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## Comparative Structural Analysis of HLA-A2 Antigens Distinguishable by Cytotoxic T Lymphocytes: Variants M7 and DR1<sup>†</sup>

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**ABSTRACT:** Comparative primary structural analyses have begun to elucidate polymorphic residues and segments of the class I antigens of the major histocompatibility complex, at least some of which presumably contribute to determinants important in immune recognition events. HLA-A2 structural variants have been described which are serologically indistinguishable from other HLA-A2 antigens, yet which can be recognized neither by HLA-A2 specific alloimmune nor by HLA-A2 restricted, virus immune cytotoxic T lymphocytes. This study utilizes double-label tryptic peptide comparisons

in combination with both conventional and microsequence analyses to investigate the structure of two such variants, M7 and DR1. We find that these variants are identical with each other and differ from the predominant HLA-A2 heavy chain species by a glutamine to arginine substitution at residue 43, by an unidentified substitution in the tryptic peptide spanning residues 147–157, and by an as yet poorly defined alteration in glycosylation. Structural information from these and other variants should be useful in precisely defining functionally important determinants on the molecule.

**T**he class I molecules encoded by the major histocompatibility complex (MHC)<sup>1</sup> are highly polymorphic cell surface antigens present on all nucleated cells (Klein, 1979). These molecules carry the foreign determinants important in immune recognition of target cells by alloreactive cytotoxic T lymphocytes (CTLs), as well as the self-determinants important in immune recognition of target cells by self-restricted CTLs. The latter has been demonstrated for CTLs responding to virally infected cells, chemically modified cells, and minor histocompatibility antigen differences (Dickmeiss et al., 1977; Goulmy et al., 1977; McMichael et al., 1977; Biddison et al., 1980a; Shearer & Schmitt-Verhulst, 1977; Zinkernagel & Doherty, 1979).

The human class I antigens (HLA-A, -B, and -C) are composed of a polymorphic, MHC-encoded heavy chain of 44 000 daltons which is found noncovalently associated with an invariant, non-MHC-encoded light chain of 12 000 daltons,  $\beta_2$ -microglobulin ( $\beta_2m$ ) (Ploegh et al., 1981). The heavy chain is a transmembrane glycoprotein consisting of a large amino-terminal extracellular portion, a short stretch of hydrophobic amino acids which are embedded in the lipid bilayer, and a small intracellular, carboxy-terminal hydrophilic region. Primary structural analysis suggests that the extracellular portion of the heavy chain may be organized into three distinct folding domains ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ) of approximately 90 amino acids each. The two amino-terminal domains,  $\alpha 1$  and  $\alpha 2$ , are polymorphic, and within these domains more or less discrete segments of sequence variation may be defined. The third of the extracellular domains,  $\alpha 3$ , is relatively nonpolymorphic; both  $\alpha 3$  and  $\beta_2m$  display high levels of sequence homology with immunoglobulin constant region domains. It is presumed that important recognition determinants will map to polymorphic segments of the amino-terminal domains. However, since even two highly cross-reactive histocompatibility antigens, HLA-A2 and -A28, differ by at least ten amino acid substitutions (Lopez de Castro et al., 1982), conclusions concerning such deter-

minants have necessarily been limited. In particular, it has not been possible to determine which sequences may contribute to serologically defined allo determinants, CTL defined allo determinants, and/or CTL defined self-determinants.

Recent studies have identified rare individuals whose cells bear HLA-A2 antigens serologically indistinguishable from those of most HLA-A2 positive individuals, yet which cannot be recognized by HLA-A2-specific CTLs (Biddison et al., 1980b,c, 1982; Goulmy et al., 1982). Cells from one such donor, M7, can be lysed neither by HLA-A2-restricted, H-Y immune CTLs nor, when influenza virus infected, by HLA-A2-restricted, influenza virus-immune CTLs. Further, they are lysed only very inefficiently by HLA-A2-specific alloimmune CTLs. Preliminary biochemical comparisons, including a number of similarly defined aberrant cell lines, have demonstrated a 100% correlation between such aberrant reactivities in cellular assays and structural differences among target cell nonglycosylated HLA-A2 heavy chains, as assessed by isoelectric focusing (Biddison et al., 1980c, 1982). The HLA-A2 heavy chains of two variants, M7 and DR1, are each more basic than those of most other HLA-A2-positive cell lines, by the equivalent of a single charge.

In the present study, double-label tryptic peptide comparisons coupled with both conventional and microsequence analyses are used to assess the true level of structural divergence among "normal" and "variant" HLA-A2 heavy chains. M7-HLA-A2 and DR1-HLA-A2 are compared to JY-HLA-A2, for which extensive primary structural data are already available. The data obtained are consistent with M7- and DR1-HLA-A2 being identical and differing from JY-HLA-A2 by a glutamine to arginine substitution at position 43 of the heavy chain, by an unidentified substitution in the tryptic peptide spanning residues 147–157, and by a poorly defined glycosylation change. Structural information obtained from these and other variant HLA-A2 antigens will be useful in

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<sup>†</sup> Abbreviations: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte;  $\beta_2m$ ,  $\beta_2$ -microglobulin; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; NP40, Nonidet P40; NaDdSO<sub>4</sub>, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; anti-H, anti-heavy chain serum; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; endo-H, endo- $\beta$ -N-acetylglucosaminidase H; CNBr, cyanogen bromide; TLC, thin-layer chromatography; IEF, isoelectric focusing; A, alanine; D, aspartic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; P, proline; Q, glutamine; R, arginine, S, serine.

defining functionally important determinants on the molecule.

### Experimental Procedures

**Cell Lines and Metabolic Labeling.** The human B lymphoblastoid cell lines JY (Terhorst et al., 1976) (HLA-A2, -B7 homozygous), M7 (Biddison et al., 1980b) (HLA-A2, -A3, -Bw35, -Bw53), and DR1 (Biddison et al., 1982) (HLA-A2, -A11, -B7, -Bw44) were maintained as suspension cultures as described (Krangel et al., 1979). [ $4,5\text{-}^3\text{H}$ ]Lysine and -leucine, [ $2,3\text{-}^3\text{H}$ ]proline and -alanine, [ $\text{G-}^3\text{H}$ ]serine, and  $\text{U-}^{14}\text{C}$ -labeled amino acids were obtained from either New England Nuclear or ICN. In general, cells were metabolically labeled with  $^3\text{H}$ - or  $^{14}\text{C}$ -labeled amino acids in RPMI 1640 lacking the appropriate amino acid (GIBCO, Select-Amine Kit), supplemented with 10% dialyzed fetal bovine serum. Labeling with [ $^3\text{H}$ ]- or [ $^{14}\text{C}$ ]alanine was performed in medium lacking glutamic acid. Metabolic labeling with [ $6\text{-}^3\text{H}$ ]- or [ $\text{U-}^{14}\text{C}$ ]-glucosamine (New England Nuclear) was performed in RPMI 1640 containing 0.2% (w/v) glucose supplemented with dialyzed fetal bovine serum.

