Primary Structure of Papain-Solubilized Human Histocompatibility Antigen HLA-B27†

Angel Ezquerra,‡ Rafael Bragado,‡ Miguel A. Vega,‡ Jack L. Strominger,§ James Woody,∥ and José A. López de Castro*,‡

Department of Immunology, Fundación Jiménez Diaz, 28040 Madrid, Spain, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, and Naval Medical Research Center, Bethesda, Maryland 20814

Received July 23, 1984

ABSTRACT: The complete amino acid sequence of papain-solubilized HLA-B27, an antigen that presents a very strong association to the development of ankylosing spondylitis, has been determined. The overall sequence homology with the cross-reactive allelic products HLA-B7 and HLA-B40 (Bw60) is 93% and 92%, respectively. Half of the differences between HLA-B27 and -B7 are located in segments 63–83 and 113–116. Most of the known HLA class I antigens are different in these segments, and it is suggested that the corresponding residues may be involved in the alloantigenic determinants of HLA-B27. A free cysteine residue is present at position 67, and it is at least partially exposed to solvent. In addition, other differences are found in various areas of the two N-terminal domains. The comparison with available HLA class I sequences allows an evaluation of their contribution to the antigenic polymorphism of these molecules. The relevance of these data is discussed in connection with the mapping of functional sites of HLA class I antigens and with the association between HLA-B27 and ankylosing spondylitis.

Human class I histocompatibility antigens are membrane glycoproteins encoded by the HLA-A, -B, and -C loci of the major histocompatibility complex (MHC).[1] They are expressed on the cell surface of virtually all human cells and display an exceptional degree of genetic polymorphism. This polymorphism is probably related to their role as restriction elements for the recognition of foreign antigens expressed on the surface of virus-infected or otherwise modified syngeneic cells (McMichael et al., 1977; Dickmeiss et al., 1977) by cytolytic T lymphocytes (CTL).

Class I HLA antigens are composed of a MHC-encoded heavy chain, noncovalently bound to β_2-microglobulin (β_2m), an invariant polypeptide (Grey et al., 1973). A large portion of the molecule, which includes about 80% of the heavy chain and β_2m, is on the outer face of the cell surface. This portion may be isolated in soluble form (HLA_{pap}) after papain treatment of cell membranes (Sanderson & Batchelor, 1968; Cresswell et al., 1973). The heavy chain spans the lipid bilayer and extends its carboxyl-terminal portion into the cytoplasm. The extracellular region is thought to be organized in three distinct domains designated as α1 (residues 1–90), α2 (residues 91–182), and α3 (residues 183–275) on the basis of protein and gene structural studies (Orr et al., 1979; Malissen et al., 1982). The two amino-terminal α1 and α2 domains are highly variable among different HLA class I specificities. By contrast, α3 is significantly more constant. This domain is probably the main interaction site for β_2m (Yokoyama & Nathenson, 1983).

Structural analyses of HLA polymorphism have been carried out by means of amino acid sequence comparisons of several HLA-A, -B, and -C specificities as determined by protein or DNA sequencing. These studies have outlined a number of segments where variable positions are found clustered (López de Castro et al., 1984). The great complexity of the structural polymorphism that emerges from such comparisons makes it difficult to establish a precise correlation between the various portions of the molecule and particular serological specificities. Nevertheless, previous comparative analyses of strongly cross-reactive specificities such as HLA-A2 and -A28 or HLA-B7 and -B40 (López de Castro et al., 1982, 1983) have provided a valuable insight into the nature and topography of several alloantigenic sites. These studies, in combination with the biochemical characterization of serologically immunoselected HLA mutants (Krangel et al., 1983a) and naturally occurring HLA variants distinguishible by specific CTL (Krangel et al., 1982, 1983b), are a powerful tool for the mapping of putative functional epitopes.

In this report, the amino acid sequence of the extracellular portion of a HLA-B27 antigen is presented and compared to that of cross-reactive alleles HLA-B7 and -B40 (-Bw60) to outline the structure of its alloantigenic determinants. The study was also undertaken in view of the fact that HLA-B27 antigen is strongly associated to the development of ankylosing spondylitis and Reiter's syndrome (Woodrow, 1980). This association is the strongest among the many diseases linked to HLA (Svejgaard et al., 1983), and thus it may constitute

†Supported by grants from the Spanish Ministerio de Educación y Ciencia (3448-79), Fondo de Investigaciones Sanitarias de la Seguridad Social (83/0694), and the National Institutes of Health (AI-10736). A.E. and M.A.V. are fellows from the C.A.M.P. of Madrid and the F.I.S., respectively. This work was also supported in part by the U.S. Naval Medical Research and Development Command (Work Unit M0095-PN001-0045) and by Contract Y001 CP0052 from the National Cancer Institute.

‡Fundación Jiménez Diaz.

§Harvard University.

∥Naval Medical Research Center.

[1] Abbreviations: MHC, major histocompatibility complex; CTL, cytolytic T lymphocytes; β_2m, β_2-microglobulin; HLA_{pap}, papain-solubilized HLA antigen; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; Pth, phenylthiohydantoin; Tris-HCl, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-1733$01.50/0

the most suitable system to investigate possible molecular mechanisms of HLA disease association.

Materials and Methods

Purification of the HLA-B27$_{pap}$ Heavy Chain. The human lymphoblastoid cell line LG-2 (HLA-A2,2;B27,27) was used as the source of material. Purification of membranes, papain solubilization, and purification of HLA-B27 protein was carried out as described in detail elsewhere (López de Castro, 1984). Preparations were homogeneous as judged by polyacrylamide gel electrophoresis (Laemmli, 1970) in sodium dodecyl sulfate ($NaDodSO_4$).

Separation of the HLA heavy chain and β_2m was performed by gel filtration in a column (1.6 × 100 cm) of Sephadex G-75 superfine (Pharmacia) equilibrated with 1 M acetic acid. Fractions containing the heavy chain were pooled and lyophilized.

Reduction and Alkylation. The purified heavy chain was completely reduced and ^{14}C-carboxymethylated in the presence of 6 M guanidine as described (Parham et al., 1977). The reduced and alkylated protein was desalted by gel filtration under conditions described above and lyophilized. Preparations were pure as assessed by $NaDodSO_4$ electrophoresis.

