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The Immunoglobulin-Like Structure of Human Histocompatibility Antigens

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Serologically defined histocompatibility antigens which govern allograft acceptance in man and other higher animals were described in humans about 15 years ago. In man these HL-A antigens (Human Leucocyte Antigens) are the products of two closely linked genetic loci. They have been detected in all tissues of the human body. Because they are expressed codominantly, all cells of a heterozygous individual bear four specificities. They are the products of the two well established 'LA' and 'Four' loci. Some evidence for the existence of a third serologically defined locus has been presented, but nothing is presently known about the chemistry of its products.

Early attempts to purify and characterize these antigens have been reviewed (1). Considerable progress has been made possible by: 1) The development of a serological inhibition assay (based on HL-A antibody and complement-dependent lysis of ^{51}Cr-labelled lymphocytes) which made possible the rapid quantitation of minute amounts of antigen (2, 3), 2) the discovery that these antigens could be solubilized by proteolytic enzymes, especially papain, while retaining virtually all of their immunological activity (4, 5), 3) the use of non-ionic detergents to solubilize a native form of the antigen, and development of a means of assaying solubilized in-

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hibitory activity in the presence of detergent (6, 7), 4) preparation of radioactive HL-A antigens and isolation of radioactive immune complexes (8), and 5) the development of cultured human lymphoblastoid lines (9) which provide a reproducible source of large quantities of HL-A antigens. In fact, the discovery that these cultured cells contain 20–50 times as much HL-A antigen as peripheral blood or spleen lymphocytes has greatly facilitated the purification of these antigens. In the present review we will describe the efforts which have been made in our laboratories to obtain pure HL-A antigens and to investigate their structure.

PURIFICATION OF HL-A ANTIGENS AFTER SOLUBILIZATION WITH PAPAIN

Two independent studies in our laboratory led simultaneously to the discovery that the HL-A antigens purified from cultured lymphoblastoid cells after solubilization with papain contained two polypeptide chains (8, 10). First efforts were made to obtain radioactively labelled HL-A antigens from cultured lymphoblast cells grown in the presence of radioactive amino acids or carbohydrates. These molecules appear to be in an active metabolic state and are rapidly resynthesized after removal from the surface of intact cells with papain (11). After methods were developed for incorporation of maximum radioactivity the surface proteins were solubilized with papain. After a preliminary purification on a Sephadex G-150 column the radioactive HL-A antigens present in this mixture were treated with specific HL-A antisera to form immune complexes. These specific immune complexes could then be separated from the other solubilized materials on Sephadex G-150 by virtue of their considerably higher molecular weight. Analysis of these radioactive immune complexes by SDS gel electrophoresis, by chromatography on agarose columns in the presence of 6 M guanidine hydrochloride or by gel electrophoresis in 8 M urea revealed that all of the complexes contained two polypeptide chains, one of molecular weight 34,000 which contained both amino acids and carbohydrates, and one of approximately 12,000 which contained only amino acids (Figure 1). The small polypeptides from different HL-A specificities have been found to be identical by the techniques which have been so far employed, but the large glycopeptide units of different HL-A specificities differ from each other in electrophoretic mobility (8, 12). The immunological specificity, therefore, presumably resides in the 34,000 molecular weight glycopeptide.

At the same time, efforts were made to purify HL-A antigens from cultured human lymphoblastoid cells (10, 13). This material offered the advantage of availability in relatively large amount and in reproducible supply of the identical histotype as compared with earlier efforts to obtain

these materials from individual human spleens. They were furthermore greatly aided by the discovery that cultured human lymphoblastoid cells contained a representation of HL-A antigens on a per cell basis of 20–50 times that found in normal peripheral blood lymphocytes, or a 10–25 fold increase when account is taken of the larger surface area of cultured cells as compared to peripheral lymphocytes (Figure 2) (14). Moreover, the preparation of homogenous membranes from these cells has greatly simplified the preparation of pure HL-A antigens. The procedure employed was essentially that described earlier for their preparation from human spleen (5, 15), but it yielded purer materials. Three different preparations have been obtained from two cell lines. From the cell line RPMI 4265 (HL-A 2, 7, 12) a preparation of HL-A 2 and a preparation of HL-A 7, 12 were obtained. From the cell line IM-1 a preparation of HL-A 3, 10, W25, 27 was obtained. In each case, the final product, like the radioactive material, contained two polypeptides, with molecular weights 34,000 and 12,000 in equimolar amounts and was free of other materials (Figure 3). The yields have been surprisingly high, of the order of 1–1.5 mg of pure HL-A antigens being obtained from 50 gms of cells. In the case of RPMI 4265 cells the yields were about 16 per cent in the overall purification, and the amount present initially may have been about 6 mg per 50 gms or 100–150 mg per kg of cells. It has also been shown in the case of the HL-A 2 antigen preparation that this material is immunochemically pure,

