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Structure of HL-A A and B Antigens Isolated from Cultured Human Lymphocytes

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The major histocompatibility complex (MHC) is a genetic region that encodes the major barrier to transplantation in man and in all other species studied so far. It was first identified through mouse breeding studies which led to the identification of the H-2 region, the second transplantation region discovered in these studies and the strongest barrier to transplantation in this species. There are at least 15 genetic loci which determine the ability of mice to exchange grafts. Little is known about the minor loci. HL-A is the human homologue of the H-2 region and, correspondingly, the strongest of the transplantation barriers in man. The existence of other loci greatly increases the complexity of our ultimate understanding of the phenomenon of graft acceptance. The sixth human chromosome (or 17th mouse chromosome) contains genetic regions HL-A *A, B,* and *C* (analogous to the H-2 *K* and *D* loci in the mouse). There are multiple alleles in the population at each of these loci. The existence of this polymorphism and its meaning for the natural function of this region (which certainly did not evolve to prevent the exchange of surgical grafts) pose the most intriguing questions about this system. This paper will deal mainly with the products of the HL-A *A* and *B* loci. Little is known about the products of the HL-A *C* locus at present. The products of the HL-A *D* locus are defined by the mixed leukocyte culture (MLC), and information about the probable product of this locus is discussed elsewhere (Springer et al.)

The products of the HL-A *A* and *B* genes are measured using specific alloantisera, the major source of which is pregnancy antisera, i.e., sera from women who have been pregnant several times and who have been immunized against paternal antigens. Indeed, one of the most interesting, incompletely solved problems in the field is why pregnant females do not reject the commonest graft in the species, i.e., the fetus in utero. Pregnancy alloantisera, in the presence of complement, lyse ^{51}Cr-labeled lymphocytes of appropriate specificity. Soluble or membrane-bound antigen is measured by its ability to inhibit this lysis by combination with antibody.

PURIFICATION OF HL-A ANTIGENS FROM HUMAN LYMPHOCYTES

A number of attempts have been made to purify HL-A antigens from human lymphocytes, notably by Sanderson and Batchelor (1968), who used splenic lymphocytes. Very small amounts were obtained from this source. Cultured lymphoblasts appeared to be a preferable source, both because they have the potential of being available in larger amounts than human spleens and because an experiment with a single human spleen cannot be repeated. Also, cultured lymphoblasts do not seem to have any altered HL-A specificities. Remarkably, these lymphoblasts also contain far more HL-A antigens than splenic or peripheral blood lymphocytes. This was shown by absorption experiments with HL-A antisera using three types of cells, all from the same individual (RH; McCune et al. 1975). Peripheral blood lymphocytes from RH had a very low absorptive capacity for these antisera. Phytohemagglutinin (PHA)-stimulated lymphocytes had a greatly enhanced capacity, and RH lymphocytes transformed by Epstein-Barr virus (EBV) and growing continuously in cultures had an enormously enhanced absorptive capacity for HL-A antisera. The representation of HL-A antigens on the surface of cultured lymphocytes transformed by EBV was 20–50-fold greater than for peripheral blood lymphocytes; all four of the HL-A specificities of RH cells were similarly affected. This enhanced representation was specific in that other membrane markers, such as 5′-nucleotidase or radioiodinatable surface protein, were increased in the transformed cell only two- to threefold, the same as the increase in surface area. One interpretation is that the virus itself induced or enhanced expression of the antigen in some way. Alternatively, the virus may have selected for transformation a subpopulation of B lymphocytes that already had an enhanced representation. The explanation is not known, but the fact that the HL-A antigens are so much more densely represented on the cultured lymphoblasts has made possible their isolation in relatively large amounts.