In other experiments 2 mg of HLA-B27$_{pap}$ was dialyzed against 0.2 M Tris-HCl buffer and 2 mM EDTA, pH 8.0, and incubated with 50 μCi of iodo[^{14}C]acetamide at a final concentration of 5 mM alkylating agent, without prior reduction of the protein. The reaction was carried out at room temperature for 20 min in the dark, and the heavy chain was subsequently separated from β_2m and salts by gel filtration as described above. Alternatively, 1 mg of HLA-B27$_{pap}$ was dialyzed against water, lyophilized, dissolved in 2 mL of 0.2 M Tris-HCl buffer, 2 mM EDTA, pH 8.0, and 6 M guanidine, and incubated with iodo[^{14}C]acetamide under the same conditions. The heavy chain was isolated and desalted by gel filtration.

Preparation of Chemically Derived Fragments. Cleavage of the HLA-B27 heavy chain at the acid-labile peptide bonds was carried out by mild acid hydrolysis essentially as described elsewhere (López de Castro et al., 1979). Briefly, about 5 mg of reduced and ^{14}C-carboxymethylated protein was incubated in 70% formic acid containing 0.1% 2-mercaptoethanol for 31 h at 37 °C, and the reaction mixture was fractionated by gel filtration in Sephadex G-75 superfine equilibrated with 1 M acetic acid. Fractions containing the purified fragments were pooled and lyophilized. Preparation of CNBr fragments was performed as previously described (Gross, 1967; López de Castro et al., 1983).

Tryptic Digestion. Protein samples were suspended in 2 mL of 0.05 M NH_4HCO_3, pH 8.5, and incubated with TPCK–trypsin (Worthington). In general, trypsin was added in 0.001 M HCl at an enzyme:substrate ratio of 1:100 (w/w) 3 times at 2-h intervals. Incubation was carried out at 37 °C for a total period of 6 h. In some experiments, trypsin was added at an enzyme:substrate ratio of 1:20 (w/w), followed by a second addition after 1 h at a ratio of 1:40, and incubated at 37 °C for a total period of 4 h. After digestion, samples were immediately lyophilized.

Peptide Purification. Peptides were separated by microbore ion-exchange chromatography following procedures detailed elsewhere (López de Castro et al., 1979). Reverse-phase high-performance liquid chromatography (HPLC) was also employed as an alternative method, using a Waters instrument equipped with a Waters μBondapak C_{18} column (0.39 × 30 cm) under previously described chromatographic conditions (Bragado et al., 1982). Peptide detection in the effluent fractions was carried out by a fluorescamine assay (Nakai et al., 1974). *S*-([^{14}C]Carboxymethyl)cysteine-containing peptides were identified by liquid scintillation counting in Aquasol (New England Nuclear).

Amino Acid Analysis. Peptide samples were hydrolyzed at 110 °C for 24 h in constant boiling 5.7 N HCl (Pierce) in the presence of 2-mercaptoethanol (50 μL/mL). Amino acid analyses were performed on a Beckman 121M analyzer.

Automatic Sequence Analysis. Automatic sequencing was carried out in an updated Beckman 890B or 890C sequenator in the presence of 3 mg of polybrene (Tarr et al., 1978), with 0.1 M Quadrol programs. An initial blank cycle was regularly performed in all runs by omitting the coupling step, in order to wash out impurities contained in the polybrene.

Pth Determination. Conversion of the anilinothiazolinone fractions to the phenylthiohydantoins (Pth's) and Pth analyses by HPLC were performed as described in a previous report (López de Castro et al., 1983).

Carboxypeptidase Analysis. Samples were dissolved in 0.2 mL of 0.02 M *N*-ethylmorpholine containing 5 nmol/mL norleucine and incubated at 25 °C with carboxypeptidase A (Sigma) at an enzyme:substrate ratio of 1:10 (w/w). Aliquots were removed after 30 and 60 min, lyophilized immediately, and analyzed in a Beckman 121M automatic amino acid analyzer. Results were normalized to the added internal standard of norleucine.

Segmental Variability Analysis. A computer program was devised to scan the segments of highest variability through the amino acid sequence of HLA proteins. The program was essentially identical with that described by Hopp & Woods (1981) for calculating protein hydrophilicity profiles, but it used the variability parameters of Wu & Kabat (1970), which are computed as the ratio of the number of different residues at a given position to the frequency of the most common residue at that position. This frequency is defined as the number of times the most common residue occurs divided by the total number of proteins being considered. A variability value was assigned to each position, taking into account the following available sequences: HLA-B7 (Orr et al., 1979), -B40 (López de Castro et al., 1983), -A2 (López de Castro et al., 1982), -A28 (López de Castro et al., 1982), -A3 (Strachan et al., 1984), -Cw3 (Sodoyer et al., 1984), and -B27. These numbers were repetitively averaged down the length of the polypeptide sequence in groups of six consecutive amino acids, generating a series of local average variability values. A span of six residues was chosen because this is the approximate size of an antigenic determinant (Kabet, 1968; Atassi, 1975). Segmental average variability was plotted vs. position at each third amino acid of the hexapeptide averaging groups. A reference line was also plotted at the average segmental variability value of residues 1–194, where the vast majority of polymorphic positions are located (López de Castro et al., 1984). The program was encoded in Basic and run in a HP-85 computer equipped with an automatic plotter.

Results

Purification of Chemically Derived Fragments. The strategy used for the sequence determination of the HLA-B27 heavy chain was based on previous experience with other HLA-B antigens (Orr et al., 1979; López de Castro et al., 1979, 1983) and consisted of the isolation of large fragments generated by a combination of selective hydrolysis at a labile Asp–Pro bond and of CNBr cleavage (Figure 1). The separation of fragments after partial acid hydrolysis is shown in Figure 2A. The heavy chain (peak 1) was cleaved with a yield greater than 90%. Two major fragments were generated

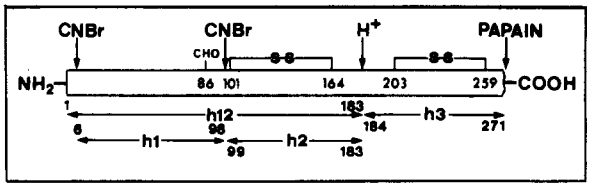

FIGURE 1: Diagram with the location of chemically derived fragments of the HLA-B27$_{pap}$ heavy chain. The alignment and dimensions of the fragments as established by sequence analysis are given. Figures are residue numbers.

