The immunoglobulin-like structure of human histocompatibility antigens[1,2]

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In this paper we will summarize what is known about the structure of human histocompatibility antigens and try to emphasize those points which suggest the possibility that they may be structurally related to the circulating immunoglobulins. The major histocompatibility antigens are products of two genetic loci, the SD-1 and SD-2 loci in man, analogous to the H-2D and H-2K loci in the mouse. These genetic loci are highly polymorphic. There are in the order of 15 or more alleles presently known at each locus in man and, since the genes are expressed codominantly, each individual cell will bear four gene products arising from these two loci. Considerable success has been obtained in their purification. However, one of the major problems in the field is the separation of the allelic specificities from each other. A major goal in undertaking this work was to attempt to elucidate the chemical basis of the extreme polymorphism. This goal is still somewhat in the distance, but the materials to approach it are now available.

REPRESENTATION OF HISTOCOMPATIBILITY ANTIGENS ON CULTURED LYMPHOBLASTS

Initially, it was thought that only a few micrograms of pure human histocompatibility antigens might be isolated from human lymphocytes (i.e., splenic lymphocytes) after a great deal of effort. The fact that the representation of histocompatibility antigens on the surface of cultured human lymphoblastoid cells is greatly enhanced relative to peripheral lymphocytes has made it possible to isolate *milligrams* of highly purified human histocompatibility antigens (6). In absorption curves (Fig. 1), the amount of histocompatibility antigens is the reciprocal of the number of cells required to absorb 50% of the cytotoxicity of the antiserum. Peripheral blood lymphocytes have, in this type of experiment, 1/20th to 1/50th of the representation of histocompatibility antigens as lymphocyte lines established from the donor (RH) of the peripheral blood lymphocytes. This line was established by transformation with Epstein-Barr virus. Phytohemagglutinin stimulation of lymphocytes also increased the amount of human histocompatibility antigens. This increase was specific since two other membrane markers (5′-nucleotidase and total iodinatable surface protein) were increased in amount in the transformed cell only about twofold, the same as the increase in surface area. The increase in histocompatibility antigens applied to all four specificities of RH cells, HL-A 3, 28, 7, and 27 (Table 1). This increased representation on cultured lymphoblasts made possible the isolation of these antigens in relatively large amounts.

PURIFICATION OF HISTOCOMPATIBILITY ANTIGENS AFTER SOLUBILIZATION WITH PAPAIN

Several methods have been used to purify histocompatibility antigens. Nathenson and Davies (8) working with the H-2 system in the mouse discovered that autolysis released H-2 antigen from mouse lymphocytes. They later used papain as a more controlled means of proteolysis to release H-2 antigens. The purification of HL-A antigens from a cultured lymphoblastoid line (RPMI 4265: HL-A 2,2; 7, 12) after solubilization with papain is shown in Table 2 (15). The purification was not extremely difficult and required only three or four steps to obtain what is believed to be pure histocompatibility antigen. The amount obtained in early attempts was in the order of 1 mg/50 g of lymphocytes. The yield has been improved so that as much as 4 mg/50 g of lymphocytes (80 mg/kg) can be obtained. The overall purification required was slightly less than 100-fold, i.e., histocompatibility antigens represent 1–2% of the total membrane protein in cultured lymphocytes. They are a major protein component of the membrane.

Separation of specificities is a big problem. The last step in the purification, DE-52 chromatography, separated HL-A2 (which has unique charge properties distinguishing it from other histocompatibility antigens) from HL-A7, 12. HL-A 7 and HL-A 12 have not been separated from each other and, since this cell line is homozygous for HL-A2, a fourth specificity does not exist. The SDS gel of the purified HL-A 2 antigen revealed that it contained

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Abbreviations: SD, serologically defined; SDS, sodium dodecyl sulfate; Ia, I-region associated; PHA, phytohemagglutinin-stimulated; and PHAL, phytohemagglutinin-stimulated lymphocyte.

