Fragmentation of the Human Transplantation Antigen Heavy Chain by Limited Proteolysis, Acid Cleavage, and Cyanogen Bromide Treatment†

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ABSTRACT: Highly purified, papain-solubilized HLA-A, -B, and -C antigens comprising a mixture of a great number of allelic forms from at least three loci have been fragmented by limited proteolysis, acid cleavage, and cyanogen bromide treatment. Limited proteolysis of ^{125}I-labeled HLA-A, -B, and -C antigens with trypsin, chymotrypsin, thermolysin, and pepsin resulted in the production of two large fragments. One fragment was associated with β_2-microglobulin and contained all of the carbohydrate. The other fragment, which had a molecular weight of about 13 000, is most probably derived from the COOH-terminal part of the heavy chain. Acid cleavage of the HLA antigen heavy chain gave rise to two main fragments with molecular weights of 22 000 and 11 000. Both fragments contained disulfide bonds. Two minor components, representing further cleavage products of the 22 000-dalton fragment, were also observed. Cleavage of the HLA antigen heavy chain at methionyl residues gave rise to one carbohydrate-containing, cysteine-free 14 000-dalton fragment and one 20 000-dalton fragment that contained all cysteines but no carbohydrate. NH_2-terminal amino acid sequence analyses demonstrated that the 22 000-dalton acid cleavage fragment and the 14 000-dalton cyanogen bromide fragment were derived from the NH_2-terminal part of the HLA antigen heavy chain.

The HLA-A, -B, and -C antigens are cell surface glycoproteins displaying extensive genetic polymorphism (see Thorsby, 1974). They are all composed of one invariant subunit, β_2-microglobulin, and one alloantigenic chain (Rask et al., 1976; Snary et al., 1977). The alloantigenic chain traverses through the membrane and has its COOH terminus on the cytoplasmic side of the cell membrane (Walsh & Crumpton, 1977; Springer & Strominger, 1976; Peterson et al., 1976; Robb et al., 1978). Due to the hydrophobic properties of the membrane-integrated portion of the HLA[1] antigens, the intact glycoproteins can only be dealt with in detergent solutions (see Helenius & Simmons, 1975). However, limited papain digestion of the cell membrane releases most of the HLA antigen molecule in a water-soluble form (see Mann et al., 1969). Extensive work in several laboratories have shown that the proteolytically derived HLA antigens are well suited for detailed structural investigations (see Möller, 1975). Most laboratories have studied HLA antigens from in vitro grown lymphoblastoid cell lines, which preferably have been homozygous in the MHC[2] region (cf. Turner et al., 1975). As such material can only be obtained at high costs, we have explored the possibility of using HLA antigens isolated from cadaveric spleens and peripheral blood lymphocytes.[3] Papain-solubilized HLA antigens derived from these sources are homogeneous in size, but as they comprise a great number of allelic forms from at least three loci they do not display a single amino acid sequence. Although variations in amino acid sequence between different allelic forms of the molecules render structural work difficult, the heterogeneous material is excellently suited to answer the question whether the allelic amino acid differences are localized to certain regions of the HLA antigen molecules or whether such differences are dispersed throughout the entire sequence. To answer this question, ways have to be found to reproducibly cleave the papain-solubilized HLA antigens into large fragments which subsequently can be dealt with individually. Preliminary studies on HLA antigens of defined specificities have shown that limited proteolysis (Peterson et al., 1975), acid cleavage, and CNBr fragmentation (Terhorst et al., 1977) may be useful procedures to obtain large fragments. This communication presents detailed data demonstrating that limited proteolysis, acid cleavage, and CNBr treatment produce fragments of the HLA antigen heavy chain, comprising a great number of allelic forms that are well suited for detailed structural analyses. The cleavage points were identified and the ordering of the isolated fragments has been accomplished.

Materials and Methods

Proteins and Enzymes. β_2-Microglobulin was isolated as described (Berggård & Bearn, 1968). Papain-solubilized HLA antigens were purified from cadaveric spleens and from large quantities of peripheral blood lymphocytes. The isolation procedure was identical with that outlined elsewhere.[3] All proteolytic enzymes were obtained from Worthington (Freehold, NJ). Light immunoglobulin chains were prepared from pooled IgG (Kabi AB, Stockholm) according to standard procedures (Fleischman et al., 1962). *Lens culinaris* hemagglutinin was isolated as described by Hayman & Crumpton (1972).

Special Materials. Sephadex G-200, G-100, and G-25 and Sepharose-6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were prepared according to the instructions supplied. Guanidine hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO) and treated with activated charcoal prior to use. [^{14}C]Iodoacetic acid (57 mCi/mmol) was the product of Amersham (Buckinghamshire, England).

Concentration of Proteins. Concentration of proteins was accomplished by ultrafiltration (Berggård, 1961) or by lyophilization after exhaustive dialysis against distilled water.