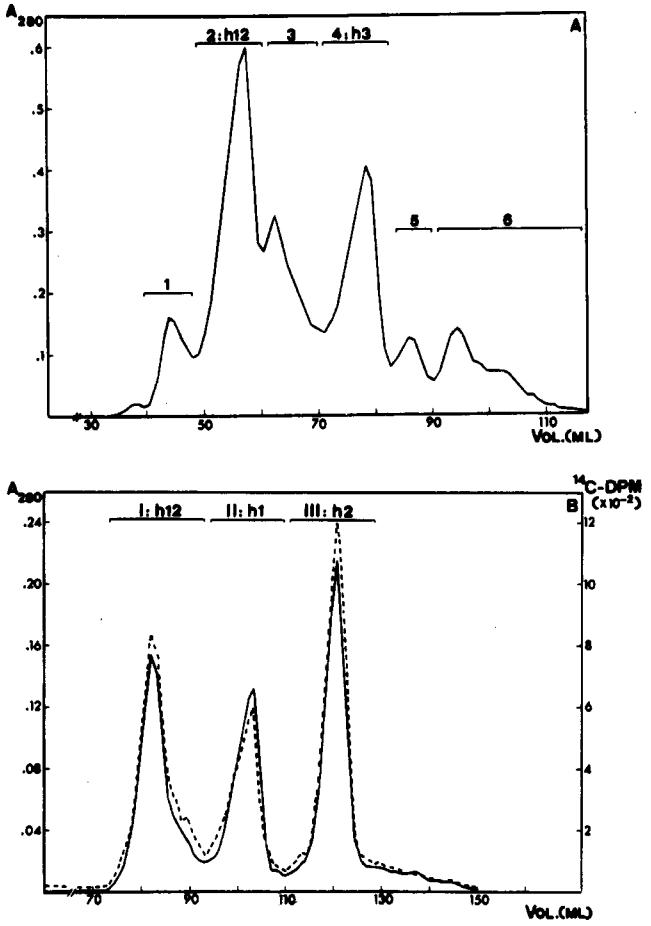

FIGURE 2: (A) Gel filtration of fragments obtained after partial acid hydrolysis of the reduced and ^{14}C-carboxymethylated HLA-B27$_{pap}$ heavy chain. (B) Gel filtration of the CNBr digestion products of the h12 fragment.

(peaks 2 and 4) that consisted of h12 and h3, respectively (Figure 1), as shown by subsequent sequence analysis. Peaks 3, 5, and 6 were the result of heterogeneous acid cleavage at a variety of peptide bonds, frequently involving aspartyl residues, as established after HPLC fractionation and sequence analysis of the peptides contained in these peaks.

Cleavage of the h12 fragment with CNBr was obtained with a yield of 65%. The digestion products were fractionated by gel filtration as shown in Figure 2B. Peak I corresponded to the undigested h12 fragment. Peaks II and III were shown by sequence analysis to consist of fragments h1 and h2 (Figure 1), respectively. Thus, the cleavage pattern of the HLA-B27 heavy chain was identical with that of HLA-B7. However, in contrast with HLA-B7 and -B40 (López de Castro et al., 1979, 1983), radiolabeling was observed in h1 (Figure 2B), suggesting the existence of one or more groups susceptible to reduction and/or ^{14}C alkylation in this fragment.

Isolation and Characterization of Peptides. Tryptic peptides of the HLA-B27$_{pap}$ heavy chain were obtained after digestion of the h1, h2, and h3 fragments and were purified by ion-exchange chromatography (Figure 3). A tryptic digest of the h12 fragment was fractionated by HPLC (not shown) to separate some of the coeluting peptides shown in Figure 3 and to obtain an overlapping peptide, T-11, for h1 and h2. A set of 34 major tryptic peptides was obtained, whose amino acid composition is presented in Table I. A few additional peptides were also detected in small amounts, mainly as a result of low-yield cleavage at positions in which Arg or Lys is flanked by acidic residues or when two consecutive Arg residues were present in the sequence. These peptides (T-9a, -9b, -13a, -14a, -17a, and -19a) were not included in Table I. The

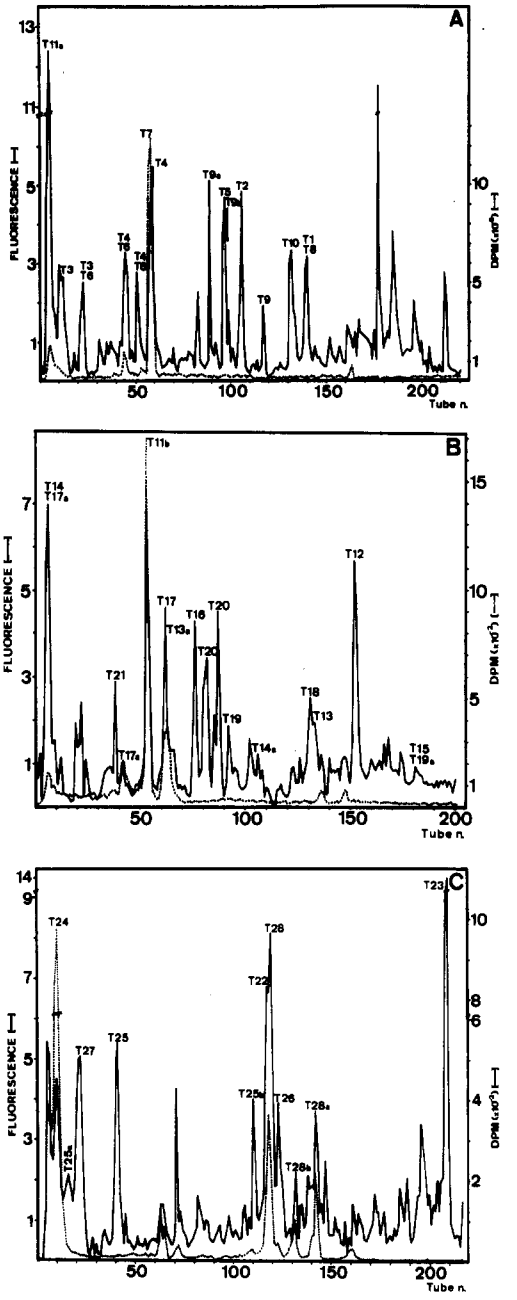

FIGURE 3: Purification of tryptic peptides by ion-exchange chromatography of tryptic digests from (A) h1, (B) h2, and (C) h3 fragments (see Materials and Methods). Peptides identified in each peak are stated. The numeration corresponds to the final alignment and is the same as that in Table I. Nonassigned peaks at the end of the h1 chromatography were shown to have no detectable peptide material by amino acid analysis of the corresponding hydrolysates. Some contamination of the h3 fragment with h12 resulted in a higher chromatographic background as well as in the excluded, nonassigned peak in (C). This peak was a mixture of several h12 peptides.

