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Hypertonic Salt Extraction of HL-A Antigens: Assessment of Protease Activity[1] (37995)

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Human histocompatibility antigens (HL-A) are genetically segregating cell surface markers which differ from person to person. The gene products of the HL-A locus thus provide a set of chemical markers of biological individuality which can be used in studying antigenic recognition of foreign graft and immune responsiveness to organ and tissue transplantation. Because of their potential usefulness, many efforts have been made during the last decade to solubilize these antigens from their hydrophobic site on the cell membrane in order to determine their chemical and molecular structure (1). Of the solubilization methods that have been tried, hypertonic salt (3 *M* KCl) extraction of cultured human lymphoid cells has proved to be the most simple, reproducible, and effective (2). This method is now widely used for the solubilization of H-2 (3) and a variety of tumor-specific antigens (4).

The objective of this study was to find out how this method solubilizes HL-A antigens from their hydrophobic sites on the cell surface. Specifically, we wanted to determine whether the method's effectiveness is attributable to the cleavage of protein fragments by intracellular proteases or to the dissociation of hydrophobic bonds brought about by the chaotropic effect of KCl.

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Materials and Methods. Cellular extracts. The WI-L_2 cultured cell line was propagated in our laboratory and we obtained cell lines RPMI 1788 and RPMI 4098 from Associated Biomedic Systems, Buffalo, NY. Cultured cells were washed three times with saline and then extracted with 3 *M* KCl at 4°C (2).

Protease substrates. *N*,*N*-^{14}C-Dimethyl casein (sp. act. 25,500 cpm/mg) (a gift from H. Drucker of Battelle Northwest Laboratories, Richland, WA) was diluted with 2% Hammersten quality casein (Nutritional Biochemicals) to a final concentration of 0.3% (5). Five tenths percent acid-denatured hemoglobin (Reheis Chemical Co.) and azocasein (Calbiochem) were also used as substrates to assay protease activity.

Protease inhibitors. Pepstatin (kindly provided by C. G. Cochrane, Scripps Clinic and Research Foundation), a potent cathepsin D inhibitor, (6, 7) was made up in a stock solution (10^{-4} *M*) with dimethyl sulfoxide and used at a final concentration of 2×10^{-5} *M* at pH 3.8 and pH 7.0.

Phenyl methane sulfonyl fluoride (Calbiochem) (8) was substituted for diisopropyl phosphofluoridate in an attempt to inhibit both chymotryptic and tryptic proteases. A stock solution of 0.1 *M* phenyl methane sulfonyl fluoride was prepared in 2-propanol and used at 1 m*M* final concentration. In order to insure the homogeneous distribution of the inhibitors in the extraction mixtures, all the inhibitors were mixed with the 3 *M* KCl solution prior to the extraction of cells.

Measurement of proteolytic activity. Acid-denatured hemoglobin was digested with

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aliquots of KCl extracts from the cultured cell lines, either at 4°C for 20 hr or at 37°C for 1 hr. The digests were then treated with 5% TCA for 1 hr at 4°C and the resulting precipitates were removed by centrifugation at 1000*g* for 15 min (International Centrifuge, Model PR-2). Aliquots of the clear supernatant were assayed by the Lowry method (9) for TCA-soluble materials after excess TCA had been neutralized with 1 *N* NaOH. Lysozyme was used as a standard. Azocasein was digested in the same manner and assayed by the method of Charney and Tomarelli (10).

TCA-soluble ^{14}C-digest. A standard curve depicting the proteolytic activity of thermolysin on *N,N*-^{14}C-dimethyl casein was established with 0.1–1.0 μg of enzyme (Calbiochem, A grade, sp act = 13,000 μm/mg) per 0.5 ml of *N,N*-^{14}C-dimethyl casein, prepared according to Drucker (1).