Affinity Chromatography. Affinity chromatography columns, containing covalently bound *Lens culinaris* hemagglutinin or antibodies against β_2-microglobulin (Peterson

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[1] For brevity, HLA-A, -B, and -C antigens are called HLA antigens in this article.

[2] Abbreviations used: MHC, major histocompatibility complex; PAS, periodic acid–Schiff reagent; $NaDodSO_4$, sodium dodecyl sulfate.

[3] L. Trägårdh, B. Curman, K. Wiman, L. Rask, & P. A. Peterson, unpublished results.

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et al., 1969), were prepared according to Cuatrecasas (1970). The columns were equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Elution of bound material was accomplished by including 10% α-methyl mannoside (Sigma) in the equilibrating buffer when lectin columns were used and by lowering the pH to 3.0 by elution with 0.05 M sodium citrate buffer containing 0.5 M NaCl when the immunosorbent columns were employed.

Radioactive Labeling. HLA antigens and fragments of the HLA antigen heavy chains were labeled with ^{125}I or ^{131}I by a slight modification of the Chloramine-T procedure of Hunter & Greenwood (1962). Protein, about 1 mg/mL, was alkylated with [^{14}C]iodoacetic acid after extensive reduction with 0.01 M dithiothreitol in 1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride and 10 mM EDTA. The reduction proceeded for 30 min at room temperature. Usually about 50 μCi of the [^{14}C]iodoacetic acid was then added. After 15 min in the dark, 0.025 M (final concentration) unlabeled iodoacetic acid was added and the alkylation reaction was terminated after another 30 min by dialysis against distilled water. The dialyzed protein was subsequently lyophilized.

Limited Proteolytic Digestion. ^{125}I-labeled HLA antigens were mixed with 2 mg of IgG light chains in 0.2 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Trypsin, chymotrypsin, and thermolysin were separately added at protein to enzyme ratios ranging from 1000:1 to 25:1. After 30 min at 37 °C the results of the enzymic digestions were evaluated by gel chromatography. The greatest yields of large HLA antigen fragments were obtained with protein to enzyme ratios of 100:1 for trypsin and 200:1 for chymotrypsin and thermolysin. Papain digestions were carried out in 0.02 M Tris-HCl buffer, pH 7.5, containing 5 mM cysteine, and pepsin digestions were performed in 0.05 M sodium acetate buffer, pH 4.5. The range of protein to enzyme ratios tested was the same as for the other enzymes. While papain did not cleave the HLA antigens, pepsin generated large fragments with the greatest yield at a protein to enzyme ratio of 200:1.

Acid Cleavage and CNBr Treatment. HLA antigen heavy chains, usually 2 mg/mL, were incubated in 70% formic acid at 37 °C for various periods of time. After 24 h of incubation, the recovery of large fragments was greatest. CNBr, twice the amount of protein (w/w), was added to the protein dissolved in 70% formic acid. Cleavage was allowed to proceed for 12 h at 15 °C in the dark. These conditions did not allow the HLA antigen heavy chains to be quantitatively cleaved but minimized unwanted cleavage at aspartyl–prolyl bonds. The acid and CNBr treatments were terminated by lyophilization.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of reduced proteins, if not stated otherwise, was carried out as described by Laemmli (1970). Gels containing radioactively labeled proteins were sectioned into 2-mm wide segments in a Gilson automatic gel slicer. Gels containing chemical amounts of protein were stained either with Coomassie Brilliant Blue or by PAS treatment (Neville & Glossman, 1974). Destained gels were evaluated for absorbance in a Gilford linear transport gel scanner.

Isolation of HLA Antigen Heavy Chain Fragments for Amino Acid Sequence Determination. Papain-solubilized HLA antigens and acid- and CNBr-treated HLA antigen heavy chains, trace labeled with ^{125}I, were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis after extensive reduction and alkylation with [^{14}C]iodoacetic acid. After completed electrophoresis, the protein and protein fragments were localized by their content of ^{125}I radioactivity. The materials to be subjected to amino acid sequence analyses were eluted from the sectioned gel with 0.01% $NaDodSO_4$ in water. The eluted proteins were passed over a Sephadex G-25 column equilibrated with 0.1% NH_4OH (v/v) and 10% propanol (v/v) in water. Material eluted in the void fraction was lyophilized.

Amino Acid Sequence Determination. NH_2-terminal amino acid sequence determinations were carried out with the automatic solid phase method (Laursen, 1971). The lyophilized protein to be sequenced was mixed with 5% (v/v) of triethylamine in water. The triethylamine was subsequently removed by lyophilization. This procedure was repeated once. The protein was dissolved in 0.2 mL of 2% $NaDodSO_4$ in water, heated at 90 °C for 5 min, and mixed with 0.2 mL of 0.2 M $NaHCO_3$, pH 9.2. One hundred milligrams of the diisothiocyanate derivative of *N*-(2-aminoethyl)-3-aminopropyl glass, prepared according to Bridgen (1976), was added to the protein solution. The mixture was gently stirred for 45 min at 45 °C under an atmosphere of nitrogen. Ethanolamine (100 μL) and 300 μL of 1% $NaDodSO_4$ in 0.2 M $NaHCO_3$, pH 9.2, were added to block excess DITC groups, and the stirring was continued for another 45 min at 45 °C under an atmosphere of nitrogen. The glass beads were washed in sequence with 1% $NaDodSO_4$ in water, distilled water, and methanol. The washed beads were dried in a desiccator under vacuum. Approximately 5 mg of the glass beads was hydrolyzed in 6 N HCl at 110 °C in vacuo for 20 h. Liberated amino acids were analyzed on a Beckman 121 M amino acid analyzer to quantitate the amount of protein bound to the beads. The coupling efficiency was usually 70–90%.