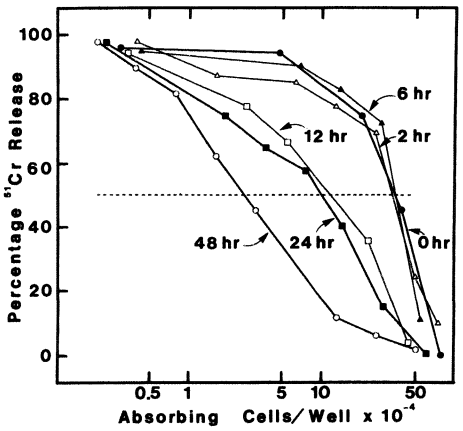


Figure 1. Comparison of absorptive capacity of peripheral blood (PBL), phytohemagglutinin-stimulated (PHAL), and Epstein-Barr virus-transformed (RH-1) lymphocytes for BC alloantisera (from donor BC) (6). The PHALs were cultured for 48 hr with 10 μg PHA/ml. Absorption endpoints (number of cells for 50% absorption) are indicated. The ratios are: PBL/PHAL = 13; PBL/RH-1 = 38.

two polypeptide chains, a discovery which was also made using radioactive antigens (Fig. 2) (3 and see also 14). One of these polypeptide chains was a glycoprotein which had a molecular weight of about 34,000; and the other polypeptide had a molecular weight of 12,000. In the HL-A7, 12 preparation two major glycoprotein bands around 35,000 molecular weight were seen in addition to the 12,000 molecular weight polypeptide. One of them may be HL-A7 and the other HL-A12 but, since they cannot be separated under nondenaturing conditions, there is at present no way of establishing this point.

Despite the apparent purity on SDS gels, the HL-A2 antigen and other preparations were heterogeneous in isoelectric focusing. HL-A2 activity appeared in at least four bands. There were a number of possible explanations for this heterogeneity, including the possibility that the histocompatibility antigens might contain a variable region. However, differences in extent of amidation of carboxyl groups or differences in the sialation of the carbohydrate chain of the heavy subunit could also account for it. The latter was in fact shown to be the correct interpretation; the heterogeneity was entirely due to variability in the number of sialic acid residues (9). Indeed the purification of histocompatibility antigens is considerably sharpened if the sialic acid residues are first removed. The preparation had an average of two sialic acid residues per mole but it consisted of a mixture of molecules with 3, 2, and 1 residues (Fig. 3). Treatment with neuraminidase resulted in a preparation that contained no sialic acid residue and was homogeneous in isolectric focusing. The microheterogeneity that had also been observed by others (5) is due to variability in the carbohydrate side chain.

What is the evidence for purity of the antigen preparations? The HL-A2 antigen preparation was labeled by reductive methylation with formaldehyde and sodium [^{3}H]borohydride, adding an average of about two [^{3}H]methyl groups per molecule to the lysine residues (10). Under those conditions, no immunological activity was lost. By gel filtration it could be shown that no immune complex was formed from this [^{3}H]HL-A2 antigen with normal serum or with a specificity control serum (HL-A8) but in the presence of several HL-A2 antisera all of the radioactivity was complexed, thus providing evidence of immunological purity of the preparation. Some evidence of chemical purity was also obtained. All of the HL-A antigen preparations examined had a single unique *N*-terminal glycine residue in the heavy chain in addition to the *N*-terminal residue of the 12,000 mol wt polypeptide (10).

PURIFICATION OF HISTOCOMPATIBILITY ANTIGENS AFTER SOLUBILIZATION WITH DETERGENT

So far two points of homology to circulating immunoglobulins have been described: *1*) The molecules are stable to papain proteolysis and immunological activity is not destroyed, even after prolonged treatment with papain; and *2*) Like the immunoglobulins, the histocompatibility antigens appear to contain both a heavy chain and a light chain. However, since HL-A antigens were prepared after papain proteolysis the question remained whether the two chains arose from cleavage of a single polypeptide during treatment with papain or whether there really were two

TABLE 1. Absorption of anti-HL-A alloantisera with PBL, PHAL, and RH-1 Cells

Specificity	Serum	Absorption endpoints, cells/well × 10^{-4}			Ratio of absorption endpoints	
		PBL	PHAL	RH-1	PBL/PHAL	PBL/RH-1
HL-A3	BC	34	2.6	0.9	13	38
W-28	MWS	14	1.5	0.8	9	18
HL-A7	SAN	15	1.2	0.4	12	38
HL-A27	DAL	21	1.8	0.4	11	52
				Averages	11[a]	36[a]

[a] These values correspond to an average sixfold increase in HL-A expression per unit surface area (Table 2) for PHALs relative to PBLs and an average 13-fold increase in HL-A antigen density on RH-1 cells relative to PBLs. PBL, peripheral blood lymphocytes; PHAL, phytohemagglutinin-stimulated lymphocytes; RH-1, Epstein-Barr virus transformed.