TCA-soluble ^{14}C-digests were assayed as follows: Aliquots of 20, 40, 60, 80, and 100 μl of antigen extract were diluted to a final volume of 500 μl with water and incubated for 1 hr at 37°C with equal volumes (500 μl) of *N,N*-^{14}C-dimethyl casein substrate solution. Five percent TCA precipitation was allowed to proceed for 1 hr at 4°C. After excess TCA had been extracted twice with equal volumes of ether, the residual ether was left to evaporate overnight at room temperature. The TCA-soluble ^{14}C counts released from the *N,N*-^{14}C-dimethyl casein were measured in 10 ml of aquasol (New England Nuclear) with a Beckman (LS 200) liquid scintillation counter.

Results. In order to find out if intracellular proteases affect the solubilization of HL-A antigens from cultured human lymphoid cells, we measured proteolytic activity during the course of antigen extraction (3 *M* KCl at 4°C). When aliquots were taken at 30 min, 2 hr, 6 hr, and 21 hr, we found that no significant percentage of protein had been digested as judged by TCA-soluble ^{14}C counts.

Similar experiments were performed using azocasein. However, here again, TCA-soluble materials could not be detected by spectrophotometric analyses in the visible region (440 nm). Apparently any protease(s) present in the extract could not digest a substrate which has either modified ϵ-NH_2 groups or a blocked amino terminus.

Finally, a 0.5% solution of acid-denatured hemoglobin was employed as substrate, because it had been shown to be the most suitable for detecting catheptic activity in bovine spleen lymphocytes (11). The results obtained with this substrate are shown in Table I. Although the actual percentage of TCA-insoluble large peptides present in the digestion mixtures is not known, our results show that at best only 2–3% of the total substrate protein was digested into TCA-soluble peptides. A very similar percentage of TCA-soluble material was obtained when cultured cells were extracted with 3 *M* KCl at 4°C and aliquots removed at various time intervals to be incubated at 37°C without any further addition of protease substrates. Protease activity ($\sim$1%) was found maximal after 2 hr of this endogenous digestion when, as we reported earlier (2), only 8% of the total HL-A antigen is solubilized. Even this minute protease activity seems to decrease near the end of the antigen extraction period (20 hr), when maximum solubilization of antigen occurs. Thus, protease activity does not appear to be directly correlated with solubilization of antigen.

In order to examine the possible effects of salt concentration and length of incubation on the total protease activity, we assayed aliquots of cellular extracts taken after 3 hr of extraction, i.e., when maximum protease activity should be detected; salt concentra-

TABLE I. Time Course Study of Protease Activity[a] During the 3 *M* KCl Extraction of Cultured Lymphocytes.

	% Protein digested			
	Time of assay (hr)			
Cell lines	½	2	8	20
WI-L2	0.3	1.0	0.9	0.7
RPMI 4098	2.4	1.3	0	0

[a] Acid-denatured hemoglobin (0.5%) was used as substrate. Activity was checked after 1-hr incubation at 37°C at each time point.

TABLE II. Effect of Salt Concentration and Temperature on Proteolytic Activity[a] of Antigen Extract.

Salt concentration (M)	Temperature of digestion (°C)	Digestion time (hr)	% Total protein digested
0.15	37	1	1.6
0.15	4	20	3.4
2.7	37	1	0.40
2.7	4	20	0.95

[a] Acid-denatured hemoglobin (0.5%) was used as substrate.

tion was either 2.7 M or 0.15 M and digestion was allowed to proceed at 37°C for 1 hr or at 4°C for 20 hr. As shown in Table II, any protease activity present in this cellular extract was inhibited by high concentrations of KCl. Thus, the actual percentage of protein digested under the conditions of maximum antigen extraction (3 M KCl for 20 hr at 4°C) was much lower (0.95%) than the percentage shown in Table I (2.4%), where digestion was done in 3 M KCl for 1 hr at 37°C.