Table I: Amino Acid Composition of HLA-B27$_{pap}$ Tryptic Peptides[a]

amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	T-11	T-11a
Asp[b]			2.3 (2)	2.1 (2)		1.4 (1)			2.3 (2)		3.5 (4)	2.0 (2)
Thr			2.0 (2)			0.5 (0)	1.0 (1)		0.9 (1)	0.9 (1)	1.1 (1)	1.0 (1)
Ser				1.6 (2)							2.0 (2)	1.5 (2)
Glu[b]		0.9 (1)	0.3 (0)		2.0 (2)	3.2 (4)	2.2 (2)		2.1 (2)		4.1 (3)[f]	3.2 (3)
Pro	1.0 (1)	1.3 (1)		1.2 (1)	1.1 (1)	2.2 (2)					1.0 (1)	
Gly[c]	0.9 (1)	1.0 (1)	1.4 (1)		0.4 (0)	1.0 (1)	0.5 (0)				3.5 (4)	1.2 (1)
Ala				2.0 (2)		0.9 (1)		0.9 (1)	0.8 (1)		1.3 (1)	1.1 (1)
Cys[d]							0.8 (1)				nd (1)[e]	
Val			2.8 (3)								0.8 (1)	
Met											nd (1)[e]	nd (1)[e]
Ile			0.8 (1)			1.0 (1)	1.0 (1)					
Leu			1.0 (1)						0.9 (1)	2.0 (2)	1.5 (1)	1.4 (1)
Tyr			1.0 (1)			0.8 (1)					2.0 (3)	1.6 (2)
Phe			1.6 (2)	0.7 (1)								
Lys						0.4 (0)	0.9 (1)	1.1 (1)				
His											0.9 (1)	1.0 (1)
Trp						nd (2)[e]						
Arg	1.1 (1)	0.8 (1)	1.1 (1)	1.3 (1)	0.9 (1)	1.4 (1)			1.9 (2)	1.0 (1)	1.1 (1)	
% yield	22	95	36	48	48	17	25	36	31	48	65	83
residue no.	15–17	18–21	22–35	36–44	45–48	49–62	63–68	69–70	71–79	80–83	84–108	84–98

amino acid	T-11b	T-12	T-13	T-14	T-15	T-16	T-17	T-18	T-19	T-20	T-21
Asp[b]	2.2 (2)		1.5 (2)	4.4 (4)			0.5 (0)		1.0 (1)		1.0 (1)
Thr				2.6 (3)	0.3 (0)					1.0 (1)	
Ser				2.1 (2)		0.4 (0)					
Glu[b]	0.5 (0)		0.9 (1)	4.6 (3)[g]	1.2 (1)	2.0 (2)	2.9 (3)		1.1 (1)	2.1 (2)	
Pro	1.1 (1)			0.3 (0)	0.4 (0)						
Gly[c]	2.8 (3)		2.4 (2)	0.7 (0)	1.2 (0)	0.4 (0)	1.2 (1)		1.0 (1)	0.8 (0)	0.7 (0)
Ala			1.2 (1)	4.3 (5)	1.8 (2)	1.1 (1)	1.6 (1)				0.9 (1)
Cys[d]	0.9 (1)						0.5 (1)				
Val	1.0 (1)				0.5 (0)	0.9 (1)	0.6 (1)				
Met											
Ile				1.5 (2)							
Leu		1.8 (2)		1.8 (2)	0.4 (0)	1.0 (1)	1.7 (2)		0.9 (1)	1.0 (1)	
Tyr	0.9 (1)		1.5 (2)	0.8 (1)			1.0 (1)		0.8 (1)		
Phe					0.5 (0)						
Lys			1.2 (1)		1.0 (1)				1.0 (1)		
His			1.2 (1)								
Trp				nd (1)[e]	nd (1)[e]						
Arg	1.0 (1)	1.2 (1)		1.0 (1)	1.0 (1)	1.0 (1)	1.3 (2)	1.0 (1)		1.0 (1)	
% yield	30	36	7	30	7	37	8	25	13	30	54
residue no.	99–108	109–111	112–121	122–145	146–151	152–157	158–170	170	171–176	177–181	182–183

amino acid	T-22	T-23	T-24	T-25	T-25a	T-25b	T-26	T-27	T-28	T-28a	T-28b
Asp[b]		1.1 (1)		4.2 (4)	3.0 (3)	1.2 (1)					
Thr		2.9 (3)	2.1 (2)	2.9 (3)	3.0 (3)		0.9 (1)		1.7 (2)	1.1 (1)	1.1 (1)
Ser		1.0 (1)			0.4 (0)			1.0 (1)			
Glu[b]		1.1 (1)	2.2 (2)	4.9 (5)	5.0 (5)		1.2 (1)	3.0 (3)	2.6 (2)	2.1 (2)	2.1 (2)
Pro	1.9 (2)	0.9 (1)	1.0 (1)	1.1 (1)		0.9 (1)		0.9 (1)	1.8 (2)	1.2 (1)	2.1 (2)
Gly[c]			1.1 (1)	2.1 (2)	1.1 (1)	1.0 (1)		1.1 (1)	1.2 (1)	1.1 (1)	1.1 (1)
Ala		1.0 (1)	1.9 (2)	1.2 (1)		0.9 (1)		2.0 (2)			
Cys[d]			0.7 (1)						0.8 (1)	0.8 (1)	0.7 (1)
Val		0.9 (1)		0.9 (1)	0.5 (1)			1.6 (3)[h]	0.9 (1)	0.9 (1)	0.8 (1)
Met											
Ile		0.9 (1)	0.8 (1)								
Leu		0.9 (1)	1.8 (2)	0.9 (1)	1.0 (1)				1.8 (2)	1.0 (1)	1.8 (2)
Tyr			0.9 (1)						0.9 (1)	0.6 (1)	0.7 (1)
Phe			0.9 (1)				0.8 (1)				
Lys	1.1 (1)						1.0 (1)		1.1 (1)	0.9 (1)	0.8 (1)
His		4.3 (4)							2.0 (2)	1.8 (2)	2.5 (2)
Trp			nd (2)[e]					nd (1)[e]			
Arg		1.0 (1)	1.3 (1)	1.8 (2)	1.0 (1)	1.0 (1)		1.0 (1)		0.6 (0)	
% yield	47	33	43	13	20	27	13	40	9	8	3
residue no.	184–186	187–202	203–219	220–239	220–234	235–239	240–243	244–256	257–271	257–268	257–270

[a] Values are given in residues per mole. In parentheses are the values as determined by sequencing. [b] Aspartic and glutamic acid values include those of Asn and Gln, respectively. [c] Increased Gly values that were found occasionally are assumed to have an exogenous origin since no additional Gly was found by sequence analyses. [d] Determined as *S*-(carboxymethyl)cysteine. [e] nd, not quantitatively determined. [f] The high value of Glu was due to a low-level contamination of this peptide with T-6. [g] The high value of Glu in this peptide is due to contamination with low amounts of peptides T-11a and T-17. [h] Not corrected for incomplete hydrolysis.

