Brief Definitive Report

IN VITRO MUTAGENESIS AT A SINGLE RESIDUE INTRODUCES B AND T CELL EPITOPES INTO A CLASS I HLA MOLECULE

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Class I proteins of the major histocompatibility complex play a central role in the lysis of virus infected or allogeneic cells by cytotoxic T lymphocytes (CTL) (1). Analysis of mutant class I molecules generated in vivo or in vitro has defined residues involved in recognition by T cell receptors and monoclonal antibodies. In general the determinants recognized by antibodies (B cells) and T cells have been found to be different. For example, naturally occurring mouse and human class I variants detected by CTL display few or no changes in antibody binding (2, 3). Substitutions at positions of variability dispersed throughout the α_1 and α_2 domains of human and murine class I molecules have been shown to affect CTL recognition. These include positions 147–157, a region having a complex pattern of substitutions in the mouse bml mutant and in HLA-A2 and HLA-A3 subtypes, and position 116, which is altered in five of the bm mutants and in HLA-B7 subtypes (4–6).

Similarly, mutations in class I molecules which affect antibody reactivity do not always alter the recognition by CTL (7). For example HLA-A2 mutants obtained by negative selection with the HLA-A2, Aw69-specific monoclonal antibody, BB7.2, were indistinguishable by CTL from the wild type molecule, although they no longer bound BB7.2 (7). Structural analysis indicated that changes at position 161 and in the peptide corresponding to positions 98–108 occurred separately in two of the mutants (5). Comparison of amino acid sequences has shown that HLA-A2 and HLA-Aw69, which are the only two molecules to bind BB7.2, share a tryptophan at position 107 within this latter region (8). All other HLA class I molecules analyzed contain glycine at this position (9). These results suggested that tryptophan 107 is crucial in forming the epitope recognized by BB7.2. Here we report experiments to directly test this hypothesis and to determine how substitution at this position affects recognition by alloreactive CTL.

Materials and Methods

Mutagenesis. Site-directed mutagenesis was performed as described (10) using a 5.1 kb Hind III fragment of the HLA-Aw68 gene (8), which was subcloned into the vector

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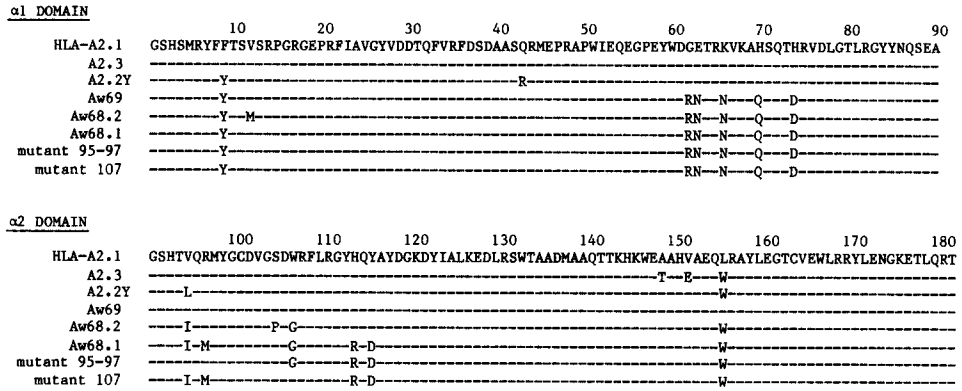

FIGURE 1. Amino acid sequences of the α_1 and α_2 domains of the HLA-A2/A28 family (17) and of the two mutants derived from HLA-Aw68.1. All sequences are compared to HLA-A2.1.

pTZ19 (Pharmacia Fine Chemicals, Piscataway, NJ). Oligonucleotides were synthesized with an Applied Biosystems automated synthesizer.

Transfection. Class I genes were subcloned into the vector pHEBO (11) using the Hind III site. 20 μg of DNA was transfected into 5×10^6 Hmy 2 CIR B lymphoblastoid cells by electroporation (12). Hmy 2 CIR is an HLA-A, B–negative derivative of LicrLon Hmy 2 (13). After 4 wk in culture with 200 μg/ml hygromycin B, >90% of transfected cells reacted with the HLA-A2, A28-specific monoclonal antibody CR11 351 (14) in flow cytometry. Cell cultures continued to stably express the transfected genes thereafter.

