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The Structure of Products of the Major Histocompatibility Complex in Man

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Introduction

The major histocompatibility complex (MHC) is a genetic region which encodes the major barrier to transplantation in man and in all other species which have been studied. 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. HLA is the human analog 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 seventeenth mouse chromosome) contains genetic regions HLA-A, HLA-B and HLA-C (analogous to the H2-K and H2-D loci in the mouse). There are multiple alleles 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) poses the most intriguing questions about this system. This paper will deal mainly with the products of the HLA-A and HLA-B loci. Little is known about the products of the HLA-C locus at present. The products of the HLA-D locus are defined by the mixed leucocyte culture (MLC) and are probably the human analog(s) of the mouse Ia antigen(s); some information about the probable product of this locus will also be presented.

The products of the HLA-A and HLA-B genes are measured using specific alloantisera, the major source of which is pregnancy antisera, i.e., sera from women who have been multiply pregnant and who have been immunized against paternal antigens. Indeed, one of the most interesting, incompletely solved problems in the field is the reason 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 Cr^{51}-labeled lymphocytes of appropriate specificity. Soluble antigen is measured by its ability to inhibit this lysis by combination with antibody.

Purification of HLA Antigens from Human Lymphocytes

A number of attempts had been made to purify HLA antigens from human lymphocytes, notably by Sanderson and Batchelor (1) who used splenic lymphocytes. Very small amounts were obtained from this source. Cultured lymphoblasts appeared to be a preferable source, both because they were available in potentially larger amounts than human spleens and because an experiment with a single human spleen cannot be repeated. However, cultured lymphoblasts are available in reproducible supply and do not seem to have any altered HLA specificities. Remarkably,

Table 1. Purification of papain-solubilized HLA antigens from 100 gm of JY cells

Purification step	Protein (mg)	Total number of inhibitory units recovered		Specific activity (inhibitory units/mg)		Purification
		HLA-A2	HLA-B7	HLA-A2	HLA-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 dilution	360	956,000	288,000	2,560	800	∿6
4. Sephadex G-150 chromatography	26	780,000	236,000	30,000	9,100	∿70
5. DE-52 chromatography	4.1 (HLA-A2) 4.9 (HLA-B7)	900,000	216,000	220,000	44,000	∿300

these lymphoblasts also contained far more HLA antigens than splenic or peripheral blood lymphocytes. This was shown by absorption experiments with HLA antisera using three types of cells, all from the same individual (2). Peripheral blood lymphocytes from RH had a very low absorptive capacity for these antisera. PHA-stimulated lymphocytes had a greatly enhanced capacity and RH lymphocytes transformed by EBV and growing continuously in cultures had an enormously enhanced absorptive capacity for HLA antisera, i.e. the representation of HLA antigens on the surface of the cultured lymphocyte transformed by EBV was in the range of 20-50-fold greater than peripheral blood lymphocytes; all four of the HLA 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 2-3-fold, 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 which already had an enhanced representation. The explanation is not known but the fact that the HLA antigens are so much more densely represented on the cultured lymphoblasts has made possible their isolation in relatively large amounts.

Preparation of the HLA Antigens after Papain Solubilization

Two principle methods have been used for solubilization of HLA antigens. These are: (a) treatment with papain and (b) solubilization with detergent. Preparation of HLA from the cell line JY after papain solubilization is shown in Table 1. The use of papain to solubilize HLA antigens goes back to the pioneering studies of Shimada and Nathenson (3) who first solubilized H-2 antigens in this way. The procedure for purification of HLA has been improved considerably over that previously reported (4) and yields of about 4-5 mg per 50 gm batch can now be obtained. The procedure for isolation is not difficult, requiring only four steps. The most interesting feature is that only about 70-fold purification was needed to obtain pure HLA antigen from cell membranes, i.e., something in the order of 1-2 % of the total membrane protein in the cultured lymphoblast is HLA antigen. That is a very large representation

of a single protein on the lymphocyte membrane surface. The cells used were homozygous for HLA-A2 at the first histocompatibility locus and for HLA-B7 at the second. HLA-A2 has a charge difference which distinguishes it from most of the other specificities. It is readily separated on DEAE-cellulose chromatography from HLA-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 HLA 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 which is the JY cell line. HLA-A2 and a mixture of HLA-B7,12 have previously been obtained from the cell line RPMI 4265 (4).