isolated tryptic peptides covered the whole extent of the polypeptide chain except the 14 amino-terminal residues. Because of the presence of a Met residue at position 5, the N-terminal peptide is generated during CNBr cleavage and its purification was not pursued. Tryptic cleavage of the h1 fragment (residues 6–98) is heterogeneous at its amino terminus due to the presence of Arg at position 6. Free Arg was encountered in the shoulder of peak T-10 (Figure 3A), but the amino-terminal peptide (residues 7–14) was not found, presumably because of its very low yield. A single radioactive peptide, T-7, was found in the h1 tryptic digest (Figure 3A) that accounted for the radioactivity of the h1 fragment (Figure

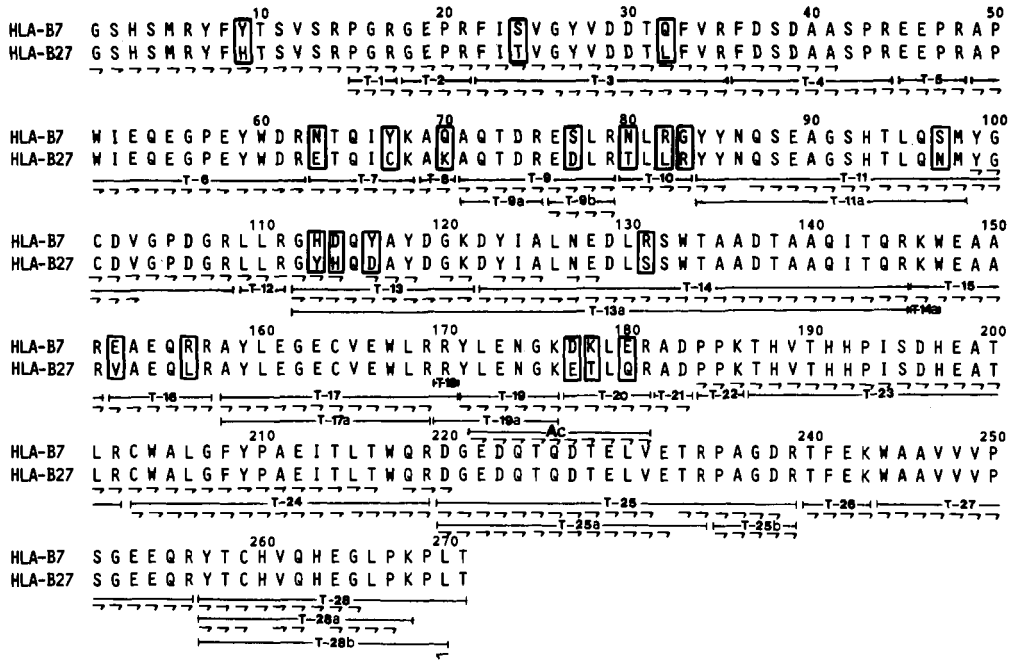

FIGURE 4: Amino acid sequence of the HLA-B27$_{pap}$ heavy chain aligned with that of HLA-B7. Identified tryptic peptides are indicated. Arrows indicate residues directly identified after Edman degradation (—) or by carboxypeptidase A analysis (⌐). The series of arrows starting at positions 1, 99, and 184 and placed directly under the residue letters correspond to the Edman degradations of the intact heavy chain and the h2 and h3 fragments, respectively. Differences between both molecules are boxed. The following one-letter code for amino acids was used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

2B). The amino acid composition of this peptide (Table I) revealed the presence of a *S*-([^{14}C]carboxymethyl)cysteine residue. Papain cleavage at both position 270 and position 271 was reflected in the generation of two carboxyl-terminal peptides, T-28 and T-28b (Table I), whose composition differed only in a Thr residue, as was previously observed in HLA-B7 and -B40 (Orr et al., 1979; López de Castro et al., 1983).

Amino-Terminal Sequence of the HLA-B27 Heavy Chain and Sequence of h1. This portion encompasses residues 1–98. Automated Edman degradation of the intact heavy chain provided the amino acid sequence of the amino-terminal 41 residues (Figure 4). Twelve tryptic peptides were also used to confirm the last part of the amino-terminal sequencer run and to complete the sequence of h1. They were aligned by homology with HLA-B7 (Figure 4). The radioactive peptide T-7 that spanned residues 63–68 contained a Cys residue at position 67. Peptide T-11 provided the carboxyl-terminal sequence of h1 and the overlap with h2. The amino acid composition of this peptide (Table I) indicated that it spans residues 84–108, but its sequence was determined only up to residue 103. The Asn residue at position 86 was not directly identified and was assigned on the basis of the following evidence: (a) T-11 and the corresponding peptide T-11a from h1 (residues 83–98) were glycopeptides as deduced from the detection of glucosamine in their amino acid analyses. (b) The amino acid composition of peptide T-11a showed two Asx residues (Table I), but only one, at position 97, was detected by sequencing. (c) A blank was obtained at position 86 in the Pth analysis of the organic phase, which is consistent with the retention of the glyco moiety in the aqueous phase during Pth extraction. (d) A glycosylated Asn residue is present in all known HLA and H-2 class I antigens. The presence of a Ser residue at position 88 provides the necessary N-linked glycosylation sequence Asn-X-Ser/Thr (Hubbard & Ivatt, 1981).

Sequence of h2 and h3. The amino acid sequence of the h2 fragment (residues 99–183) was established by a combination of automatic sequencing of the whole fragment and of eight tryptic peptides (Figure 4). Peptides T-13 and T-14 were aligned on the basis of the h2 amino-terminal run. They allowed the completion of the assignments at the end of the run and extended the sequence up to residue 145 (Figure 4). Peptides T-15–T-17 were aligned by homology with the HLA-B7 sequence. They established the sequence up to residue 169 (Figure 4). Position 170 was assigned as Arg on the basis of the characterization of T-18 as free Arg and of the detection of heterogeneous tryptic cleavage at this position, which resulted in the generation of peptides T-17, T-17a, and T-19a (Figures 3B and 4). This is the only region of h2 with an Arg-Arg sequence. In addition, the sequence of a peptide designated as Ac that was isolated after HPLC fractionation of the acid cleavage byproducts (Figure 2A, peak 6) provided an overlap for peptides T-19 and T-20 (Figure 4). This peptide confirmed the sequence at positions 177–180, where a cluster of amino acid differences with HLA-B7 (and -B40) was found. The atypical structure of T-21 allowed its assignment as the carboxyl-terminal peptide of h2.