Analysis. Cell binding radioimmunoassays were performed with monoclonal antibodies MB40.5, PA2.1, CR11-351, BB7.2, and Tu160 (gift of Dr. Andreas Ziegler) as described (14, 15). CTL were derived from the line AJY and assayed as described (16). In inhibition assays peptides were preincubated for 30 min with 5×10^3 CTL (clones AP23.1 and ATL8.1) or 3×10^3 CTL (clone AP31.1) before addition of 10^3 ^{51}Cr-labeled target cells. The peptides were present throughout the assay period of 3–5 h (9).

Results and Discussion

Analysis with antibodies and CTL has defined a series of HLA-A molecules related to HLA-A2 and called the HLA-A2/A28 family (17). The amino acid sequences of six members of this family in the two most variable domains (α_1 and α_2) are shown in Fig. 1. Within this family, HLA-A2.1 and HLA-Aw68.1 are least homologous, differing by six amino acids in both the α_1 and α_2 domains (Fig. 1). Other members of the family (HLA-A2.2Y, -A2.3, -Aw68.2) contain intermediate numbers of substitutions. HLA-Aw69 is a recombinant composed of an α_1 domain of HLA-Aw68.1 and α_2 and α_3 domains of HLA-A2.1 (8). Correlation of antibody reactivity with the primary structure identified tryptophan 107 as a candidate for involvement in epitopes shared by HLA-A2.1, -A2.2Y, -A2.3, and -Aw69, but not present in HLA-Aw68.1 and -Aw68.2. This hypothesis was directly tested by introducing this residue into HLA-Aw68.1 by site-directed mutagenesis. A second mutant was generated by replacing isoleucine and methionine residues at positions 95 and 97 of HLA-Aw68.1 with the corresponding valine and arginine residues of HLA-A2.1 (Fig. 1).

The two mutants and the wild type HLA-Aw68.1 gene were transfected into the B lymphoblastoid cell line Hmy2 CIR, which was chosen for its low level of class I antigen expression. The binding of monoclonal antibodies to these transfectants is shown in Fig. 2. Monoclonal antibodies such as CR11-351, which