Preparation of HL-A Antigens after Papain Solubilization

Two principal methods have been used for solubilization of HL-A antigens. These are (1) treatment with papain and (2) solubilization with detergent. Preparation of HL-A from the cell line JY after papain solubilization is shown in Table 1. The use of papain to solubilize HL-A antigens goes back to the pioneering studies of Shimada and

Table 1. Purification of Papain-solubilized HL-A Antigens from 100 g JY Cells

Purification step	Protein (mg)	Total number of inhibitory units recovered		Specific activity (inhibitory units/mg)		Fold purification
		HL-A A2	HL-A B7	HL-A A2	HL-A B7	
1. Cell membrane	1760	640,000	280,000	360	159	1
2. Papain digestion	1480	504,000	248,000	340	167	1
3. DE-52 batchwise elution	360	956,000	288,000	2560	800	~6
4. Sephadex G-150 chromatography	26	780,000	236,000	30,000	9100	~70
5. DE-52 chromatography	4.1 (HL-A A2) 4.9 (HL-A B7)	900,000	216,000	220,000	44,000	~300

Nathenson (1967), who first solubilized H-2 antigens in this way. The procedure for purification of HL-A has been improved considerably over that previously reported (Turner et al. 1975), and yields of about 4–5 mg per 50 g of cells can now be obtained. The procedure for isolation is not difficult, requiring only four steps. The most interesting feature is that only about 150-fold purification is needed to obtain pure HL-A A plus B antigen from cell membranes, i.e., something on the order of 0.5–1% of the total membrane protein in the cultured lymphoblast is HL-A antigen. That is a very large representation of a single protein on the lymphocyte membrane surface. The cells used were homozygous for HL-A A2 at the first histocompatibility locus and for HL-A B7 at the second. HL-A A2 has a charge difference which distinguishes it from most of the other specificities. It is readily separated on DEAE-cellulose chromatography from HL-A B7 at the last step of purification. Separating the allelic specificities from each other is one of the biggest problems in this field. It is relatively easy to obtain pure HL-A antigens, i.e., a mixture of the four specificities. Of course, the most interesting part of the chemistry requires that they be separated. Starting with doubly homozygous cell lines greatly reduces the problem. Several such lines have been started from homozygous individuals in the Indiana Amish community, an inbred religious sect; one of them is the JY cell line. HL-A A2 and a mixture of HL-A B7,12 were obtained previously from the cell line RPMI 4265 (Turner et al. 1975).

Our first interesting finding was that these antigens contain two subunits (Cresswell et al. 1973, 1974): a heavy chain which is glycoprotein and a light chain which is now known to be β_2-microglobulin (a protein first isolated from human urine). Sodium dodecyl sulfate (SDS) gels of the HL-A A2 antigen preparation showed the heavy chain to have a molecular weight of 34,000 and the light chain to have a molecular weight of 12,000 (β_2-microglobulin). SDS gels of the HL-A B7,12 mixture showed a doublet at 34,000 m.w. in addition to β_2-microglobulin. One of the doublet glycoproteins may be HL-A B7 and the other HL-A B12. However, since these gels are denaturing gels, the glycoproteins could not be recovered to prove this.

Despite the apparent purity of the HL-A antigen preparations, isoelectric focusing revealed considerable heterogeneity (Turner et al. 1975). For example, in the HL-A A2 preparation, at least four bands with HL-A A2 antigenic activity were seen. The most interesting possible interpretation of this heterogeneity was that it resided in the amino acid sequence, and therefore that some kind of variable region might exist in the HL-A antigens. However, the heterogeneity turned out to be due to variability in the number of sialic acid residues on the molecule (Parham et al. 1974). The HL-A A2 preparation was treated with neuraminidase as a function of time. The initial preparation contained a species with two sialic acid residues as the major component, but there were also species with three and species with one sialic acid residue. As the result of treatment, a sialic-acid-free preparation was obtained and all heterogeneity disappeared. The same result was obtained for other HL-A preparations.

