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ISOLATION FROM HUMAN URINES OF A MUCIN WITH BLOOD GROUP Sd^a ACTIVITY.

Jean-Pierre Cartron, Michel Kornprobst, Marguerite Lemonnier, Patrick Lambin, Friedrich Piller and Charles Salmon.

INSERM U76, Centre National de Transfusion Sanguine - 6, rue Alexandre Cabanel, 75739-PARIS Cedex 15, France.

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Summary. A mucin with Sd^a blood group activity was isolated from human group O urines by a multistep procedure including an affinity chromatography on Helix pomatia - Ultrogel. About 8 mg of active material was obtained from 100 litres of urines. The purified substance of apparent molecular weight 340,000 dalton is not stained by Coomassie blue but gave a single periodic acid-Schiff positive band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analytical composition indicated the absence of mannose, a high content of N-acetylgalactosamine and a molar ratio galactose : N-acetylgalactosamine: N-acetylglucosamine: sialic acid of 2:2:1:2. Amino acid composition is typical of a mucin substance with high values of serine, threonine, proline and alanine. The urinary mucin inhibited human anti-Sd^a antibodies as strongly as the Tamm-Horsfall glycoprotein isolated from Sd(a+) urines. However, the two substances clearly have different composition and properties. It is suggested that oligosaccharide chains with Sd^a blood group activity might be carried by different glycoconjugates of human urines and tissues.

INTRODUCTION. The Sd^a blood group antigen occuring on red cells and in urines of 96% human individuals is inherited as a dominant autosomal character (1,2,3).

Numerous sources of Sd^a substances have been discovered in body fluids and tissues from several species but is is not found in birds (4) and therefore chickens were chosen for successful immunisation and production of anti-Sd^a antibodies (5).

A great variation in the strength of the red cell Sd^a antigen from one individual to an other was noticed in the earliest reports, and the rare Cad antigen (non-A_1, Dolichos biflorus positive red cells) first described by Cazal et al. (6) was later on recognized as a very strong form of Sd^a and therefore termed "super Sd^a" or Sd(a++) cells (7).

However, there is some controversy about the identity between Cad and Sd^a antigens (5,8) which could be solved only by purification and analysis of the respective substances.

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Haemagglutination-inhibition of Sd(a++) cells by the Dolichos biflorus lectin have suggested that the chief structural determinant of Cad specificity is N-acetyl-D-galactosamine (7,9), a sugar already involved in blood group A, Tn, P and Forssman receptors (10,11,12).

However, N-acetyl-D-galactosamine (GalNAc), the A active disaccharide GalNAc(α1-3)Gal, the Forssman glycolipid and glycopeptides containing a terminal N-acetylgalactosamine α-linked to serine or threonine, did not inhibit either the agglutination of Sd(a+) or Sd(a++) red cells by human anti-Sd^a antibodies (3,13). The carbohydrate nature of the Sd^a antigen was demonstrated (13,14) since the partially purified material isolated from human urines was shown to be resistant to proteolytic cleavage but destroyed by periodate oxidation. More recently it was suggested that the Sd^a activity is closely associated with the Tamm-Horsfall (T-H) glycoprotein identified in all human urines for many years (15,16). Biochemical analysis indeed demonstrated clearly a striking difference in the GalNAc content of the T-H glycoprotein from Sd(a+) and Sd(a-) individuals (15). Since the T-H is synthetized by distal tubules of the kidney (17) it is further suggested that this glycoprotein might be of importance in the graft survival following renal transplantation in Sd(a-) individuals (16,18).

In the present report we describe the composition and some properties of a new component of human urines which, in addition to T-H glycoprotein, contribute to the Sd^a blood group activity.

MATERIALS AND METHODS. Human anti-Sd^a serum was a generous gift from Dr. L. Messeter, Blood Bank University Hospital, Lunds, Sweeden. Strongly reacting group Sd(a++) red cells were provided by Dr. M. Monis, Centre de Transfusion Sanguine de Montpellier, France.

Helix pomatia lectin-Ultrogel was purchased from IBF-Pharmindustrie, Villeurbanne, France. Other lectins such as Dolichos biflorus, Salvia sclarea, Helix pomatia and Ulex europaeus were crude seed extracts from the Commercial Division of the Centre National de Transfusion Sanguine, Paris, France.

Reagent grade chemicals were used throughout. The chemicals used for polyacrylamide gel electrophoresis were acrylamide, N,N,N',N'-tetramethyleethylene diamine from Eastman-Kodak Corp. (Rochester, New-York) and sodium dodecylsulfate from Prolabo (Paris, France).