Our first interesting finding was that these antigens contain two subunits (5, 6): 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). SDS gels of the HLA-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 HLA-B7,12 mixture showed a doublet at 34,000 molecular weight in addition to β_2-microglobulin. One of the doublet glycoproteins may be HLA-B7 and the other HLA-B12. However, since these gels are denaturing gels, the glycoproteins could not be recovered to prove that point.

Despite the apparent purity of the HLA antigen preparations, isoelectric focusing revealed considerable heterogeneity (4). For example, in the HLA-A2 preparation at least four bands with HLA-A2 antigenic activity were seen. The most interesting possible interpretation of this heterogeneity was that there was heterogeneity in the amino acid sequence and, therefore, that some kind of V region might exist in the HLA antigens. However, the heterogeneity turned out to be due to variability in the number of sialic acid residues on the molecule (7). The HLA-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 preparation which was sialic acid-free was obtained and all the heterogeneity disappeared. The same result was obtained for other HLA preparations.

Are these antigen preparations really pure? To approach that question, the HLA antigen preparations were labeled by reductive methylation with formaldehyde and sodium borohydride (8). About two methyl groups/mol were introduced on the ε-amino groups of lysine of the molecule. This treatment did not alter immunological activity at all. Essentially 100 % of these labeled preparations of HLA-A2 antigens formed a specific complex with HLA-A2 antisera. No significant complexation was observed with normal serum or with specificity controls (HLA antisera with specificities other than HLA-A2).

However, when the HLA-B7,12 preparation or another HLA antigen preparation containing HLA-A3, AW25, B12 and B27 were used, only about 70 % of the total antigen could be complexed (8). With the HLA-B7,12 preparation, about 40 % complexation was obtained with HLA-B7 antiserum, 30 % with HLA-B12 antiserum and a total of 70 % with a mixture of antisera. What does that mean? The residual 30 % of material could be the product of the HLA-C locus or other unidentified HLA antigens, denatured antigens, or some other unrelated material co-purifying with HLA.

Table 2. Amino acid composition of papain solubilized HLA2 and HLA7+12 from cell line RPMI 4265 and papain-solubilized HLA2 and HLA7 from cell line JY in mol/100 mol. Each analysis was done in duplicate for 24, 48 and 72 h

Amino acid	$HLA2_{JY}$	$HLA2_{4265}$	$HLA7_{JY}$	$HLA7+12_{4265}$
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
CMCys	1.5	1.6	1.6	1.7
Trp[d]	3.2	3.2	3.1	3.2

[a] Ammonia not determined. [b] Extrapolated zero-time values. [c] 72 h value only. [d] Determined spectrophotometrically.

Another evidence of purity is the single common amino terminal group found in the HLA antigen preparations (8). 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. The analyses of four preparations of the heavy chains of HLA 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 HLA-A2 from JY and HLA-A2 from RPMI 4265 cells. Very small differences between HLA-A7 and the HLA-B7,12 mixture were found. However, on the order of 20 to 30 amino acid differences between HLA-A2 and HLA-B7 may be estimated. The degree of relatedness of these proteins can be examined from their amino acid analyses by a statistical method described by Marchalonis and Weltman (9). In this method, none of several thousand unrelated proteins had $S\Delta Q$ values of less than 100. A $S\Delta Q$ value of less than 50 was therefore considered to suggest a significant relatedness between the proteins. By this method, HLA-A2 from JY was identical to

Table 3. Relatedness among HLA antigens and β_2-microglobulin as determined from the amino acid compositions by the SΔQ method

Protein	HLA2 (4265)	HLA2 (JY)	HLA7 (JY)	HLA7+12 (4265)	β_2-M (human)	β_2-M (mouse)
$HLA2_{4265}$	0					
$HLA2_{JY}$	1	0				
$HLA7_{JY}$	13	16	0			
$HLA7+12_{4265}$	14	18	4	0		
$\beta_2\text{-}M_{human}$	172	183	183	173	0	
$\beta_2\text{-}M_{mouse}$	138	147	153	141	70	0

HLA-A2 from 4265 cells (Table 3). HLA-A2 was very closely related to HLA-B7 or to HLA-B7,12. However, the heavy chains of the various HLA 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 larger SΔQ values. The lowest SΔQ was obtained in comparison with IgD heavy chain but it was in the order of 70 (the same value as was obtained in comparison of human β_2-microglobulin with either mouse β_2-microglobulin or with the Fc fragment of Eu myeloma protein (an IgG)). There appears to be a relatedness of the heavy chain of the HLA antigens and the heavy chain of the IgD, but it may not be very extensive.

