Detergent-Soluble Products of the HLA Region

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THE major histocompatibility complex (MHC) of higher vertebrates is a genetic region that contains the major histocompatibility antigens and a series of closely linked genes[1] whose products seem either to be present on cell surfaces, to be involved in the immune system, or both. The classical histocompatibility antigens of humans are codominantly expressed products of two closely linked, well studied loci, HLA-A and HLA-B, and a third less well serologically defined locus, HLA-C. The HLA-A and B gene products are highly homologous, and these genes must have arisen by duplication.[2] A fourth HLA locus, HLA-D, is the strongest mixed lymphocyte culture (MLC) stimulating locus. It may, at least in part, code for HLA-linked B-cell-specific antigens.[3] The HLA loci are extremely polymorphic, with at least 20 alleles at HLA-A and B.[4] Since such a high degree of polymorphism could only have arisen by strong selective pressure, histocompatibility antigens may have an important function, probably quite apart from their role in transplantation rejection.

Two types of molecules coded for by the MHC are involved in graft rejection. Ia antigens in the mouse and HLA-linked B-cell antigens in the human, both found primarily on B lymphocytes, appear important in MLC stimulation, an in vitro analogue of the sensitization phase of graft rejection. The classical histocompatibility antigens, H-2 antigens in the mouse and HLA-A,B antigens in the human, are important as the target of killing in cell-mediated lympholysis, an in vitro analogue of the destructive phase of graft rejection.[3] The importance of these antigens in graft rejection may be related to their normal biologic functions. Ia antigens and immune response functions genetically comap,[1] and Ia antigenic determinants are present on antigen-specific factors that mediate B- and T-lymphocyte cooperation.[5] Recognition of H-2 antigens is required for killing of cells bearing viral,[6] minor histocompatibility,[7] or chemically modified[8] antigens. These antigens, therefore, appear to play an important, yet, incompletely understood, role in the immune system, and their structure, particularly after solubilization in intact form, is of great interest.

PURIFICATION

Antigens were solubilized from cultured RPMI 4265 lymphoblastoid cells (HLA-A2, 2; B7, 12) with nonionic detergents and purified by *Lens culinaris* lectin affinity chromatography and Bio-Gel A-5m filtration. Dithiothreitol (DTT) was included in buffers to prevent oxidation of sulfhydryls, resulting in HLA antigen dimer formation.[9] In the last step, HLA-A and B antigens were separated from HLA-linked B-cell antigens. The specificity HLA-A2 could be separated from specificities HLA-B7, 12 by column isoelectric focusing.[10]

HLA-A, B ANTIGEN STRUCTURE

Purified HLA-A,B antigens (p44,12) contained two polypeptides (Fig. 1A, gel 1), a glycoprotein of 44,000 mol wt (p44), and a protein of 12,000 mol wt (p12) that was shown to be identical to β_2-microglobulin (β_2-m).[11] β_2-m had previously been isolated from nephrotic urine and shown to have sequence homology with immunoglobulin-constant domains.[12] The N-terminal se-

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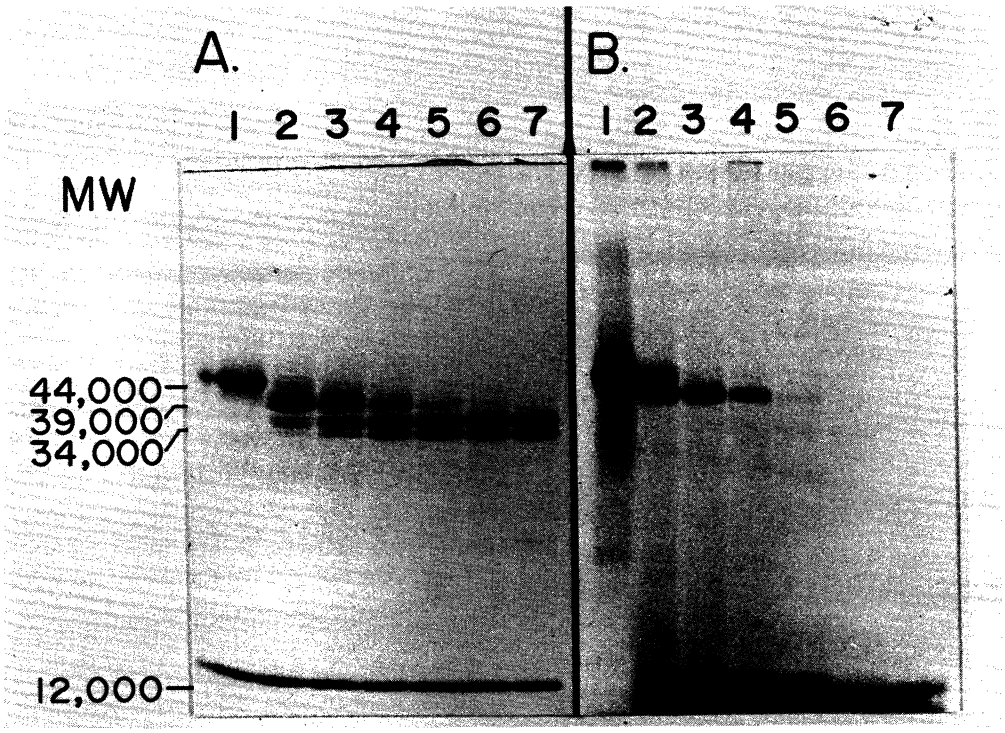