Neutral hexoses, N-acetylhexosamines and N-acetylneuraminic acid were quantitated by gas-liquid chromatography according to Chambers and Clamp (19). Trimethysilyl derivatives of O-methyl glycosides were separated on a glass column packed with 3% SE.30 on Chromosorb 80-100 mesh HAW, DMCS. Mannitol was

added as internal standard. The chromatograph Hewlet Packard 5750 was equipped with a flame ionization detection system and was temperature programmed from 100°C to 200°C at 1°C/minute.

Proteins (60μg) were hydrolyzed with HCl 6N, at 105°C for 18, 24 and 48 hours under vacuo. Amino acid analysis was performed with a Jeol 6 AH amino acid analyzer on a single column (cation exchange resin M72). Amino acids were eluted with sodium citrate buffers (pH 3.22, 0.2M; pH 4.7, 0.2M and pH 7.45, 1.6N). Norleucine was added as internal standard. For performic acid oxidation, samples were first dissolved in 0.06 ml HCOOH (89%) and 0.01 ml H_2O_2 (30%) was added. Following continuous stirring for 2 hours at 4°C, the samples were diluted, freeze dried and hydrolyzed in HCl 6N for 2 hours. The cysteic acid content was determined on the Jeol 6 AH amino acid analyser as described above.

SDS-Polyacrylamide gel electrophoresis was carried out in 3-20%(w/v) acrylamide gradient as previously described (20) and the gels were stained for protein with Coomassie Blue R250 and for carbohydrate with periodic acid-Schiff (PAS) reagent.

Direct haemagglutination and inhibition tests were carried out in conventional assays (21). Initial steps of purification were followed by haemagglutination inhibition of Sd(a++) cells against a chicken serum whereas final steps were controlled using the human Sd^a antibody.

RESULTS AND DISCUSSION. The Sd^a active material was isolated from 104 litres of blood group O urines collected from four unrelated Sd(a+) individuals

Eight batches of 13 liters of urines were separately processed before the affinity chromatography step on HPA-Ultrogel. Each batch collected on toluene (4 ml/2000 ml urines) was diluted with one volume of cold NaCl 0.15M and centrifuged at 10,000 g for 15 mn after standing no more than 24 hours at 4°C. The clear supernatant was concentrated about 100 times on hollow fiber (Amicon, H_1DP10 membrane) and dialysed at 4°C against Tris-HCl 0.1M pH 8.0 containing 0.25M NaCl and 0.01% NaN_3 (buffer A). Insoluble material was removed by low speed centrifugation (10 mn at 5000 g). The dialysate was loaded at 4°C on a Sephacryl-S 200 column (3 x 95 cm, Pharmacia, Uppsalla, Sweeden) equilibrated in Tris-HCl 0.1M pH 8.0, 0.25M NaCl, 0.01% NaN_3 and eluted with the same buffer at a rate of 19 ml/hour. The Sd^a active fractions were eluted in the void volume dialysed against distilled water and freeze dried.

The lyophilisate was redissolved in 20 ml of distilled water and extracted by 9 volumes of a chloroform-methanol mixture (2:1, by volume). The delipidated material was recovered from the upper aqueous phase, diluted to the initial volume with distilled water and freeze dried. The lyophilisate was redisolved in buffer A, loaded on a Sepharose-4 B- column (2,5 x 100 cm) equilibrated in the same buffer and eluted at 4°C at the rate of 20 ml/hour. The

three fractions obtained at Ve/Vo ratio of 1.05,2.0 and 2.5 (Ve=elution volume of the fraction, Vo=void volume of the column) were pooled, extensively dialysed against distilled water and freeze dried. A significant part of the Sd^a activity, as estimated by hemagglutination inhibition with the chicken serum was observed in the intermediate fractions (Ve/Vo=2.0). The intermediate fractions collected from eight different batches of urines were pooled and rechromatographied on the Sepharose 4B column. After extensive dialysis against distilled water, 450 mg of dried material was obtained. At this step, about 0.08 μg of the substance was enough to inhibit 4 hemagglutinating doses of the human anti-Sd^a antibody.

The urinary substance was further purified by affinity chromatography on Helix pomatia-Ultrogel column (2 x 6 cm, 20 ml of gel) equilibrated in PBS. Five columns were run, each loaded with about 90 mg of dried material dissolved in 5 ml of cold PBS. Unbound substances were removed by extensive washings of the column with cold PBS. The column was then eluted at 4°C with 25 ml of N-acetylgalactosamine 0.1M in PBS, followed with 25 ml of PBS.