N-terminal sequence data (Table 4) (10) showed no differences between th two HLA-A2 antigen preparations. HLA-B7 and the HLA-B7,12 mixtures were very similar and the latter showed heterogeneity at only one position. However, there was only one difference in the first 25 amino acids between HLA-A2 and HLA-B7, although there may be only in the order of 20-30 amino acid differences 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 HLA is homologous to the heavy chain of immunoglobulin then one would expect four half cystine residues in a molecule of 29,000 molecular weight. Using [^{3}H]carboxymethyl cysteine-labeled HLA antigens in sequence studies up to 40 residues, well past where one would expect the first cysteine residue, no significant counts were found. This is beyond the place that one might expect to find the first cysteine residue if there was strong homology to immunoglobulins.

There are several important points which can be made from these data.

1. The products of the two loci (HLA-A and HLA-B) although separated by a distance equivalent to several thousand genes are remarkably similar to each other and they 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 HLA-A and HLA-B with that obtained in several laboratories for mouse H2-D and H2-K antigens reveals

Table 4. Partial amino acid sequence analysis of heavy chains from HLA antigens: HLA-2 (JY and RPMI 4265), HLA-7,12 (RPMI 4265) and HLA-7 (JY). Partial sequence analysis of the N-terminal residues was obtained in the Beckman 890B Automatic Sequencer. The sequences of the two HLA-2 preparations were identical

	Amino acid identified		
Step Number	HLA-2	HLA-7,12	HLA-7
1	Gly	Gly	Gly
2	Ser	Ser	Ser
3			
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

strong homologies between the human and murine antigens. These similarities are discussed in other papers in this volume and have recently been reviewed editorially (11) in connection with recent N-terminal sequence data for additional HLA antigens (12, and see also 12a). The latter are strikingly similar to those presented here.

. Several points which may indicate similarities between HLA antigen and immunoglobulins may be summarized: (a) Limited proteolysis by papain; (b) A two-chain structure with sequence homology between the light chain of HLA (β_2-microglobulin) and some heavy chain immunoglobulin domains (6, 13, 14, 15, 16). (c) However, from the compositional and sequence data available so far, there is little apparent homology between the heavy chain of HLA antigens and immunoglobulins. The cys-

Table 5. Purification of detergent solubilized HL-A from 150 g of J. Yoder cells

Purification step	mg	% Recovery of inhibitory units		Specific activity (inhibitory units/mg)		Fold purification
		HL-A2	HL-A7	HL-A2	HL-A7	
Detergent-solubilized membrane	750	100	100	330	140	(1)
Lectin column chromatography	111	85	83	1890	760	5.6x
Anti-β_2-microglobulin column	-	63	59	-	-	
Bio-gel A-5m column	7	54	50	19000	7300	55x

teine content, is however, relevant. The heavy chain of papain-solubilized HLA antigens is equal to two immunoglobulin domains in size. Like immunoglobulins there are two cysteine residues in intrachain disulfide linkage for each polypeptide "domain" of approximately 14,000 daltons. There are four half cystines for each polypeptide size of about 29,000 daltons in papain-solubilized HLA antigens and these half cystines are all present in intrachain disulfide linkages. Knowledge of the linkage and distribution of these 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 HLA Antigens After Detergent Solubilization

Most membrane proteins are solubilized by detergents which have 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 HLA antigens appeared similar to the solubilization of bacterial membrane proteins except for the fact that a group of relatively hydrophilic Brij detergents appeared to be relatively selective in solubilizing HLA antigens (18).