Fig. 1. SDS slab gel electrophoresis of iodo-[^{14}C]acetate-labeled HLA-A,B antigen. Antigen was digested with the indicated ratios of papain to protein: 1, none; 2, 1:3840, 3, 1:1920; 4, 1:960; 5, 1:480, 6, 1:240; 7, 1:120. Each aliquot was divided into halves and electrophoresed on 2 sides of an 11% acrylamide SDS slab gel. One-half of the slab gel was stained (A); the other half was autoradiographed (B).

quence of p44 was Gly-Ser-?-Ser-Met[13] and was identical to that of p34 of papain-solubilized HLA-A,B antigens (p34,12).[2] Therefore, papain digestion removes peptides totaling 10,000 mol wt from the C-terminus. Alkylation of HLA-A,B antigens with iodo- [^{14}C] acetate after mild reduction resulted in incorporation of radioactivity into p44 but not p12 (Fig. 1B, gel 1). When digested with graded amounts of papain (Fig. 1), p44,12 was first converted to p39,12, then to p34,12. Peptides containing [^{14}C] carboxymethyl cysteine were removed at each stage and were then susceptible to further cleavage. [^{14}C] carboxymethyl cysteine was present in p44, to a lesser extent in p39, and was absent from p34 and p12. These cysteines on p44 could also be oxidized to form dimers, which have been observed to be formed by p44,12, but not by p34,12.[9] To purify p44, p39, and p34, HLA-A,B antigens were chromatographed on Bio-Gel A-1.5m, either after treatment with no papain, 1/3000 papain yielding p39,12 and contaminated with some p34,12, or 1/125 papain yielding p34,12. p39,12 was separated from contaminating p34,12 during gel filtration, since p39,12 (as well as p44,12) binds to a large detergent micelle and chromatographs at an apparent mol wt of 400,000, while p34,12, which does not bind, chromatographs at 45,000 mol wt. The proteins were completely reduced and alkylated, and large subunits were separated from p12 by filtration on Sepharose CL 6B in 6*M* guanidine HCl. The pools containing p44, p39, and p34 were extensively dialyzed, lyophilized, hydrolyzed, and subjected to amino acid analysis (Table 1).[13] Subtractive com-

Table 1. Amino Terminal Sequences

	Residue														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
p16	Thr	Glu	Thr			Phe				Lys	Phe		Pro	Asp	
p29	Gly	Asp	Thr	Pro			Phe	Leu	Glu	Gln					
p34	Ile	Trp	Glu	Glu		Val	Ile		Glu	Ala	Glu	Phe	Tyr	Leu	Asn

Peptides (10–30 nmol) were sequenced using ^{35}S-phenylisothiocyanate in an updated Beckman 890 B Sequencer. Phenylthiohydantoins were identified after thin-layer chromatography on polyamide sheets by visual inspection and autoradiography. Arginine, lysine, isoleucine, and leucine were identified on the amino acid analyzer after back hydrolysis. Peptides were also sequenced using the SDS-Edman-dansyl technique.[20]