Are these antigen preparations really pure? To approach that question, the HL-A antigen preparations were labeled by reductive methylation with formaldehyde and sodium borohydride (Parham et al. 1975). About two methyl groups/mole were introduced on the ϵ-amino group of the lysines of the molecule. This treatment did not alter immunological activity at all. Essentially 100% of these labeled preparations of HL-A A2 antigens formed a specific complex with HL-A A2 antisera. No significant complexing was observed with normal serum or with specificity controls (HL-A antisera with specificities other than HL-A A2). However, when the HL-A B7,12 preparation or another HL-A antigen preparation containing HL-A A3, Aw25, B12, and B27 was used, only about 70% of the total antigen could be complexed (Parham et al. 1975). With the HL-A B7,12 prep-

Table 2. Amino Acid Composition of Papain-solubilized HL-A A2 and HL-A B7,12 from Cell Line RPMI 4265 and Papain-solubilized HL-A A2 and HL-A B7 from Cell Line JY in Moles/100 Moles

Amino acid	HL-A A2 (JY)	HL-A A2 (4265)	HL-A B7 (JY)	HL-A B7,12 (4265)
Asp[a]	7.9	7.9	9.7	9.4
Thr[b]	7.6	7.5	6.2	6.9
Ser[b]	5.2	5.2	5.2	5.0
Glu[a]	13.9	13.9	14.8	14.4
Pro	4.3	4.5	5.4	5.4
Gly	7.9	7.6	8.0	6.9
Ala	8.8	8.5	8.0	7.8
Val[c]	6.3	6.4	5.0	5.0
Met	1.3	1.5	1.1	1.0
Ile[c]	1.6	1.8	2.6	2.9
Leu	6.2	6.3	6.4	6.7
Tyr[b]	4.8	4.7	4.5	5.3
Phe	2.9	2.9	2.3	2.4
Lys	4.2	4.3	3.7	3.6
His	4.8	4.5	3.5	3.3
Arg	7.6	7.8	8.8	9.3
CM-Cys	1.5	1.6	1.6	1.7
Trp[d]	3.2	3.2	3.1	3.2

Each analysis was done in duplicate for 24, 48, and 72 hr.
[a] Ammonia not determined.
[b] Extrapolated zero time values.
[c] Seventy-two-hour value only.
[d] Determined spectrophotometrically.

aration, about 40% complexation was obtained with HL-A B7 antiserum, 30% with HL-A B12 antiserum, and a total of 70% with a mixture of antisera. What does this mean? The residual 30% of material could be the product of the HL-A *C* locus or other unidentified HL-A antigens, denatured antigens, or some other unrelated material copurifying with HL-A.

Another evidence of purity is the single, common, amino-terminal group found in the HL-A antigen preparations (Parham et al. 1975). The five preparations available all had glycine as the N-terminal amino acid of the heavy chain. An isoleucine residue was also found; it is the N-terminal residue of β_2-microglobulin.

With confidence that these preparations were pure, the heavy and light chains were reduced and alkylated by treatment with iodoacetic acid and then separated by gel filtration. Amino acid analysis of the heavy chain has been carried out and sequence studies initiated (Terhorst et al. 1976). The analyses of four preparations of the heavy chains of HL-A antigens obtained from RPMI 4265 cells and JY cells are shown in Table 2. First of all, there were no significant differences between the heavy chain of HL-A A2 from JY and HL-A A2 from RPMI 4265 cells. Very small differences between HL-A B7 and the HL-A B7,12 mixture were found. However, approximately a 20 to 30 amino acid difference between HL-A A2 and HL-A B7 may be estimated. The degree of relatedness of these proteins can be examined from their amino acid analyses by an empirical method described by Marchalonis and Weltman (1971). In this method, none of several thousand unrelated proteins had SΔQ values of less than 100. An SΔQ value of less than 50 was therefore considered to suggest a significant relatedness between the proteins. By this method, HL-A A2 from JY was identical to HL-A A2 from RPMI 4265 cells (Table 3). HL-A A2 was very closely related to HL-A B7 or to HL-A B7,12. However, the heavy chains of the various HL-A-antigen preparations and β_2-microglobulin were not related (SΔQ ~ 170). This calculation carried out with various classes of immunoglobulin heavy chains (IgG, IgA, IgD, and IgM) also resulted in relatively large SΔQ values. The lowest SΔQ was obtained in comparison with IgD heavy chain, but it was on the order of 70 (the same value obtained when human β_2-microglobulin was compared with either mouse β_2-microglobulin or with the Fc fragment of Eu myeloma protein). There might be a relatedness of the heavy chain of the HL-A antigens and the heavy chain of the IgD, but it may not be very extensive.

