfection and NP was shown to be a target antigen for the majority population of murine CTL that crossreact with different virus subtypes (7, 8). Epitopes were localized by transfecting fragments of NP into target cells (9), and when appropriate peptides were synthesised, they were recognized by CTL (10).

Similar studies were carried out in man. Using a set of recombinant vaccinia viruses to express each viral protein in appropriate target cells, human polyclonal CTL were shown to recognize the conserved internal proteins of influenza A virus: NP, basic polymerase (PB2), and the matrix protein (M1) (11). Further, peptides were identified that were derived from the influenza NP and matrix protein and were recognized by virus-specific human CTL in association with HLA-B37 and HLA-A2, respectively (10, 12). Thus results in mouse and man showed that viral proteins are recognized as peptide fragments that probably bind specifically to class I MHC molecules.

To elucidate the nature of the tripartite interaction between virus, MHC molecule, and TCR, further experiments have been performed where HLA-A2 was the restricting element. The three-dimensional structure of this molecule (13, 14), revealed a groove on its surface between two long α helices (α1 and α2) lying across...
an eight-stranded β-pleated sheet. An unidentified electron density, probably a mixture of peptides, was seen in the groove (13, 14).

Experiments with naturally occurring variants of HLA-A2 and deliberately constructed mutants of HLA-A2 containing single amino acid changes (15) have identified residues within the groove of the HLA-A2 molecule that may be important for binding the influenza matrix peptide. However, the results also showed that the viral peptide must interact with the MHC molecule with some flexibility (16).

To determine the way in which this matrix peptide fits into the proposed antigen-binding site of HLA-A2, a series of peptide analogues, each containing a point mutation, was synthesized. Each was assayed for its ability to sensitize target cells and to compete with the natural sequence for recognition by CTL. These experiments could distinguish between the residues necessary for recognition by the antigen receptor of the T cell and those needed to bind to the restriction element.

Materials and Methods

**Peptides.** The index or native matrix peptide 57–68 (sequence: lys, gly, ile, leu, gly, phe, val, phe, thr, leu, thr, val), a series of analogue peptides with single amino acid substitutions within the sequence and a set of shortened peptides, were synthesized by solid phase techniques on a peptide synthesiser (model 430A; Applied Biosystems, Inc., Foster City, CA). Commercially available amino acid phenylacetamidomethyl resins and t-Boc-suitably protected amino acids were used. All couplings were performed using a 2.5-mol excess of t-Boc-amino acid and dicyclohexyl-carbodiimide over the number of milliequivalents of amino acid on the resin. The peptides were deprotected and removed from the resin simultaneously by treatment with anhydrous hydrogen fluoride in the presence of anisole, dimethyl sulphide, and indole. The peptides were separated from the various organic side products by extraction with ether and isolated from the resin by extraction with 5% acetic acid and subsequent lyophilization. The purity of the crude product was determined by HPLC on a C-18 reverse-phase column. All of the peptides contained >90% of the desired product.

**Blood Donor.** JM was a healthy male member of laboratory staff and was HLA typed by standard techniques by Dr. A. Ting and associates, Nuffield Department of Surgery, Oxford. His tissue type was HLA A2; B15; B51; C3; DR4. In this report all cells used carried the common type of HLA-A2, also known as HLA-A2.1.

**CTL Lines.** CTL lines were prepared from influenza A virus-stimulated cells from the HLA-A2 donor JM, prepared as described previously (17). The cells were restimulated once a week with equal numbers of irradiated (3,000 rad) autologous lymphoblastoid cells that had been pretreated with matrix peptide 57–68 (60 μM for 1 h), and rIL-2 (Cetus Corp., Emeryville, CA) at 10 U/ml. Cells were also fed twice weekly with medium containing 10 U/ml rIL-2. CTL lines have been maintained for >12 wk and were highly specific for HLA-A2 matrix peptide 57–68.

**CTL Clones.** CTL clones were prepared as has been previously described (16). Briefly, cells from a JM CTL line established for 14 d were plated by limiting dilution onto autologous peptide-pulsed irradiated feeder cells, 104 well, in U-bottomed 96-well microtitre trays in medium containing rIL-2 to 10 U/ml rIL-2. 8:48 wells seeded at 10 cells/well, and 5:36 wells seeded at 1 cell/well showed growth after 2–3 wk. They maintained their growth for 6–8 wk. Clones had individual fine specificities, shown here and previously (16), that were constant throughout their life. They are referred to as 'clones' because they grew out at low frequency, but more formal proof of monoclonality of each has not yet been obtained.