The large amount of material with a significant Sd^a activity which did not stick to the column was discarded. The substance eluted with N-acetylgalactosamine from each of the five affinity columns were pooled, extensively dialysed against distilled water and lyophilised. The lyophilisate, redissolved in buffer A, was finally chromatographied on Sephacryl S 300 (2.7 x 18 cm Pharmacia, Uppsalla, Sweeden) in the same buffer and was recovered in the void volume of the column.

The purified material (8 mg dry weight) exhibited a strong Sd^a activity very similar to that of the T-H glycoprotein isolated from Sd(a+) urines, but was completely devoided of any blood group H activity as seen by the absence of inhibition of the Ulex europaeus lectin (Table 1).

Following SDS-polyacrylamide gel electrophoresis the Sd^a preparation failed to react with Coomassie blue R250 but gave a single band of 340,000 daltons apparent molecular weight strongly stained by the periodic acid-Schiff reagent (not shown).

Table 1. Immunological specificity of the Sd^a active substances isolated from human urines (1).

Component	Human antibody inhibition Anti-Sd^a Sd(a+)	Cad	Anti-A A_2	Lectin inhibition(2) DBA	HPA	SSA	UEA
Urine substance	0.003	0.006	0.8	0.2	0.4	0.8	>10
T-H Sd(a+)	0.005	0.01	>10	0.3	>10	0.6	
T-H Sd(a-)		> 10			>10	1.3	
GalNAc	>100	> 100	>100	6	12	0.8	>100
GlcNAc	>100	> 100	>100	>100	24	>100	>100

(1) Minimum amount of substance ($\mu g/40\mu l$) giving complete inhibition.

(2) Agglutination inhibition of A_1 red cells by Dolichos biflorus (DBA) and Helix pomatia (HPA) lectins. Inhibition of In red cells by Salvia sclarea (SSA) agglutinin and of O red cells by Ulex europaeus (UEA) agglutinin.

Analytical composition of the 340,000 daltons component was simultaneously determined with that of the T-H glycoprotein, as indicated in Table 2. The sugar composition of the substances was different since the 340,000 daltons material contained no detectable mannose and fucose and gave a molar ratio of Gal:GalNAc: GlcNAc:NeuAc of 2:2:1:2:(whereas it was close to 2:1:3:2: for T-H). Very likely the high GalNAc content of the 340,000 daltons explains its weak reactivity with group A and "A-like" reagents (Table 1). Amino acid analysis also revealed a striking difference between T-H and the 340,000 daltons material since the latter had a typical composition of a mucin with a low content of cysteine and methionine but large amount of serine, threonine, alanine and proline.

Finally, it was observed that, at variance of the T-H glycoprotein, the urinary mucin did not react with an anti-uromucoid serum in immunodiffusion test (not shown) which further substantiated the difference between these two Sd^a active substances.

From these results, it is concluded that both the T-H glycoprotein and the mucin isolated from Sd(a+) urines carry oligosaccharide chains of still unknown structure, with Sd^a blood group activity.

Table 2. Analytical composition of the Sd^a active substances isolated from human urines.

Component	Mucin substance (340,000 daltons)		T-H (82,000 daltons)
	µg/ml	molar ratio	molar ratio
Fucose	0	-	0.13
Mannose	10	0.04	1.05
Galactose	250	1	1
Glucose	0	-	0.10
N-acetylglucosamine	140	0.51	1.55
N-acetylgalactosamine	280	1.02	0.35
N-acetylneuraminic acid	290	0.90	0.85
Total amino acids	600		
		residues per 100 residues aminoacids	
Asp	48.3	8	11.3
Thr	59.2	11.2	7.1
Ser	51	11.2	8
Glu	49.3	7.4	10
Pro	61.2	12.1	6.7
Gly	26.7	9	9.8
Ala	46.5	12.5	7.3
1/2 Cys	4.2	0.8	7.7
Val	35.5	6.9	6.2
Met	nd	nd	2
Ile	10.36	1.8	2.6
Leu	24.9	4.2	7.1
Tyr	9	1.0	2.8
Phe	14.9	2.0	2.8
His	26.2	3.6	2.5
Lys	16.2	2.4	2.8
Arg	32.6	3.8	5.0

nd= not detectable.

That different macromolecules may carry a similar determinant is in agreement with our present chemical knowledge of other blood group substances (10) as well as with the wide distribution of Sd^a in tissues (22,23).

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