Automated Edman degradation of the h3 fragment (residues 184–271) was carried out for 37 cycles. In addition, eight tryptic peptides were used to establish the amino acid sequence of this portion of the molecule. They were aligned by homology with HLA-B7 (Figure 4). Automatic peptide sequencing allowed direct assignments up to residue 267. The sequence of carboxyl-terminal residues 268–271 of the heavy chain was established from the following evidence (Figure 4): (a) Two carboxyl-terminal peptides, T-28 and T-28b, were found that differed in a Thr residue only (Table I). This allowed the assignment of Thr-271. (b) Carboxypeptidase A digestion of T-28b resulted in the selective release of Leu. This result established the identity of residue 270 and suggested that residue 269 should be Pro, Arg, or Lys, since these are the only residues that are not cleaved by this enzyme (Ambler, 1967). (c) The amino acid composition of peptide T-28a lacks only a Leu residue and a Pro residue when compared to that of

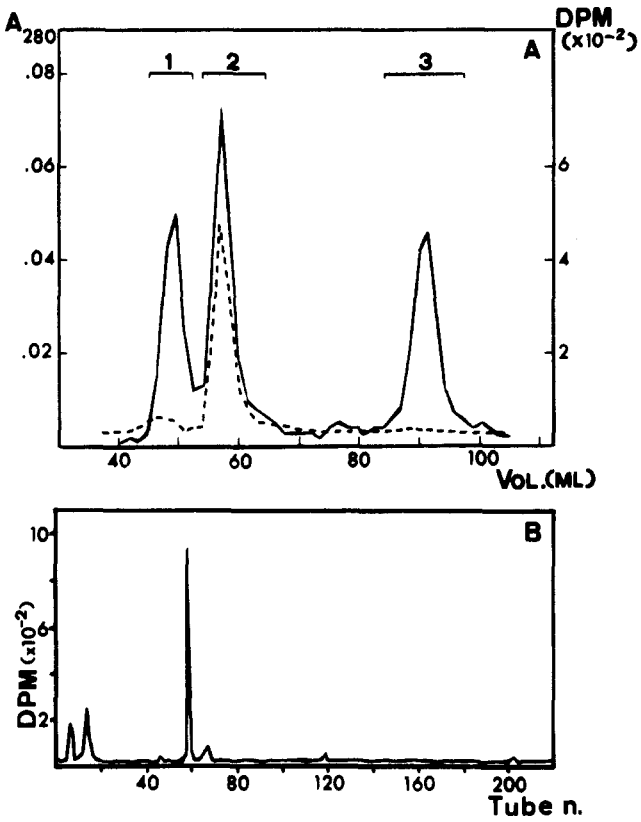

FIGURE 5: (A) Gel filtration of nonreduced, ^{14}C-carboxamidated HLA-B27$_{pap}$ in the presence of 6 M guanidine. The separation was carried out in a column (1.6 × 100 cm) of Sephadex G-75 superfine equilibrated with 1 M acetic acid. The labeled peak is the HLA-B27$_{pap}$ heavy chain (see text). (B) HPLC purification of radioactive tryptic peptides from the nonreduced, ^{14}C-alkylated heavy chain (see text).

T-28b (Table I), which indicates that immediately amino terminal to Leu-270 should be a Pro residue. Since T-28a is a Lys peptide, this feature establishes the identity of residue 268. Taken together, these data indicate that HLA-B27 is cleaved by papain in an identical fashion to that of HLA-B7 and other HLA class I specificities (Orr et al., 1979; Lôpez de Castro et al., 1982, 1983).

The proposed amino acid sequence of the papain-solubilized HLA-B27 heavy chain is presented in Figure 4 along with the peptides used for its determination. The sequence is compared to that of HLA-B7, which was used for alignment of many HLA-B27 peptides. The number of residues, the position of the single glycosylation site, and the disposition of the disulfide bonds are the same in both molecules, as well as in all other known HLA class I antigens.

HLA-B27 Has a Free Cysteine in the α1 Domain. The data shown above revealed the presence of a Cys residue at position 67 that was amenable to carboxymethylation after reduction of the heavy chain (Table I and Figure 3A). In order to determine whether or not this residue consisted of a free Cys in the chemically unmodified protein, HLA-B27$_{pap}$ was treated with iodo[^{14}C]acetamide without previous reduction in the presence of 6 M guanidine (see Materials and Methods). After alkylation, the heavy and light chains were separated by gel filtration, as shown in Figure 5A. Peak 1 was a disulfide-bonded heavy-chain dimer as established by NaDodSO$_4$ electrophoresis both in the absence and in the presence of 2-mercaptoethanol (not shown). Peak 2 corresponded to a monomeric heavy chain that was selectively labeled. Peak 3 was β_2m. The ^{14}C-alkylated heavy chain was digested with trypsin, and the resulting peptide mixture was fractionated by HPLC (Figure 5B). A major radioactive peak was obtained,

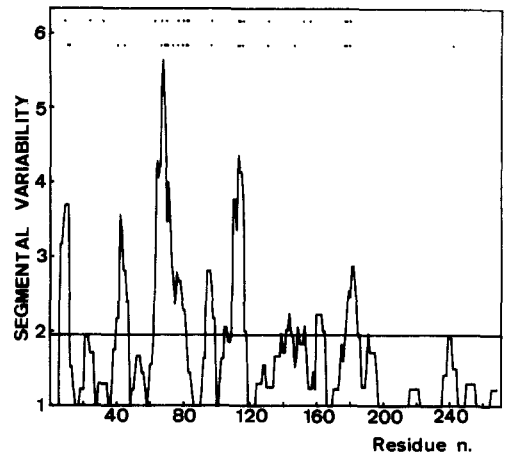

FIGURE 6: Segmental variability analysis of HLA class I antigens. The average variability of hexapeptide segments was computed and plotted vs. position as described under Materials and Methods. Dots represent amino acid sequence positions in which HLA-B27 is different from HLA-B7 (upper dotted line) or from HLA-B40 (lower dotted line).

with an elution position corresponding to that of peptide T-7 (residues 63–68). In addition, two minor labeled peaks were obtained at the beginning of the chromatography that were not identified.

^{14}C-Carboxamidation of HLA-B27$_{pap}$ was also performed under nondissociating conditions, following an otherwise analogous procedure. Selective labeling of the heavy chain was also obtained on the native protein, but the specific activity of the labeled polypeptide was about 2.5 times smaller than that obtained in the presence of 6 M guanidine. Tryptic peptide analysis of the labeled heavy chain was performed by HPLC as in the previous experiment. The result was analogous to that shown in Figure 5B: Once again a major radioactive peak was obtained at the elution position of the T-7 peptide.