TABLE 2. Purification of papain-solubilized HL-A antigens from RPMI 4265 cells

Purification step	Protein, mg	Recovery of inhibitory units, %		Specific activity, inhibitory units per mg		Purification
		HL-A2	HL-A7	HL-A2	HL-A7	
Cell membrane	225	100	100	3,150	320	(1)
Papain digest	45	32	41	5,000	600	1.5–2
CM-52 chromatography		22	28			
Sephadex G-150 chromatography	6	22	28	25,800	3,330	8–10
DE-52 chromatography	0.5 (HL-A2) 0.5 (HL-A7,12)	16	14	253,000	20,000	60–80

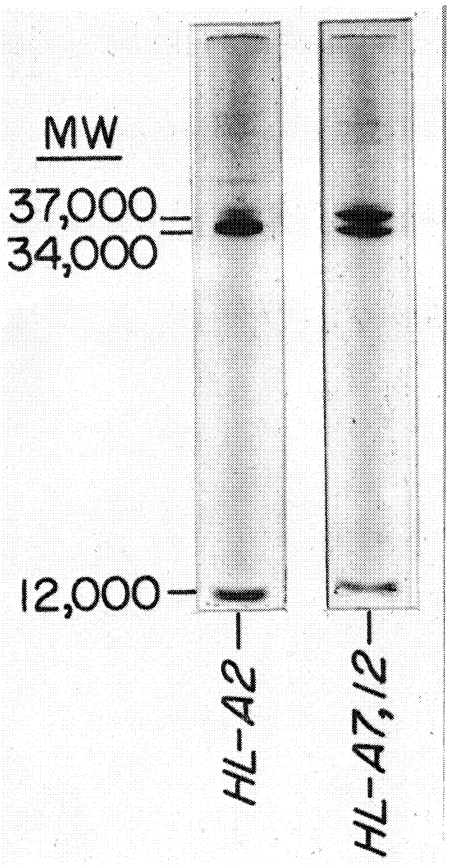
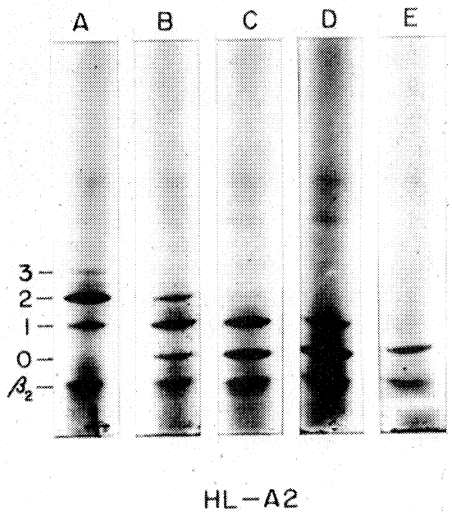


Figure 2. SDS gel electrophoresis (10% acrylamide) of purified HL-A2 and HL-A7, 12 preparations, stained with Coomassie brilliant blue. MW, molecular weight.

chains in the original antigen preparation. To answer that question and some others the purification of HL-A antigens after solubilization with detergents was undertaken. The detergents that solubilized membrane proteins from bacteria were usually in a hydrophobic-lipophilic balance range of 12–13.5. Some relatively hydrophilic detergents also appeared to solubilize HL-A antigens, perhaps selectively, from lymphoid cell membranes. Starting with that observation, the purification to homogeneity of the detergent solubilized HL-A antigen preparation has also been accomplished (12). The steps involve solubilization, lectin affinity chromatography, the use of an anti-β_2-microglobulin immunoabsorbant column and finally, filtration on an agarose 5M column (Table 3). Several polypeptides appeared to follow HL-A in various purifications steps, but the immunoabsorbent column removed them, leaving the heavy chain with a molecular weight of 44,000 and the smaller polypeptide (β_2-microglobulin) with a molecular weight of 12,000 (Fig. 4). Therefore, the two-chain structure is not the consequence of treatment with papain since it also occurs in the detergent solubilized molecule.

