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PURIFICATION AND CHARACTERIZATION OF HL-A ANTIGENS FROM HUMAN PLATELETS, SOLUBILIZED BY THE NON-IONIC DETERGENT NP-40

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SUMMARY

Soluble HL-A antigens can be extracted with high yields from whole human platelets or platelet membrane preparations by the non-ionic detergent NP-40. The active molecules have high molecular weights ($\geqslant$200000) and the HL-A specificities present in the preparation were not separated by electrofocusing. An increase of the detergent/protein ratio gives rise to active molecules with lower molecular weight (15000–30000). In this case the two specificities studied (HL-A2 and HL-A7) could be separated by electrofocusing.

INTRODUCTION

A series of procedures have been introduced to solubilize histocompatibility antigens from cell membranes[1] and most of them generally allowed good reproducibility: but the yield of highly purified molecules carrying alloantigenic specificities remained relatively low. Therefore only a small number of studies devoted to the molecular aspects of these substances have been achieved. Recently, Schwartz and Nathenson[2] reported that the extraction with the non-ionic detergent Nonidet P-40 (NP-40) allowed nearly total solubilization of alloantigens from the cell membrane.

The present paper describes the application of the procedure of solubilization to HL-A antigens from human platelets.

MATERIALS AND METHODS

Human platelets

Human platelets used throughout this study were obtained by differential centrifugation (P A Plasmapheresis double, Blood Pack 500 ml, acid–citrate–dextrose, Fenwal Laboratories) from normal donors. Tissue types of the donors were determined by lymphocytotoxicity and platelet complement fixation tests. (For HL-A types and characterization of platelet preparations, see Table II).

Solubilization procedure: preparation of solubilized extract

The solubilization of the antigens was realized either from platelet suspensions

or from platelet membranes purified by the procedure of De Duve[3]. The concentrations of NP-40 (Compagnie Française des Produits Chimiques Shell, Paris) of the various preparations ranged between 0.1–0.5% (v/v): the incubation time was always 30 min at 4 °C, with agitation. Insoluble material was removed by high-speed centrifugation (165000 $\times g$ for 90 min).

Elimination of the detergent

An extensive dialysis of the crude extract on Diaflo-membranes, PM-10 (Amicon), eliminated about 90% of the detergent. No toxic phenomenon could be observed in lymphocytotoxicity tests with the doses used (generally less than 1 μl per test).

Detection of antigen specificities

During all the purification steps, the antigenic activity was monitored by inhibition of lymphocytotoxicity tests[4]. Various amounts of extract (usually 0.01–1 μl) were tested for their inhibitory activity against 0.5, 1, 2 and 3 antibody units (one antibody unit being the smallest amount of immune serum needed to kill 100% of lymphocyte target cell in the test). The percent cytotoxicity was determined in each of the four tests for each extract dose and the sum of percent toxicity was obtained by adding the four percentages.

The percent inhibition for an extract dose was determined by the expression:

$$\frac{\text{sum of percent toxicity (test without extract)} - \text{sum of percent toxicity (test with extract)}}{\text{sum of percent toxicity (test without extract)}}$$

The 50% inhibition dose (ID_{50}) of an extract was then determined graphically. The antisera used for this study are indicated in Table I.

Purification of the soluble extract

The purification was achieved by electrofocusing[5] (LKB 8101 column of 110 ml capacity; carrier ampholytes pH 3–10; 40–45 h; 200–600 V; 2-ml fractions). The

TABLE I

CHARACTERISTICS OF THE ANTISERA USED

Antisera	*Specificity*	*Dilution*	r^*	N^*
Rousse	HL-A2	1:3	0.97	100
Dubois	HL-A5	1:1	0.98	126
Lamarq	HL-A5	1:3	0.95	100
Fische	HL-A9	1:1	0.97	50
8751	HL-A7	1:3	0.95	100

* Correlation coefficient r between the reactivity of the antiserum and the distribution of antigens in a panel of N individuals.

absorbance and the pH of each fraction were determined with a Beckman DU spectrophotometer at 280 nm and with a Beckman pH-meter, respectively.

Determination of the molecular weight

Molecular weight determinations of active molecules were performed on Sephadex G-200 and G-75 columns, with a 0.05 M Tris–0.14 M NaCl buffer, pH 7.3.

Polyacrylamide gel electrophoresis

It was carried out either at pH 8.9, gel porosity 7.5%[6], or at pH 7.5, gel porosity 7.5%[7] (4 mA/tube; 30–40 min; coloration with amidoschwarz).

RESULTS

Human platelet suspensions and platelet membrane preparations were submitted to different concentrations of NP-40. Table II summarizes the characteristics of the platelet preparations which were used and indicates the recoveries of the antigenic activities after elimination of the detergent.

With NP-40 concentrations between 0.1 and 0.5%, high molecular weight fragments were characterized carrying the activities tested in the starting material (Fig. 1).