**Target Cells.** EBV-transformed B lymphoblastoid cell lines were prepared as described previously (18) for use as target cells. These were labeled with 51Cr (Amersham International, Amersham, UK) before use in lytic assays.

**The Lytic Assay.** This was performed as described previously (4). CTL were used as effector cells by mixing with 104 51Cr-labeled target cells at different ratios. Peptides were added to a final concentration of 0.01–10 μM in the assay. Supernatants were harvested after 5 h and
specific lysis was calculated from the formula: $100 \times (E - M/D - M)$, where $E$, experimental release; $M$, release in presence of culture medium; $D$, release in presence of 5% Triton X-100.

**Competition between Peptides.** Blocking assays were performed by adding the native matrix peptide 57-68 at concentrations from 0.01 to 10 μM, together with an analogue peptide that was not recognized by CTL, in the lytic assay. The analogue or 'blocking' peptide was used at a final concentration of 300 μM. Conditions for optimal blocking were defined before these experiments and were identical to those described by Bodmer et al. (19).

**Blocking at the Level of the Target Cell.** ⁵¹Cr-labeled target cells were preincubated with competitor peptides at 300 μM with or without the index peptide 57-68 at concentrations from 0.1 to 10 μM. Preincubation was at 37°C for 1 h, after which time target cells were washed three times before use in the cytolytic assay.

**Results**

**Determination of the Optimal Length of Matrix Peptide Recognized by Influenza-specific CTL Lines and Clones.** Comparisons were made of recognition by a matrix peptide-specific polyclonal CTL line of the matrix peptides 57-68 or 56-68 and four other peptides, 58-68, 59-68, 60-68, and 56-67. As shown in Fig. 1A, peptides 57-68, 58-68, and 59-68 were seen in an almost identical fashion, whereas peptide 60-68 required 100-fold higher concentration for equivalent lysis. Fig. 1B shows that peptide 56-67 was also seen 100-fold less well than peptide 56-68. Thus, the amino acids at position 59 (isoleucine) and 68 (valine) were shown to be important for recognition; the shortest optimal peptide was 59-68. Similar experiments using three clones, K6, K2, and D10, derived from the JM CTL line, showed (Fig. 2, A–C) that there were fine differences in specificity between the clones, most notably in recognition of the 9 amino acid peptide 60-68 that was only recognized by CTL clone K2. However,
even though the polyclonal line comprised a number of fine specificities, there was a major common feature: specific recognition of the matrix peptide with HLA A2.

**Identification of Critical Residues for T Cell Recognition.** To approach the physical and chemical requirements for peptide recognition by T cell clones and binding to the HLA-A2 molecule of the target cell, an analysis was made of recognition of peptide
Recognition of Alanine Substituted Peptides

The analogue peptides each had a single amino acid substitution at a position within the sequence. Each peptide was titrated at concentrations from 0.01 to 10 μM and an example of the full data is shown for the individual alanine substitutions in Fig. 3. Each peptide was incubated in the lytic assay with the JM CTL line and HLA-A2-matched lymphoblastoid target cells. Alanine substitution at different positions had dramatically different effects on recognition. An alanine substitution at position 59 (I→A) had no effect on recognition. Substitutions of alanine at positions 62 (F→A), 66 (L→A), 60 (I→A), 64 (F→A), 65 (T→A), and 63 (V→A) decreased recognition, and a substitution at position 61, a conservative substitution of alanine for glycine, completely abrogated recognition.

The results summarised in Fig. 4 are from experiments where all of the analogue peptides were tested. Because they include data from three experiments, results are shown in each case as a percentage of the lysis obtained with the native 57–68 matrix peptide. Individual substitutions at each position are ranked at the top of the figure in the same order as they appear vertically on the bar chart. Each peptide was tested...
at a range of concentrations from 0.01 to 10 μM, but for clarity, only the 10-μM result is shown. It was found that a greater latitude was possible for substitutions at positions near the NH₂ or COOH termini of the peptide compared with the central positions. There was a core of six residues, 60-65, where few substitutions were allowed. Recognition of peptide by the JM CTL line required a glycine at position 61.

The four CTL clones, D10, K2, K6, and 01, were assayed for their ability to recognize the analogue peptides in order to determine whether there were differences in specificity imposed by their different TCRs (Table I). In most cases the reactivity of the clones reflected that of the line; for example, none of the clones were able to lyse target cells in the presence of substitutions at position 61. Similar recognition was also apparent with substitutions at positions 63, 67, and 68. However, for several substitutions at positions 59, 60, 62, 64, 65, and 66, different reactivity was seen for individual clones. In each case where differential recognition was seen, at least one clone was capable of lysing target cells sensitized with the peptide. Consequently, the analogues were capable of binding to the A2 molecule, and the most likely explanation for the differences are sequence variations present in the TCRs of the clones.