Segmental Variability Analysis. HLA-B27 is the seventh human class I antigen whose primary structure has been determined. When HLA-B27 is compared with all other available sequences (Lôpez de Castro et al., 1984), there are 68 nonidentity positions, almost all of them being located in the N-terminal 194 residues. Thus, the definition of variability areas in these molecules is becoming increasingly difficult. A clearer outline of the segments of highest variability was attempted by a modification of the variability analysis of Wu & Kabat (1970), consisting of calculating the average variability of short segments along the sequence (see Materials and Methods). The procedure tends to emphasize those areas with several very close variable positions, where most proteins are likely to be different. The results are shown in Figure 6. There are three major areas of variability in the α1 domain centered around positions 10, 42, and 68. These three areas have been previously identified by direct sequence comparisons (Lôpez de Castro et al., 1984), but this analysis clearly identifies the segment centered at residue 68 as the most variable segment of the class I molecules. The second highest variable segment is located in the α2 domain, centered around residue 113. In addition, two other variable segments are centered in residues 95 and 181, respectively. Other variable areas, particularly the previously outlined one spanning residues 137–163 (Lôpez de Castro et al., 1984), are not above the average variability of the α1 and α2 domains.

This analysis improves the definition of hypervariable regions in HLA molecules and, as discussed below, may be helpful in defining putatitve HLA-B27 alloantigenic sites. The

chosen average span takes into account the approximate size of an antigenic determinant (see Materials and Methods). However, the spatial relationship among different variable positions is obviously ignored.

Discussion

Knowledge of the structure of the HLA-B27 antigen is relevant to the understanding of HLA biology for at least three reasons: (1) It constitutes only the third HLA-B locus product whose amino acid (or DNA) sequence has been determined, the other two being the cross-reactive HLA-B7 and -B40 antigens (Orr et al., 1979; Lôpez de Castro et al., 1983). A comparison of these sequences allows for localization of polymorphic areas that may contribute to the structure of the alloantigenic determinants. (2) HLA-B27 antigens are heterogeneous. Two subgroups are distinguished by an allospecific monoclonal antibody (Grumet et al., 1982), and at least three subgroups are identified by alloreactive (Bruer-Vriesendrop et al., 1984) or virus-immune, HLA-B27-restricted CTL (Toubert et al., 1984). Thus, the structure of one such antigen provides a necessary reference for future biochemical studies aimed at the characterization of functional sites, now in progress in our laboratory. (3) HLA-B27 shows a very strong association to ankylosing spondylitis and Reiter's syndrome (Woodrow, 1980). Any molecular approach seeking to explore the putative role of this antigen in determining disease susceptibility must take into account its chemical structure.

HLA-B27 differs from HLA-B7 in 20 positions through the extracellular portion, all of them being located in $\alpha 1$ and $\alpha 2$ domains (Figure 3). When compared with HLA-B40, HLA-B27 differs in 23 positions. Thus, the overall homology (92–93%) among these proteins is analogous to that previously reported for HLA-B7 and -B40 (Lôpez de Castro et al., 1983). These three antigens are included in the same cross-reacting group, but the degree of cross-reactivity between HLA-B27 and -B40 is smaller than that of HLA-B27 vs. HLA-B7 or HLA-B7 vs. HLA-B40, suggesting that it is not only the number of differences that determines serological relatedness but also their nature and/or location, as noted in a previous report (Lôpez de Castro et al., 1984).

As shown in Figure 4, most differences between HLA-B27 and -B7 are clustered in segments 63–70, 77–83, 113–116, and 177–180. In addition, there are five differences scattered through the sequence at positions 9, 24, 32, 97, and 131 and two more at positions 152 and 156. The seven differences included in segments 63–70 and 77–83 might be forming a single spatial cluster consisting of two continuous antiparallel β strands (Vega et al., 1984). These segments, which include the free cysteine residue at position 67, are located in the area of maximum local variability (Figure 6), where all known HLA class I antigens differ (Lôpez de Castro et al., 1984). Therefore, they are probably major contributors to the HLA-B27 allospecificity. The cluster of three differences within residues 113–116 is coincident with the second highest segment of local variability (Figure 6). Most known HLA class I antigens have nonidentical sequences in this segment, and hence, it is likely that these substitutions may also be involved in the HLA-B27 allospecificity. The substitution at position 97 has been suggested as being close to residues 113–116 on the basis of the predicted folding of the protein chain (Vega et al., 1984), further contributing to the polymorphism of this epitope. Whether substitutions at residues 63–83 and 113–116 form two separate determinants or are spatially related is not known. Other variable positions are less likely to be major contributors to the HLA-B27 "private" specificity. This is the case for the substitutions at positions 152 and 156 (Figure 4) that involve a strong change in local polarity and could probably result in a different antigenic determinant. However, HLA-B27 and -B40 are identical in these positions, suggesting that these residues could be important in determining a cross-reactive ("public") determinant. A similar situation occurs at residues 177–180, where HLA-B27 differs from both HLA-B7 and HLA-B40 at three positions (Figures 4 and 6). However, HLA-B27 is identical with HLA-Aw24 in and around segment 177–184 (C. Nguyen and B. R. Jordan, personal communication), where other HLA-A and -B specificities are difficult (Lôpez de Castro et al., 1984). It is interesting to consider the structural relationship among different antigens in this segment in relation to the diallelic system of the HLA-B locus supertypic specificities Bw4 and Bw6 (Ayres & Cresswell, 1976). HLA-B27 is included in the Bw4 subgroup whereas both HLA-B7 and HLA-B40 are in Bw6 (Schreuder et al., 1980). Most HLA-A specificities do not cross-react with these supertypic antigens, the only three exceptions being HLA-Aw23, -Aw24, and -Aw32, which are associated to Bw4 (Kostyu et al., 1980; Müller et al., 1982). Thus, it is tempting to speculate that segment 177–184 could be related to the Bw4 determinant. Clearly, more structural information is needed to confirm this point.

The putative contribution of polymorphic residues 9, 24, and 32 to the HLA-B27-specific determinants is difficult to assess. Position 9 is included in a short variability cluster spanning residues 9–12, where most known class I antigens differ (Lôpez de Castro et al., 1984). The other two positions are included in segment 13–40 in which HLA-B27 and -B40 are identical. However, at least positions 9 and 24 may be spatially related by the folding of the polypeptide chain (Vega et al., 1984). Thus, the possibility remains that they may participate in a single polymorphic epitope.

