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24 February 1971

Salt Extraction of Soluble HL-A Antigens

Abstract. *Extraction of cultured human lymphoid cells with hypertonic salt solutions (3 molar potassium chloride) resulted in high recoveries of membrane-associated histocompatibility (HL-A) antigens in soluble form with potent activity and marked immunologic specificity. The active principle was purified by preparative acrylamide-gel electrophoresis. Application of the hypertonic salt extraction method is now yielding sufficient HL-A antigen to begin the elucidation of the molecular basis of transplantation individuality.*

The elucidation of the HL-A locus, which governs the major histocompatibility factors in human transplantation, stimulated a search for effective methods of solubilizing the corresponding cell membrane antigens (*1*). Soluble HL-A antigens have a number of applications: (i) for the pretreatment of graft recipients to deceive their immunologic system into accepting foreign tissues, as has been shown for animal systems; (ii) for production and standardization of potent monospecific tissue-typing alloantiserums; and (iii) for clarification of the relation between the HL-A locus and its gene products (*2*).

Several unique aspects make the solubilization of HL-A antigens now amenable to attack. (i) Hosts develop reactivity against foreign transplantation antigens resulting in an array of specific alloantiserums; (ii) HL-A determinants can be rapidly and sensitively detected by their ability to inhibit the cytotoxic reactions of these serums; and (iii) human lymphoblastic cell lines, derived from the peripheral lymphocytes of a normal donor and perpetuated in tissue culture, provide abundant source material with greater amounts of antigen per cell than any other tissue. Thus, there has been a demand for a solubilization method which most effectively utilizes the cell lines to obtain potent inhibitors of specific HL-A alloantiserums.

Exposure to sonic energy is a successful technique for solubilization of transplantation antigens from a variety of cell sources (*3*). Soluble human histocompatibility antigens, obtained by exposure of cells from several cultured lines to low frequency sound, inhibited the cytotoxic reactions of 21 specific alloantiserums in a pattern consistent with the phenotype of the cell line donor (*4*). Although this technique provided as good a yield as any described method (*2*), greater recoveries were necessary to obtain enough antigen for biologic and chemical characterization.

Initial experiments revealed that hypertonic salt extraction with 3*M* KCl alone, or in conjunction with sonication, efficiently solubilized HL-A antigens from cultured lymphoblasts (*5*). We now report the method, yields, and specific activities of soluble HL-A antigens extracted with 3*M* KCl and purified by preparative, discontinuous polyacrylamide electrophoresis.

The source materials were cell line RPMI 1788, derived from the peripheral lymphocytes of a normal donor and propogated in culture for 2 years (*6*), and cell line WI-L2 initiated from a spleen which had been removed from a patient with hereditary spherocytic anemia, and perpetuated in culture for 3 years (*7*). Absorption typing, and the direct cytotoxic reactions of 21 specific alloantiserums were used to establish the phenotype of the peripheral lymphocytes of the RPMI 1788 donor (*4*). The phenotype of the RPMI 1788 cultured cells, as determined by absorption and by fluorochromasia techniques (*8*), was identical to that of the donor's peripheral lymphocytes. On the other hand, the WI-L2 phenotype, as ascertained by testing the cultured cells with the absorption and fluorochromasia techniques, could not be confirmed with the donor's peripheral lymphocytes since he was unavailable.

Up to 50×10^9 dispersed, cultured human lymphoid cells were suspended in phosphate-buffered saline containing 3*M* KCl, *p*H 7.4 (20 ml of solvent per 10^9 cells) and gently agitated for 16 hours at 4°C on an Eberbach shaker; they were then centrifuged at 163,000*g* (average) for 1 hour (maximum, 235,000*g*). During dialysis (24 hours) against three changes of 200 volumes of saline, a gelatinous material formed, which comprised 18 percent of the ultracentrifugal supernatant and contained primarily DNA. This gelatinous fraction was removed by centrifugation at 1500*g* for 20 minutes; DNA could not be detected in this supernatant (1500*g*) either by the diphenylamine test of a hot trichloroacetic acid extract (*9*), or by radioactivity labeling experiments. For example, the antigen which was contained in the 1500*g* supernatant was from two generations of cultured WI-L2 lymphoid cells that had been uniformly labeled with [2-^{14}C]thymidine (New England Nuclear, 50 mc/mmole; 0.1 μc per 5×10^5 cells); this antigen contained less than 1 percent of the total labeled DNA.