Amino acid sequence determination was carried out in an Anachem (Luton, England) APS 2400 peptide sequencer with a double-coupling, double-cleavage program. The pH of the coupling buffer was raised to 9.2 to minimize histidine previews. The released anilinothiozolinones were taken to dryness and converted to phenylthiohydantoin derivatives by the addition of 200 μL of 1 NCl containing 0.1% ethanethiol and by incubating at 80 °C for 10 min. The phenylthiohydantoin derivatives were identified after extraction with 2 × 700 μL of ethyl acetate by high pressure liquid chromatography on a Waters instrument equipped with a Spectrophysics SP 4000 integrator. The reversed phase C18 column was eluted with a 14-mL linear gradient of acetonitrile from 18% to 45% in 0.01 M sodium acetate buffer, pH 5.1, followed by 18 mL of the 45% acetonitrile solution. This procedure separated all derivatives except valine-methionine and isoleucine-phenylalanine. These residues were identified by amino acid analysis after back hydrolysis (Mendez & Lai, 1975).

Other Methods. Indirect immunoprecipitations were carried out as described (Österg et al., 1976). Protein was determined by the modified Folin procedure (Lowry et al., 1951).

Results

Limited Proteolysis of Papain-Solubilized HLA Antigens. Papain-solubilized HLA antigens, labeled with ^{125}I and mixed with unlabeled human immunoglobulin light chains, were separately digested with various amounts of chymotrypsin, thermolysin, papain, trypsin, and pepsin. At protein to enzyme ratios of 1000:1 and 500:1, very little digestion was observed during 30 min at 37 °C. However, at protein to enzyme ratios of 100:1 to 200:1, a significant portion of the HLA antigens were digested by most enzymes to yield a few large fragments (see below). At higher ratios, the enzymatic digestions were extensive and substantial amounts of low molecular weight peptides were generated. The optimal conditions for the digestions, yielding maximal amounts of large fragments, were

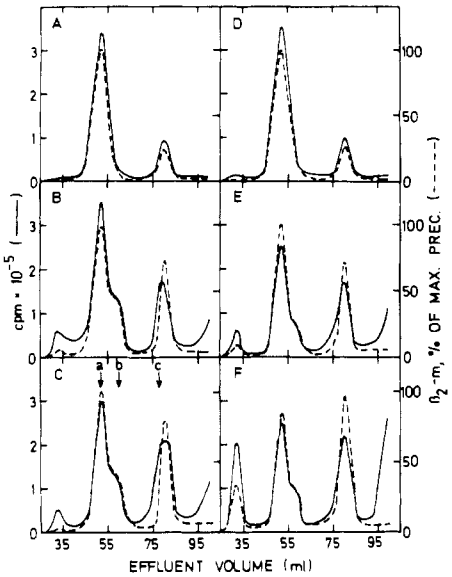

FIGURE 1: Gel chromatography of ^{125}I-labeled HLA antigens subjected to limited proteolytic digestion with chymotrypsin (B), thermolysin (C), papain (D), trypsin (E), and pepsin (F). As a control, undigested HLA antigens were also chromatographed (A). The digestions were carried out as described under G-100 and Methods. The columns (100 × 1 cm) of Sephadex G-100 were equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Fractions of 1 mL were collected at 12-min intervals. Total radioactivity (—) and the occurrence of β-microglobulin (- - -) were determined in each fraction. β_2-Microglobulin was estimated on aliquots subjected to indirect immunoprecipitation. The arrows denoting a, b and c refer to fractions containing material used in subsequent analyses.