Inhibition of Recognition by JM CTL of the Native Matrix Peptide by Analogue Peptides. Peptides containing amino acid substitutions that were not recognized by JM CTL line were used to compete with the native peptide in the lytic assay. If the substituted peptides inhibited or blocked recognition by CTL of target cells, these peptides must bind to HLA A2, suggesting that the substitutions that abrogate recognition face the TCR.

Substitutions at positions 60 (L→T), 64 (F→I), and 65 (T→K) resulted in peptides that inhibited recognition (Fig. 5). Substitutions at positions 61, 62 (F→E), and 63 (V→F or E), which resulted in analogue peptides not recognized by the CTL line, did not block recognition of the native peptide. Therefore, we can model the peptide with positions 60, 64, and 65 facing the TCR and positions 61, 62, and 63 interacting with the A2 molecule.

Blocking of Recognition of the Native Peptide by Competitor Peptides at the Level of the Target Cell. To confirm that the previous inhibition experiments reflected competition for binding to HLA-A2 on the target cell rather than for the TCR, target cells were preincubated with peptide analogues containing substitutions at positions 60, 61, 64, and 65 together with the native peptide. After washing, the targets were incubated with the CTL in the lytic assay. Similar inhibition of recognition was seen (Fig. 6) to that shown in Fig. 5, indicating that the peptides substituted at positions 60, 64, and 65, present in molar excess compared with the index peptide during preincubation, must bind to the target cell. Such results further support the hypothesis that positions 60, 64, and 65 within the matrix peptide interact with the antigen receptor of the CTL.

Discussion

To understand the molecular basis of HLA restriction, we have attempted to identify the critical residues for binding the previously defined (12) influenza A virus matrix peptide, 56-68, to HLA-A2. A necessary prerequisite to the identification of the exact position of the peptide in the antigen-combining site is the determination of the conformation and orientation of the bound peptide. The strategy that we have adopted was to assay the ability of peptide analogues, each containing a
**Clonal Differences in Recognition of Substituted Peptides**

| Substituted peptides | JM CTL line | JM CTL Clones |
|----------------------|-------------|---------------|
|                      | D10 | K2 | K6 | O1 |
| K59                  | +   | +  | +  | +  |
| F                    | ±   | +  | ±  | +  |
| A                    | +   | +  | ±  | +  |
| Y                    | ±   | +  | ±  | +  |
| E                    | +   | +  | +  | ±  |
| S60                  | +   | ±  | ±  | +  |
| N                    | +   | +  | -  | +  |
| T                    | ±   | ±  | -  | +  |
| A61                  | -   | -  | -  | -  |
| S                    | -   | -  | -  | -  |
| T                    | -   | -  | -  | -  |
| V                    | -   | -  | -  | -  |
| D                    | -   | -  | -  | -  |
| E                    | -   | -  | -  | -  |
| K62                  | -   | -  | -  | -  |
| E                    | -   | -  | -  | -  |
| Q                    | -   | -  | -  | -  |
| I                    | +   | +  | ±  | ND |
| V                    | +   | +  | -  | ±  |
| Y                    | +   | +  | +  | -  |
| D                    | +   | -  | -  | -  |
| F63                  | -   | -  | -  | -  |
| Y                    | -   | -  | -  | -  |
| E                    | -   | -  | -  | -  |
| R                    | ±   | -  | +  | -  |
| N64                  | +   | ±  | +  | -  |
| S                    | +   | +  | -  | -  |
| Y                    | ±   | ±  | +  | -  |
| Q                    | -   | -  | -  | -  |
| K65                  | -   | -  | ±  | -  |
| G                    | +   | ±  | +  | ±  |
| S66                  | +   | +  | +  | ±  |
| F                    | +   | ±  | +  | +  |
| R                    | +   | ±  | +  | ±  |
| E                    | +   | ±  | ±  | ±  |
| D67                  | +   | +  | +  | +  |
| A                    | +   | +  | +  | +  |
| K68                  | +   | +  | +  | +  |

Clones D10, K6, K2, and O1 derived from the JM CTL line were tested for recognition of autologous B lymphoblastoid target cells in the presence of the peptides indicated at 10 μM final concentration in the assay. Comparisons were made with recognition of the index matrix peptide 57–68 and were scored: + +, lysis >90% of that with matrix peptide 57–68; +, lysis 50–90% of that with matrix peptide 57–68; ±, lysis 10–50% of that with matrix peptide 57–68; −, lysis <10% of that with matrix peptide 57–68. The index peptide 57–68 scored ++ in all categories.
point substitution, either to act as targets for influenza A virus matrix-specific CTL lines or clones, or to inhibit recognition of the native sequence.