Further structural definition of the HLA-B27 serological determinants is to be provided by the comparative biochemical analysis of HLA-B27 antigens distinguishable by a B27-specific monoclonal antibody, M2 (Grumet et al., 1982). This antibody recognizes a majority of B27-positive cells, including the LG-2 line, the source for the antigen whose sequence is presented in this paper (E. Gomard, personal communication). Data from our laboratory indicate that two different HLA-B27 antigens which do not react with the M2 antibody differ from the M2+ sequence in at least one substitution within positions 76–79 (M. S. Vega and J. A. Lôpez de Castro, unpublished results), suggesting that residues in or close to this segment may contribute to the HLA-B27 allospecificity. In addition, at least three subgroups are distinguished by alloreactive (Breur-Vriesendorp et al., 1985) or virus-immune, HLA-B27-restricted CTL (Toubert et al., 1984). They have been designated as B27W, -K, and -C and B27.1, -.2, and -.3, respectively. B27.1, to which the present protein sequence belongs (E. Gomard, personal communication), appears to be the most common subgroup in Caucasians. It is probably equivalent to the B27W subgroup defined by alloreactive CTL that may also be identified by some polyclonal alloantisera (De Waal et al., 1984). B27.2 and -.3 may be related to the B27K and -C subgroups, respectively, but the precise relationship is less clear. B27.2 is included in the serological M2-negative subset. B27.3 (and B27C), a subtype that may be prevalent in orientals, is not clearly correlated with M2 reactivity. Interestingly, it has been suggested that HLA-B27 behaves as a dominant restriction element over other class I antigens, including HLA-B7, in CTL responses against influenza and Epstein–Barr viral antigens. At least HLA-B27.1 and -B27.2 subgroups function with similar high efficiency of restriction

in these systems (Gomard et al., 1984). Thus, the data presented in this report, together with ongoing structural studies on antigens from the other subgroups, will enable us to achieve a progressive definition of HLA-B27 functional sites and to clarify the relationship between serologically and CTL-defined allospecific determinants.

The relationship between the molecular properties of HLA-B27 and its association to ankylosing spondylitis and Reiter's syndrome remains obscure. Two basic mechanisms have been proposed whose critical assessment is essential in evaluating the putative relevance of the HLA-B27 antigen structure to the pathogenesis of these arthropathies: (1) HLA-B27 itself is just a fortuitous marker for a susceptibility gene segregating in strong linkage disequilibrium. (2) HLA-B27 plays a functional role in disease pathogenesis, either in a direct manner or in conjunction with some additional, perhaps environmental, factor.

Against the involvement of a separate susceptibility gene is the fact that HLA-B27 shows a strict correlation to ankylosing spondylitis irrespective of race (Woodrow, 1980) and haplotype (Truog et al., 1975). Thus, it would be necessary to postulate a remarkable uniformity in the degree of linkage disequilibrium of the hypothetical second gene with HLA-B27 in different ethnic groups.

A number of immunological studies suggest that the HLA-B27 antigen may play a direct role in the pathogenesis of B27-associated arthropathies. These studies are based on the observation that certain enterobacteria, such as *Klebsiella pneumoniae*, are found more frequently in patients with ankylosing spondylitis (Ebringer et al., 1978). Furthermore, the development of Reiter's syndrome and Reiter's-like reactive arthritis may follow infection by *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Shigella flexneri* (Aho et al., 1974; Friis & Svejgaard, 1974; Simon et al., 1981). Geczy et al. (1983) have reported that anti-*Klebsiella* antibodies may react with B27 lymphocytes from patients with ankylosing spondylitis but not with B27 cells from healthy individuals. They suggest that an exogenous factor(s) of bacterial origin may be associated to the B27-linked antigenic modification. The persistence of such cross-reactions in cultured cells from B27-positive patients suggests that a genetically transmissible element might be involved.

A comparison between HLA-B27 antigens from healthy individuals and patients with ankylosing spondylitis failed to demonstrate any differences by isoelectric focusing and peptide mapping (Karr et al., 1982). The chemical structure of HLA-B27 presented in this paper shows no obvious anomaly as compared with other HLA class I antigens, the only unusual feature being the existence of a free, apparently exposed, cysteine residue at position 67, within the most conspicuous hypervariable region of the molecule. Such residue may endow the molecule with a potential for establishing intermolecular disulfide bonds with other surface components under appropriate conditions. It should be noted, however, that similar cysteine residues are present in variable areas of some other class I antigens, such as Cys-9 in HLA-Cw3 (Sodoyer et al., 1984) and Cys-121 in H-2K^b (Coligan et al., 1981) and that no intermolecular complexes have been detected during isolation of HLA-B27 from several lymphoblastoid cell lines (M. A. Vega, unpublished results). Further insight into the putative connection between HLA-B27-specific determinants and the development of disease will require supplementing the present data with the biochemical characterization of other HLA-B27 subgroups, as well as evaluating the possible differential linkage of these subgroups to ankylosing spondylitis. So far, no preferential association has been found between this disease and any particular CTL subtype (Breur-Vriesendrop et al., 1984), suggesting that at least some naturally occurring structural variation of HLA-B27 that affects CTL recognition might have no effect on disease susceptibility.

No information is presently available on the structure of the carbohydrate moiety of HLA-B27 or on the existence of putative differences in the carbohydrate of HLA-B27 from healthy individuals and those with ankylosing spondylitis. Such differences could conceivably change the antigenicity of HLA-B27 in a way not easily distinguishable by allospecific sera, since these are mostly directed against polypeptide structures (Ploegh et al., 1981). Still, these differences could have an effect on the degree of cross-reactivity of HLA-B27 with cell surface bacterial antigens.

Further studies should address the possibility of biochemical modification of cell surfaces from HLA-B27 patients with ankylosing spondylitis, involving either HLA-B27 itself or additional membrane components. These studies would substantiate the above-mentioned findings concerning the antigenic relationship between such cell surfaces and bacterial products. Finally, it is expected that the data presented in this paper may help in the identification and cloning of the HLA-B27 gene, a necessary step for further studies aimed at probing the putative existence of closely linked genetic elements responsible for disease susceptibility.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Bill Lane, Protein Chemistry Laboratory, Harvard University. We thank our colleagues Bertrand Jordan, Elisabeth Gomard, Jean-Jacques Metzguer, Gijs van Seventer, and Pavol Ivanyi for making available to us their data before publication and Gabriel Herrero and Fernando Ortiz for comments on the manuscript. We give special thanks to Dr. E. Gomard for kindly performing cellular and serological typing of LG-2 cells and to Dr. F. Ortiz for sustained support.

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