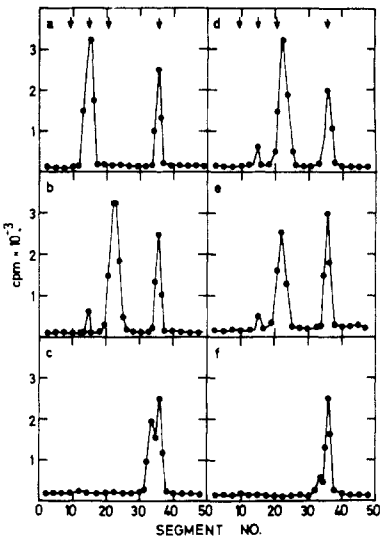

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{125}I-labeled HLA antigen fragments obtained after limited digestion with thermolysin and separation by gel chromatography (cf. Figure 1C). Material in the fractions denoted with the arrows in Figure 1C was directly subjected to electrophoresis (a–c). Glycoproteins in fraction b of Figure 1C that were retained by a *Lens culinaris* hemagglutinin column were desorbed and analyzed by electrophoresis (d). Material from fractions b and c of Figure 1C which bound to an anti-β_2-microglobulin antibody column was also electrophoresed (e and f). The arrows from left to right denote the migration positions of ^{131}I-labeled marker IgG heavy chains, HLA antigen heavy chains, IgG light chains, and β_2-microglobulin.

determined for each enzyme. Labeled HLA antigens digested under such conditions were subjected to gel chromatography. Figure 1A demonstrates that prior to the digestions the labeled HLA antigens emerged from the column as one dominating peak, representing intact antigens, and one small peak comprising free β_2-microglobulin, which obviously had been released from the HLA antigen heavy chain as a result of the labeling procedure. Chymotrypsin, thermolysin, trypsin, and pepsin-digested HLA antigens gave rise to very similar chromatograms (Figure 1B,C,E,F). Apart from material occurring in the void fraction, which represented minor amounts of radioactivity except for the pepsin digest, three main radioactive peaks in addition to the small peptides eluted in the total volume of the column were observed. β_2-Microglobulin occurred coincident with the two first eluted peaks. However, free β_2-microglobulin was generally eluted somewhat later than the radioactivity in the last eluted peak (cf. Figure 1C). Papain did not seem to digest the HLA antigens (Figure 1D), although this enzyme was as efficient as any of the others to cleave the immunoglobulin light chains into variable and constant domains (not shown).

To analyze the molecular weights of HLA antigen fragments generated by the limited proteolysis, material from each gel chromatography peak of the various enzymic digests was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The results were very similar for all digests and are therefore depicted only for the thermolysin-cleaved HLA antigens. Figure 2a shows that the gel chromatography peak denoted "a" in Figure 1C comprised undigested HLA antigens as revealed by the occurrence of 33 000- and 12 000-dalton polypeptide chains. Fraction b in Figure 1C consisted of molecules with apparent molecular weights of about 20 000 and 12 000 (Figure 2b) and fraction c in Figure 1C contained two polypeptide chains with very similar molecular weights of about 12 000 (Figure 2c).

Table I: Carbohydrate and β_2-Microglobulin Content of ^{125}I-Labeled HLA Antigen Fragments[a]

	material bound to			
	L. culinaris hemagglutinin		anti-β_2-microglobulin	
fraction	cpm	%	cpm	%
fraction b	9600	82	6400	55
fraction c	<100	<2	1900	38

[a] Materials in fraction b (11 7000 cpm) and c (5000 cpm) of Figure 1C were separately subjected to affinity chromatography on *Lens culinaris* hemagglutinin and anti-β_2-microglobulin antibody columns. Bound material was desorbed with α-methyl mannoside and a low pH (3.0), respectively. Desorbed radioactivity was measured.

The distribution of carbohydrate on the enzymatically produced fragments was examined by *Lens culinaris* hemagglutinin affinity chromatography. Material corresponding to fractions b and c in Figure 1C was separately applied to the column. It can be seen in Table I that about 80% of the labeled material in fraction b bound to the column and was desorbed by elution with α-methyl mannoside. No material in fraction c bound to the lectin column. The bound and desorbed material derived from fraction b in Figure 1C was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Figure 2d shows that the carbohydrate-containing material was representative for all the material in fraction b (cf. Figures 2b and 2d). This result is consistent with the existence of a single carbohydrate moiety for the HLA antigens (Parham et al., 1977).

The association between β_2-microglobulin and the enzymatically produced fragments of the HLA antigen heavy chain was examined by subjecting material corresponding to fractions b and c in Figure 1C to immunosorbent chromatography on anti-β_2-microglobulin antibody columns. Table I demonstrates that about 55% of the radioactive material in fraction b and

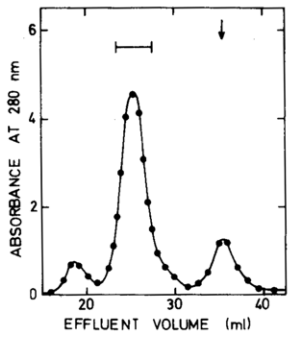

FIGURE 3: Gel chromatography of papain-solubilized HLA antigens on a column (1.2 × 40 cm) of Sepharose 6B equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 6 M guanidine hydrochloride. Fractions of 0.6 mL were collected at 20-min intervals. The bar denotes fractions containing the HLA antigen heavy chain that were pooled, dialyzed, and lyophilized. The arrow shows the elution position for ^{131}I-labeled β_2-microglobulin which was used to calibrate the column.