parison of p44, p39, and p34 showed that upon conversion of p44 to p39, a highly hydrophilic C-terminal peptide was released (polarity, 64%), while conversion of p39 to p34 released a highly hydrophobic penultimate peptide (polarity, 21%). The finding that p44 and p39 have a hydrophobic region, while p34 does not, correlated with the observation that p44,12 and p39,12 bind to detergent micelles, while p34,12 does not. The hydrophobic nature of the penultimate peptide, and its role in detergent binding, indicate that it anchors HLA-A,B antigens to the cell membrane through hydrophobic bonds. Since the C-terminal peptide is highly hydrophilic, it is presumably present in the aqueous phase. Structural resemblance of HLA-A,B antigens to glycophorin[14] suggests that the hydrophilic peptide lies inside the cell, although a more complicated U-shaped arrangement is also possible. The orientation and integration of HLA-A,B antigens in the membrane are summarized in Fig. 2, which incorporates the hypothesis that HLA-A,B antigens span the membrane. Capping experiments have suggested that a transmembrane link exists between histocompatibility antigens and the cytoskeleton.[15] Such a link could be provided by the C-terminal hydrophilic peptide, if, as suggested, it lies inside the cell.

HLA-LINKED B-CELL-SPECIFIC ANTIGEN STRUCTURE

As mentioned previously, B-cell-specific antigens copurified with HLA-A,B antigens during lectin affinity chromatography but chromatographed at a higher molecular weight than HLA-A,B antigens during Bio-Gel A-5m filtration. B-cell-specific antigens have also been isolated from papain digests of membranes and contained polypeptides of 23,000 and 30,000 mol wt.[16] Detergent-soluble B-cell-specific antigens contain two polypeptide chains of 29,000 (p29) and 34,000 mol wt (p34) that always remained associated with one another during purification. In SDS gel electrophoresis experiments (Fig. 3, A and B), it was found that in the presence of SDS at 21°–38°C, p 29 and p34 remained noncovalently associated in a

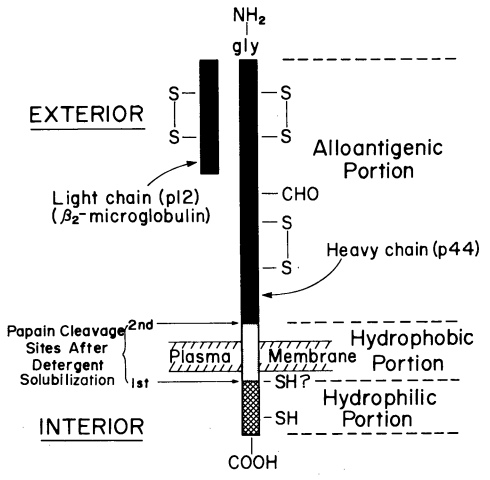

Fig. 2. Arrangement of HLA-A,B antigen in the membrane with the hydrophilic peptide hypothetically shown inside the cell membrane. The position of β_2-microglobulin relative to the alloantigenic portion of the heavy chain is not known. The possible presence and position of a second sulfhydryl, shown near the first papain cleavage site, is unclear. It may be present on only some HLA specificities, or the exact position of papain cleavage relative to it may vary.