Attempts were also made at abolishing protease activity and then determining whether HL-A antigens could still be solubilized. The results of these experiments are shown in Table III. It is quite evident that protease inhibitors such as EDTA, iodoacetamide, or phenylmethane sulfonyl fluoride had absolutely no influence on the small amount of detectable protease activity. However, pepstatin, a specific inhibitor for cathepsin D, did exert a potent inhibitory effect (85% inhibition). Pepstatin is only effective at pH 3.8 and requires a concentration (8×10^{-5} M) which is four magnitudes greater than that used with other acid–protease systems (12). Pepstatin did not show any inhibitory effect on protease activity at pH 7. It is neither practical nor relevant to test the effect of this particular protease inhibitor at an acid pH since the actual KCl extraction was done at pH 7.4 and antigenic activity is irreversibly destroyed below pH 5.0 (unpublished observation).

We think it is interesting that antigenic yields were drastically reduced in the presence of the various protease inhibitors utilized (Table IV). Phenylmethane sulfonyl fluoride mixed with iodoacetamide as well as EDTA resulted in a marked loss in antigen yield. From these data we can see that the presence of the protease inhibitors resulted in low antigenic yields without any detectable effect on protease activity. However, the yield of soluble HL-A antigen was not wholly determined by the effects of protease inhibitors. In fact, even 2-propanol, which is not a protease inhibitor but is commonly used to dissolve phenylmethane sulfonyl fluoride, caused a 75% loss of

TABLE III. Proteolytic Activity in the Presence of Protease Inhibitors During the 3 M KCl Extraction of WI-L2 Cultured Cells.

Inhibitor	% Total protein digested			
	Time of assay (hr)[a]			
	½	2	8	20
None	0.3	1.0	0.9	0.7
None[b] (+2-propanol, 1% v/v)	0.27	0.9	0.9	0.63
PMSF[c] (10^{-3} M)	0.3	1.0	0.9	0.6
IAA[d] (10^{-2} M)	0.25	1.0	0.9	0.7
PMSF + IAA	0.3	1.0	0.8	0.7
EDTA (10^{-2} M)	0.3	1.0	0.9	0.7

[a] Casein (2%) was used as substrate in the protease assay.

[b] 2-Propanol occupied 1% (v/v) of the extraction mixture when phenyl methane sulfonyl fluoride was added.

[c] PMSF: phenyl methane sulfonyl fluoride.

[d] IAA: Iodoacetamide.

TABLE IV. Effect of Protease Inhibitors on HL-A Antigenic Activity During 3 *M* KCl Extraction.

Inhibitors	ID_{50}[a] (μg)	% Solubilization (activity)	% Inhibition of proteolytic activity at 24 hr[b]
None	0.16	100.0	0
2-propanol (1% v/v)	0.70	22.9	10
PMSF[c] (10^{-3} *M*)	0.80	20.0	14
IAA[d] (10^{-2} *M*)	0.50	32.0	0
PMSF + IAA	0.50	32.0	0
EDTA (10^{-2} *M*)	1.70	9.4	0

[a] ID_{50}: Inhibition dose required to halve the cytotoxic power of a specific HL-A alloantisera.
[b] Casein (2%) was used as substrate in the protease assay.
[c] PMSF: phenylmethane sulfonyl fluoride.
[d] IAA: Iodoacetamide.

antigenic yield at a 1% (v/v) concentration. Since protease inhibitors did not inactivate the antigen directly once it was solubilized from the membrane, they, as well as 2-propanol, seem to interfere with the release of HL-A antigen from the membrane.

The small amount of protease activity detectable in the KCl extract seems to be mainly attributable to cathepsin D, since it can only be completely inhibited by pepstatin at pH 4. Other cathepsin D-like properties exhibited were heat instability at 60°C (15 min), pH optimum at 4.0, and the inability to digest substances which blocked N-terminals or substituted E-amino groups.

Discussion. Quantitative analyses of detectable protease activity in 3 *M* KCl extracts of cultured human lymphoid cells revealed minimal proteolytic activity and digestion of only 2–3% of the protein. This result was achieved under optimal conditions of proteolysis, i.e., incubation at 37°C for 1 hr at 0.15 *M* salt concentration (Table II). However, when actual extraction conditions (2.7 *M* KCl for 20 hr at 4°C) were used, less than 1% of the substrate was digested, apparently due to the cellular protease cathepsin (13).