Experiments to determine optimum conditions revealed that 3*M* KCl extracted more antigen than either 0.3*M*

Table 1. Solubilization of HL-A antigens from cultured lymphoid cells (RPMI 1788).

Sample	HL-A2 (ID_{50} units)*		HL-A7 (ID_{50} units)*		HL-A2 specificity ratio†	Recovery (%)‡	
	Per milligram	Per 10^9 cells	Per milligram	Per 10^9 cells		HL-A2	HL-A7
1	25,000	450,000	9,000	162,000	70	37	29
2	33,000	462,000	5,500	170,500	75	38	31
3	20,000	620,000	5,500	165,000	70	51	30
4	40,000	920,000	11,000	341,000	100	85	64

* The reciprocal of the soluble protein antigen which inhibits cytotoxic antiserums TO 11.30 (anti-HL-A2) and Cutten (anti-HL-A7) at zero cytotoxic units to 50 percent. † Ratio of the concentration of antigen required to inhibit an indifferent antiserum compared to that required for a homologous antiserum. ‡ The percentage of recovery is equal to $(AD_{50}/CE_{50}) \times 100$.

or 1.0*M* and that a 16-hour extraction period gave maximum yield. Since repeated extractions of the cell pellet yielded only small amounts of antigen without any increase in specific activity, this maneuver was not considered practical. Crude antigen preparations were stable for at least several months when stored in 0.9 percent NaCl at −20°C.

Preparative, discontinuous acrylamide-gel electrophoresis was used to purify the antigenic principle. The 1500*g* supernatant was dialyzed against tris-phosphate buffer, *p*H 6.7 (0.045*M* tris, 0.032*M* H_3PO_4); and from 50 to 75 mg protein was applied to a Buchler "Polyprep 100" column. Electrophoresis was performed at 0°C in system "B" of Rodbard and Chrambach (*10*) (*p*H 9.6, 7½ percent acrylamide gel) at a constant current of 35 ma. Fractions (8 ml) were collected at a flow rate of 0.8 ml/min, with a tris-HCl elution buffer [0.138*M* tris, 0.18*M* HCl, *p*H 8.2, containing 10 percent (weight/volume) sucrose]. Only the fraction eluting at R_F 0.78 to 0.80 exhibited specific antigenic activity. Upon reelectrophoresis in an analytical gel system (Fig. 1) (7½ percent acrylamide gel, 0°C, *p*H 9.6), the active fraction contained but a single electrophoretic component, as had been previously shown with HL-A antigens solubilized with sonication (*5*).

In order to estimate minute amounts of antigen, an isotope labeling technique was combined with Kjeldahl nitrogen analysis. Cultured cells (10^9) in the log phase of growth (concentration 2×10^7 cell/ml) were incubated for 4 hours at 37°C with a mixture of ^{3}H-labeled amino acids (2.5 mc) in Eagle's minimum essential medium that contained only 1 percent of its normal amino acid content. Approximately 15 percent of the tritium was incorporated into the cells, while 2 percent was found in the supernatant after ultracentrifugation and 0.04 percent in the purified antigen.

The inhibitory activity of the isolated antigen was assayed by a modification of the Terasaki-McClelland technique (*11*) in which inverted phase microscopy is used to assess target cell viability. Preliminary incubation of alloantibody with homologous antigen results in diminished cytotoxic activity of the serum.

The soluble antigens inhibited alloantiserums in specific fashion; they blocked only the cytotoxic reactions of serums directed against specificities present on the corresponding cell source. Thus, the RPMI 1788 antigen with HL-A specificities (2+, 5−, 7+) inhibited antiserum TO 11.03 directed against HL-A2 and antiserum Cutten directed against HL-A7, but had no effect upon TO 38.14 directed against HL-A9 nor upon antiserum D-66 directed against HL-A5. Similarly, the WI-L2 antigen with HL-A specificities (2+, 5+) inhibited antiserums TO 11.03 and D-66, but was ineffective against antiserums Cutten and TO 38.14.