N-terminal sequence data (Table 4) showed no differences between the two HL-A A2 preparations. HL-A B7 and the HL-A B7,12 mixture were very similar, and the latter showed heterogeneity at only one position. However, there was also only one difference in the first 25 amino acids between HL-A A2 and HL-A B7, and there may be only about a 20–30 amino acid difference in the whole molecule. There are four half-cystine residues per heavy chain in the papain-derived product (see below). The heavy chain has a polypeptide molecular weight of about 29,000. If the heavy chain of HL-A

Table 3. Relatedness among HL-A Antigens and β_2-Microglobulin as Determined from the Amino Acid Compositions by the SΔQ Method

Protein	HL-A A2 (4265)	HL-A A2 (JY)	HL-A B7 (JY)	HL-A B7,12 (4265)	β_2-Microglobulin human	β_2-Microglobulin mouse
HL-A A2 (4265)	0					
HL-A A2 (JY)	1	0				
HL-A B7 (JY)	13	16	0			
HL-A B7,12 (4265)	14	18	4	0		
β_2-Microglobulin (human)	172	183	183	173	0	
β_2-Microglobulin (mouse)	138	147	153	141	70	0

Table 4. Partial Amino Acid Sequence Analysis of Heavy Chains from HL-A Antigens

	Amino acid identified		
Step number	HL-A A2	HL-A B7,12	HL-A B7
1	Gly	Gly	Gly
2	Ser	Ser	Ser
3			His
4	Ser	Ser	Ser
5	Met	Met	Met
6	Arg	Arg, Val	Arg
7	Tyr	Tyr	Tyr
8	Phe	Phe	Phe
9	Phe	Tyr	Tyr
10	Thr	Thr	Thr
11	Ser	Ala	Ser
12	Val	Val	Val
13	Ser	Ser	Ser
14	Arg	Arg	Arg
15	Pro	Pro	Pro
16	Gly	Gly	Gly
17			
18	Gly	Gly	Gly
19	Glu	Glu	Glu
20			
21			
22	Phe	Phe	Phe
23	Ile	Ile	Ile
24	Ala	Ala	
25	Val	Val	Val

Partial sequence analysis of the N-terminal residues was obtained in the Beckman 890B automatic sequencer. The sequences of the two HL-A A2 preparations were identical. HL-A A2 (JY and RPMI 4265); HL-A B7,12 (RPMI 4265); HL-A B7 (JY).

is homologous to the heavy chain of immunoglobulin, then one would expect four half-cystine residues in a molecule of 29,000 m.w. Using [^{3}H] carboxymethyl-cysteine-labeled HL-A antigens in sequence studies up to 40 residues, which is well past the place where one would expect to find the first cysteine residue if there was strong homology to immunoglobulins, no significant counts were found.