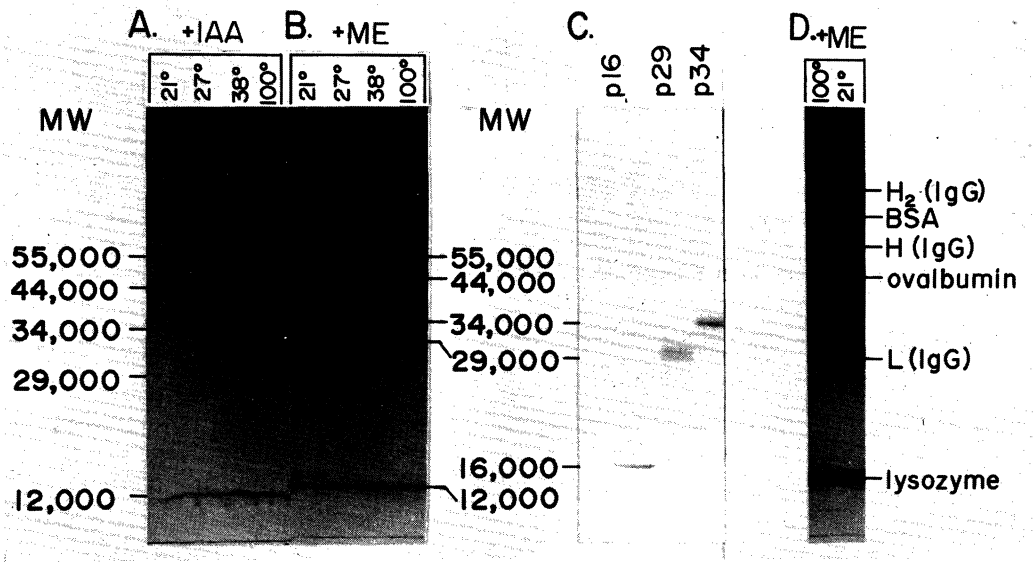

Fig. 3. SDS gels of p29,34 preparations after various treatments. (A) SDS gel electrophoresis in 10% acrylamide SDS slab gels of material purified in the absence of DTT by lectin affinity chromatography and gel filtration[9,10] after incubation with 0.6% SDS and 10 mM IAA for ½ hr at 20°, 27°, and 38° or for 5 min at 100°. (B) Same as (A), except incubation was in the presence of 5% ME instead of IAA. (C) Two μg of each polypeptide eluted from gels was electrophoresed in an SDS 7%–15% acrylamide gradient gel. (D) A mixture of standards (IgG, bovine serum albumin (BSA), ovalbumin, and lysozyme) were electrophoresed after treatment as in (B).

p29,34 complex, which calibrated at an apparent mol wt of 55,000. When heated at 100°C, however, in the absence as well as in the presence of 2-mercaptoethanol (ME), p29,34 were dissociated into the p29 and p34 subunits, indicating the absence of interchain S-S bridges. The preparation shown in Fig. 3 had been isolated in the absence of DTT and contained HLA p44 dimers.[9] Interestingly, immunoglobulin H chains remained associated noncovalently (at the ME concentration employed[17]) as H_2 dimers at 21° and were dissociated at 100° (Fig. 3D). The B-cell-specific antigens were treated with a cleavable cross-linking reagent, dimethyl dithiobis (propionimidate) to investigate noncovalent subunit associations. A cross-linked product of 64,000 mol wt was obtained, which was shown to contain p29 and p34 by cleavage with ME of the S-S bond in the reagent.[18] The apparent mol wt of 55,000 in SDS gel electrophoresis of the unboiled complex was lower than the mol wt of 64,000 observed for boiled, cross-linked p29,34. p29,34 in SDS at 21° retained a folded conformation (see below) and, therefore, would be expected to possess electrophoretic properties different from cross-linked p29,34, which had been unfolded by heating at 100°.

Since p29 and p34 remained noncovalently associated at low temperatures in the presence of SDS, it was hypothesized that their conformation might closely resemble that of native p29,34. Immunization of rabbits with p29,34 purified by preparative SDS gel electrophoresis elicited an antiserum with a cytotoxic titer of 500 and a high degree of specificity for B lymphocytes.[19] The separated subunits failed to elicit an antiserum. Anti-p29,34 reacts with the same immunodeterminants present on B lymphocytes as an antiserum (anti-p23,30) prepared against papain-solubilized B-cell-specific antigens, since both antisera were completely inhibited in their lysis of B cells by SDS-gel-purified p29,34, but not by dissociated p29 or p34 (J. Kaufman, un-

Table 2. Amino Acid Composition

	Residues/Molecule					moles/100 moles			
	HLA-A,B Antigen						B-Cell Antigen		HLA-A,B
Amino Acid	p44	p39	p34	p44–p39	p39–p34	p16	p29	p34	p44
CM-Cys	4.5	4.2	3.6	0.3	0.6	0.55	1.50	1.21	1.31
Asp	30	22	21	8	1	12.14	7.87	8.93	8.73
Thr*	24	19	19	5	0	10.78	6.70	6.65	6.87
Ser*	23	18	13	5	5	7.32	6.87	5.32	6.77
Glu	44	36	36	8	0	7.80	12.62	11.61	12.65
Pro	18	14	12	4	2	4.37	4.82	6.26	5.06
Gly	27	22	12	4	4	7.04	7.93	6.89	7.67
Ala	29	23	22	6	1	7.16	4.85	5.27	8.32
Val‡	21	21	15	0	6	7.90	8.14	8.48	6.19
Met†	5	5	4	0	1	0.63	1.52	1.46	1.51
Ile‡	12	12	6	0	6	5.31	3.33	4.89	3.45
Leu	24	24	17	0	7	5.94	8.56	9.17	6.94
Tyr†	15	13	13	2	0	3.58	3.76	2.73	4.32
Phe	11	10	7	2	3	6.21	5.31	5.98	3.06
His	10	10	10	0	0	1.34	2.83	2.70	2.91
Lys	13	11	9	2	2	5.79	3.88	4.45	3.87
Arg	25	21	21	4	0	3.24	7.01	5.56	7.19
Trp	N.D.§	N.D.	N.D.	N.D.	N.D.	2.90	2.51	2.44	N.D.
Polarity	49.2	46.7	50.7	63.7	20.7	48.4	47.8	45.3	49.0

Determinations of residues/molecule are rounded to the nearest integer, except for CM-Cys.