Figure 3. Isoelectric focusing gels in 6 M urea of HL-A2 antigen preparation treated with neuraminidase. Gel A shows the untreated antigen. Gels B–E show the result of increasing digestion with neuraminidase.



During papain proteolysis of the detergent solubilized molecule (12), the 44,000 mol wt glycoprotein was first degraded to a 39,000 mol wt intermediate and finally to the 34,000 mol wt glycoprotein which is obtained directly on papain proteolysis of membranes. Material totaling 10,000 mol wt was removed in at least two steps of 5,000 mol wt each. The light chain (β_2-microglobulin) appeared to be unaffected by papain.

IDENTIFICATION OF THE LIGHT CHAIN OF HL-A ANTIGENS AS β_2-MICROGLOBULIN

It was observed that anti-β_2 microglobulin serum precipitated from labeled lymphocyte membranes not only a 12,000 mol wt polypeptide, but also a polypetide of mol wt 44,000 (4). There were no other major polypeptides that were precipitated from detergent solubilized human lymphocyte membranes by anti-β_2-microglobulin serum. The anti-β_2-microglobulin serum was also used to make an immune complex which was purified on Sephadex G-150. When that immune complex was examined by SDS gel electrophoresis, it also contained 44,000 and 12,000 mol wt subunits. A variety of immunological and chemical techniques carried out in several laboratories (2, 7, 11) have shown that β_2-microglobulin and the small subunit of histocompatibility antigens are identical. For example, β_2-microglobulin antiserum was lytic for lymphocytes. This lysis was inhibited by β_2-microglobulin and in a quantitatively similar manner by either the papain or the detergent solubilized HL-A antigen preparations. β_2-Microglobulin was first isolated from human urine. It has been sequenced and has a strong homology to the domains of the circulating immunoglobulins, especially to the C_3H domain of IgG. The third point of homology to immunoglobulins is, therefore, the fact that the light chain of the histocompatibility antigens is β_2-microglobulin.

MULTIMERIC STRUCTURE OF HISTOCOMPATIBILITY ANTIGENS

The next question was whether, like IgG and other immunoglobulins, the histocompatibility antigens might exist as more than a two-chain structure. Are there disulfide bridges linking heavy chains and/or light chains? On the agarose column, using a high affinity fraction from the lectin column, HL-A antigenic activity ap-

TABLE 3. Purification of detergent-soluble HL-A antigens from IM-1 cells

Purification step	Protein, mg	Recovery of inhibitory units, %		Specific activity inhibitory units per mg		Purification
		HL-A27	HL-A3	HL-A27	HL-A3	
Cell membrane	2,400	100	100	11	6	(1)
Detergent: solubilization and lectin column	81	23	34	84	50	8
Anti-β_2-microglobulin immunoabsorbent column		11	11			
Agarose 5M chromatography	2	5	4	640	250	40–60