The purification of the active molecules present in the extracts was achieved by electrofocusing: no separation of the specificities could be obtained. HL-A2 and HL-A5 specificities (extracts Lehideux Nos 2 and 4) were found in the same fraction, with an isoelectric point of 6.2 and a molecular weight $\geqslant 200000$; a single protein band after polyacrylamide gel electrophoresis at pH 7.5 was detected (R_F 0.42).

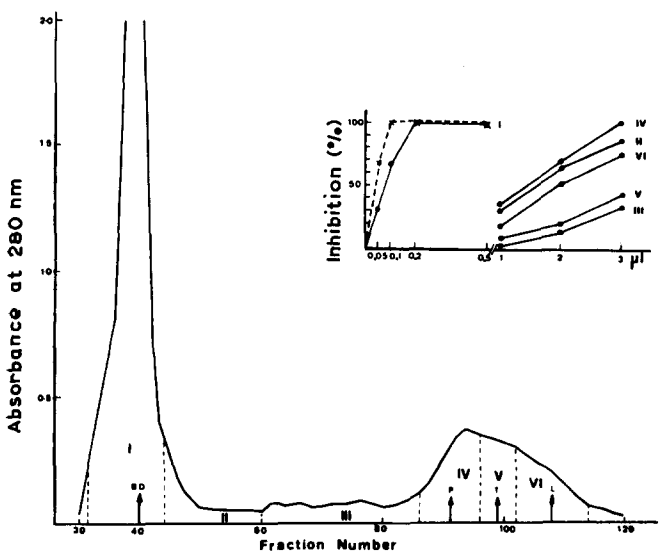

Fig. 1. Filtration of extract Dupuis No. 2 on Sephadex G-200 (100 cm × 2.2 cm); 0.05 M Tris–0.14 M NaCl buffer, pH 7.3; 2-ml fractions. ↑ : test substances used for column calibration: BD = dextran blue 2000; P = pepsin; T = trypsin; L = hen egg-white lysozyme. Insert: aniigenic activity of pools I–VI from Sephadex column, after concentration to 2 ml. ●—●, HL-A2; ×---×, extract.

TABLE II

CHARACTERISTICS OF PREPARATIONS USED AND ANTIGENIC ACTIVITY TESTED AFTER EXTRACTION WITH NP-40

Donor	*Extract No.*	*HL-A type*		*Starting material*		*Storage**	*NP-40* (%)	*Extract vol.* (*ml*)	*Activity*** *inhibition units* (ID_{50})		*Specificity ratio****
		First locus	*Second locus*	*Whole platelets*	*Platelet membranes*				*HL-A2*	*HL-A5*	
Lehideux	1	HL-A2	HL-A5	$1 \cdot 10^{11}$		14	0.1	4.0	13 000	1 500*	n.d.
	2	W-28	W-5	$1 \cdot 10^{11}$		7	0.5	1.5	60 000	15 000	40(2) 10(5)
	3			$1 \cdot 10^{11}$		90	0.5	2.0	67 000	20 000	n.d.
	4			$1 \cdot 10^{11}$		30	0.5	2.0	80 000	80 000	n.d.
									HL-A2	*HL-A9*	
Dupuis	1	HL-A2	HL-A12	$2 \cdot 10^{11}$		18	0.5	2.0	40 000	200 000	5(2) 25(9)
	2	HL-A9	W-18		$1 \cdot 10^{11}$	12	0.3	2.0	40 000	40 000	20(2) 20(9)
									HL-A2	*HL-A7*	
Gargallo		HL-A2	W-22		$1 \cdot 10^{11}$	34	0.5	2.0	40 000	80 000	20(2) 40(7)
		HL-A11	HL-A8								
									HL-A2	*HL-A5*†	
Luthy	1	HL-A1	HL-A5		$1 \cdot 10^{11}$	130	0.3	4.0	10 000	20 000	5(2) 10(5)
	2	HL-A2	HL-A7		$1 \cdot 10^{11}$	150	0.5	1.6	36 000	36 000	20(2) 20(5)

* Time in days during which platelet suspensions were kept at 4 °C between separation from whole blood and preparation of extract.

** ID_{50}= 50% inhibition dose. The figure represents the absolute number of ID_{50} in the extract. For definition of ID_{50}, see text.

*** Specificity ratio[8]= ratio of ID_{50} determined in a non specific system (antibody reacting with an antigen not present in the extract), to ID_{50} determined in a specific system (antibody directed against an antigen present in the extract). Number between parentheses refers to the HL-A specificity tested. n.d.= not done.

† Extracts Lehideux No. 1, 2 and 3, were tested with anti HL-A5 Dubois; extract No. 4 was tested with anti-HL-A5 Lamarcq. The latter was also employed for tests concerning extracts Luthy.

Similarly the specificities HL-A2 and HL-A9 of extract Dupuis No. 1 (Fig. 2) were characterized in the same fraction, with an isoelectric point of 5.5 and again a molecular weight $\geqslant 200000$.