*Extrapolated to zero time values.

†24-hr values.

‡72-hr values.

§Not determined.

published information). Since p23,30 inhibits alloantisera recognizing the HLA-linked, B-cell-specific antigen, both p29,34 and its papain product, p23,30, must be at least in part a product of the HLA region. p29,34 must retain a conformation in SDS similar to its native state, since antibodies made against it react with the native form of p29,34 on the surface of B lymphocytes, and this reaction could be inhibited by SDS-gel-purified p29,34, but not its dissociated subunits.

For chemical characterization, p29, p34, and p16 (another protein present in the Bio-Gel A-5m pool) were purified after heating at 100° by preparative SDS gel electrophoresis in preeluted gels.[18,20] Analytical SDS gel electrophoresis showed them to be homogeneous (Fig. 3C). N-terminal sequencing by the SDS Edman-dansyl technique,[20] as well as automated sequencing, also showed the proteins to be homogeneous. The N-terminal sequences of p16, p29, and p34 (Table 1), as well as the p12[12] and p44,[2,31] are all quite different. However, the possibility of a low degree of N-terminal homology of p29 to p44 and of p34 to p12 has not been ruled out, and a decisive answer must await further sequencing.

HOMOLOGIES

Although limited N-terminal sequencing did not establish homologies among p29, p34, and p44, homologies between polypeptides of dissimilar size would not necessarily commence at the N-terminus. Therefore, two methods were used for detection of homologies: statistical comparison of amino acid compositions and peptide mapping.

The amino acid compositions (Table 2) of p29 and p34 are quite similar to one another and to that of p44. p29, p34, and p44 are also glycoproteins, since they stained

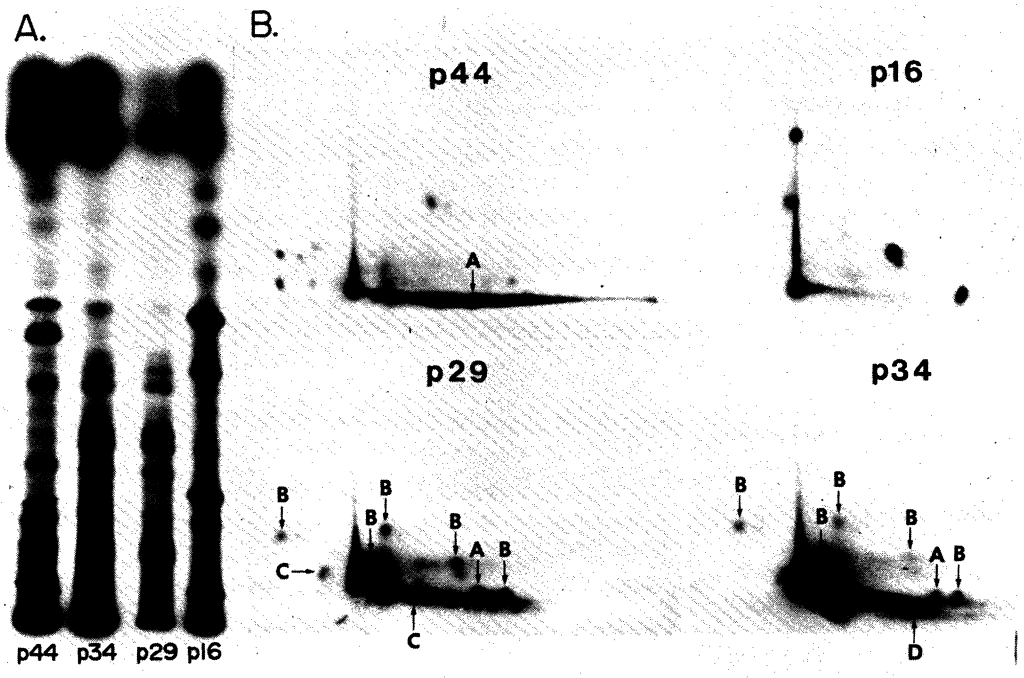

Fig. 4. ^{125}I-tyrosine-labeled tryptic peptide maps of p16, p29, p34, and p44. Peptides were labeled with ^{125}I in the presence of SDS, digested with trypsin, and chromatographed on silica gel G.[23] HLA antigens were prepared as described,[10] and p44 was separated from p12 by preparative SDSgel electrophoresis.[20] (A) Chromatography dimension only. (B) Chromatography (upward) followed by electrophoresis (anode to right). The origin is the intensely radioactive spot near the lower left corner. Labeled spots: A, peptides common to p29, p34, and p44; B, peptides common to p29 and p34; C, peptides unique to p29; D, peptides unique to p34.