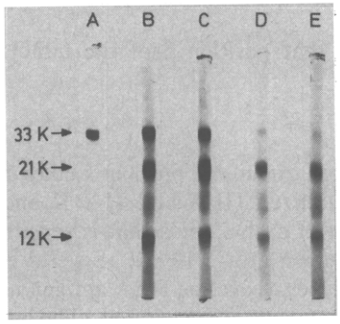

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified HLA antigen heavy chain before (A) and after treatment with 70% formic acid at 37 °C for 15 (B), 24 (C), 38 (D), and 48 (E) h. The gels which were run under reducing conditions were stained with Coomassie Brilliant Blue.

about 38% of the radioactive material in fraction c were bound by the antibodies. The desorbed protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Figure 2e shows that in addition to β_2-microglobulin the 20 000-dalton fragment from fraction b had been retained by the column. Only β_2-microglobulin but not the other polypeptide chain in fraction c had been bound by the antibodies (Figure 2f). These data suggest that β_2-microglobulin may be associated with the 20 000-dalton fragment of the HLA antigen heavy chain.

Acid Cleavage of the Antigen Heavy Chain. The HLA antigen heavy chain was separated from β_2-microglobulin by gel chromatography under denaturing conditions. Figure 3 demonstrates that the two chains were well resolved. The heavy chain was pooled, as indicated in the figure, exhaustively dialyzed against distilled water, and lyophilized.

The lyophilized HLA antigen heavy chain fraction comprised a single molecular species, as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 4A). Acid cleavage of the heavy chain was attempted by incubating it with 70% formic acid at 37 °C for various periods of time. Figure 4 (B–E) shows that the acid cleaved primarily a few peptide bonds which resulted in the generation of some large fragments. The main fragments had apparent molecular weights of about 22 000 and 11 000, respectively, and quantitatively less pronounced species occupied the molecular weight positions 18 000 and 16 000. The yield of the fragments appeared to be optimal after about 24 h of incubation (Figure 4C). The fragment patterns were very similar on sodium dodecyl sulfate–polyacrylamide gel electrophoresis regardless

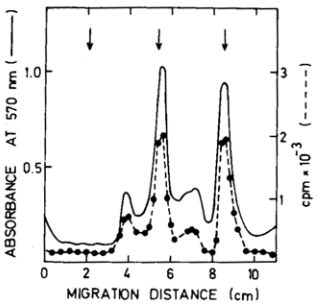

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ^{14}C-carboxymethylated, acid-cleaved HLA antigen heavy chains. The picture is composed of data from two gels run in parallel. One gel was stained with Coomassie Brilliant Blue, destained, and scanned at 570 nm. The other gel was segmented into 2-mm wide slices which separately were subjected to radioactivity measurements. The arrows, from left to right, denote the migration positions of marker IgG heavy and light chains and β_2-microglobulin.

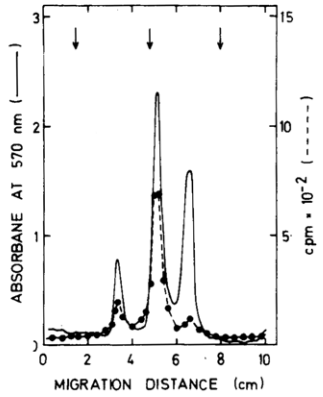

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ^{14}C-carboxymethylated, CNBr-cleaved HLA antigen heavy chains. The figure is composed of data from two gels run in parallel as described in the legend of Figure 5. The arrows have the same meaning as in Figure 5.

of whether the analyzed material had been reduced prior to the electrophoresis or not. However, in the absence of reduction the 18 000- and 16 000-dalton fragments could not be visualized. This observation suggests that the cleavage point giving rise to the 22 000- and 11 000-dalton fragments does not reside inside a disulfide bridge.

After acid cleavage, the HLA antigen heavy chain was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. PAS staining of the gel revealed carbohydrate-containing material in positions corresponding to approximate molecular weights of 33 000, 22 000, 18 000, and 16 000. No stained material was visualized in the 11 000-dalton position, suggesting that the 11 000-dalton acid cleavage fragment is devoid of carbohydrate.

The acid-cleaved HLA antigen heavy chain was reduced and alkylated with [^{14}C]iodoacetic acid. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed radioactive components with molecular weights of 33 000, 22 000, 18 000, 16 000, and 11 000, as can be seen in Figure 5. As none of the components were alkylated by the radioactive compound in the absence of reduction (not shown), it seems reasonable to conclude that all acid-cleavage fragments contained disulfide bridges.