There are several important points that can be made from these data: (1) The products of the two loci (HL-A *A* and *B*), although separated by a distance equivalent to several thousand genes, are remarkably similar to each other and must have arisen by gene duplication. (2) Allelic products at a single locus do not differ very much from each other, i.e., the immunological difference is not reflected in a very large difference in amino acid composition or in N-terminal sequence. (3) Comparison of the sequence data for HL-A A and B antigens with that obtained in several laboratories for mouse H-2D and K antigens reveals strong homologies between the human and murine antigens. These similarities are discussed in other papers in this volume and have recently been reviewed (Howard 1976) in connection with recent N-terminal sequence data for additional HL-A antigens (see also Bridgen et al. 1976; Appella et al. 1976). The latter are strikingly similar to those presented here. (4) Several points that may indicate similarities between HL-A antigens and immunoglobulins can be summarized: (a) limited proteolysis by papain; (b) a two-chain structure with sequence homology between the light chain of HL-A (β_2-microglobulin) and some heavy-chain immunoglobulin domains (Grey et al. 1973; Nakamuro et al. 1973; Cresswell et al. 1974; Peterson et al. 1974; Cunningham and Berggård 1974). (c) From the compositional and sequence data available so far, there is little apparent homology between the heavy chains of HL-A antigens and immunoglobulins. The cysteine content is the same as that found in two immunoglobulin domains, and all four cysteines are intrachain disulfide linked. The size of the papain-solubilized heavy chain, however, is equal to roughly two and one-half immunoglobulin domains. Knowledge of the linkage and location of these cysteines will therefore be of great interest, and further data will greatly clarify our knowledge of the structure and evolution of these interesting proteins.

Preparation of HL-A Antigens after Detergent Solubilization

Most membrane proteins are solubilized by detergents having HLB (hydrophilic lipophilic balance) numbers in the range of 12–14. The HLB number is an empirical measure of a detergent's tendency to make oil-in-water or water-in-oil emulsions. The solubilization of HL-A antigens appeared similar to the solubilization of bacterial membrane proteins except that a group of relatively hydrophilic Brij detergents appeared to be relatively selective in solubilizing HL-A antigens (Springer et al. 1976a).

The purification of detergent-soluble material using an anti-β_2-microglobulin immunoabsorbent column is summarized in Table 5 (Robb and Strominger 1976). An earlier procedure (Springer et al. 1974) yielded partially purified material. After membrane preparation and solubilization in detergent, the next steps are passage through a lectin affinity column, absorption on an anti-β_2-microglobulin affinity column and subsequent elution with purified soluble β_2-microglobulin, and removal of the excess β_2-microglobulin on a Bio-Gel A-5m column. The purification from detergent-solubilized membrane protein required to obtain pure material was only about 50-fold, and the yields were on the order of 50%. About 7 mg of HL-A antigen was prepared from 150 g of cells. The anti-β_2-microglobulin column has also been used successfully without the lectin column step (Robb and Strominger 1976). Alternatively, repeated agarose gel filtration after passage through the lectin column also yielded pure antigen and may be more applicable to large-scale work (Springer et al. 1976a). The detergent-solubilized HL-A antigens also contained two poly-

Table 5. Purification of Detergent-solubilized HL-A from 150 g of JY Cells

Purification step	Protein (mg)	Percent recovery of inhibitory units		Specific activity (inhibitory units/mg)		Fold purification
		HL-A A2	HL-A B7	HL-A A2	HL-A B7	
Detergent-solubilized membrane	750	100	100	330	140	1
Lectin column chromatography	111	85	83	1890	760	5.6
Anti-β_2-microglobulin column	–	63	59	–	–	–
Bio-Gel A-5m column	7	54	50	19000	7300	55

peptides, a heavy chain of 44,000 daltons, and a light chain of 12,000 daltons, thus showing that the previously observed structure was not the result of proteolysis by papain.