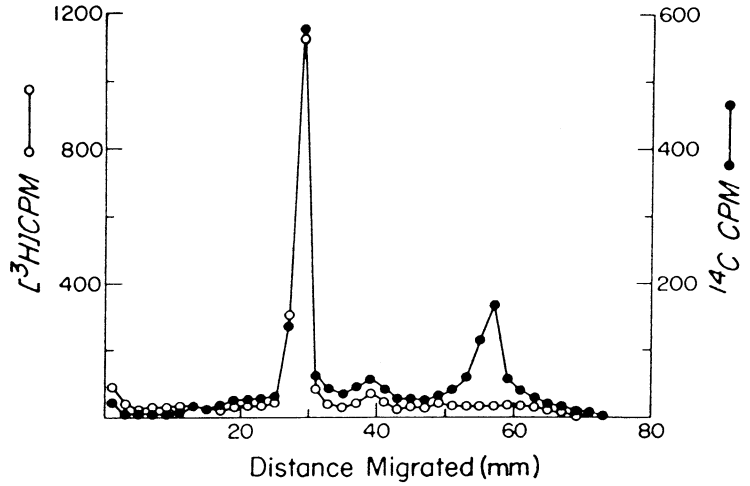

Figure 1. SDS gel (10 per cent acrylamide) electrophoresis of a mixture of [^{3}H]glucosamine-labelled and [^{14}C]aminoacid-labelled HL-A7 (Jackson) complexes (8). The two subunits are shown.

i.e., that all of the HL-A 2 antigen (which had been tritiated by reductive methylation) would form a complex with specific HL-A 2 antisera (Figure 4). Similar studies are now being carried out with the other antigen preparations.

A second question has to do with microheterogeneity in these preparations which had been observed on isoelectric focusing in this and other laboratories (16). Charge heterogeneity was also suggested by the broad activity peaks observed on chromatography on DEAE-cellulose. One possible source of microheterogeneity was the occurrence of variable regions in the polypeptide chain such as occurs in immunoglobulins. However, it has now been shown that the heterogeneity is due entirely to the presence of molecules containing different numbers of sialic acid residues and that it is entirely lost upon removal of sialic acid residues with neuraminidase (17) (Figure 5). This procedure may be of great importance in sharpening separations of molecules bearing different HL-A specificities, e.g. preparations from the IM-1 cell line.

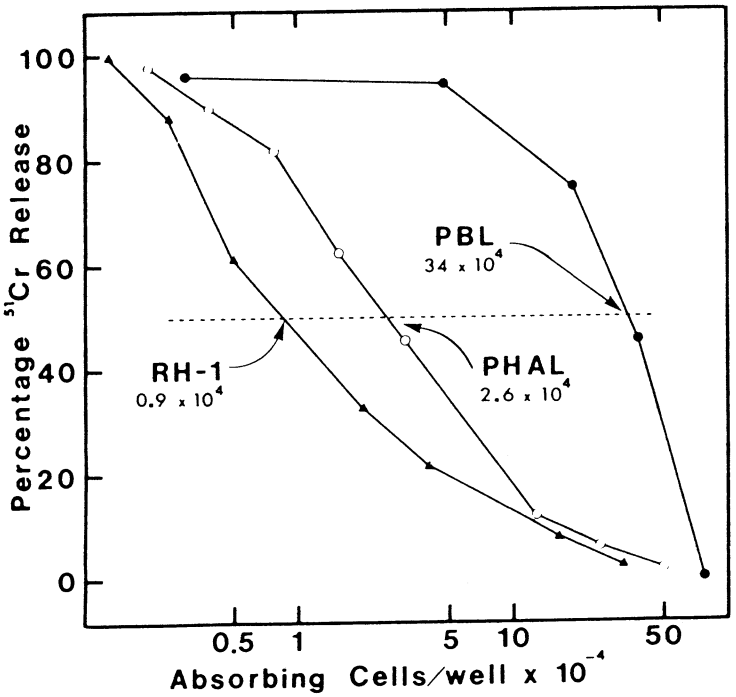

Figure 2. Comparison of absorptive capacity of peripheral blood (PBL), phytohemagglutinin-stimulated (PHAL), and EBV-transformed (RH-1) lymphocytes for BC alloantisera (HL-A3) (14). PHAL's were cultured for 48 hr with 10 μg PHA/ml. Absorption endpoints (number of cells for 50 per cent absorption) are indicated. The ratios are: PBL/PHAL = 13; PBL/RH-1 = 38.