with the periodic acid-Schiff reagent and contained similar amounts of sialic acid. The amino acid compositions were compared by a statistical method[21] to estimate evolutionary relationships. By this method, p29 and p34 were highly related, to the same extent as HLA-A and HLA-B antigens,[22] and were related to a lesser extent to p44. p12 and p16 were not shown to be related to each other or to the other proteins.

Carboxymethylated p16, p29, and p34, as well as p44 purified by the same technique, were heated at 100°C in SDS, labeled with ^{125}I, digested with trypsin,[23] and compared on one-dimensional (Fig. 4A) and two-dimensional (Fig. 4B) maps.[18] p29 and p34 share six major tyrosine tryptic peptides, and each protein also contains unique peptides (Fig. 4B), in good agreement with calculations from the amino acid compositions that show that p29 and p34 contain eight and seven tyrosines, respectively. p44 contained many unique peptides, as well as one peptide in the same position as those of p29 and p34. The data show striking homology between p29 and p34 and suggest a low extent of homology with p44. If the tyrosine tryptic peptides are representative of the remaining three-quarters of the tryptic peptides of these molecules, p29 and p34 would be identical throughout most of their sequences. Several pieces of evidence showed that p29 could not have been derived from p34 by proteolytic cleavage. p29 contained extra serine, tryosine, and tyrosine tryptic peptides. Proteolysis was never observed during purification; in SDS gels, the ratio of p29 to p34 remained constant, and equimolar amounts of p29 and p34 were recovered from preparative gels.

Proteolysis would have been expected to also yield $(p29)_2$ molecules; these were not detected in cross-linking or isoelectric focusing experiments. Also, protease digestion by papain converted one polypeptide to p30 and the other to p23, suggesting them to be unique. p29 and p34 are different at their N-terminals, and it will be interesting to see whether differences are confined to this region. The high homology between p29 and p34 is most likely due to a gene duplication, as is the case for HLA-A and HLA-B antigens.[2] The alternative would be that a common portion is coded for by one gene, and unique portions, probably at the N-terminus, are coded for by different genes (a two-gene, one-polypeptide model). The low degree of homology with p44 suggests that the genes for p29,34 and p44 of the A and B loci may have arisen from a single ancestral gene.

GENE-PRODUCT RELATIONSHIPS

Both HLA-A,B antigens (p44,12) and HLA-linked B-cell-specific antigens (p29,34) have been found to be composed of two noncovalently-associated, nonidentical subunits. The HLA-A and B genes on chromosome six[24] encode p44, which expresses the alloantigenic activity.[25] Its antigenic determinants,[2] as well as those of other MHC products,[26] appear to be expressed by their protein, rather than carbohydrate, moieties. p12(β_2-m) is invariant and is encoded by a gene on chromosome 15.[27] The partial sequence of p44[2,13] has provided no evidence for homology with p12(β_2-m) or immunoglobulins.

The alloantiserum inhibition experiments[16] have shown that at least one of the B-cell-specific antigen subunits is encoded by one of the two B-cell-specific antigen loci in HLA that are separable by crossing-over.[28] p29 and p34 contain different N-terminals, but peptide mapping studies showed that a large portion of their sequences are identical. p29 and p34 are not necessarily both HLA products, since homologous protein subunits are often found to be specified by unlinked genes, as is the case for immunoglobulin L and H chains[29] and hemoglobin α and non-α chains.[30] It will be important to determine whether genes for both p29 and p34 are present in HLA and to which of these subunits alloantisera are directed. The inability so far to obtain separated subunits that retain antigenic activity hinders a simple answer to these questions. However, it should be possible to determine whether different antisera recognize determinants present on the same supramolecular structure through the use of lysostrip, capping, blocking, and precipitation experiments.

It is increasingly evident that many products of the MHC function in the immune system.[1,51,31] The finding that MHC-linked B-cell antigens have a structure composed of homologous subunits is suggested to have implications for such a possible role. Proteins with this type of structure often can be composed of any of a set of interchangeable subunits that are specified by duplicated genes. Since many unique combinations can be made using a much smaller number of unique subunits, great diversity in functional specificity can be generated in an economical fashion.

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