Cyanogen Bromide Cleavage of the HLA Antigen Heavy Chain. The HLA antigen heavy chain was subjected to cleavage with CNBr in 70% formic acid. To minimize acid cleavage, the reaction was allowed to proceed for only 12 h and at 15 °C rather than at room temperature. After lyophilization, the CNBr-treated HLA antigen heavy chain was

Table II: Amino Acid Sequence in the NH_2 Terminus of the Intact Papain-Solubilized HLA Antigen Heavy Chain and of Its Acid Cleavage and CNBr Fragments

material[a]	amino acid residue found in position									
	1[b]	2	3	4	5	6	7	8	9	10
33 K		Ser	His	Ser	Met	Arg	Tyr	Phe	Tyr Phe	Thr
22 K (acid)		Ser	His	Ser	Met	Arg	Tyr	Phe	Tyr Phe	Thr
18 K (acid)		Ser	His	Ser	Met	Arg	Tyr	Phe		
16 K (acid		Ser	His	Ser	Met	Arg	Tyr	Phe	Tyr Phe	
11 K (acid)		Pro	*b*	Thr	His	Val	Thr	His	His	Pro
20 K (CNBr)		Leu Gly	CM-Cys	Asp	Val	Gly	Pro Ser	Asp	Gly	
14 K (CNBr)		Tyr	Phe	Tyr Phe	Thr	Ala	Val	Ser	Arg	Pro

[a] The intact HLA-antigen chain, 0.8 mg, and the acid-cleaved (0.3–1.1 mg) and CNBr (0.4 and 0.6 mg) fragments, isolated as described under Materials and Methods, were subjected to automatic degradation in a solid phase sequencer. The liberated and converted phenylthiohydantoin derivatives were identified by high pressure liquid chromatography. CM-cysteine was also identified by its content of ^{14}C. The repetitive yield in these analyses averaged 92%. [b] Due to the coupling procedure employed, amino acid residues containing α-NH_2 and ϵ-NH_2 groups were not released from the glass beads.

subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Figure 6 demonstrates that two main fragments had arisen. The same two fragments were also evident when the electrophoresis was carried out with material that had not been reduced. In the latter case, the two fragments were not as well resolved, as shown in Figure 6. Reduction and alkylation with [^{14}C]iodoacetic acid resulted in the incorporation of radioactivity into the 20 000-dalton CNBr fragment, whereas the lack of radioactivity in the 14 000-dalton component strongly suggests that this fragment is devoid of cysteine (Figure 6). Staining with PAS of the CNBr fragments after electrophoretic separation in sodium dodecyl sulfate revealed that the 33 000- and 14 000-dalton components contained carbohydrate, whereas the absence of stained material in the 20 000-dalton position of the gel indicated that the larger of the two CNBr fragments lacked carbohydrate.

NH_2-Terminal Amino Acid Sequence of the Intact HLA Antigen Heavy Chain, Acid Cleavage, and CNBr Fragment. The intact HLA antigen heavy chain and the various fragments obtained from the heavy chain after acid cleavage and CNBr treatment, respectively, were subjected to NH_2-terminal amino acid sequence analyses in a solid-phase sequencer. The fragments, trace-labeled with ^{125}I, were isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The amino acid sequence results are summarized in Table II. Due to scarcity of material, only seven to nine amino acid residues in the NH_2 terminus could be positively identified in each case. It can be seen in Table II that the 22 000-, 18 000-, and 16 000-dalton acid cleavage fragments displayed amino acid sequences that were identical with that of the intact HLA antigen heavy chain. The NH_2-terminal amino acid sequences obtained for the 20 000-dalton CNBr fragment and for the 11 000-dalton acid cleavage component were distinctly different and did not correspond to the NH_2-terminal sequence of the intact HLA antigen heavy chain. These results are consistent with the two latter fragments being the COOH-terminal fragments after cleavage with acid and CNBr, respectively. The 14 000-dalton CNBr fragment obviously has the amino acid residue occupying position 6 of the intact heavy chain as the NH_2-terminal residue. This is in complete agreement with

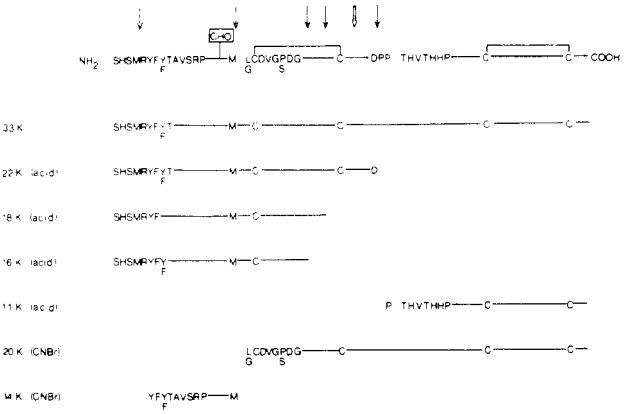

FIGURE 7: Tentative model for papain-solubilized HLA antigen heavy chain. The amino acid sequence data are given in the one letter code (see Dayhoff et al., 1976). The open arrow denotes the approximate cleavage position for proteases. The solid arrows represent the acid cleavage positions. The broken arrows indicate the positions of the methionyl residues. For further details, see text and Table II.

the observation that position 5 of the intact protein is a methionine.