For labeling, batches of  $5 \times 10^6$  or  $10^7$  cells were washed once with the labeling medium, were resuspended at  $10^6/\text{mL}$  in labeling medium, and were allowed to preincubate at  $37^\circ\text{C}$  for 1 h.  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled amino acids were added to 100–250 and 25  $\mu\text{Ci}/\text{mL}$ , respectively, and incubation at  $37^\circ\text{C}$  was continued for 6–12 h. [ $^3\text{H}$ ]Glucosamine and [ $^{14}\text{C}$ ]-glucosamine were added to 70 and 100  $\mu\text{Ci}/\text{mL}$ , respectively, and incubation at  $37^\circ\text{C}$  was for 18–20 h. Following incubation, radiolabeled cells were harvested by centrifugation, and a fresh batch of preincubated cells was immediately added to the used labeling medium and incubated as described above. In this manner, two or three successive labelings were usually performed with each batch of labeling medium. When required, cells were labeled in the presence of 4  $\mu\text{g}/\text{mL}$  tunicamycin (a kind gift of Eli Lilly Co.) as described (Krangel et al., 1979).

**Purification of Radiolabeled HLA-A2 Heavy Chains.** Labeled cells were washed once with ice-cold phosphate buffered saline and were solubilized by incubating on ice for 45 min at  $10^7$  cells/mL in 10 mM Tris-HCl (pH 7.5), 2% (v/v) NP40, 1 mM DTT, and 0.1 mM PMSF. Insoluble debris was pelleted by centrifugation at 13000g for 5 min in an Eppendorf microfuge, and the supernatants were stored at  $-70^\circ\text{C}$  until use. Detergent lysates were precleared with 10  $\mu\text{L}$  of normal rabbit serum/ $10^7$  cell equiv of lysate and formalin-fixed, heat-killed *Staphylococcus aureus* (Cowan I strain) (Kessler, 1975) as previously described (Krangel et al., 1979), except that centrifugation was at 13000g for 1 min in an Eppendorf microfuge. The HLA-A2 specific mouse monoclonal antibody PA2.1 (a kind gift from Dr. P. Parham) (Parham & Bodmer, 1978) coupled to agarose A50M beads (Bio-Rad) was then added at 35  $\mu\text{L}$  of packed beads/ $10^7$  cell equiv for two successive 4-h incubations while mixing at  $4^\circ\text{C}$ . The two batches of beads were washed as described (Krangel et al., 1979) and were each eluted by boiling in 100  $\mu\text{L}$  of 1%  $\text{NaDODSO}_4/(1-3) \times 10^7$  cell equiv for 3 min. Eluates were diluted with 4 volumes of 10 mM Tris-HCl (pH 7.5) and 2.5% (v/v) NP40 on ice. Rabbit anti-HLA heavy chain serum (anti-H) (Krangel et al., 1979) was then added at 15–20  $\mu\text{L}/10^7$  cell equiv to the diluted eluate from the initial PA2.1 incubation and at half that concentration to the diluted eluate from the second incubation. After 2–4 h on ice immune complexes were harvested by using *Staph. aureus*, and an abbreviated series of washes was performed. The supernatant from the anti-H and *Staph. aureus* precipitation of the diluted eluate from the initial PA2.1 incubation was then reprecipi-

tated with 7–10  $\mu\text{L}$  of anti-H/10<sup>7</sup> cell equiv and washed in a similar fashion. The three resulting aliquots of washed immunoprecipitates were then each eluted by boiling in 200  $\mu\text{L}$  of 2%  $\text{NaDODSO}_4$  for 3 min. The ratio of cpm in the three eluates was typically 4:1:1 for the homozygous cell line and 4:1:0.2 for the heterozygous cell line. Following anti-H immunoprecipitations, goat anti-human  $\beta_2$ -microglobulin coupled to agarose beads could be used to isolate the light chain.

**Peptide Mapping.**  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled HLA-A2 heavy chain preparations were combined and precipitated on ice in the presence of 5 volumes of acetone. A minimum of 40 000 cpm of  $^3\text{H}$ -amino acid labeled and 12 000 cpm of  $^{14}\text{C}$ -amino acid labeled heavy chains was used for analytical peptide maps. However, both the absolute amounts and ratio of  $^3\text{H}:^{14}\text{C}$  cpm varied. Following centrifugation at 4000g for 10 min, pellets were dried under a stream of  $\text{N}_2$  and were redissolved by boiling in 200  $\mu\text{L}$  of 350 mM Tris-HCl (pH 8.3), 3.5 mM EDTA, 2%  $\text{NaDODSO}_4$ , and enough ovalbumin to bring the total amount of carrier protein to 0.5 mg. DTT was added to 3 mM, and tubes were gassed with  $\text{N}_2$ , stoppered, and incubated at  $37^\circ\text{C}$  for 2–3 h. Iodoacetamide was then added to 8 mM, and tubes were gassed with  $\text{N}_2$ , stoppered, and incubated at  $22^\circ\text{C}$  for 30 min in the dark. The reaction was subsequently quenched by the addition of a drop of  $\beta$ -mercaptoethanol, and protein was precipitated at  $-20^\circ\text{C}$  by the addition of 5 volumes of acetone and 0.5 volume of 45% (w/v) trichloroacetic acid. Following centrifugation at 4000g for 10 min, pellets were washed once with ice-cold acetone and were dried under a stream of  $\text{N}_2$ ; 200  $\mu\text{L}$  of 50 mM ammonium bicarbonate (pH 7.7) was added to the pellet and trypsinization utilizing diphenylcarbamoyl chloride treated bovine trypsin (Sigma Chemical Co.) was performed as described (Kaufman et al., 1980). Trypsinized samples were either frozen or used immediately.

High-pressure liquid chromatography (HPLC) was performed utilizing a 4 mm  $\times$  25 cm Zorbax C-8 column (Du Pont) and a Waters HPLC system with a Model 660 solvent flow programmer. Samples were spun at 10000g for 1 min in a Beckman 152 microfuge to remove particulate material prior to injection. Following injection the column was washed with 10 mM ammonium acetate (pH 6.5), eluted with a 55-min concave gradient 0–30% acetonitrile in the same buffer, and then washed isocratically with first the final buffer and then 100% acetonitrile. Gradient 8 on the 660 programmer was used for all runs except [ $^3\text{H}$ ]- vs. [ $^{14}\text{C}$ ]leucine maps, which employed gradient 7. Flow rate was 1.5 mL/min at all times, and fractions were collected at 0.4-min intervals. For analytical runs fractions were collected directly into 1-dram vials and evaporated to dryness.

Ion-exchange chromatography was performed on a 4 mm  $\times$  20 cm Beckman microbore column containing AA20 cation-exchange resin, utilizing a gradient of pyridine and acetic acid, as described (Kaufman et al., 1980).

**Liquid Scintillation Counting.** Samples to be counted were dissolved in 100  $\mu\text{L}$  of water and 3 mL of Liquiscint (National Diagnostics) and were counted in a Beckman LS255 liquid scintillation counter. Samples were counted by utilizing variable windows. Spillover of  $^{14}\text{C}$  cpm into the  $^3\text{H}$  channel was typically 20–30%, and spillover of  $^3\text{H}$  cpm into the  $^{14}\text{C}$  channel was typically 0.1–0.2%.