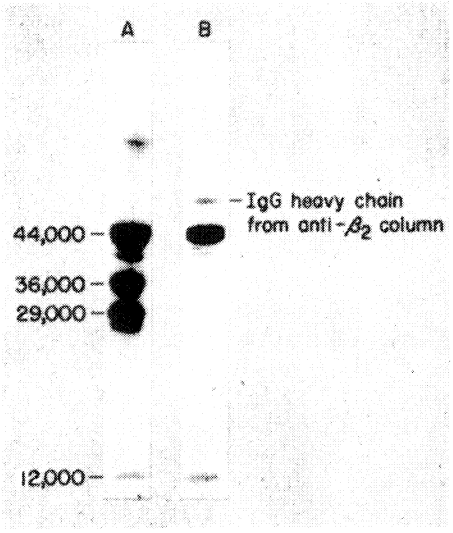
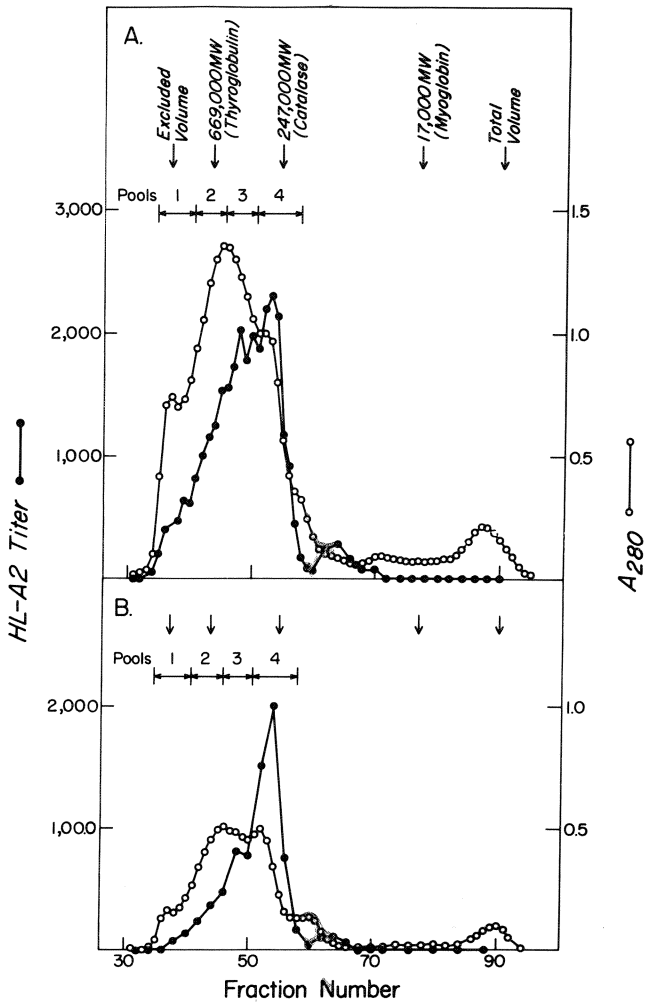


Figure 4. SDS gels of HL-A antigens from IM-1 cells (a cultured lymphoblastoid cell line) before *A*) and after *B*) chromatography on an anti-β_2-microglobulin immunoabsorbent column.

peared to "tail" toward the high molecular weight end (13). Several pools were made as indicated (Fig. 5), radioiodinated, and then subjected to double antibody precipitation using specific HL-A antiserum and goat anti-human IgG. When this was done with the low molecular weight pool, peaks of 44,000 and 12,000 were obtained whether or not the material had been reduced (Fig. 6). In the intermediate pool, both a dimer of the heavy chain (mol wt 85,000) and a higher multimer were present in addition to monomer and β_2-microglobulin. In the high molecular weight pool virtually no labeled monomer (mol wt 44,000) or dimer (mol wt 85,000) were present; most of the material was in a very high molecular weight form which on reduction gave only the 44,000 mol wt subunit. These multimeric forms could also be seen by two-dimensional gel electrophoresis (Fig. 7), the first dimension of which was an SDS gel *without* mercaptoethanol and the second dimension of which contained mercaptoethanol. Any protein that is not interchain S-S linked appeared on a diagonal line, while S-S linked dimers and higher multimers lay below this diagonal.

The HL-A antigens appear to exist both as a two-chain structure with one heavy and one light chain and also as higher multimers of this basic unit. One important question is whether the dimerization or polymerization occurred during isolation or existed in the membrane. Are the S-S bridges artifacts? A similar experiment was carried out by Cresswell and Dawson (1) using direct precipitation from solubilized membranes without purification. They obtained similar results. Therefore, if the dimerization is an artifact, it occurs very quickly on detergent solubilization of these molecules.

Figure 5. Agarose 5M filtration of pools from lectin column. The high affinity fraction is above, and the low affinity fraction is below.



INTRACHAIN AND INTERCHAIN S-S BRIDGES IN HL-A ANTIGENS

A preliminary study of S-S bridges in the heavy chain of the molecule has been carried out (13). From the sequence work carried out in several laboratories, it was known that there was one intrachain S-S bridge in β_2-microglobulin. This S-S bridge provided a useful reference standard. In the heavy chain obtained after papain cleavage of three different preparations, there were four half-cystine residues involved in intrachain disulfide bridges, showing that there must be two intrachain disulfide bridges for each heavy chain. Since each heavy chain consists of an amount of polypeptide equivalent to two immunoglobulin domains (estimated polypeptide mol wt of about 22,000 (3)), these two intrachain bridges may be located in the molecule in such a way that there is one of them in each of the putative domains of the heavy chain.