Table 2. Electrophoretic purification (EP) of HL-A antigen (WI-L2).

	Nitrogen per 10^9 cells (mg)	ID_{50} units	
		Per milligram of nitrogen	Total
Antigen before EP	3.89	41,666	162,080
R_F 0.78 fraction	0.089	833,333	74,166

Antigen yield was estimated by quantitative absorption studies. The relative absorbing capacity of antigen preparations was determined, that is, the ratio of the number of donor cultured cells (AD_{50}) required to obtain a 50 percent reduction of the titer of the cytotoxic alloantiserum to the number of cell equivalents (CE_{50}) of soluble antigen required to achieve the same degree of serum inhibition [percent yield is equal to (AD_{50}/CE_{50}) × 100]. Table 1 illustrates that the HL-A2 and HL-A7 specificities were recovered in good yield (35 to 80 percent) from four cell batches representative of the 16 lots extracted. Two representative antigenic extracts from the WI-L2 cell line showed 80 percent or more recoveries of HL-A2 and HL-A5. The antigenic activity was as high as 200,000 ID_{50} (inhibition dose) units per milligram of protein. One preparation isolated from a batch of cells with 23 percent viability yielded only 9 percent of a soluble antigen that had poor immunologic potency (the specificity ratio was 18). On the other hand, all extracts shown in Table 1 were obtained from cultured cells with 90 to 95 percent viability. Only 3 to 8 percent of HL-A antigen was extracted in soluble form by KCl treatment of cell membranes obtained by freeze-thawing and hypotonic elution (*12*) or by decompression (*13*) of cultured lymphoid cells.

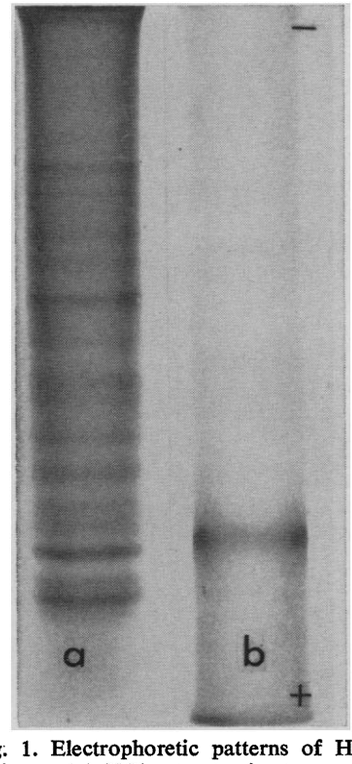

Fig. 1. Electrophoretic patterns of HL-A antigens. (a) KCl extract prior to preparative gel electrophoresis. (b) R_F 0.78 fraction isolated by preparative gel electrophoresis.

Table 2 shows the yield and activity of the acrylamide purified HL-A antigen. Approximately 2 percent of the nitrogen and 45 to 60 percent of the total antigenic activity applied to the gel column were recovered in the antigenic component with a 20-fold increase in the number of ID_{50} units per milligram of nitrogen.

Hypertonic salt extraction yields higher recoveries of potent HL-A antigens from lymphoid cell lines as compared to other methods. The active principle in the KCl extract was soluble because (i) it did not sediment upon ultracentrifugation at 163,000*g*; (ii) it could be passed through a Millipore filter (0.45 μ); (iii) it did not show any ultrastructural components upon electron microscopic analysis (*14*); and (iv) it could be purified to a well-defined protein by preparative acrylamide gel electrophoresis. These HL-A antigens were immunologically potent because (i) they inhibited alloantiserums in specific fashion, and (ii) they induced blastic transformation of histoincompatible, but not of isogeneic, leukocytes in vitro (*15*).