**Carbohydrate Analysis.** Digestion with endo-H (a kind gift from Drs. C. Hubbard and P. Robbins) was performed as described (Krangel et al., 1979). Pronase digestion and Pronase glycopeptide analysis were kindly performed by Dr. C. Hubbard.

**Thin-Layer Chromatography.** Radiolabeled peptides were hydrolyzed at 110 °C for 20 h in constant boiling 5.7 N HCl. Chromatography of radiolabeled amino acids and unlabeled standards was performed on 0.1 mm MN-cellulose 300 layers (Brinkmann Instruments). Chromatograms were developed with 1-butanol-acetone-diethylamine-water (10:10:2:5) and stained with ninhydrin (Brenner et al., 1969). The position of radiolabeled species was determined by scraping off and counting sections of cellulose.

**Preparation of Unlabeled Heavy Chain Fragments.** Papain-solubilized HLA-A2 antigens were isolated from JY and M7 cells as described (Parham et al., 1977). Heavy chain isolation, CNBr digestion without reduction and alkylation, and CNBr fragment separation were performed as described (Orr et al., 1979a). CNBr-5 was used directly in sequence analysis. CNBr-2 and CNBr-3 were each digested with trypsin as described (Lopez de Castro et al., 1979), and tryptic peptides were separated by HPLC as detailed above for radiolabeled peptides, using gradient 8. Peptides were monitored by absorbance at 214 nm, and the peaks with mobilities similar to the difference peptides were subjected to further analysis.

**Peptide Composition and Sequence Analysis.** Amino acid compositions of unlabeled peptides were determined as described (Lopez de Castro et al., 1979). Sequence analysis was performed on a Beckman 890 C liquid phase sequencer, as described (Orr et al., 1979b). For radioactive samples, 25 nmol of sperm whale apomyoglobin was applied with the sample to act as a carrier. The butyl chloride extracts were dried and counted directly. For unlabeled samples, anilinothiazolinones were converted to phenylthiohydantoin amino acids which were identified by HPLC.

## Results

**Strategy.** Double-label tryptic peptide analysis was used to assess structural differences among the HLA-A2 heavy chains synthesized by the cell lines JY, M7, and DR1. Although radiolabeled lysine and arginine would together label every tryptic peptide but the carboxy-terminal one, this approach could not be taken due to an inability to incorporate sufficient amounts of radiolabeled arginine. An alternative approach involved metabolic labeling with five different amino acids, which together labeled overlapping sets of peptides encompassing virtually all of the JY-HLA-A2 heavy chain. The JY-HLA-A2 amino acid sequence has been determined to 90% of the 271-residue papain-solubilized molecule (Lopez de Castro et al., 1982), and 32 residues of the intracellular carboxy-terminal portion have also been assigned (Robb et al., 1978). Tentative assignment of remaining residues based upon homology with HLA-A28 (96% homologous), or with HLA-B7 (86% homologous) when HLA-A28 is also unassigned, would suggest that 37 of 41 tryptic peptides, encompassing 98% of the detergent-solubilized HLA-A2 heavy chain, should be labeled by the amino acids used. Radiolabeled lysine, leucine, serine, proline, and alanine would be expected to label 11, 16, 11, 11, and 17 tryptic peptides, respectively. These calculations must be regarded with some caution pending definitive assignments at all positions in the molecule.

Purification of radiolabeled HLA-A2 heavy chains was by a two-step immunoprecipitation procedure, the first with an HLA-A2-specific monoclonal antibody and the second with an anti-HLA heavy chain heteroantiserum. The first step resulted in a partially purified preparation which consisted of actin, HLA-A2 heavy chains, and  $\beta_2$ -microglobulin, as well as other minor components. Following denaturation, anti-heavy chain immunoprecipitation from this mixture resulted in a preparation of HLA-A2 heavy chains of greater than 95% purity,

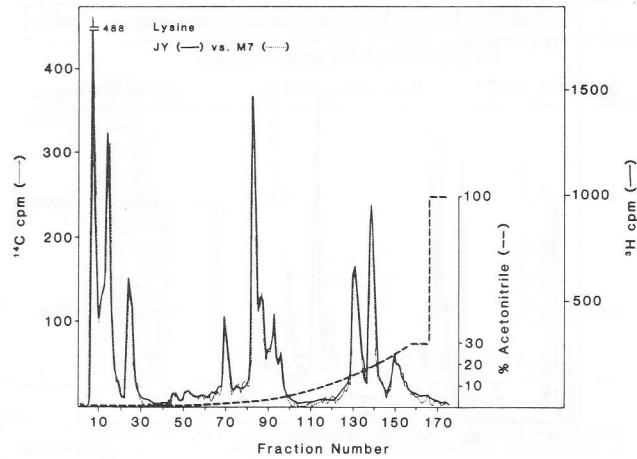


FIGURE 1: HPLC comparison of lysine-labeled tryptic peptides: [<sup>3</sup>H]lysine-labeled JY (—) vs. [<sup>14</sup>C]lysine-labeled M7 (---).

with an occasional trace of actin as the major contaminant (data not shown). Yields were typically 0.04% from total radioactivity in the detergent lysate of the heterozygous cell line and 0.08% from the homozygote.

<sup>3</sup>H- and <sup>14</sup>C-labeled heavy chain preparations were combined, reduced, alkylated, and digested with trypsin; tryptic peptides were then resolved by reverse-phase HPLC. The accuracy and sensitivity of this method of comparison was assessed as follows. Total recovery of counts applied to the column was typically 50%. However, the chromatograms generally displayed the expected number of peaks, albeit with varying yields. Further, only a small percentage of the applied radioactivity flowed through the column. Thus, most tryptic peptides were resolved by this technique. When [<sup>3</sup>H]Leu- and [<sup>14</sup>C]Leu-labeled JY HLA-A2 heavy chains were compared, no difference in the chromatograms, other than some single-tube shifts in peak fractions, were observed. However, when [<sup>3</sup>H]Leu-labeled HLA-B7 was compared to [<sup>14</sup>C]Leu-labeled HLA-A2, only 6 of 16 peaks were found to coelute. Excluding the flow-through peak and a peak eluted with 100% acetonitrile, this was precisely the number of peptides expected to be shared based upon amino acid sequence analysis. Hence, this appeared to be a sensitive method to compare most tryptic peptides in the molecule; those few peptides which might be either insoluble or otherwise of extremely low yield or which might flow through the column would of course be refractory to this analysis.