The heavy chain of HL-A antigens is therefore 10,000 daltons larger than the heavy chain isolated from the papain-solubilized product, i.e., the papain cleaves a piece of 10,000 daltons from the molecule. Moreover, the detergent product has one, or possibly two, additional sulfhydryls present in easily reducible form which are not found on the papain product. These easily reduced sulfhydryl groups can be specifically labeled with [^{14}C]iodoacetic acid. The kinetics of papain cleavage of this material (Fig. 1) clearly reveals that it proceeds in two steps (Springer et al. 1974; Springer and Strominger 1976). An intermediate of 39,000 daltons is first formed, followed by cleavage to a final product of about 34,000 daltons. The N-terminal sequence of the 44,000-dalton peptide is the same as that of the 34,000-dalton peptide. This shows that the small peptides are cleared from the C terminus. The easily reduced cysteines which had been labeled are removed in two steps, one at the first step of the cleavage and the remainder at the second step. The amino acid compositions of the 44,000-, 39,000-, and 34,000-dalton polypeptides have been obtained. The difference between p44 and p39 is the composition of the first peptide released, and the difference between p39 and p34 is the composition of the second peptide released (Table 6). The first peptide released is extremely hydrophilic, much more hydrophilic than the protein as a whole. The second peptide released is very hydrophobic and contains large numbers of leucine, isoleucine, and valine residues. The hydrophobic region is likely to be inserted in the membrane, whereas the hydrophilic region is likely to be internal (although a hairpin structure is not excluded by the data available).

Do HL-A Antigens Have a Four-chain Structure?

Since immunoglobulins have a four-chain structure (i.e., two heavy chains and two light chains), it seemed reasonable, in view of the other indications of homology, to ask whether or not HL-A antigens also existed in this form. A number of observations in several laboratories, including our own (Strominger et al. 1974; Peterson et al.

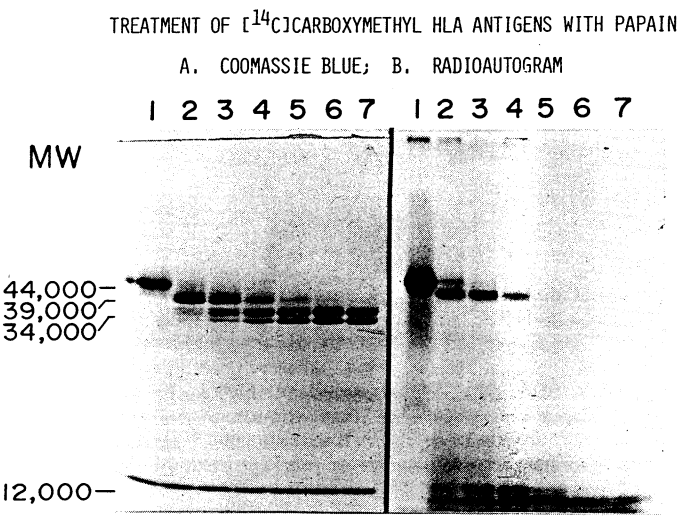

Figure 1. SDS slab gel electrophoresis of [^{14}C]carboxymethyl HL-A antigen. Antigen was digested with the indicated ratios of papain to protein: *(1)* none; *(2)* 1:3840; *(3)* 1:1920; *(4)* 1:960; *(5)* 1:480; *(6)* 1:240; *(7)* 1:120. Each aliquot was divided into halves and electrophoresed on two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

Table 6. Amino Acid Composition, Residues/Molecule

Amino acid	Δ p44–p39	Δ p39–p34
CM-Cys	0.3	0.6
Asp	8	1
Thr[a]	5	0
Ser[a]	5	5
Glu	8	0
Pro	4	2
Gly	4	4
Ala	6	1
Val[c]	0	6
Met[b]	0	1
Ile[c]	0	6
Leu	0	7
Tyr[b]	2	0
Phe	2	3
His	0	0
Lys	2	2
Arg	4	0
Trp	N.D.	N.D.
Polarity[d]	63.7	20.7

Determinations are rounded to the nearest integer, except for CM-Cys. (N.D. indicates not determined.)
[a] Extrapolated to zero time values.
[b] Twenty-four-hour values.
[c] Seventy-two-hour values.
[d] The polarity of p44 was 49.2; of p39, 46.7; and of p34, 50.7.