Preparation of HL-A antigens after solubilization with detergent

A second approach to the purification of HL-A antigens involved solubilization from membranes with detergent and subsequent purification. An important first step in accomplishing this objective was to develop a method for assaying antigenic activity in the presence of detergent since detergent

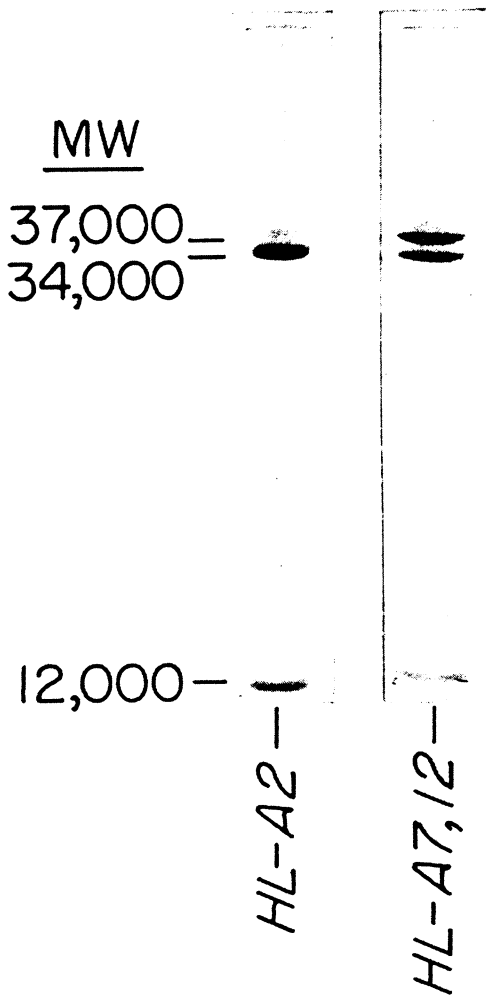

Figure 3. SDS gel electrophoresis (10 per cent acrylamide) of purified HL-A2 and HL-A7, 12 preparations, stained with Coomassie brilliant blue (10).

itself lyses the [^{51}Cr] labelled lymphocytes employed as target cells in the cytotoxicity assay. This assay was accomplished simply by adsorption of detergent on bovine serum albumin prior to assay of HL-A inhibitory activity (7). A partial purification of the HL-A antigenic activity has been obtained, roughly to 50 per cent purity. With this material it was possible

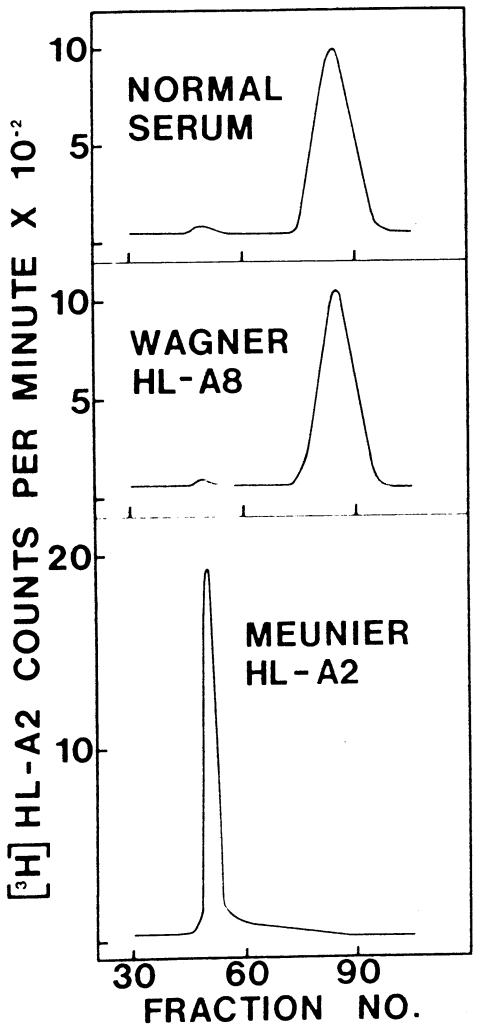

Figure 4. Immunological purity of HL-A2 antigen. The preparation was labelled by reductive methylation with formaldehyde and [^{3}H]-$NaBH_4$. A specific HL-A2 antiserum (Meunier) complexed 100 per cent of this material. Specificity controls (Wagner HL-A8 and normal serum) are also shown. Gel filtration was carried out on Sephadex G-150. The immune complex is excluded from the gel. (P. Parham, unpublished).

to establish that the detergent solubilized HL-A antigen contained two polypeptide chains of 44,000 and 12,000 molecular weight. This finding was of considerable importance since it established that the two polypeptide chains found in the papain product were not the consequence of proteolysis by papain but were both constituents of the native molecule found in the membrane. It was moreover shown that the 44,000 molecular weight protein was hydrolyzed by papain in two steps yielding a 39,000 molecular weight intermediate and a final product of 34,000 molecular weight identical to the product obtained by papain treatment of cell membrane directly (Figure 6, 7). Efforts to obtain a homogeneous product from the detergent soluble preparation are in progress.