The experiments were carried out with CTL lines that were originally stimulated in vitro with influenza A virus and subsequently grown on matrix peptide 56–68-pulsed autologous cells in the presence of IL-2. These lines were composed of at least four clonal specificities, all directed at peptide plus HLA-A2. These could be distinguished when individual clones were isolated when differences between them were apparent (e.g., Fig. 2), although they shared specificity for matrix peptide and HLA-A2. In experiments where recognition was completely abrogated, it is likely that all component CTL clones were affected (see Table I).

A number of characteristics can be identified from the analysis of T cell recognition of a large number of analogue peptides. There are some positions, particularly at the NH₂ and COOH termini of the peptides that were permissive in their chemical requirements. For example, the amino acid at position 68, normally a valine, was shown to be necessary for optimal activity, but substitution with markedly different
amino acids such as lysine and aspartic acid did not reduce recognition by CTL. In contrast, other positions have strict requirements for a particular type of amino acid. The most extreme example was glycine-61 where none of the other seven amino acids tested, including alanine, generated an analogue that was capable of being recognized by CTL or of inhibiting recognition of the native sequence. Intermediate requirements were apparent at some positions, such as 62, where a branched hydrophobic amino acid in place of the natural phenylalanine gave a functioning peptide. The sequence corresponding to residues 61–65 appeared to be the most critical for recognition, because it was contained in the shortest peptides that were recognized and at least one substitution in each position resulted in complete loss of recognition. A central core of critical residues has been identified in other helper and cytotoxic T cell epitopes suggesting that the ends of these determinants have less conformational constraints.

To distinguish those amino acids in the native peptides that interact with HLA-A2 from those that are directly recognized by the TCR, analogues that could not sensitize target cells were used in competitive inhibition assays with the native peptide.
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The conditions necessary for these assays were found to be identical to those
described by others (19), particularly that the competing peptide must be in large molar
excess and that it must be added before, or at the same time as, the native peptide.
Peptides containing substitutions at positions 60, 64, and 65 when incubated in the
presence of the native peptide, competed effectively, whereas those with changes at
residues 61–63 did not inhibit. We argue that competition occurs at the level of binding
to HLA-A2 because when target cells were incubated with native and analogue pep-
tide and then washed before adding to the CTL, inhibition was still observed. There
was no evidence of nonspecific toxicity because background chromium release of
these targets was in the normal range, <25% of detergent release. These experiments
allow us to conclude that the peptides with substitutions at positions 60, 64, and
65 bind effectively to HLA-A2 and, because they are so similar to the native peptide,
we suggest that they adopt a similar conformation and position in the peptide-binding
groove. The loss of CTL recognition of these peptides would then be due to failure
of interaction with the TCR. This is explained most simply if the substituted amino
acids point directly at the TCR. However, we cannot completely exclude the possi-
bility that one or more of these residues could point into the groove and affect the
fine positioning of the peptide, much as a mutation at position 9 in HLA-A2 does
(16). We consider this unlikely because these substitutions at residues 60, 64, and
65 abrogated recognition by all JM CTL lines and clones, unlike the HLA-A2 posi-
tion 9 mutation (16). The analogue peptides, with substitutions at positions 61–63,
which did not compete, identify residues that interact with HLA-A2.

A second approach to the identification of the residues that point towards the TCR
was to analyze recognition by CTL clones (Table I) (20). It was found that particular
substitutions at positions 59, 60, 62, 64, 65, and 66 gave disparate results with different
T cell clones; in each case these was at least one analogue that was recognized strongly
by one T cell clone and not by another. The simplest interpretation of these findings
is to orientate all of these positions towards the TCR. This would agree with our
interpretation of the competition experiments for positions 60, 64, and 65, but not
for position 62. One possible explanation is that the peptide can bind to HLA-A2
in two or more completely different ways. We consider this unlikely because there
appear to be relatively few epitopes in the 10 proteins of influenza virus that are
recognized by CTL; only the matrix protein and a single epitope in that molecule
interact with HLA-A2 to be recognized by CTL (11). Thus, it is unlikely that this
matrix peptide would contain more than one HLA-A2-associated epitope. A second
possibility is that position 62 points towards HLA-A2 and that the substitutions of
isoleucine, aspartic acid, or tyrosine for the natural phenylalanine affect the fine posi-
tioning of the peptide, or have an effect on the conformation of HLA-A2, and thereby
abrogate recognition by clone K6 but not by the other clones. This would be an-
alogous to our finding that a mutation at position 9 of HLA-A2, which is in the floor
of the groove, affected recognition of the matrix peptide by T cell clones K2 and
01 and not by the others (16); because it is unlikely that the TCR would reach posi-
tion 9, this result is best explained by an effect on fine positioning of the peptide.
A similar explanation for the results with the peptide substituted at position 62 allows
us to give equal weight to both types of experiment, competition, and differential
recognition by T cell clones.