Are there also interchain S-S bridges? There are no free SH groups in the molecule and there are no interchain S-S bridges in the papain product. However, the detergent product contained two additional half-cystine residues which were easily reduced and could be involved in interchain bridges (13). These putative interchain bridges must be

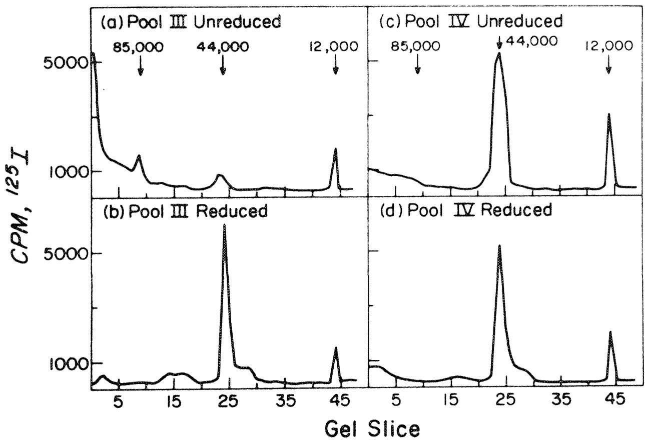


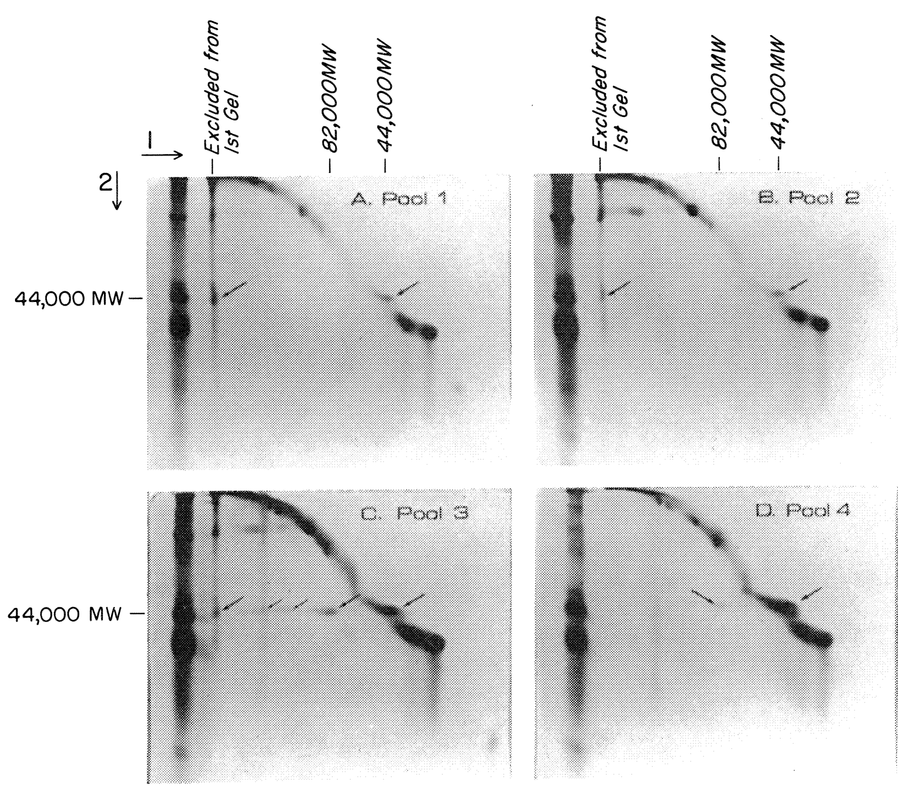
Figure 6. SDS gels of immunoprecipitated [^{125}I]HL-A from 4265 cells.

present in that part of the molecule that was removed by papain, i.e., presumably in the hydrophobic region of the molecule located in the membrane.