Identity of the small subunit of papain or detergent solubilized HL-A antigens with β_2-microglobulin

β_2-microglobulin is a small protein first isolated from the urine of patients with cadmium poisoning. It has a moderate degree of sequence homology

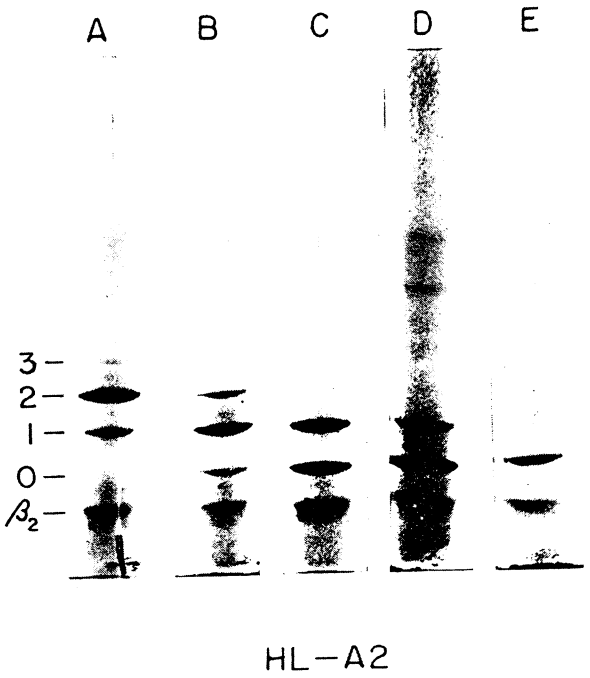

Figure 5. Isoelectric focusing gels in 6 M urea of HL-A2 antigen preparation treated with neuraminidase (17). Gel A shows the untreated antigen. Gels B–E show the result of increasing digestion with neuraminidase.

to constant region domains of immunoglobulin polypeptide chains, and moreover contains the same intrachain disulfide linkage as is found in each of the domains of immunoglobulin molecules (for references, see other papers in this issue). Several experiments have shown that the small subunit of the HL-A antigens is β_2-microglobulin (18, 19). Initially lymphocytes were radiolabelled with ^{125}I by the lactoperoxidase catalyzed reaction and the membrane proteins were solubilized with the detergent Nonidet P-40. Double antibody immunoprecipitation was performed on these lysates with rabbit anti-β_2-microglobulin serum and goat anti-rabbit gamma globulin as the coprecipitating agent. The precipitates were reduced, alkylated and subjected to electrophoresis on SDS gels. Two polypeptides were observed,

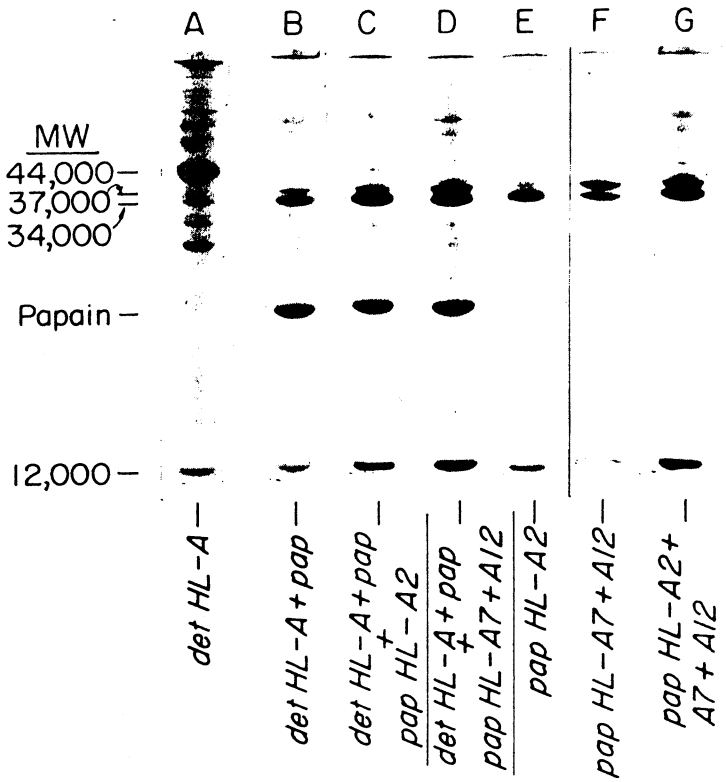

Figure 6. Identity of detergent-soluble HL-A antigen digested with papain and HL-A antigen solubilized from membranes with papain (7). Detergent-soluble HL-A antigen (8 μg) was run on SDS-6 M urea Laemmli 12 per cent gels with no papain (pap) digestion (A) or after digestion with 1/10 papain (B, C, D). HL-A antigen solubilized with papain from membranes and purified (5 μg of either HL-A2 (C, E, G) or a mixture of HL-A7 and A12 (D, F, G) was run either alone or mixed with the above as indicated on the figure.

one with molecular weight about 44,000 and the other with molecular weight of about 12,000. It therefore appeared that membrane-bound β_2-microglobulin was associated with a larger polypeptide of the same molecular weight as the large glycopeptide of detergent-solubilized HL-A antigens.