**Peptide Comparisons of M7- with JY-HLA-A2.** Lysine-, leucine-, serine-, proline-, and alanine-containing tryptic peptides from JY- and M7-HLA-A2 heavy chains are compared in Figures 1-5, respectively. Particular peptides in Figures 1-5 will be referenced based upon the labeled amino acid, i.e., K, L, S, P, or A, as well as fraction number. No differences can be observed in either the lysine-containing or proline-containing peptides (Figures 1 and 4, respectively). However, a number of distinct types of differences can be observed in the other peptide comparisons. These consist of differences in either peak height or elution position. Apparent changes in peak height may be observed in peptides L64 and L68 (Figure 2), S18, S79, S81, S84, S87, S125, S132, and S146 (Figure 3), and A85, A88, and A91 (Figure 5). Changes in peak position occur in peptides L55, L116, and L120 (Figure 2), S72 and S76 (Figure 3), and A74 and A76 (Figure 5).

Observed differences can be classified as either real or artifactual based upon <sup>3</sup>H-labeled JY vs. <sup>14</sup>C-labeled JY control comparisons. Thus, as can be seen in Figure 6, the differences in Figure 3 peptides S18, S81, S84, S125, S132, and S146

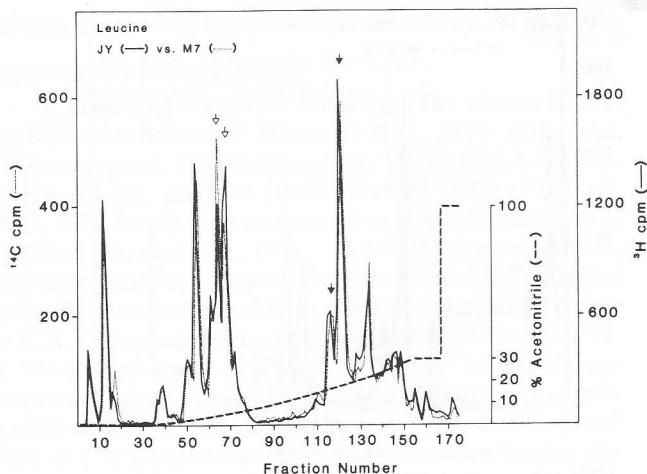


FIGURE 2: HPLC comparison of leucine-labeled tryptic peptides: [<sup>3</sup>H]leucine-labeled JY (—) vs. [<sup>14</sup>C]leucine-labeled M7 (...). Note that the gradient used for this comparison differed from that used in other comparisons, as described under Experimental Procedures. The single tube shift in peptide L55 was not reproducible and hence is not considered significant. Remaining differences are distinguished by whether they were (solid arrows) or were not (open arrows) evident in control comparisons.

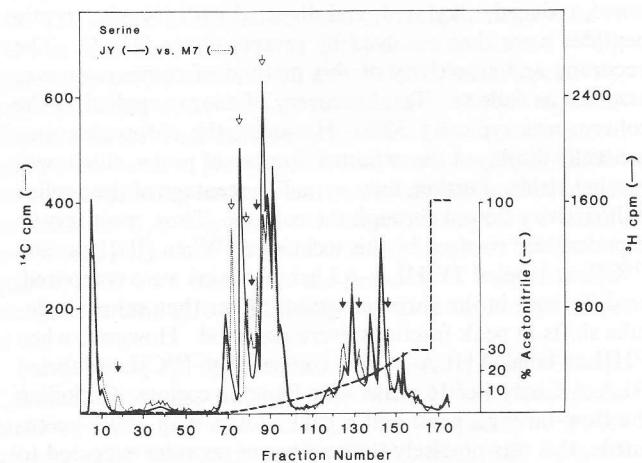


FIGURE 3: HPLC comparison of serine-labeled tryptic peptides: [<sup>3</sup>H]serine-labeled JY (—) vs. [<sup>14</sup>C]serine-labeled M7 (...). Differences were distinguished by whether they were (solid arrows) or were not (open arrows) evident in control comparisons (see Figure 6).

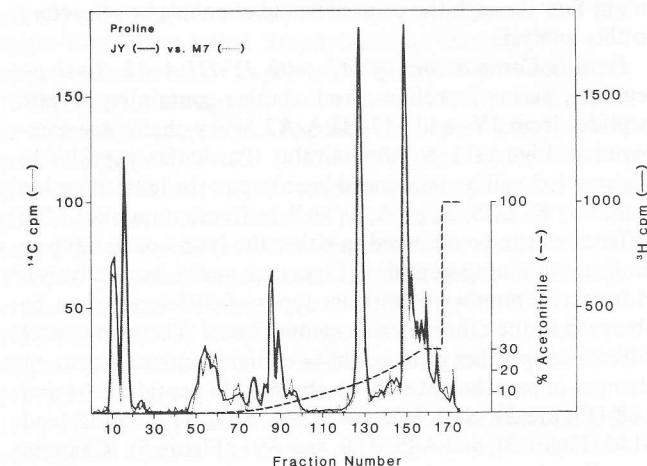


FIGURE 4: HPLC comparison of proline-labeled tryptic peptides: [<sup>3</sup>H]proline-labeled JY (—) vs. [<sup>14</sup>C]proline-labeled M7 (...).

(solid arrows) are still evident, whereas the other differences are not. Those differences which persist in this control comparison are the result of metabolic conversion of radiolabeled

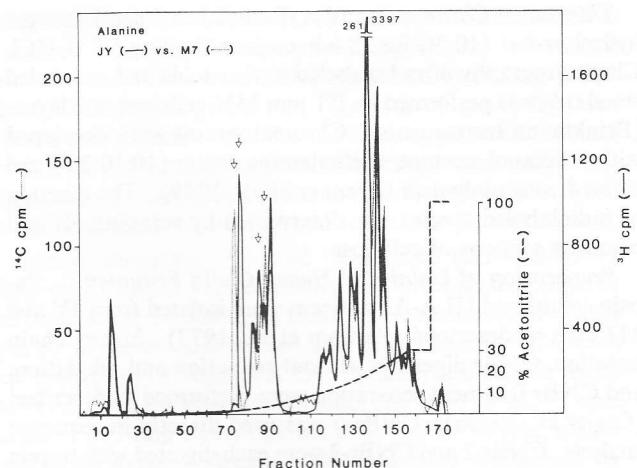


FIGURE 5: HPLC comparison of alanine-labeled tryptic peptides: [<sup>3</sup>H]alanine-labeled JY (—) vs. [<sup>14</sup>C]alanine-labeled M7 (...). The apparent peak height change in peptide A91 was not considered significant when radioactivity was carefully summed across the peak. Remaining differences are denoted by open arrows.