Since HL-A antigen comprised only 1% of the total protein present in the cellular extract (14), this small protease activity can account for only 0.02–0.03% of the total of 80% of the cells' detectable surface antigen which was solubilized when a complete chaotropic extraction was done. This can be further substantiated by earlier work of other investigators who found that autolysis by endogenous cellular proteases solubilized only 1–2% of the HL-A antigen after 3-hr incubation at 37°C (15). In our instance, the high concentration of salt, neutral pH, and the low temperature used during 3 *M* KCl extraction seemed to be detrimental to native cathepsin-like proteases. Indeed, we found that the best pH for proteolysis was pH 4, at which point enzymatic activity was at least one magnitude greater than at pH 7.2. Interestingly, biologically active HL-A antigen could not be obtained at pH 4.0 since all antigenic activity is irreversibly destroyed under this condition. If we stipulate that even the minute amount of proteolysis activity at pH 7.4 may be responsible for HL-A antigen solubilization, we have to assume that the enzyme(s) is specific for the release of soluble HL-A antigens. This is difficult to imagine because we found that there seems to be no direct correlation between proteolysis and the degree of antigen solubilization. Actually, maximum solubilization requires 16–20 hr of extraction in 3 *M* KCl at 4°C, whereas saline extraction under similar conditions (which should be more favorable for native protease action) can solubilize at best only 10% of the antigen.

The fact that protease inhibitors reduced antigenic yield without influencing protease activity brings out an interesting point, i.e., that these inhibitors and related compounds

seem to alter mammalian cell membranes in such a way that the release of soluble HL-A antigens by 3 *M* KCl is adversely affected. In fact, we have found (Pellegrino and Reisfeld, unpublished observations) that HL-A antigen cannot be solubilized by 3 *M* KCl from membranes isolated by the zinc chloride fixation procedure developed by Brunett and Till (16). However, essentially all the HL-A antigen initially detectable on the whole cultured cells was still found on the isolated membranes, i.e., 3 *M* KCl simply could not solubilize the cell surface antigens once the membrane structure had been altered. The fact that the condition of the cell membrane per se is crucial for the solubilization of HL-A antigens is also substantiated by our findings that lyophilization, freezing, or any decrease in cell viability of whole cultured cells subsequently causes a sharp drop in the yield of soluble HL-A antigen (2). In addition, Warren *et al.* have pretreated tissue cultured cells with sulfhydryl-blocking agents such as fluorescein mercuric acetate and 5,5′-dithiobis-2-nitrobenzoic acid which resulted in the hardening of cell membranes, facilitating their isolation (17, 18). EDTA has also been employed for the more efficient isolation of red cell ghosts (19) and of plasma membranes (20).

Those procedures utilizing the addition of relatively large amounts (E/S = 1:1–1:100) of proteolytic enzymes, e.g., papain, yield 15–25% antigen, irrespective of the condition of the cell membrane (21). This observation itself suggests that solubilization of HL-A antigen in the presence of hypertonic salt is due to a different mechanism than proteolysis. The direct effect of protease inhibitors on cell membranes and their indirect effect on HL-A antigen solubilization may explain the findings of others (22), who concluded, that the low HL-A antigen yield obtained in the presence of protease inhibitors in 3 *M* KCl was due to inhibition of protease activity instrumental to HL-A antigen solubilization without making any direct measurement of protease activity.

We conclude that the actual mechanism underlying the solubilization of HL-A antigen from cultured human lymphoid cells with 3 *M* KCl does not involve the action of cellular proteases but probably depends on the chaotropic effects of KCl. Thus, it seems to us that the solubilization of HL-A antigen from lymphoid cell membranes takes place when the chloride ion, by disordering the structure of water, breaks hydrophobic bonds responsible for cell membrane integrity.

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