That this anti-β_2-microglobulin precipitated complex was in fact HL-A antigen was shown by the following experiments. Immune complexes were formed from ^{3}H-labelled papain digests of cultured lymphocytes with *anti-β_2-microglobulin* serum. All of the specific HL-A antigens in the papain digest of RPMI 4265 cells (HL-A 2, 7 and 12) were present in this immune complex. The amount of immune complex was about 4 times that formed with any individual HL-A antiserum. SDS gels of immune complexes formed with anti-β_2-microglobulin serum showed the same 34,000 and 12,000 molecular weight polypeptides as were found in immune complexes formed with HL-A antisera. Further quantitative immunological data for the identity of the small peptide of HL-A antigens with β_2-microglobulin included: 1) the lysis of peripheral blood lymphocytes by anti-β_2-microglobulin sera and the inhibition of this lysis by HL-A antigens, 2) the inhibition by HL-A antigens of precipitation of [^{125}I]-β_2-microglobulin with rabbit antiserum to β_2-microglobulin and 3) double diffusion in agar. These

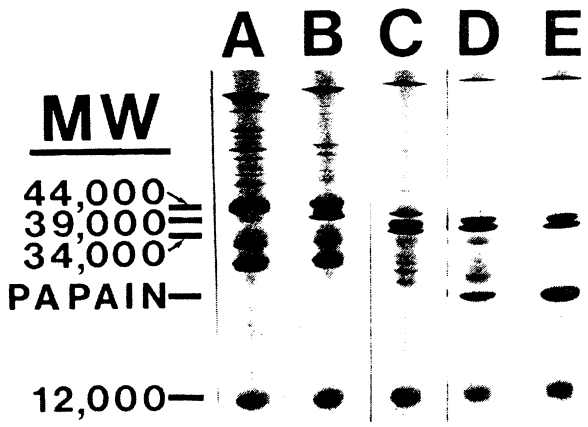

Figure 7. SDS gels of detergent-soluble HL-A antigens purified through lectin affinity chromatography and its papain digestion products. Protein was digested with 0, 1/1000, 1/100, 1/10 or 1/3 ratios of papain to protein in gels A-E, respectively. The 39,000 MW intermediate product (7) is shown.

data indicated virtually total cross reactivity between the small subunit of HL-A antigens and β_2-microglobulin. Moreover, these two small polypeptides were identical by SDS and SDS-urea gel electrophoresis and by isoelectric focusing. Data obtained in two other laboratories led to the identical conclusions (20, 21).

A dimeric structure for the HL-A antigen complex

The fact that the small subunit of HL-A antigens was β_2-microglobulin and the moderate sequence homology between β_2-microglobulin and immunoglobulins, including the presence of an intrachain disulfide bridge in β_2-microglobulin, thus led to the speculation that HL-A antigens might have an immunoglobulin-like structure and therefore, that the molecule might be composed of 4 polypeptide chains, two of the heavy glycopeptide (44,000 MW in the detergent solubilized molecule or 34,000 MW in the papain solubilized molecule) and two of the small polypeptide (β_2-micro-

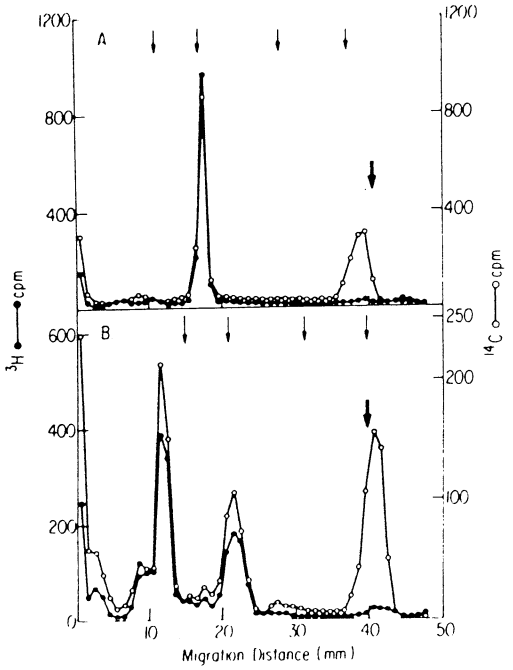

Figure 8. SDS gel electrophoresis of radioactive immune complexes obtained with anti-β_2-microglobulin serum from detergent solubilized membranes of IM-1 cells. (22). The labels were [^{3}H]glucosamine and [^{14}C]amino acids.
Above: 9 per cent acrylamide + 5 per cent 2-mercaptoethanol.
Below: 7.5 per cent acrylamide, *no 2-mercaptoethanol.*

globulin). Several kinds of experiments have led to the conclusion that this is indeed the case. Earlier preparations of HL-A antigens were made under conditions which might be expected to dissociate such a dimeric structure, including the presence of reducing agents, moderate salt concentrations, and the use of ion exchange chromatography. Several experiments have been carried out in which these conditions were avoided.