The purification of detergent-soluble material using an anti-β_2-microglobulin immunoabsorbent column is summarized in Table 5 (19). An earlier procedure (17) 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 required to obtain pure antigen was only about 50-fold over the detergent-solubilized membranes and the yields were on the order of 50 %. About 7 mg of HLA antigen were prepared from 150 g of cells. The anti-β_2-microglobulin column has also been used succesfully without the lectin column step (19). Alternatively, repeated agarose gel filtration after passage through the lectin column also yielded pure antigen and may be more applicable to large-scale work (18). The detergent-solubilized HLA antigens also contained two polypeptides, a heavy chain of 44,000 daltons and a light

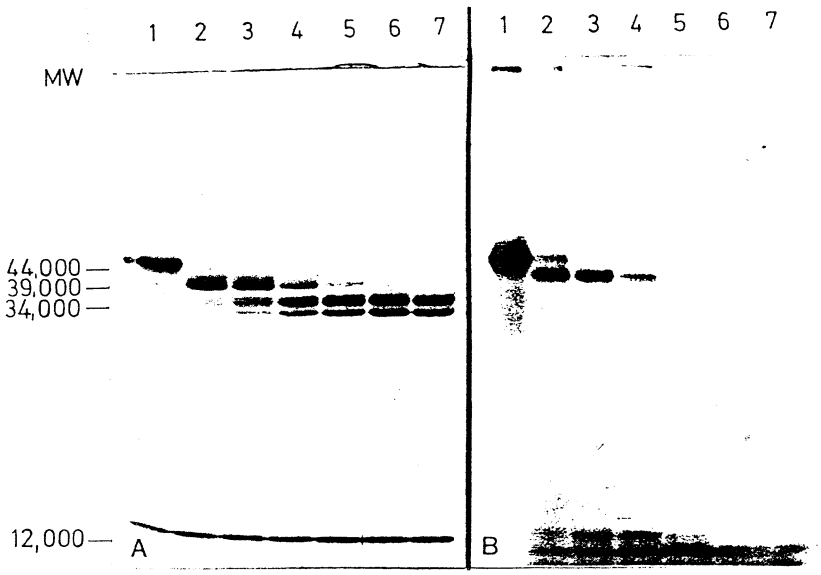

Fig. 1A and B. SDS slab gel electrophoresis of iodo-[^{14}C]acetate-labeled HLA antigen. Antigen was digested with the indicated ratios of papain to protein: 1: none; 2: 1/3840; 3: 1/1920; 4: 1/960; 5: 1/480; 6: 1/240; 7: 1/120. Each aliquot was divided into halves and electrophoresed on two sides of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

chain of 12,000 daltons, thus showing that the previously observed structure was not the result of proteolysis by papain.

The heavy chain of HLA 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 reduced form (and therefore not in intrachain linkage), 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 (17, 20). An intermediate of 39,000 daltons is first formed, followed by cleavage to a final product of about 34,000 daltons. 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

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. = not determined.

[a]Extrapolated to zero time values. [b]24-h values. [c]72-h values.
[d]The polarity of p44 was 49.2; of p39, 46.7; and of p.34, 50.7.

been obtained. The difference between p44 and p39 is the composition of the first peptide released, and the difference between the 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 contain large numbers of leucine, isoleucine and valine residues. The hydrophobic region is likely to be inserted in the membrane and the hydrophilic region is likely to be internal (although a hair-pin structure is not excluded by the data available).

Do HLA 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 HLA antigens also existed in this form. A number of observations in several laboratories including our own (21-24) indicated that dimers and oligomers of the two-chain unit could occur. What is the significance of these observation? Is the polymerization an artifact which occurs after isolation

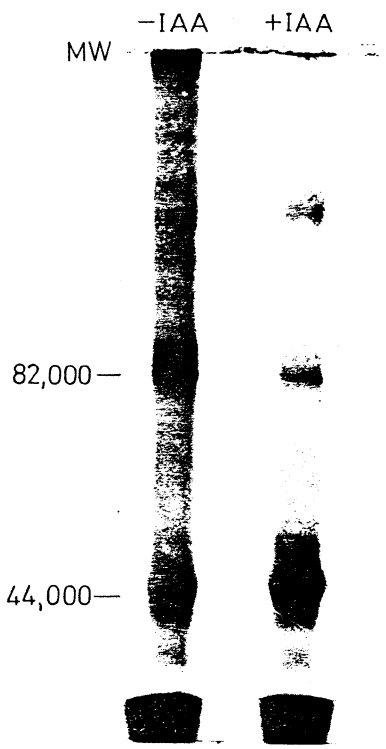

Fig. 2. SDS gel electrophoresis of HLA prepared in the presence and absence of iodoacetamide. Aliquots of the preparation were electrophoresed on Laemmli 7.5 % polyacrylamide SDS gels in the absence of ME