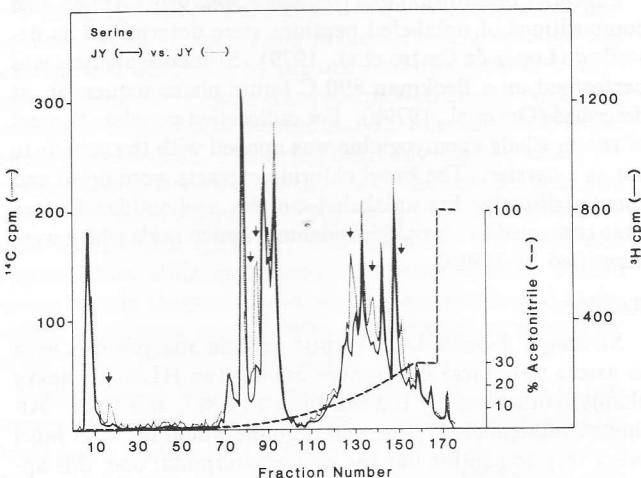


FIGURE 6: Control HPLC comparison of serine-labeled tryptic peptides: [<sup>3</sup>H]serine-labeled JY (—) vs. [<sup>14</sup>C]serine-labeled JY (...). Differences are denoted by solid arrows.

serine to glycine. The evidence for this consists of the following. NH<sub>2</sub>-Terminal sequence analysis of radiolabeled  $\beta_2$ -microglobulin was performed to detect any interconversions. When  $\beta_2$ m isolated from [<sup>14</sup>C]serine-labeled cells was analyzed, radioactivity was released at cycles assigned either serine or glycine, consistent with 32% conversion of [<sup>14</sup>C]serine to [<sup>14</sup>C]glycine. No other interconversions could be detected. When the appropriate HPLC fractions (solid arrows, Figure 3) were hydrolyzed and the radioactivity was analyzed by thin-layer chromatography, most radioactivity migrated as glycine, and not serine, and that migrating as glycine had an unusually high ratio of <sup>14</sup>C:<sup>3</sup>H. Differential loss of radioactivity would necessarily result from the metabolic conversion of [U-<sup>14</sup>C] and [G-<sup>3</sup>H]serine to glycine, which thus explains the peak height differences in the fractions in question. Therefore, the apparent differences in peptides S18, S81, S84, S125, S132, and S146, are artifacts of this interconversion. Some slight peak shift differences also persisted in control comparisons. Two such examples, peaks L116 and L120 (solid arrows, Figure 2), quite consistently exhibit a changing ratio of <sup>3</sup>H to <sup>14</sup>C across the peak, with <sup>14</sup>C trailing. We have no conclusive explanation for this phenomenon but suppose that HPLC may be partially resolving these peptides on the basis of isotope differences alone. Some other minor differences

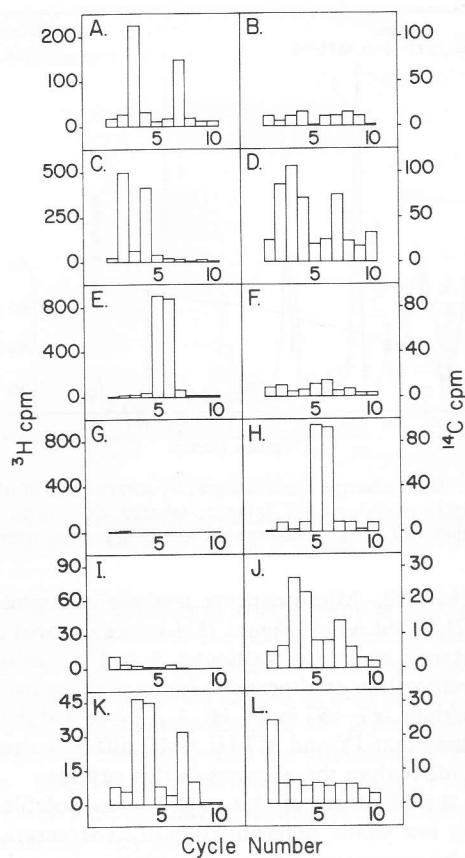


FIGURE 7: Microsequence analysis of difference peptides. Chromatogram fractions containing difference peptides from double-label comparisons were subjected to amino acid sequence analysis as described under Experimental Procedures. Radioactivity released in the chlorobutane extract at each cycle is presented. (A) and (B) represent corrected  $^3\text{H}$  and  $^{14}\text{C}$  channel radioactivities, respectively, for the S76 sequence (Figure 3). (C) and (D) represent corrected  $^3\text{H}$  and  $^{14}\text{C}$  channel radioactivities, respectively, for the S69 plus S72 mixture sequence (see text) (Figure 3). (E) and (F) represent corrected  $^3\text{H}$  and  $^{14}\text{C}$  channel radioactivities, respectively, for the A76 sequence (Figure 5). (G) and (H) represent corrected  $^3\text{H}$  and  $^{14}\text{C}$  channel radioactivities, respectively, for the A74 sequence (Figure 5). (I) and (J) represent corrected  $^3\text{H}$  and  $^{14}\text{C}$  channel radioactivities, respectively, for the A130 sequence (Figure 8). (K) and (L) represent corrected  $^3\text{H}$  and  $^{14}\text{C}$  radioactivities, respectively, for the A165 sequence (Figure 8). The  $^{14}\text{C}$  radioactivity at cycle one in panel L is not considered informative, since it is not unusual to observe radioactivity washing out of the cup at the first cycle.

were not reproducible, as noted in the figure legends.

Remaining differences (open arrows) were deemed significant and were analyzed by microsequence analysis in order to identify the peptides in question. In addition, a difference occasionally observed as an excess of  $[^3\text{H}]$ leucine radioactivity around fraction L128, which was not obvious in Figure 2, was also investigated. Automated Edman degradation of the JY peptide S76 yielded radioactivity at cycles 3 and 7, suggesting that the peptide contained serines at these positions (Figure 7A,B). Hydrolysis of this peptide followed by TLC of the hydrolysate confirmed that all radioactivity in this peptide migrated as serine, and not glycine. Automated Edman degradation of the JY peptide A76 suggested that this peptide contained alanine at positions 5 and 6 (Figure 7E,F). When compositional information (i.e., no leucine, proline or lysine) and sequence information were compared with those of the tryptic peptides expected based upon the known JY-HLA-A2 amino acid sequence (Table I), it became clear that peptides A76 and S76 were identical and corresponded to the tryptic peptide spanning residues 36–44. The amino acid sequence

Table I: Serine- and Alanine-Labeled Tryptic Peptides<sup>a</sup>

Serine-Labeled Tryptic Peptides		
residue no.	serine position(s)	other residue(s)
1–6	2, 4	
7–14	5, 7	
36–44	3, 7	A
83–97	6, 10	A, L
98–108 <sup>b</sup>	8	
132–144 <sup>c</sup>	1	A, K
187–202	9	A, L
244–256 <sup>d</sup>	8	A, P
274–309 <sup>d</sup>	3, 4, 6	A, P, L
312–315	1, 2	
317–338	3, 5, 9, 10, 12, 16, 19	A, L

Alanine-Labeled Tryptic Peptides		
residue no.	alanine position(s)	other residue(s)
23–35	3	
36–44	5, 6	S
49–62	1	P
69–75	1, 3	
83–97	8	S, L
115–121 <sup>e</sup>	3	K
122–127	4	L, K
132–144 <sup>c</sup>	4, 5, 8, 9	S, K
147–157	3, 4, 7	L
158–169 <sup>d</sup>	1	L
182–186	1	P, K
187–202	7, 13	S, L
203–219	3, 9	L, P
235–243	2	P, K
244–256	2, 3	S, P
274–309 <sup>d</sup>	15, 18, 21, 27, 30, 31	S, P, L
317–338	7, 18, 13, 22	S, L

<sup>a</sup> Data derived from Lopez de Castro et al. (1982), Ploegh et al. (1981), and Robb et al. (1978). <sup>b</sup> M. S. Krangell, unpublished evidence for D102 and R108. <sup>c</sup> By homology with HLA-A28 and -B7. <sup>d</sup> By homology with HLA-B7. <sup>e</sup> By homology with HLA-A28.

of this peptide is FDSDAASQR.