Lymphocytes were grown with radioactive amino acids or sugars. Their membranes were solubilized in 0.5 per cent sodium deoxycholate. Immune precipitates were then prepared using rabbit anti-β_2-microglobulin serum and goat anti-rabbit IgG. The precipitates were washed and applied to SDS gels in the presence or in the *absence* of 2-mercaptoethanol (which is normally employed in the SDS gel technique). In the presence of the reducing agent only the two polypeptides, MW 44,000 and 12,000, which had been observed previously, were found. However, in the absence of reducing agent, an additional polypeptide appeared with a MW of about 82,000 (Figure 8) (22). This band was cut out from a parallel gel, eluted,

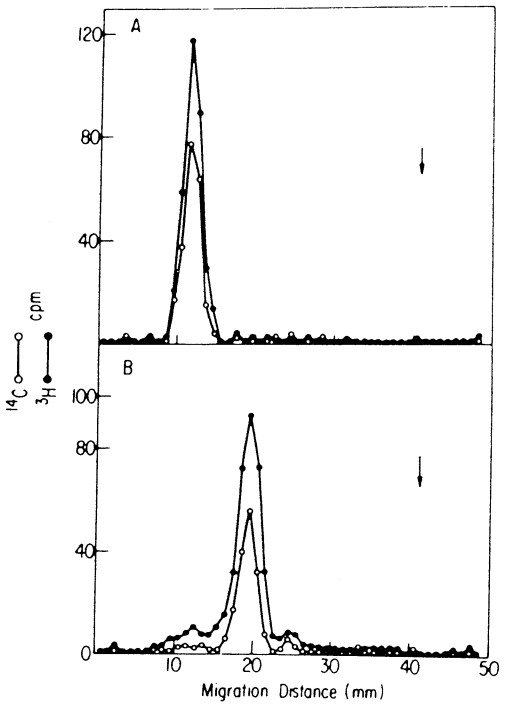

Figure 9. SDS gel electrophoresis (7.5 per cent acrylamide) of the 82,000 MW peptide (from a parallel gel to that of Figure 8) in the absence (above) or presence (below) of 2-mercaptoethanol (22).

and half of it was rerun on an SDS gel under non-reducing conditions; the other half was rerun under reducing conditions (Figure 9). It was clear that the 82,000 MW polypeptide was reduced by 2-mercaptoethanol to the 44,000 MW material.

At the same time, during purification of detergent solubilized HL-A antigens as described previously, it was observed that a second peak of HL-A antigenic activity appeared at a higher MW on agarose columns than that reported previously (see Figure 1 in (7)), and in an amount approximately equivalent to the lower molecular weight. When this material was analyzed by SDS gel electrophoresis in the absence of reducing agents, no 44,000 MW polypeptide was found as had been observed previously (7). Instead an 82,000 MW deriver and higher multimers were present. The 82,000 MW band was cut from a stained gel and rerun in the presence and in the absence of reducing agent. Again, the polypeptide of MW 82,000 was reduced to one of MW 44,000 (Figure 10) (23). Therefore, two independent lines of investigation led to the conclusion that at least some of the

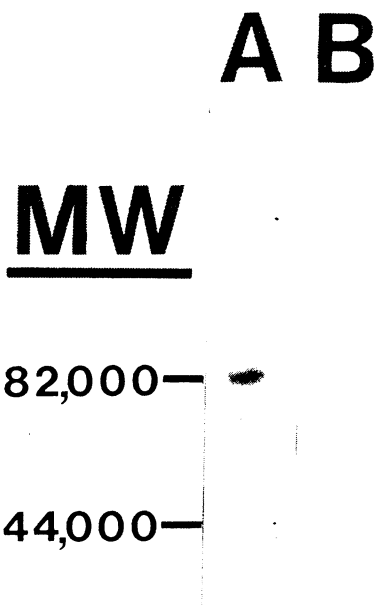

Figure 10. SDS 8 per cent Laemmli gels showing disulfide-bonded dimers (23). High MW HL-A antigenic activity was electrophoresed and the 82,000 MW band was cut out of Coomassie blue stained gels and rerun either + N-ethylmaleimide (A) or + 2-mercaptoethanol (B).

HL-A antigen present in cultured human lymphocytes and solubilized with detergent was present in a form in which the heavy polypeptide chain was found as a dimer which would be reduced to a monomer with mercaptoethanol, presumably a disulfide linked dimer. No evidence has been found that the light chain (β_2-microglobulin) is linked to the heavy chain in this manner; indeed the structural studies of β_2-microglobulin show that there is no free sulfhydryl group to form such a linkage.