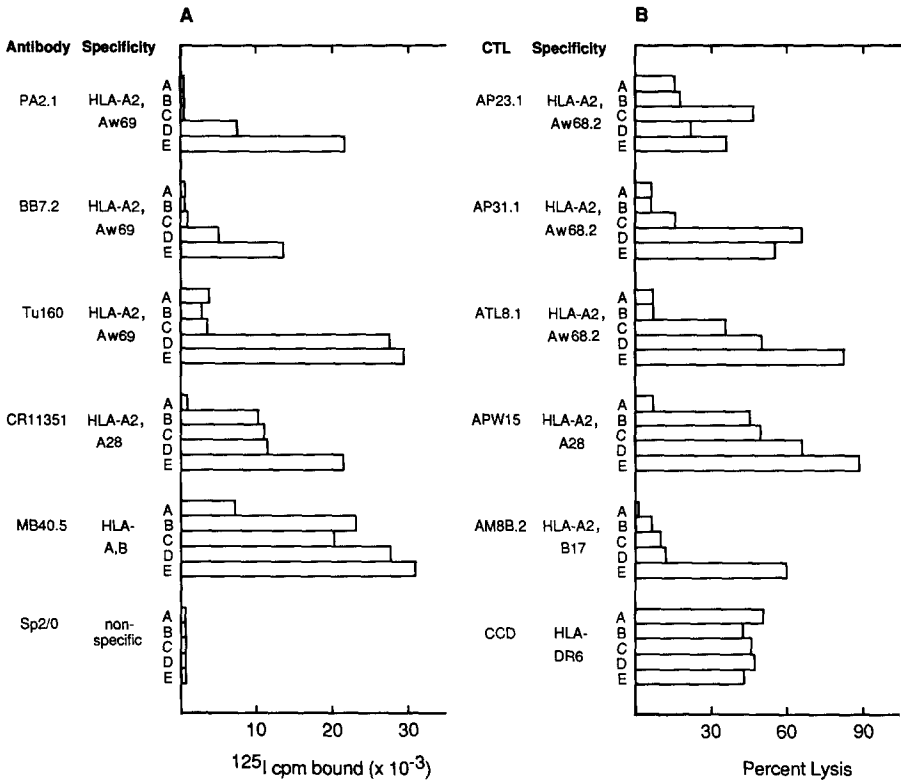

FIGURE 2. Recognition of HLA-Aw68.1 mutants by monoclonal antibodies and CTL. (*A*) RIA of Hmy CIR transfectants containing the pHEBO vector alone (*A*), HLA-Aw68.1 (*B*) mutant 95–97 (*C*) or mutant 107 (*D*) genes in pHEBO, and of the $A2^+$ B cell lines JY (*E*). (*B*) CTL analysis performed with the panel of cells used in *A*. Labeled target cells were incubated for 4 h with effector cells at the following E/T ratios: Clones ATL8.1, AP31.1, and APW 15, E/T = 1.2; clone AP23.1, E/T = 5; clone AM8B.2, E/T = 2. The class II–reactive CTL line CCD was used at E/T = 1.2. Clone AM8B.2 is reactive with an epitope in the α_1 domain of HLA-A2, which is shared with HLA-B17, but not HLA-Aw68. Clone APW 15 was used as a control to show that all three transfectants bearing HLA-Aw68.1-derived molecules could be lysed. Monoclonal antibodies directed against class I determinants present on target cells were able to block lysis by each class I–reactive clone, whereas an antibody specific for class II antigens was not (data not shown).

are specific for epitopes shared by HLA-A2, Aw68, and Aw69, bound strongly to cells transfected with HLA-Aw68.1 and both mutant genes. This showed that the transfected genes were being expressed. The HLA-A2, Aw69-specific antibodies PA2.1, BB7.2, and Tu160 bound the mutant 107 transfectant, but not the HLA-Aw68.1 or mutant 95–97 transfectants. This result proves that the determinant recognized by the HLA-A2, Aw69 specific antibodies is critically dependent upon tryptophan 107. Furthermore, the introduction of these epitopes into another class I molecule (i.e., HLA-Aw68.1) can be effected by this single amino acid substitution.

It was therefore of interest to determine if epitopes recognized by CTL were also transferred by these mutations. The transfectants expressing mutants 107 and 95–97 were tested as targets for 21 clones of CTL that lysed CIR cells transfected with the HLA-A2.1 gene (data not shown) but did not lyse or gave

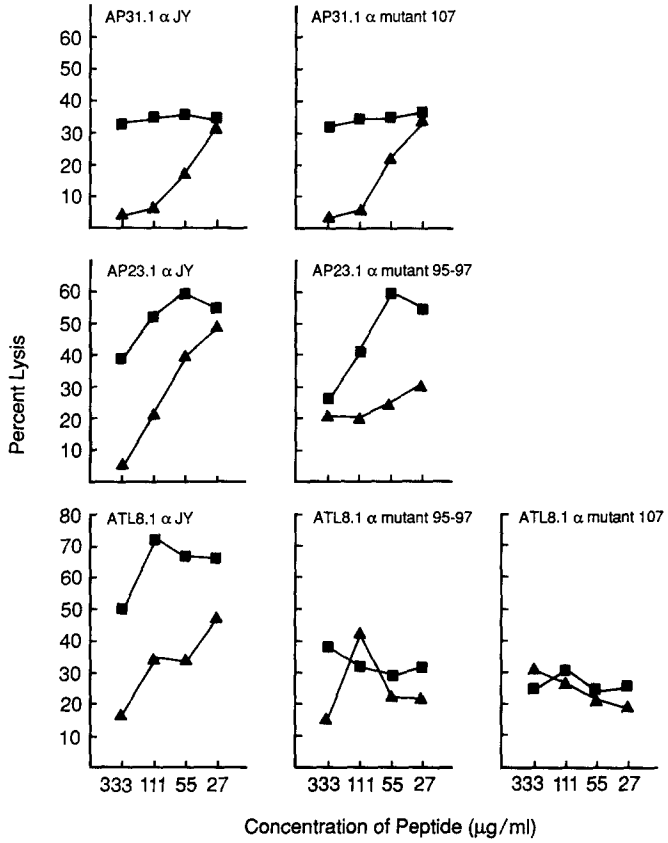

FIGURE 3. Inhibition of CTL clones by peptides. The combinations of CTL and target cell lines used are labelled above the individual graphs. Peptides used are A2.98–113 (MYGCDVGSDWRFLRGY) and Aw68.98–113 (MYGCDVGSDGRFLRGY) designated by ▲ and ■, respectively. At 55 μg/ml, the molar concentrations are 2.7×10^{-5} M for A2.98–113, and 2.9×10^{-5} M for Aw68.98–113.