Peptides S72 and A74 were shown to correspond to the M7 forms of this peptide, in similar fashion. Sequence analysis of peptide S72 was complicated by the fact that in all chromatographs subsequent to that shown in Figure 3, S72 coeluted with S69. Parts C and D of Figure 7 present  $[^3\text{H}]$ - and  $[^{14}\text{C}]$ serine, respectively, released at each cycle when this mixture was sequenced. One JY peptide and two M7 peptides were expected. The JY peptide (S69) contained serines at positions 2 and 4 (Figure 7C) and was identified as the peptide GSHSMR, residues 1–6 in the molecule.  $[^{14}\text{C}]$ Serine was released at cycles 2, 3, 4, and 7 (Figure 7D). We interpret this as a mixture of the M7 peptide corresponding to residues 1–6 (S69) with that corresponding to residues 36–44 (S72). Repetitive yield considerations are consistent with this interpretation. The sequence of peptide A74 (Figure 7G,H) indicated the presence of alanine at positions 5 and 6, identical with that of the JY form of this peptide. Thus the JY- and M7-HLA-A2 heavy chains differ at a residue or residues other than serines or alanines in the tryptic peptide spanning residues 36–44 in the JY-HLA-A2 sequence.

Due to our inability to incorporate sufficient amounts of radiolabeled arginine and aspartic acid, determination of the complete sequence of the difference peptide was not feasible by radiochemical methodology. Instead, milligram amounts of purified JY- and M7-HLA-A2 were used to isolate the difference peptides, as described under Experimental Procedures. Compositional analysis of the JY form (Table II) was consistent with the published sequence of this peptide. The M7 form was identical except that it lacked glutamine. Amino

Table II: Amino Acid Compositions of Difference Peptide<sup>a,b</sup>

	36-44	
	JY	M7
Asx	1.68 (2)	2.00 (2)
Thr		
Ser	2.17 (2)	1.93 (2)
Glx	1.39 (1)	0.04
Pro	0.24	
Gly	0.34	0.08
Ala	2.12 (2)	2.03 (2)
Val	0.05	
Met	0.12	0.11
Ile	0.11	
Leu	0.22	
Tyr	0.05	
Phe	0.81 (1)	0.94 (1)
His		0.06
Lys	0.87 (1)	
Arg	1.08 (1)	

<sup>a</sup> Values reported as moles of residue per mole of peptide.<sup>b</sup> Numbers in parentheses represent predicted compositions based upon sequence analysis.

acid sequence analysis confirmed this difference and indicated that the sequence of the M7 peptide was FDSDAASR. This change could have arisen as a result of a glutamine to arginine substitution at position 43, generating the sequence FDSDAASRR, from which the M7 peptide would have arisen by trypsin proteolysis. Alternatively, this change could have resulted simply by the deletion of Gln-43. The former possibility would necessitate only a single nucleotide change in DNA coding sequences and would account for the single charge difference which distinguishes these molecules by isoelectric focusing (Biddison et al., 1980c); the latter would require a three nucleotide deletion and would not explain the observed charge difference.

As mentioned above, a small peak of [<sup>3</sup>H]leucine radioactivity was occasionally observed at around fraction L128, although it was not clearly evident in the Figure 2 comparison. Microsequence analysis of fractions encompassing this difference peptide yielded both <sup>3</sup>H radioactivity and <sup>14</sup>C radioactivity at cycle 3, but only <sup>3</sup>H radioactivity at cycle 10. This was interpreted as a mixture of a peptide common to both cell lines with one unique to JY. However, two peptides in the molecule would be expected to have leucine at position 10. The first, spanning residues 147–157, contains in addition three alanine residues. The second, spanning residues 257–268, contains a lysine residue. Although no clear differences could be observed in this region of the gradient in either the lysine (Figure 1) or alanine (Figure 5) HPLC comparison, results obtained from work on another variant HLA-A2 antigen (unpublished observations) suggested that this difference peptide did, in fact, contain alanine. Although it was most likely obscured in the alanine HPLC comparison by high yield contaminants, it could nevertheless be easily resolved by another chromatographic procedure, cation-exchange chromatography.

Accordingly, [<sup>14</sup>C]alanine-labeled JY-HLA-A2 was compared with [<sup>3</sup>H]alanine-labeled M7-HLA-A2 by cation-exchange chromatography. Although much radioactivity flowed through the column at initial conditions, and far fewer peptides were resolved than by reverse-phase HPLC, multiple differences were apparent (open arrows, Figure 8). JY peptide A57 and an M7 component of peak A67 were shown by microsequence analysis to contain alanine at positions 5 and 6, consistent with these representing alternative forms of the previously defined difference peptide, which spans residues 36–44.

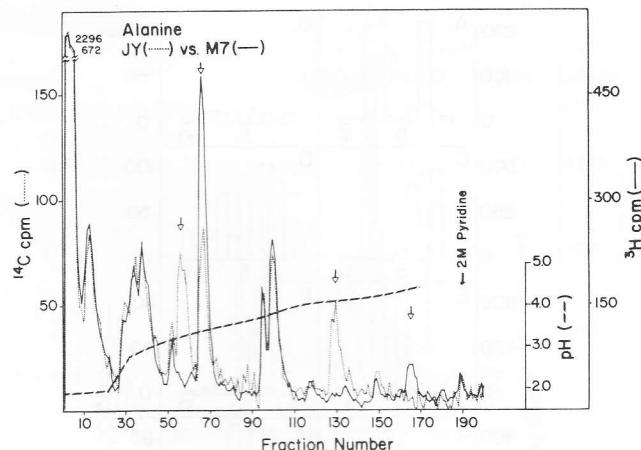


FIGURE 8: Ion-exchange chromatography comparison of alanine-labeled tryptic peptides: [<sup>14</sup>C]alanine-labeled JY (...) vs. [<sup>3</sup>H]alanine-labeled M7 (—). Differences are denoted by open arrows.

in JY-HLA-A2. Microsequence analysis of peptides A130 (Figure 7I,J) and A165 (Figure 7K,L) demonstrated that they each contained alanine at positions 3, 4, and 7, consistent with their identification as alternative forms of the peptide which spans residues 147–157 in JY-HLA-A2 (see Table I). Thus we conclude that JY and M7 HLA-A2 differ at a residue or residues other than the alanines in this peptide.

Since the M7 form of this peptide was isolable only in extremely low yields, determination of its structure was attempted by automated Edman degradation from the amino terminus of CNBr-5, which begins at residue 139. However, technical problems limited the amount of pure material available for analysis, and reliable sequence information through residue 157 could not be obtained. Hence, the precise alteration in this peptide remains unresolved.