The next question was whether the putative disulfide linkage was also present in the papain solubilized product since evidence for small amounts of a higher molecular weight form of the papain solubilized HL-A antigen had been observed on Sephadex G-150 chromatography. Conditions were found (low salt and the absence of reducing agents, including the employment of preactivated papain in the digestion) in which virtually all of the HL-A antigen was present in a position on an Agarose 15 M column which calibrated at a molecular weight of 96,000 (a value suggesting the presence of two heavy chains and two light chains in the molecule) (Figure 11) (23). However, SDS gel chromatography of this material in the presence or absence of reducing agent indicated that it contained only a heavy chain of 34,000 MW and no evidence of a disulfide-linked dimer was obtained. This result was confirmed by measurements of disulfide bridges in the

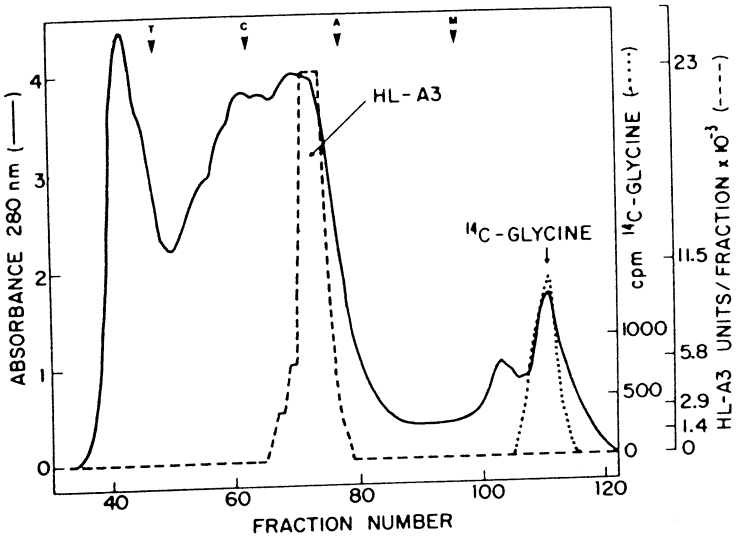

Figure 11. Agarose 15 M gel filtration of supernatant of IM-1 membrane digestion with preactivated papain, eluted with 0.01 M Tris-C1 buffer, pH 8.0. The molecular weight of the HL-A activity peak is about 96,000 as judged by calibration standards: T, thyroglobulin, C, catalase, A, serum albumin, and M, myoglobin (23).

TABLE I

Estimation of total half cysteine content of the heavy chain of HL-A antigen derived by papain cleavage (23)

HL-A antigens were treated with ^{14}C monoiodoacetic acid in the presence of 6 M guanidinium/HCl and a 200-fold excess of dithiothreitol. The number of residues was calculated from the ratio of cpm in the heavy chain and β_2-microglobulin after SDS-gel electrophoresis, assuming equimolar amounts of the two chains and one intrachain disulfide bridge in β_2-microglobulin.

Preparation	Number of residues Half-cystine*/heavy chain
HL-A 2_{pap}	3.6
HL-A 7, 12_{pap}	4.1
HL-A 3, 10, W25, 27_{pap}	4.3

* Since there are no interchain disulphides in the papain product (Table II), these half-cystines must all be present in two intrachain disulfides per 34,000 MW glycopeptide.

various preparations (see below). Thus the papain solubilized HL-A antigen may also be a molecule composed of two of the 34,000 MW glycopeptides and two of the 12,000 MW polypeptides (β_2-microglobulin). However, these polypeptides are associated entirely by noncovalent bonds which may be readily dissociated into monomers (composed of one heavy and one light chain), probably by the presence of high salt.

The presence of disulfide bridges in detergent and papain solubilized HL-A antigens

None of the HL-A antigen preparations obtained with papain contained any free sulfhydryl groups. However, the presence of intrachain (but no interchain) disulfide bridges has been demonstrated using ^3H- and ^{14}C-monoiodoacetic acid and other similar reagents (Tables I and II) (23). No free SH groups were exposed to the reagents under mild reducing conditions (30 fold excess of dithiothreitol); this result excludes the presence of interchain disulfide bridges. However, on denaturation with 6 M guanidine hydrochloride and reduction with a 200-fold excess of DTT each of the preparations of the papain solubilized HL-A antigens was found to contain two intrachain disulfide bridges in each of the 34,000 MW glycopeptides (Table I). These glycopeptides are composed of a polypeptide of molecular weight about 22–24,000 and a glycan. Thus, the glycopeptide contains one intrachain disulfide bridge for each 11–12,000 MW, i.e. one disulfide bridge for each length of molecule corresponding to an immunoglobulin domain.

TABLE II

Estimation of the free SH groups and interchain S-S bridges in the heavy chain of HL-A antigens (23)

A. HL-A antigens were treated with ^{3}H-monoiodoacetic acid in 6 M guanidinium/HCl and, after extensive dialysis, they were treated with ^{14}C-monoiodoacetic acid in the presence of 6 M guanidinium/HCl and a 200-fold excess of dithiothrejtol. The ratio of pmoles incorporated in the heavy chain was calculated after SDS electrophoresis. In this experiment ^{3}H measures free SH groups and ^{14}C both intra- and interchain disulfides.

B. HL-A antigens were treated with ^{3}H-monoiodoacetic acid in the presence of a 30-fold excess of dithiothreitol. Labelling with ^{14}C-monoiodoacetic acid and estimation of the ratio of $^{3}H/^{14}C$ were then carried out as described above. In this experiment ^{3}H measures free and interchain SH groups and ^{14}C intrachain disulfides.