of the HLA antigen, i.e., did heavy chains in the preparation become disulfide-linked to each other during isolation? Two kinds of experiment to examine that possibility have been carried out (25). If, indeed, the antigens are present in a tetrameric structure in the membrane before solubilization, then a chemical cross-linking reagent should cross-link the chains in various ways, forming at least heavy-chain dimers and light-heavy dimers. The only product obtained with cross-linking reagents was a dimer containing a light chain and a heavy chain. A dimer containing two heavy chains was not formed. In another set of experiments cells were treated with iodoacetamide to block all the free SH groups before isolating the antigens. Under these conditions little or no oligomer was present in the isolated HLA antigens (Fig. 2) (20). Both of these experiments seem to suggest that HLA antigens do not exist in the membrane as disulfide-linked oligomeric forms. The possibility remains that they may nevertheless be associated as tetramers or higher oligomers by non-covalent interactions (for a discussion, see Ref. 25).

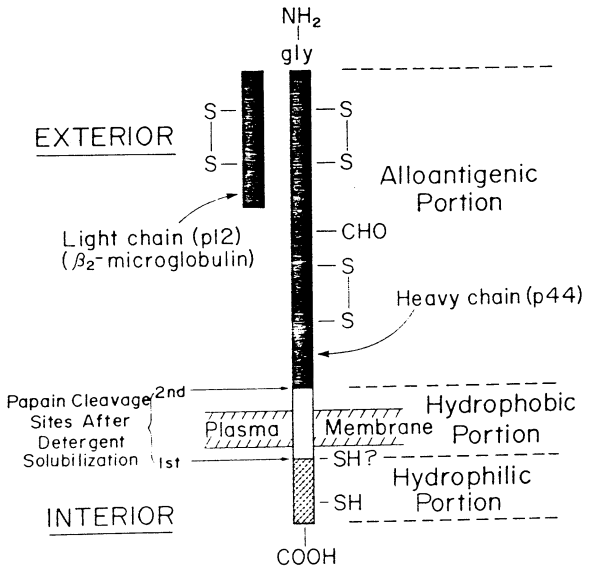

Fig. 3. Arrangement of HLA-A,B antigen in the membrane with the hydrophilic peptide hypothetically shown inside the cell membrane. The position of β_2-microglobulin relative to the alloantigenic portion of the heavy chain is not known. The possible presence and position of a second sulfhydryl, shown 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

Summary

From these data a model summarizing schematically our present knowledge of the structure and orientation of the HLA antigenic molecule in the lymphocyte membrane is shown in Figure 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 residues which have the potential for forming interchain disulfides. Whether or not these are actually formed physiologically remains an interesting question. There is the attractive possibility that whatever the physiological function(s) of HLA antigens are, these molecules provide structurally the potential for signaling from outside the cell to inside the cell because they span the membrane. It is even conceivable that this functio might be expressed via opening and closing of disulfide bridges.

B Cell-Specific Antigens from Human Lymphocytes

A second type of molecule which also has a two-chain structure and may be structurally related to HLA antigens has also been isolated. These materials are probably the products of the HLA-D genes and may be analogous to mouse Ia antigens. At an early stage of purification the HLA antigen preparations (obtained after papain solubilization) all contained impurities in varying amounts with molecular weights of 70,000; 30,000; 23,000 and 13,500. The latter was distinguishable from β_2-microglobulin. Detergent solubilized preparations contained analogous materials with molecular weights of 105,000; 34,000; 29,000 and 16,000

Papain-Solubilized B-Cell Antigens

These materials from a papain preparation were separated by careful gel filtration. In addition to the peak of HLA antigen, three addition

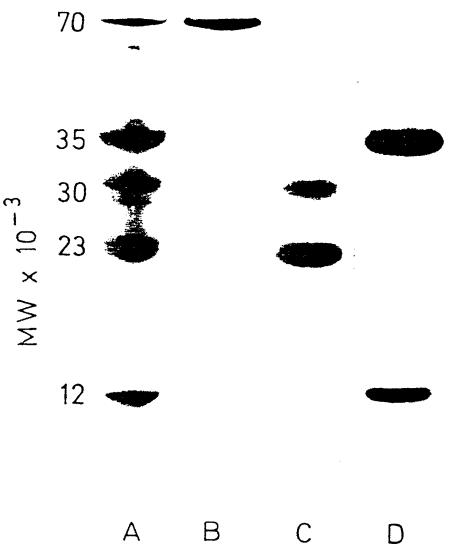

Fig. 4A-D. SDS-polyacrylamide gels of lymphocyte membrane proteins. (A) HLA antigen prepared with DTT preactivated papain containing "contaminants" of 70,000; 30,000 and 23,000 daltons. (B) Purified 70,000 daltons component. (C) Purified complex of 23,000 and 30,000 daltons components. (D) Purified HLA antigen containing 35,000 and 12,000 daltons components