1975; Cresswell and Dawson 1975; Strominger et al. 1976), indicated that dimers and oligomers of the two-chain unit could occur. What is the significance of these observations? Is the polymerization an artifact that occurs after isolation of the HL-A antigen, i.e., did heavy chains in the preparation become disulfide-linked to each other during isolation? Two kinds of experiments have been carried out to examine that possibility (Springer et al. 1976b). If, indeed, the antigens are present in a four-chain structure in the membrane before solubilization, then a chemical cross-linking reagent should cross-link the chains in various ways forming either heavy-chain dimers or light-chain dimers in addition to light–heavy-chain products. The only product obtained with cross-linking reagents contained a light chain and a heavy chain. In another set of experiments, cells were treated with iodoacetamide (IAA) to block all the free—SH groups before isolating the antigens. Under these conditions, little or no oligomer was present in the isolated HL-A antigens (Fig. 2) (Springer et al. 1976b). Both of these experiments seem to suggest that HL-A antigens do not exist in the membrane as disulfide-linked oligomeric forms. The possibility remains that they nevertheless may be associated as dimers or higher oligomers by noncovalent interactions.

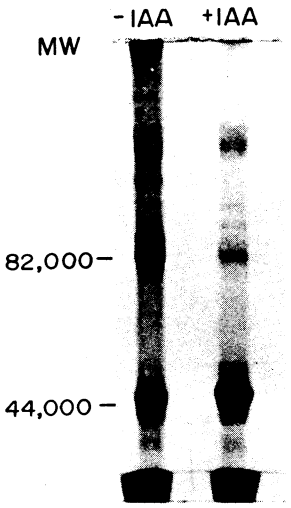

Figure 2. SDS gel electrophoresis of HL-A prepared in the presence and absence of IAA. Aliquots of the preparation were electrophoresed on Laemmli 7.5% polyacrylamide-SDS gels in the absence of mercaptoethanol (ME).

SUMMARY

From these data, a model was prepared which summarizes schematically our present knowledge of the structure and orientation of the HL-A antigenic molecule in the lymphocyte membrane (Fig. 3). It seems likely that the heavy chain spans the membrane, with the hydrophobic region inserted in the membrane and the hydrophilic C-terminus inside the cell. This C-terminal region bears one (possibly two) SH residue which has the potential for forming interchain disulfides. Whether or not these are actually formed physiologically remains an interesting question. There is the at-

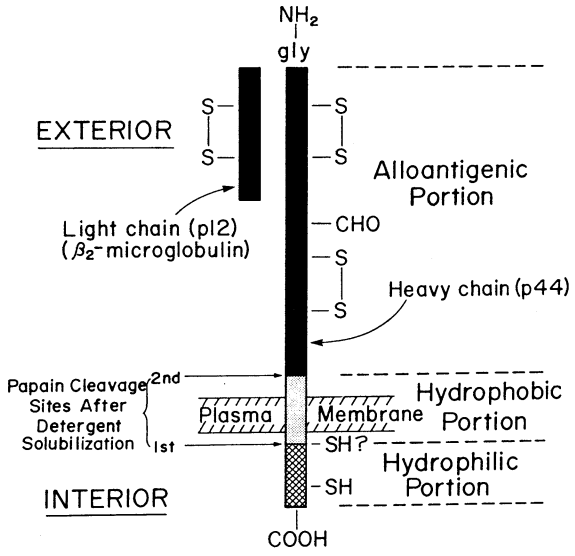

Figure 3. Arrangement of HL-A A,B antigen in the membrane with the hydrophilic peptide hypothetically shown inside the cell membrane. The position of β_2-microglobulin relative to the alloantigenic portion of the heavy chain is not known. The possible presence and position of a second sulfhydryl, shown near the first papain cleavage site, is unclear. It may be present on only some HL-A specificities, or the exact position of papain cleavage relative to it may vary.

tractive possibility that whatever the physiological functions of HL-A antigens are, structurally these molecules provide the potential for signaling from outside the cell to inside the cell because they span the membrane. It is even conceivable that this function might be expressed via the opening and closing of disulfide bridges.

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