To summarize, the structure of HL-A antigens could be represented as shown in Fig. 8. β_2-Microglobulin is a monomer with one intrachain S-S bridge. The heavy chain of the papain solubilized antigen has a polypeptide chain that is twice as large and it contains two intrachain S-S bridges. There is, in addition, a third domain of molecular weight about 10,000 which is removed under the conditions of papain proteolysis and which appears to contain two additional SH residues. Since there is evidence that these molecules may be present as dimers or even as higher oligomers, it can be speculated that the dimerization is brought about by disulfide bridges located in the hydrophobic portion of the molecule. The formation of higher oligomers could be brought about utilizing the second SH group which may be present in the hydrophobic region of the molecule.

If the HL-A antigens are homologous in structure to immunoglobulins, one might try to think of what kind of homologous function they might have. Perhaps, the association of variable and constant regions in circulating immunoglobulins was a late evolutionary event, conceivably necessitated by the secretion of this molecule into the circulation of some higher organism. At an earlier stage of evolution, these molecules may have been membrane-bound and associated with some other molecule by noncovalent bonds. Possibly the HL-A antigens also are associated in the membrane with other molecules by noncovalent interactions. One candidate for such molecules would be the Ia gene products. Therefore, one very interesting question to ask is whether in the membrane of lymphoid cells there are any other polypeptides with which HL-A antigens are associated by noncovalent bonds. Might such polypeptides be homologs of the variable regions of the circu-

Figure 7. Two-dimensional SDS gels of agarose column pools (Fig. 5). Pools from the high affinity fraction were used. SDS gels were run without mercaptoethanol in the first dimension and with mercaptoethanol in the second. Arrows indicate materials of 44,000 mol wt (MW) in the second dimension.



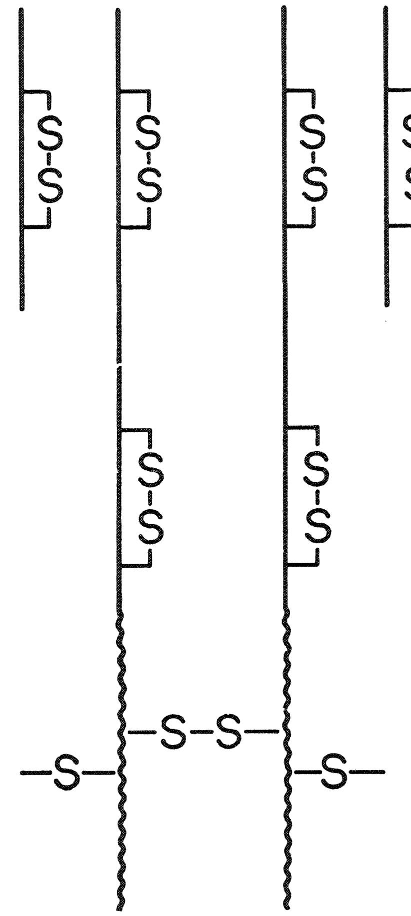


Figure 8. Possible structure for HL-A antigen.

lating immunoglobulins? In any case it seems possible that histocompatibility antigens and immunoglobulins have evolved after duplication of some common primordial ancestral gene. Further studies of their structure should clarify this point as well as many other interesting features of these molecules. FP

Note added in proof: Recently the amino acid composition and *N*-terminal sequences of four HL-A antigens have been elucidated, including products of both of the major histocompatibility loci (C. Terhorst, P. Parham, D. Mann and J. L. Strominger, *Proc. Natl. Acad. Sci. USA* (in press) March, 1976). The products of the two loci (first locus: HL-A2; second locus: HL-A7 and HL-A12) are strikingly similar to each other in so far as the *N*-terminal sequences have been elucidated, although they differ significantly in amino acid composition. They also have considerable homology to mouse H2 antigens, the structures of which are being worked on in several other laboratories. The presently available sequence data on the heavy chain of the four HL-A antigens (25 residues) indicates little homology with human immunoglobulins unless a large number of deletions are inserted. Moreover, no half cystine was found in the first 40 residues. Therefore, many more data will need to be collected to examine the possible homology to the sequence of immunoglobulins. With regard to the dimeric structure, it is noteworthy that pretreatment with iodoacetamide of membranes resulted in the isolation of HL-A antigens which were not cross-linked by S-S bridges (R. Robb, unpublished), and experiments with chemical cross-linking agents did not support the idea that two heavy chains may be located near each other in the structure (T. Springer, unpublished). These data suggest that the dimerization or polymerization of the HL-A antigens may largely occur after their solubilization.

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