Discussion

The present data extend our previous studies demonstrating that papain-solubilized HLA and H-2 K and D antigens display a stretch of amino acids that is sensitive to limited proteolysis (Peterson et al., 1975). Several enzymes with different specificities cleave the HLA antigen heavy chain so that two main fragments are generated. The larger fragment contains all of the carbohydrate and is at least partly associated with β_2-microglobulin. The yield of the fragments was relatively poor when ^{125}I-labeled antigens were digested. As the yield was even less when chemical amounts of HLA antigens were digested, limited proteolysis does not seem to be a useful procedure to obtain large fragments for future structural analyses. Therefore, acid cleavage and CNBr treatment of the HLA antigen heavy chain were attempted. Both procedures resulted in almost quantitative cleavage of the heterogeneous mixture of HLA antigen heavy chains into a few large fragments. This observation generalizes the finding of Terhorst et al. (1977) who showed that a few HLA antigens of defined specificity could be cleaved by the above procedures to yield fragments similar to those reported here.

The present data allow the construction of a tentative model for the HLA antigen heavy chain (Figure 7). As the 22 000-, 18 000-, and 16 000-dalton acid cleavage fragments display the same NH_2-terminal amino acid sequence cleavage must have occurred at the COOH terminus of these fragments. The NH_2-terminal amino acid sequence of the 11 000-dalton fragment positions this component as the COOH-terminal fragment of the intact papain-solubilized HLA antigen heavy chain. The combined molecular weights of the two main acid cleavage fragments add up to the entire weight of the papain-solubilized heavy chain suggesting that, as a result of the acid cleavage, only small peptides, if any, could have been released in addition to the main fragments.

The amino acid sequence of the CNBr fragments clearly demonstrate that the 14 000-dalton fragment is located in the NH_2 terminus and the 20 000-dalton fragment in the COOH terminus of the HLA antigen heavy chain. Obviously, a five-membered CNBr peptide located in the NH_2 terminus escaped detection in the present analyses and other peptides of similar size may also have gone undetected but it is unlikely that they represent any substantial portion of the HLA antigen

heavy chain. This is inferred from the fact that the combined molecular weights of the two main CNBr fragments amount to that of the intact papain-solubilized heavy chain.

The 14 000-dalton CNBr fragment and the 22 000-dalton acid cleavage fragment contain carbohydrate. This suggests that the carbohydrate moiety, which seems to comprise some 15 monosaccharides (Parham et al., 1977, and unpublished observations), is located within the first 100 amino acids of the HLA antigen heavy chain. Moreover, the lack of cysteines in the 14 000-dalton CNBr fragment indicates that the first cysteine occurs carboxy terminal to the prosthetic group. The most NH_2-terminal cysteine was, in fact, obtained in position 3 of the 20 000-dalton CNBr fragment.

Previous studies and unpublished observations in this laboratory have shown that each one of the two main proteolysis fragments of the HLA antigen heavy chain contains a single disulfide loop encompassing some 60 amino acids (Peterson et al., 1975). This finding together with the present information on the occurrence of [^{14}C]carboxymethylcysteine in the various acid cleavage and CNBr fragments allow the position of the disulfide bridges as shown in Figure 7.

The model of the HLA antigen heavy chain is in agreement with that suggested by Terhorst et al. (1977) based on preliminary data obtained with the B-7 antigen. As the present data were obtained with HLA antigens comprising a great number of antigenic specificities from at least three loci, it appears reasonable to conclude that most, if not all, HLA antigens, regardless of specificity, display common structure. As acid cleavage preferentially occurs at asparaginyl-prolyl bonds (see Hill, 1965) and CNBr cleaves at methionyl residues (Gross & Witkop, 1962) the great majority of the HLA antigens must have amino acid sequences that are invariant with regard to methionine and the Asp-Pro sequence. Likewise, the distribution of cysteines along the sequence seem to be identical for most if not all HLA antigens.

While the NH_2-terminal sequence of the papain-solubilized HLA antigen heavy chain is consistent with previous reports (Terhorst et al., 1977; Apella et al., 1976; Ballou et al., 1976; Bridgen et al., 1976), the amino acid sequences reported here in other positions differ considerably from those published by Parham et al. (1977) and Terhorst et al. (1977). Parham et al. isolated carbohydrate-containing tryptic peptides from HLA antigens A2 and B7 and determined the amino acid sequence for these peptides. The peptide derived from the B7 antigen contains a methionyl residue and part of the peptide should according to the model of Terhorst et al. (1977) comprise the NH_2-terminal sequence of the 20 000-dalton CNBr fragment. Our data on the NH_2-terminal sequence of the CNBr fragment only bears a slight resemblance with that of Parham et al. (1977). Among other things the CM-Cys positively identified in position 3 of our sequence is absent in that of Parham et al. (1977). This is surprising as these and previous data (Peterson et al., 1975) strongly suggest that the linear positions of the cysteines are invariant. However, HLA-B7 molecules may be distinct from most other HLA antigens in this region. Likewise, the NH_2-terminal sequence determined here for the 11 000-dalton acid cleavage fragment differs from the corresponding sequence determined for the HLA-B7 acid cleavage fragment (Terhorst et al., 1977). In this case, however, the revised sequence of the HLA-B7 antigen is apparently identical with the present one (R. Robb, personal communication).