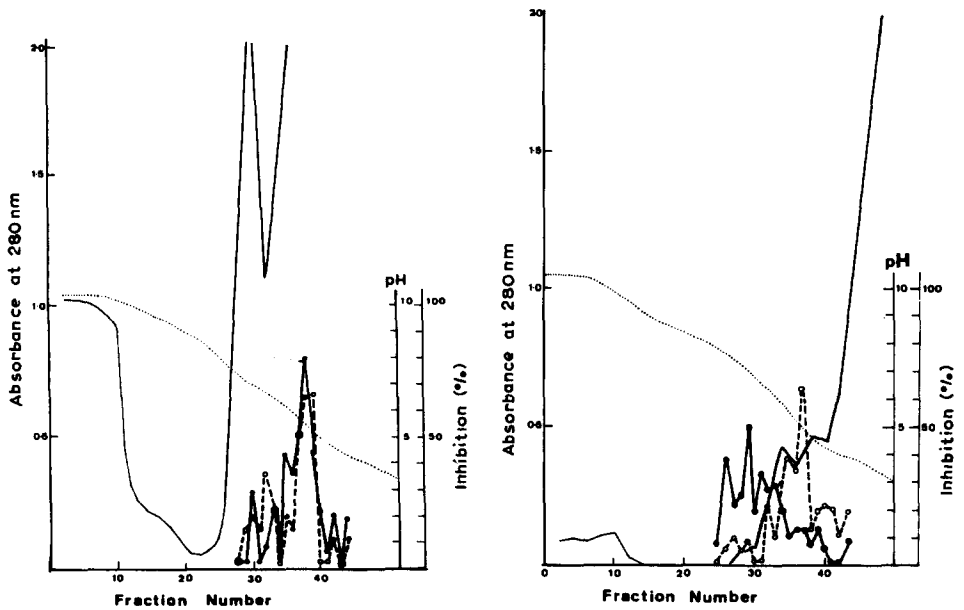

Fig. 2. Electrofocusing of extract Dupuis No. 1. 2-ml fractions. ———, absorbance at 280 nm; ·····, pH. Antigenic activity (0.2 μl) of fractions eluted from the column: ●—●, HL-A2; ○---○, HL-A9. (The strong absorbance at 280 nm of the last fractions eluted from the column is due to the remaining detergent).

Fig. 3. Electrofocusing of extract Gargallo. 2-ml fractions. ———, absorbance at 280 nm; ·····, pH. Antigenic activity (0.5 μl) of the fractions eluted from the column: ○---○, HL-A7; ●—●, HL-A2. (The strong absorbance at 280 nm of the last fractions is due to the remaining detergent.)

Using as starting material a purified platelet membrane preparation, an increase of the detergent/protein ratio (5 mg of proteins instead of 20 mg in 0.5% NP-40) gave active fragments with a lower molecular weight (15000–30000) carrying only a unique HL-A specificity; these fragments could be separated by electrofocusing as they had different isoelectric points. Fig. 3 shows such a separation: HL-A7 specificity is clearly localized in Fraction 37 (isoelectric point 5) whereas HL-A2 specificity is characterized in a larger area with a maximum in Fraction 29 (isoelectric point 7).

DISCUSSION

Several points may be noted from these results. Using NP-40 for solubilization of human platelet antigens, we obtained substances with a high inhibitory activity *in vitro*. It is difficult to evaluate with precision the yield of the method. Indeed antigens *in situ* do not have the same configuration as those present in the soluble extracts and do not present the same inhibitory effect against specific antibodies. However, if we compare the antigenic activity of these preparations with the activity

previously characterized in extracts prepared by digestion with papain of the same platelet suspension[9], we may consider the solubilization by the detergent to be at least ten times more efficient.

From Table II it can be assumed that the storage of the platelets before extraction does not influence significantly the activity of the extracts and that membrane preparations present an activity which can be compared to that obtained directly from whole platelet suspensions.

We considered the material which did not sediment at $165000 \times g$ for 90 min as soluble and we observed that it remained soluble after elimination of nearly all the detergent NP-40. By this procedure, molecules of high molecular weight ($\geqslant 200000$) were obtained comparable with those characterized when other detergents were employed[10,11]; on the contrary, molecules presenting a lower molecular weight were obtained by digestion with proteolytic enzymes[12], by sonication[13] or by treatment with salts[14,15]. Nevertheless, higher concentrations of NP-40 seemed to provoke a more important degradation, with the appearance of smaller active fragments (extract Gargallo). In all cases, when high molecular weight substances were obtained, after purification they all carried the two activities which were tested at the beginning of the preparation. Thus it might be concluded that two or several antigenic determinants could be situated on the same molecule. Moreover acrylamide gel electrophoresis of these purified active fractions showed only a single protein band. The hypothesis that one molecule of high molecular weight might carry several antigenic specificities has already been proposed, mainly by Hämmerling and co-workers[16,17]. An alternative hypothesis would be that NP-40, when used at a relatively low concentration could extract associated molecules, or that after extraction some degree of reassociation could occur. Recent results reported by Cullen *et al.*[18] show indeed that mouse H-2 antigens solubilized by NP-40 could be separated. Using an immunological method, a separation of the four main H-2 specificities carried by lymphocytes of heterozygous mice was obtained. These results are in accordance with our investigations using a relatively high concentration of NP-40.

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