peaks of protein were obtained which calibrated on the gel column at molecular weights of 50-70,000; 135,000, and in the excluded volume. The 50-70,000 molecular weight material was composed of two polypeptides with molecular weights of 23,000 and 30,000. The material which calibrated at a molecular weight of 135,000 on the Sephadex column was composed of apparently identical polypeptides of molecular weight 70,000 each. The material of very high molecular weight in the excluded volume of the column contained a single polypeptide of molecular weight 13,500, apparently highly aggregated. The purity of some of these preparations is illustrated by SDS gels (Fig. 4) (26).

Rabbits were immunized with all these preparations. The properties of the antisera which were obtained are extemely interesting. In this section we will focus on antiserum prepared to the material which contains polypeptides of 23,000 and 30,000 molecular weight. It had the following interesting properties:

Lysis of Peripheral Blood Lymphocytes and Lymphoblast Lines. Only a fraction of peripheral blood lymphocytes were lysed by the antiserum in complement-mediated cytotoxicity assays (Fig. 5). However, two B-cell lymphocyte lines, RH-1 and IM-1, were totally lysed at antiserum dilutions of 1:2000. When peripheral blood lymphocytes were separated into B, T and null cells, the T cells were not lysed at all; the B cells were completely lysed and a fraction of the null cells was lysed. In separate experiments with the null cell population, the population lysed by anti-p23,30 serum was found to bear the EAC rosette receptor and to cause the ADCC reaction (27). The null cell population which

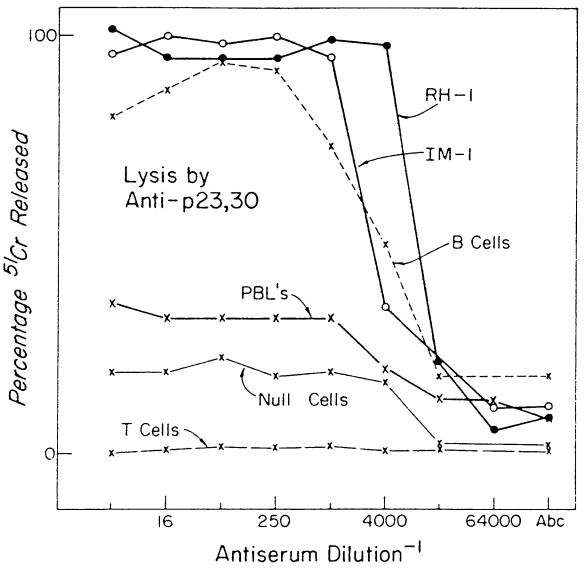

Fig. 5. Lysis of several purified populations of lymphocytes by anti-p23,30 rabbit sera. The IM-1 and RH-1 are lymphoblasts. Peripheral blood lymphocytes (PBLs) from one individual (RH) were separated into B, T, and Null lymphocyte populations

was not lysed by the anti-p23,30 serum did not bear the EAC rosette receptor and did not participate in the ADCC reaction. An anti-p23,30 serum blocked the ADCC reaction. By contrast, anti-β_2-microglobulin serum lysed all of these populations of cells.

Peripheral blood lymphocytes of 40 individuals were separated into T cells and B cells. The B cells of all of these individuals were lysed by the sera and, at the low dilution used (1:10), some of the T cells were also lysed. However, at 1:500 or 1:1000 the antiserum was absolutely specific for B cells of the separated populations. T-cell lines and B-cell lines established from the same individuals were also examined. Three such pairs of lines were available. Again, in each case only the B-cell line was lysed at high dilution (1:500). At low dilution some partial lysis of one of the T cell lines was observed.