weak lysis of the HLA-Aw68.1 transfectant. Four positive clones were found (Fig. 2). Clone ATL8.1 lysed mutant 107 at a high level, and mutant 95–97 to a lesser extent. Clone AP23.1 lysed the mutant 95–97 transfectant, but not the mutant 107 transfectants. Clone AP31.1 lysed transfectant 107 but not transfectant 95–97, whereas clone APW.15 lysed both transfectants (Fig. 2). These results demonstrate that one or two amino acid substitutions in the HLA-Aw68.1 molecule are sufficient to confer reactivity with certain HLA-A2 specific CTL clones.

Analysis of the fine specificity of CTL clones ATL8.1 and AP31.1 showed that their reactivity is distinct from that of the HLA-A2, Aw69-specific antibodies. First, neither CTL lysed cells expressing HLA-Aw69, and other CTL that recognize HLA-Aw69 failed to lyse transfectant 107. Second, these CTL lysed other members, e.g., HLA-Aw68.2 of the HLA-A2/Aw68 family, whereas the antibodies are specific to HLA-A2 and HLA-Aw69.

We have previously shown that tryptophan 107 is a critical residue for the inhibition of CTL given by synthetic peptides. A peptide (A2.98–113) corresponding to residues 98–113 inhibits lysis of HLA-A2 bearing targets by CTL

specific for HLA-A2. In contrast, a second peptide (Aw68.98–113) derived from the same region of HLA-Aw68.1, and which only differs by the tryptophan to glycine change at position 107 did not affect lysis (9). The mechanism of this inhibition is not known and may occur at the surface of either the CTL and/or the target cell. To see if inhibition was dependent upon the presence of tryptophan 107 in the target class I molecule we tested the capacity of peptides A2.98–113 and Aw68.98–113 to inhibit the lysis of mutant transfectants 107 and 95–97 by A2-specific CTL. The lysis of an HLA-A2.1 expressing cell line (JY) by all three CTL tested was specifically inhibited by A2.98–113. CTL AP31.1, which is specific for transfectant 107 but not 95–97, and CTL AP23.1, which is specific for transfectant 95–97 but not 107 were both inhibited by peptide A2.98–113 on the appropriate transfectant target. This shows that the presence of tryptophan 107 in the target class I molecule is not a necessary requirement for the peptide inhibition. Neither is it a sufficient requirement, as shown by the results with CTL ATL8.1, which was inhibited by the peptide on JY but not on mutant 107.

These results show that a region encompassing residues 95–107 at the NH_2-terminal end of the α_2 domain of HLA-A2 is important in forming epitopes recognized both by monoclonal antibodies with polymorphic specificity and by alloreactive CTL. In the case of position 107, a single substitution is capable of introducing novel B and T cell defined epitopes of HLA-A2 into the HLA-Aw68.1 molecule. We do not know whether tryptophan 107 directly interacts with the combining sites of the relevant antibodies and CTL receptors or if it produces conformational changes at a separate site that is responsible for these interactions. Prediction of secondary structure shows that position 107 has a high probability of being involved in a turn at the surface of class I molecule, favoring the model of direct interaction.

Summary

We have studied the interaction of HLA class I antigens with alloreactive cytotoxic T lymphocytes and monoclonal antibodies using site-directed mutagenesis and expression of an HLA-Aw68.1 gene. Two mutants containing distinct substitutions at polymorphic residues near the NH_2-terminal end of the α_2 domain were made. One mutant with substitutions at positions 95 and 97 corresponding to residues found in HLA-A2.1 showed no alterations in binding of HLA-Aw68- or HLA-A2-specific monoclonal antibodies, but was reactive with some HLA-A2-specific CTL clones. A second mutant, in which glycine at position 107 was replaced with tryptophan found at that position in HLA-A2.1, was recognized by HLA-A2-specific CTL clones and HLA-A2, Aw69-specific monoclonal antibodies. Thus, substitution of a single amino acid residue at position 107 of the HLA-Aw68.1 molecule generates an allospecific determinant shared with HLA-A2.1 and recognized by both B and T lymphocytes.

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Note aded in proof: Similar results have been reported by Layet et al. (18) showing that the epitope bound by the antibody BB7.2 depends on tryptophan 107.

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