This study strongly suggests that a mixture of HLA antigens of different specificities represents a suitable material for further structural work. This is ascertained by the limited amino acid sequence information. Out of the 36 residues analyzed only three positions display more than one unique amino acid. This, of course, does not necessarily imply that there exists any single HLA antigen molecule with the amino acid sequence given in Figure 7 but suggests that the sequenced portions are relatively invariant.

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Reactivity of the Antibodies to DNA Modified by the Carcinogen *N*-Acetoxy-*N*-acetyl-2-aminofluorene†

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ABSTRACT: Rabbits were immunized with native DNA modified by the carcinogen *N*-acetoxy-*N*-acetyl-2-aminofluorene. The interactions between the purified antibodies to nDNA-AAF (or the Fab fragments) and several ligands have been studied. By radioimmunoassay, nDNA-AAF, dDNA-AAF, and GMP-AAF were found to bind to the antibodies with about the same affinity. GMP-AF interacts slightly less, and GMP and *N*-OH-AAF do not interact. The values of the association constants deduced from fluorescence measurements for the binding of the Fab fragments to nDNA-AAF, dDNA-AAF, and GMP-AAF, in 50 mM NaCl, pH 7.5, are of the same order of magnitude. The values of the association constants with nDNA-AAF and dDNA-AAF depend upon salt concentration. From this variation, it is deduced that 1–1.5 phosphate groups interact by charge–charge interactions with the Fab fragments. The absorption and circular dichroism spectra of GMP-AAF, nDNA-AAF, and dDNA-AAF bound to the Fab fragments show that the Fab fragments induce similar perturbation to the three ligands. These results lead to the conclusion that the immunodeterminant group is the dGMP-AAF residue.

It is now well established that the carcinogen *N*-acetoxy-*N*-acetyl-2-aminofluorene (AAAF)[1] can react in vitro with native DNA. Two main adducts have been identified (for a general review, see Kriek, 1974). One is *N*-(2′-deoxyguanosin-8-yl)-2-(acetylamino)fluorene (dGuo-AAF) and results from the covalent binding of the carcinogen on the C(8) of guanine residues. The other is 3-(2′-deoxyguanosin-N^2-yl)-2-(acetylamino)fluorene and results from the covalent binding of the carcinogen on the amino group of guanine residues. The relative amounts of the two adducts after in vitro modification are about 80–90 and 10–20%, respectively (Kriek, 1972; Fuchs, 1978). There is evidence that the geometry of the double helix is modified by the reaction of the carcinogen on the C(8). It has been proposed that the acetylaminofluorene residues are inside the double helix while the guanine residues are outside (for general reviews, see Weinstein, 1977, and Daune & Fuchs, 1977). These modifications of the conformation may be a critical step in the carcinogenic process.

We have undertaken a study of these conformational changes of the DNA using antibodies as probes. Antibodies to nucleosides were found to react with DNA modified by AAAF (Bases et al., 1976; Sage & Leng, unpublished experiments). On the other hand, recently we have shown that native DNA after reaction with AAAF was immunogenic (Leng et al., 1978a,b). Injections in rabbits of the modified DNA led to synthesis of antibodies which react with the modified DNA and not with the unmodified DNA. The specific antibodies have been purified by affinity chromatography. Some evidence was presented, showing that the antibodies recognize the dGMP-AAF residues in the modified DNA. It seemed to us of interest to study in more detail the reactivity of the purified antibodies.

In this paper, we report complementary results which confirm the fact that the dGMP-AAF residues are the immunodeterminant groups. The experiments have been done with the purified antibodies and the Fab fragments by use of several techniques, i.e., radioimmunoassay, fluorescence, absorption, and circular dichroism.

Material and Methods

Double-stranded calf thymus DNA ($M \simeq 5 \times 10^5$) and heat-denatured samples were modified by reaction with AAAF according to a procedure already published (Fuchs & Daune, 1972). The percentage of bound AAF was determined from the analysis of the ultraviolet absorption spectrum. We will note respectively nDNA-AAF or dDNA-AAF for double-stranded and heat-denatured DNA which have been reacted with AAAF. The percentage of modified bases will be given in parentheses.

N-(Guanosin-8-yl)-2-(acetylamino)fluorene (Guo-AAF) was prepared by the reaction of guanosine (Sigma) and AAAF as described by Kriek (1969). Guo-AAF was isolated by

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[1] Abbreviations used: AAAF, *N*-acetoxy-*N*-acetyl-2-aminofluorene; AAF, *N*-acetyl-2-aminofluorene; AF, 2-aminofluorene; *N*-OH-AAF, *N*-hydroxy-*N*-acetyl-2-aminofluorene; nucleotide-AAF or AF, nucleotide substituted on C(8) with AAF or AF; DNA-AAF, DNA modified covalently with AAAF; RIA, radioimmunoassays.

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