Separation of Cells in the Fluorescence-Activated Cell Sorter. In the Herzenberg fluorescence activated cell sorter the difference between T, B and null cells was dramatically observed (26, 28). Cells were treated with anti-p23,30 serum and then with FITC-conjugated goat anti-rabbit IgG. The fluorescence-activated cell sorter yields data regarding both the number of cells and their relative fluorescence. A very high fluorescence was obtained with B cells, but no reactions was observed with T cells. A subpopulation of null cells was shown to react with the p23,30 antiserum.

Precipitation of Polypeptides from [^{35}S]Methionine Internally Labeled and Detergent-Solubilized Membranes. Another way of examining the specificity of these antisera is to radiolabel membranes and ask what polypeptides are precipitated. This question was of special interest with the p23,30 antiserum because it had been obtained by immunization with p23,30 polypeptides prepared after papain solubilization. The native form of the p23,30 complex might be different in the membrane. When radiolabeled membranes were solubilized in detergent and their extract treated with p23,30 antiserum, three polypeptides were observed in the precipitate with molecular weights of 39,000; 34,000 and 29,000.

Table 7. Inhibition by p23,30 of cytolysis of Amish cell lines by Amish antisera

Amish cell line	Cell source of p23,30 added	Amish antisera					
		35	76	192	289	590	RMB
KL	IM-1	+	-	-	+	-	+
	RPMI 4265	-	+	-	-	-	+
	JY	+	+	+	+	-	+
SL	IM-1	+	-	-	-	-	+
	RPMI 4265	-	+	-	-	-	+
	JY	+	+	+	+	-	+
PY	IM-1	+	-	-	+	-	+
	RPMI 4265	+	+	-	-	-	+
	JY	+	+	+	+	+	+
JY	IM-1	+	-	-	+	-	+
	RMPI	-	+	-	-	-	+
	JY	+	+	+	+	-	+

Apparently the p23,30 polypeptides originated from these. The precursor product relationship among these has not yet been elucidated.

Many HLA antisera contain additional antibodies specific for polypeptides other than HLA (obtained by double antibody precipitation from radiolabeled membranes). DAL (an HLA-A27 antiserum), MWS (a W28 antiserum) and BC (an HLA-A3 antiserum) all brought down a small amount of the 30,000 daltons polypeptide. BEL (an HLA-A27 antiserum) is very interesting because it contains an antibody directed against a 70,000 dalton component as well as an antibody directed against the 30,000 daltons component.

Using a rabbit anti-β_2-microglobulin serum only the 44,000 daltons heavy chain of the HLA antigens and the 12,000 daltons β_2-microglobulins were precipitated. No polypeptide corresponding to p39,34,29 were observed. At least as defined by this type of experiment, no polypeptide other than that of 44,000 daltons is associated with β_2-microglobulin in the human lymphocyte membrane.

Lysis of Various Amish Cell Lines by Amish Antisera and Its Inhibition by p23,30 Antigens. The p23,30 antigen has been obtained from three different cell lines: IM-1 (presently available as the purest of the preparations), RPMI 4265 and JY. JY is a member of the Indiana Amish community, a highly inbred human population, as mentioned previously. A number of cytolytic antisera from multiparous women of this community do not contain HLA antibodies but apparently have antibodies directed against other lymphocyte membrane components (29). These sera were used in cytotoxicity assays against four cell lines, also derived from the Amish population (Table 7) (26). The antisera used lysed all four of the cell lines. Several patterns are evident. First of all, JY p23,30 antigen inhibited lysis of the JY cell line by all of the antisera; the same was true of JY p23,30 antigens as an inhibitor of lysis of cells of his relative, PY. An interesting pair of antisera are 35 and 76. Antiserum 35, which lysed all four cell lines, was blocked by the IM-1 p23,30 antigen in each case. Antiserum 76, however, was not inhibited by the IM-1 p23,30 antigen but it was inhibited by p23,30 from RPMI 4265 cells. All of the data suggest that the p23,30 antigens

from IM-1, 4265, and JY cells are alloantigens; some of them inhibit some of the Amish alloantisera and others inhibit other Amish alloantisera. Antiserum RMB is interesting because it was inhibited by all three of the p23,30 antigens. Possibly it recognized a determinant common to all of them.

Use of p23,30 Rabbit Antisera in Purification of p23,30 Antigens. An interesting use of this antiserum is in following the p23,30 complex in a crude mixture, e.g., the separation of solubilized membrane proteins on a gel filtration column. The p23,30 complex, detected by inhibition of cytolysis, immediately preceded the HLA antigens. The p23,30 antiserum might also be useful in the purification of these materials by immunoabsorbent chromatography.