Preparation	A* pmoles ^{3}H/pmoles ^{14}C MIAA	B** pmoles ^{3}H/pmoles ^{14}C
HL-A 7, 12_{pap}	0.07	0.04
HL-A 2_{pap}	0.03	0.03
HL-A 3, 10, W25, 27_{pap}	0.09	0.10
HL-A 2, 7, 12_{det}	n.d.	0.52
HL-A 3, 10, W25, 27_{det}	n.d.	0.48

* Ratio: free-SH groups/total-S-S-bridges.

** Ratio: (free-SH groups + interchain-S-S-bridges)/intrachain-S-S-bridges.

+ Since there are a minimum of two intrachain disulfide linkages (four half-cystines) per 44,000 MW polypeptide in the detergent product (measured by ^{14}C), from the ratios shown there are at least two additional half-cystines per 44,000 MW polypeptide available for interchain disulfide bridges (measured by ^{3}H).

Studies of the detergent solubilized product are not yet complete but the data which have been obtained indicate that there are at least two free sulhydryl groups in each molecule of heavy chain exposed under mild reducing conditions (30 fold excess of DTT) in the absence of denaturing agents (Table II). These conditions are characteristic of those used for reduction of interchain disulfide bridges in immunoglobulins, and thus the detergent solubilized HL-A molecule (in contrast to the papain solubilized molecule) contains a minimum of two interchain disulfide bridges for each molecule.

Proliferation of HL-A antigens during mitogenesis

The proliferation of HL-A antigen was studied during transformation of normal peripheral lymphocytes by phytohemagglutinin and by the Epstein-Barr virus (14). In lymphocytes stimulated by PHA the increase in HL-A

antigen content began at 6–12 hours and a 10-fold increase was found at 48 hours. A 30-fold increase was found in cells transformed by the Epstein-Barr virus (Figure 2). The early proliferation induced by PHA in particular raises the question of whether this proliferation is related to the immune function of these cells.

DISCUSSION

These data including the limited sites of cleavage by papain are strikingly similar to those found for immunoglobulins and taken together they indicate that the HL-A antigens have an immunoglobulin-like structure. Much remains to be done to establish this securely, of course. The positions of the intrachain disulfide bridges are of great importance, as is of course the sequence of amino acids in the heavy chain of the HL-A antigens and its possible homology to sequence of immunoglobulins. If further studies bear out the homology of structure, then it seems likely that both HL-A antigens and immunoglobulins would have evolved from common ancestral genes. If they have diverged structurally, one may wonder how they have diverged functionally. They may both serve an immunological defense function. The circulating immunoglobulins provide a means of defence against invading foreign substances, such as bacteria, and perhaps the HL-A antigen serving some kind of surveillance function against invasion by foreign cells, e.g. tumor cells arising in the organism, or even by viruses. However, attempts to find some haptenic group (e.g. in viruses) to which HL-A might bind have so far been unsuccessful. Other possible functions are in cell-cell interactions, during differentiation, or in the B and T cell association in the immune response.

From the data which have been obtained it is unlikely that HL-A antigens contain variable regions as are found in immunoglobulins. Thus, each of the polypeptides is smaller than the IgG peptides by about one domain (12,000 MW). One could imagine that the HL-A antigens are associated in the membranes with some kind of variable region, perhaps linked to HL-A by non-covalent bonds and separated during the various precipitation and purification procedures that have been employed. Possible candidates for such variable regions which could be associated with HL-A antigens are Ir and Ia gene products which are closely linked genetically to the structural genes for HL-A antigens. Perhaps the covalent association of the constant and variable regions of immunoglobulins was a late evolutionary event, necessitated by the step in which these substances became secretory proteins and thus circulated in an aqueous environment rather than being localized in the membrane.

Among animal proteins the immunoglobulins and HL-A antigens are unique for the high degree of polymorphism which has been maintained in the population. This polymorphism must have a survival value for the species. In any case further structural studies of the HL-A antigens and studies of their association with other membrane molecules will hopefully provide clues to the function of these extremely interesting molecules.

ABSTRACT

HL-A antigens are composed of two polypeptide chains. In the case of HL-A antigens solubilized with papain, these have molecular weights of about 34,000 and 12,000, while those solubilized with detergent have molecular weights of 44,000 and 12,000. The 12,000 MW polypeptide is identical to β_2-microglobulin. In the membrane the HL-A antigenic molecule appeared to contain two each of the heavy chain and the light chain. The two heavy chains are linked by disulfide bridge(s) which are located in the portion of the molecule removed after papain proteolysis. The two light chains (β_2-microglobulin) are associated with the heavy chains by tight noncovalent bonds. There is one intrachain disulfide bridge for each 11–12,000 MW of polypeptide in the heavy chain as well as in β_2-microglobulin. Thus all of the data are compatible with an immunoglobulin-like structure for the HL-A antigens, probably one in which only constant region domains are represented.

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