One striking feature of the data is the permissiveness of peptide interaction with
HLA-A2. The latter must bind to any of the analogues that stimulates one or more T cell clones. Also, variant peptides that abrogate all response but compete, must bind. Thus, of all the analogue peptides tested, only 13 failed to bind and these were all substituted at positions 61–63. This also suggests that these three positions must make critical contact with HLA-A2.

The pattern that emerges, therefore, is that residues 61, 63, and probably 62 interact with HLA-A2 and 59, 60, 64, 65, and 66 interact with the TCR. This is reminiscent of the analysis made of the interactions between an OVA peptide and IAd by Sette et al. (21). There is no simple secondary structure of extended chain, turn, or a helix that satisfactorily meets all of these conditions and, as previously argued, makes contact with residues 9, 66, 70, 152, and 156 in HLA-A2 (16). However, most of these points would be met with the peptide in an α helix as shown in Fig. 7. This could fit into the groove in parallel with the two α helices of HLA-A2 and could reach each of the above positions in that molecule. The two bulky phenylalanines at positions 62 and 64 would point into and out of the groove, respectively, and the essential glycine at position 61 would face one side, so meeting the steric constraint of a narrow groove (13, 14). The glycine might face one of the bulky residues at positions 66 (lysine), 70 (histidine), or 74 (histidine) of HLA-A2, all of which point into the groove. Residues 61–63 would interact with the three sides of the groove and 60 and 64 would face the TCR. Other conditions imposed by the data are more difficult to satisfy, however, and weaken the model. In the helical wheel shown in Fig. 7, residue 65 points into the groove, but a substitution of lysine for threonine abrogated recognition and competed; it is possible that the size and polarity of this residue would enable its side chain to point out of the groove and interfere with T cell recognition while still binding. Residues 59 and 66 both point deep into the groove in this model but certain substitutions affected some CTL clones and not others. It is possible that these affect orientation or conformation as argued for the isoleucine at position 62 and the mutation in position 9 of HLA A2 (16). These special arguments weaken the model but do not preclude it, and at present, this seems to be the best working hypothesis. The proposal should lead to testable predictions, particularly that proline substitutions might destroy the helix and, therefore, binding; that it should be possible to complement abrogative mutations of HLA-A2 and peptide to give a positive response; and that the model should lead to successful prediction of peptide epitopes from other viruses. These are currently being tested.

Ultimately, the position and orientation of this peptide in HLA-A2 will be solved by cocrystallization of HLA-A2 with peptide and X ray diffraction studies. A solu-

**FIGURE 7.** The influenza matrix peptide 57–68 in a helical form, shown as a helical wheel. In this orientation residues 60 and 64 face the TCR and residues 61–63 face the A2 molecule.
tion will be needed to the problem being encountered of binding peptide to purified HLA-A2 molecules. This may require an understanding of how peptide enters the A2 molecule and whether it exchanges naturally. As it is unlikely that many different peptide MHC molecule complexes will be cocrystallized, a correlation between crystal structure data and functional data, of the type discussed here, will be essential. This should enable other peptide MHC interactions to be explored by this functional approach.

Summary

CTL specific for the influenza A virus matrix peptide 57–68 and restricted by HLA-A2 were studied. Their ability to recognize a set of analogue peptides, each of which differed from the natural peptide by a single amino acid, was analyzed. This revealed a core of five amino acids, 61–65, where one or more changes completely abrogated recognition. The glycine at position 61 was the only residue where no substitution was tolerated. Analogue peptides that did not induce CTL-mediated lysis were tested as competitors with the natural peptide; those with substitutions at positions 60, 64, and 65 inhibited, identifying residues that interact with the TCR. Another approach was to test a set of four CTL clones on all of the analogues. Marked differences in recognition by individual CTL clones were observed for several substituted peptides. The data indicate that most of the analogues bind to HLA-A2 with possible differences in fine positioning of the peptide. An α helical orientation for the peptide is discussed.

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