**Glycopeptide Analysis.** The remaining differences in the serine, leucine, and alanine HPLC peptide maps may all be attributable to differential glycosylation. Sequence analysis identified M7 peak S79, as well as the trailing shoulder of JY peak S76, as 83–97, the glycopeptide (serine at positions 6 and 10). Peak S87 was found to consist of a mixture of a peptide shared by both cell lines together with a form of the JY glycopeptide which had no comigrating M7 counterpart, thus explaining the peak height difference. The relationships among the various glycopeptide forms were more clearly displayed when [<sup>3</sup>H]glucosamine- and [<sup>14</sup>C]glucosamine-labeled tryptic peptides were compared (Figure 9). As could be inferred from the serine map, the earlier eluting form(s) was (were) common to both JY and M7, whereas the later eluting form(s) was (were) unique to JY. Since, in addition to serine, the glycopeptide was predicted to contain alanine and leucine as well (Table I), differences in the alanine and leucine maps could be expected. Sequence analysis of alanine-containing peptides A85 and A88 (open arrows, Figure 5) confirmed that the differences observed were indeed due to the glycopeptide. This could not be confirmed for peaks L64 and L68 (open arrows, Figure 2). However, the failure to identify the glycopeptide in these fractions may have resulted from low recovery of radioactivity during sequence analysis, due to the fact that leucine occurs at position 13 of this 15-residue peptide.

Available data suggest that HPLC is resolving glycopeptides on the basis of oligosaccharide differences, and not amino acid sequence differences. No glycopeptide differences were observed when the nonglycosylated [<sup>3</sup>H]- and [<sup>14</sup>C]serine-labeled heavy chains synthesized in the presence of tunicamycin were compared. A number of pieces of evidence indicate that

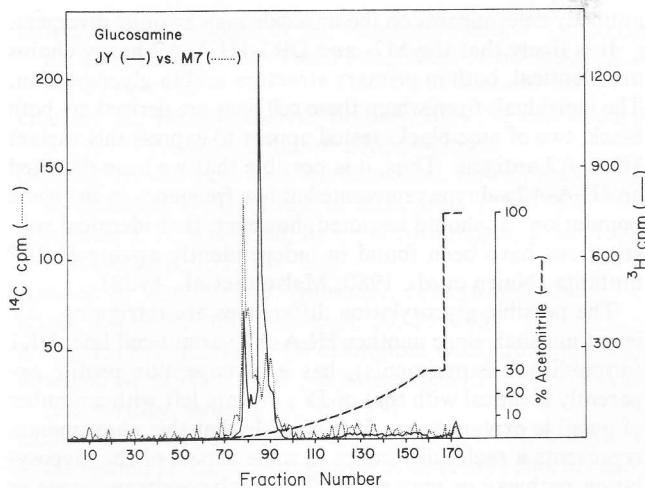


FIGURE 9: HPLC comparison of glucosamine-labeled tryptic peptides: [<sup>3</sup>H]glucosamine-labeled JY (—) vs. [<sup>14</sup>C]glucosamine-labeled M7 (....).

glycopeptide heterogeneity is not the result of partial tryptic cleavages at either end of the peptide. Finally, amino acid analyses of various JY glycopeptide forms generated from unlabeled material suggested identity in their protein portions, and the amino acid compositions of M7 glycopeptide forms were completely consistent with the published JY-HLA-A2 sequence. Thus we conclude that some of the JY- and M7-HLA-A2 heavy chains may be distinguished based upon the oligosaccharides they carry.

The nature of this difference has not been defined. It is not likely due to differential sialylation, since IEF analysis has indicated comparable ratios of zero, one, two, and three sialic acid residue containing species (Biddison et al., 1981b). It is not likely due to differential amounts of precursor, high mannose oligosaccharides, since both NaDODSO<sub>4</sub>-polyacrylamide gel electrophoresis analysis of endo-H digestion products and Pronase glycopeptide gel filtration analysis reveal almost exclusively complex oligosaccharides. In addition, it is not likely due to significant differences in oligosaccharide size, since comparative Pronase glycopeptide gel filtration analysis reveals no such differences. It is possible, however, that the harsh conditions employed for Pronase digestion, 3 days at 50 °C, may release a relatively labile moiety, which would then go undetected. Other possible differences in carbohydrate composition, linkages, and chemical modifications have not been investigated.

*Peptide Comparisons of DR1- with JY- and M7-HLA-A2.* Peptide maps comparing DR1- with JY-HLA-A2 again indicated differences in peptides 36–44 and 147–157 and the glycopeptide 83–97, but otherwise the two molecules were identical. When a direct HPLC comparison of [<sup>3</sup>H]serine-labeled DR1-HLA-A2 with [<sup>14</sup>C]serine-labeled M7-HLA-A2 was performed, the molecules proved indistinguishable (Figure 10). An ion-exchange comparison of [<sup>14</sup>C]alanine-labeled DR1-HLA-A2 with [<sup>3</sup>H]alanine-labeled M7-HLA-A2 also indicated identity. It thus appears that two cell lines derived from unrelated individuals share identical HLA-A2 molecules which are distinguishable from JY-HLA-A2 both in primary structure and in glycosylation.

#### Discussion

Previous work has identified individuals whose peripheral blood lymphocytes are serologically indistinguishable from those of most HLA-A2 positive individuals, yet which are recognized neither by HLA-A2-restricted, influenza virus-immune CTLs nor by HLA-A2-specific alloimmune CTLs.

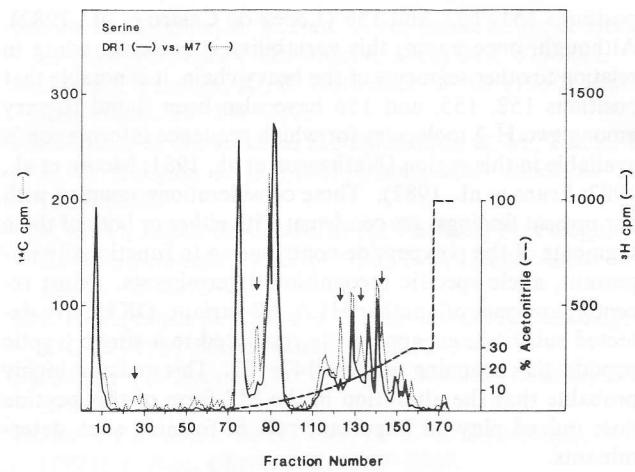


FIGURE 10: HPLC comparison of serine-labeled tryptic peptides: [<sup>3</sup>H]serine-labeled DR1 (—) vs. [<sup>14</sup>C]serine-labeled M7 (....). Differences evident in control comparisons (see Figure 6) are denoted by solid arrows.