Similarities to Mouse Ia Antigens and Antisera. Several similarities of this antiserum to mouse Ia antisera, include the fact that it blocks the MLC reaction of human lymphocytes and their Fc receptors. The molecular weights of the two polypeptides of the antigen complex are also similar to those reported for mouse Ia antigens.

Detergent-Solubilized B-Cell Antigens

Similar materials have been separated from HLA antigens after solubilization of lymphocyte membranes with detergent (30). This material was present in a complex with a molecular weight of 55,000 which could be separated into two polypeptides with molecular weight of 29,000 and 34,000 in a 1/1 ratio. Rabbit antiserum prepared against the p55 complex (i.e., p29,34) had properties very similar to that prepared against p23,30.

Amino acid analysis of separated p29 and p34 revealed that these polypeptides were strikingly similar, and they yielded very low SΔQ values when compared by the statistical method (9). Similarly, low values were obtained when p44, the heavy chain of the HLA-A and HLA-B antigens was compared to p29 and p34 (Table 8). These data suggest not only that the p29 and p34 polypeptides are similar, but that there may be similarities of structure also to p44. Further comparison were made by radioiodinating the tyrosine residues of these polypeptides with lactoperoxidase and then preparing tyrosine-labeled tryptic peptide maps of the three polypeptides. P29 and p34 shared six major tyrosyl-tryptic peptides and they each also contained unique peptides. The number of peptides obtained was in agreement with the amino acid analysis showing that p29 and p34 contain eight and seven tyrosine residues respectively. A large number of the shared tyrosyl-tryptic peptides suggest that these peptides are highly homologous in structure. However, this homology does not extend to the amino terminus because N-terminal analyses of the two polypeptides (Table 9) (30) showed little similarity. P44 contained many unique tyrosyl-tryptic peptides not found in either p29 or p34, but it also contained one peptide which was in the same position in two-dimensional peptide maps as one of the labeled peptides shared by p29 and p34. These data are further suggestive of a low degree of homology between p44 and p29 or p34. However, much more extensive data will be required to prove this point.

It is clear from the present studies that gene duplication has been involved in the evolution of the heavy chain of the HLA-A and HLA-B antigens and also in the evolution of the two chains of the B-cell specific alloantigens. At least one of the latter polypeptides is a product of the HLA-D locus, though it is not clear whether or not the other polypeptides are also specified by genes in this region. If

Table 8. SΔQ values

	p12	p29	p34
p29	103		
p34	94	16	
p44	225	27	43

Table 9. N-Terminal sequences of human HLA-linked B cell antigens (cell line RPMI 4265) (from Springer et al. (30))

	1	5	10	15
p29	GLY-ASP-THR-PRO-	-	-PHE-LEU-GLU-GLN-VAL-	
p34	ILE-LYS-GLU-GLU(ARG)VAL-ILE-		-GLN-ALA-GLU-PHE-TYR-LEU-SER-	

further study confirms the suggestion that p44 and p29,34 are structurally related, then the intriguing question might be posed as to whether many of the genes in the major histocompatibility complex on the sixth human chromosome (many of which serve some kind of immunological or cell recognition function) could have arisen from duplication of one or a few primitive ancestral genes. It is hoped that further structural studies will help to shed light on the possible biological functions of these intriguing molecules as well as on their evolutionary relationships.

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Discussion

Dr. Rajewsky: Since you are comparing the products of the MHC with immunoglobulin: have you found antibody-like heterogeneity for any of the products?

Dr. Strominger: There are two ways to answer that question. The first thing to say is that the similarity to immunoglobulins would be that the HLA antigens correspond only to the constant region domains. That is, if one added a V region of 12,000 to each of the chains of HLA antigens, then one would have something which looked much more like an immunoglobulin. The HLA antigens would correspond to immunoglobulins minus V regions. The second point is that, although the 34,000 dalton polypeptide of the D locus product may be homogenous, the 29,000

dalton product is heterogeneous on isoelectric focusing and urea-Laemmli gel electrophoresis. It remains to be clarified further what that heterogeneity means.

Dr. Rajewsky: It may be useful to say again that the latter product would correspond to an I region product in mice, wouldn't it? And in the I region the Ir genes etc. are located.