In all likelihood KCl dissociates hydrogen bonds and salt linkages releasing alloantigen, possibly in a manner analogous to the depolymerization of membrane components resulting from

sonication (*3*). These relatively mild methods seem preferable to papain digestion, which breaks peptide linkages and is difficult to control, and to extraction with complex salts and detergents, which are difficult to remove from the solubilized product. The KCl method is undoubtedly applicable to solubilization of other cell membrane activities. Indeed, analogous to the solubilization of tumor-specific antigens by sonication (*16*), 3*M* KCl has been shown to effectively solubilize tumor-specific antigenic determinants of diethylnitrosamine-induced guinea pig sarcomas (*17*).

The 3*M* KCl extraction procedure and electrophoretic purification technique, combined with the availability of cultured cell lines, should provide sufficient purified material to elucidate the chemical structure of the HL-A antigens, and thereby, the molecular basis of transplantation individuality.

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16 February 1971

Compositional Relatedness between Histocompatibility Antigens and Human Serum Lipoproteins

Abstract. *Human histocompatibility antigens and the protein part of Lp(a) lipoprotein and low density lipoprotein of human serum appear to be related when the amino acid compositions are compared. The Lp(a) lipoprotein appears to be closely related also to murine H-2^d antigen. The results support the concept of a relation between serum lipoproteins and histocompatibility antigens on cell membranes.*

We have suggested that a relation may exist between lipoproteins in the serum and histocompatibility antigens in cell membranes (*1*). In an investigation of human donors and recipients of skin grafts, we found that the grafts survived significantly longer when donors and recipients had the same phenotype within the Lp system (*2*) of inherited antigens belonging to the serum lipoproteins. The material studied was, however, of limited size. The observation by Kasukawa *et al.* (*3*) that rabbits may produce antibodies to allotypic specificities of serum lipoproteins after transplantation of skin from other rabbits appears to be compatible with, and possibly in support of, the view that a relation exists between transplantation antigens and serum lipoprotein antigens.

Zmijewski *et al.* (*4*) have presented evidence that there is a substance in human serum which is variable in quantity and may either inhibit or augment the detection of HL-A antigens by the leukocyte agglutination technique. Van Rood *et al.* (*5*) have reported that antiserum of the specificity HL-A2 can be neutralized with whole serum from people possessing the corresponding antigen.

Charlton and Zmijewski (*6*) have shown that the HL-A7 antigen is present in the soluble state in the β-lipoprotein fraction of human serum. Thus, there is now direct evidence for the existence of a connection between serum lipoprotein components and transplantation antigens.

A comparison of the protein composition of transplantation antigens and serum lipoproteins is therefore of considerable interest, but information on amino acid sequence is, unfortunately, not available for histocompatibility antigens or serum lipoproteins.

However, Metzger *et al.* (*7*) have developed a method for assessment of compositional relatedness between proteins based on the amino acid composition. The method was developed as an aid to the study of possible relations between proteins, when sequence data are not available. By means of this method, two proteins are compared by determining the difference in their fractional contents of each amino acid. From the sum of these differences, a difference index (DI) is calculated for the two proteins. Two proteins with the same composition would have a DI of 0 and two proteins with no amino acid in common would have a DI of 100. Metzger *et al.* (*7*) conducted 630 comparisons of different proteins or subunits of proteins and listed the resulting difference indices. The lowest value observed was 9.1, four (0.6 percent) of the 630 values were lower than 10, and 32 values (5 percent) were 13.5 or lower. Metzger *et al.* (*7*) also compared some proteins or protein subunits which are known to be related. For instance, a DI of 7.5 was found in the comparison of triose phosphate dehydrogenase of man and of *Escherichia coli*.

The amino acid composition of the main lipoprotein fractions—the low density lipoprotein (LDL) and high density lipoprotein (HDL) of human serum—has been studied in several laboratories. Simons *et al.* (*8*) reported the amino acid composition of the Lp(a) lipoprotein, and Mann *et al.* (*9*) reported amino acid analysis of two samples of human histocompatibility antigen and two samples of murine histocompatibility antigen. Thus, the question of a possible compositional relatedness between cellular histocompatibility antigens and serum lipoproteins may now be approached by the method of Metzger *et al.* (*7*).

I now report the result of an analysis where the data of Simons *et al.* (*8*) were used for the different lipoproteins and data of Mann *et al.* (*9*) were used for human and murine histocompatibility antigens. More amino acids had