Isoelectric focusing has revealed HLA-A2 heavy chain structural alterations which appear to correlate with these CTL recognition anomalies. This report demonstrates that the HLA-A2 heavy chains of two such individuals, M7 and DR1, may each differ from the predominant HLA-A2 heavy chain species by a glutamine to arginine substitution at position 43, an undefined alteration in the peptide spanning residues 147–157, and an as yet poorly understood change in glycosylation. Any or all of these differences may be crucial in determining CTL recognition determinants. The possibility that other alterations exist, but cannot be detected by the methods employed, cannot be eliminated, however.

Primary structure analysis of HLA-A, -B, and -C antigens, as well as their murine homologues H-2K, D and L, have been in part aimed at elucidating regions of structural polymorphism, which presumably contribute to functionally important determinants on the molecule. Comparisons among HLA-B7, -A2, and -A28 reveal striking, but not absolute, conservation in the amino- and carboxy-terminal portions of the papain-solubilized molecule, with the majority of differences falling between residues 65 and 194. These form three distinct clusters, encompassing residues 65–80 in  $\alpha 1$  (the amino terminal domain), residues 105–116 in  $\alpha 2$  (the second domain), and residues 177–194 in the junction between  $\alpha 2$  and  $\alpha 3$  (the second and third domains), as well as less well-defined clusters in  $\alpha 2$ , encompassing residues 95–98, 138–145, and 151–156 (Lopez de Castro et al., 1982).

Comparisons of the amino-terminal 100 residues of a number of H-2 molecules reveal additional polymorphism in the amino-terminal portion of the molecule (Nathenson et al., 1981). Small clusters are evident spanning residues 18–24, 30–32, and 41–45. Clearly, as more sequences become available, our perceptions of polymorphic segments may be modified. However, the segment spanning residues 41–45 appears to be unique among the variable segments in the amino-terminal 64 residues because it carries multiple HLA substitutions (43 and 45) as well as H-2 substitutions (41, 43, and 45). It should be further noted that the substitution at position 45 is the only HLA substitution within the amino-terminal portion requiring two base changes in DNA coding sequences. Both HLA substitutions are nonconservative, and that at 45 involves a charge difference.

Polymorphism among three HLA sequences has also been demonstrated in the region encompassing residues 151–156. Substitutions involving charge differences occur at each of

positions 151, 152, and 156 (Lopez de Castro et al., 1982). Although, once again, this variability is not outstanding in relation to other segments of the heavy chain, it is notable that positions 152, 155, and 156 have also been found to vary among two H-2 molecules for which sequence information is available in this region (Nathenson et al., 1981; Moore et al., 1982; Evans et al., 1982). These considerations, coupled with our present findings, are consistent with either or both of these segments of the polypeptide contributing to functionally important, allele-specific recognition determinants. Most recently, analyses of another HLA-A2 variant, DK1, have detected substitutions apparently restricted to a single tryptic peptide, that spanning residues 147–157. This makes it highly probable that the alteration in the M7 form of this peptide does indeed play an important role in forming such determinants.

The nature of the glycosylation difference, and its possible effects on CTL determinants, remains unclear. Since we have been unable to directly demonstrate structural alterations in the oligosaccharide, any conclusions must be regarded with caution. It should be borne in mind that whereas glycosylation has been shown not to influence the serologic activity of HLA-A2 (Parham et al., 1977), it is not known whether it has any effect on CTL recognition.

Nathenson and colleagues have analyzed structurally a series of spontaneously arising H-2K<sup>b</sup> mutants which have been detected by screening for unexpected skin graft rejections (Nairn et al., 1980). The single or double amino acid substitutions found among these molecules are scattered over the amino-terminal half of the molecule. Two of these mutants may be particularly relevant to interpreting the effects of the substitutions found in M7-HLA-A2. One, bm8, has a substitution at residue 23, coincident with one of the small, amino-terminal variable clusters in H-2, described above. Thus, residues in the relatively nonpolymorphic amino-terminal portion of the molecule may be important in forming determinants important in CTL recognition. The other, bm1, has substitutions at residues 155 and 156. Whereas both mutants are among the more distantly related of all the K<sup>b</sup> mutants to the parent molecule as assessed by their reactivities with alloimmune CTLs, the bm1 mutant is the only one of the structurally characterized mutants that is easily distinguishable from H-2K<sup>b</sup> by H-2K<sup>b</sup>-restricted, virus-immune CTLs (Klein, 1978; Melief et al., 1980; Nairn et al., 1980; Sherman, 1980). The apparent structural and functional correlations between the bm1 mutant and the HLA-A2 variants discussed here are striking.

Interestingly, it has not been possible to distinguish M7 from JY-HLA-A2 by either allospecific monoclonal antibodies or conventional typing sera. This argues against any gross conformational differences between these molecules. The failure to distinguish these molecules may be due to the fact that HLA-A2-specific antibodies are not usually generated against the determinant involved, but is more likely the result of the limited number of monoclonal antibodies available for testing and the inadequacy of relatively complex human alloantiseria in detecting such subtle differences. Thus, if only one of many HLA-A2-specific serological determinants were altered in M7, it might be very difficult to detect. Careful serological characterization of H-2K<sup>b</sup> mutants have resulted in the definition of quantitative differences in particular determinants in some instances (Klein, 1978). However, for whatever the reasons, the inability to easily distinguish JY- and M7-HLA-A2 serologically suggests strongly that the number and/or localization of HLA-A2-specific CTL and

antibody determinants on the molecule may be quite divergent.

It is likely that the M7- and DR1-HLA-A2 heavy chains are identical, both in primary structure and in glycosylation. The individuals from whom these cell lines are derived are both black; two of nine blacks tested appear to express this variant HLA-A2 antigen. Thus, it is possible that we have detected an HLA-A2 subtype represented at low frequency in the black population. It should be noted, however, that identical substitutions have been found in independently arising H-2K<sup>b</sup> mutants (Nairn et al., 1980; Melvold et al., 1982).

The possible glycosylation differences are intriguing. JY is not unusual, since another HLA-A2 variant cell line, DK1 (unpublished experiments), has a glycopeptide profile apparently identical with that of JY. We are left with a number of possible explanations. It is possible that this phenomenon represents a racial difference in some aspect of the glycosylation pathway or may result from a glycosyltransferase or glycosidase in genetic linkage disequilibrium with the variant HLA-A2 allele. Alternatively, the amino acid substitutions at position 43 and/or in the peptide spanning 147–157 may somehow affect the processing and modifications of the oligosaccharide, which is attached at Asn-86. The latter hypothesis would suggest that some of these residues may be in close proximity in the native molecule. Although we cannot distinguish among these possibilities at present, a detailed structural analysis of the oligosaccharides derived from HLA-A2, as well as other specificities on these and other black- and caucasian-derived cell lines, should be helpful in clarifying this issue.

It has been estimated that there may be as many as 100 alleles each at the K and D loci in the H-2 complex (Klein, 1979). The levels of polymorphism currently recognized in man at the HLA-A and -B loci are substantially lower, there being approximately 18 alleles at the A locus and 32 at the B locus. The results of this and other studies (Biddison et al., 1980b, 1981, 1982) suggest that many of the specificities defined by conventional serology may consist of families of structurally similar but nonidentical molecules, which may display profound differences in biological activity.

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