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ISOLATION AND CHARACTERIZATION OF PARTIALLY PURIFIED LEPTOSPIRAL ANTIGENS (1)

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SUMMARY

The methanol extract of **Leptospira interrogans** serovar **canicola** was purified by precipitation with acetone or acetone and chloroform. The antigenicity of the antigen was not altered by heating or treatment with pepsin and pronase. However the antigenicity was lost when the antigen was treated with periodic acid. Chemical analysis revealed the presence of 40% carbohydrate (22% methylpentose, 28% hexoses), 4% protein, 20% lipid and 2,7% phosphate. The complement fixation test with sera from patients with leptospirosis agreed with the microscopic agglutination reaction.

KEY WORDS: **Leptospira**; Antigens; Purification; Diagnosis.

INTRODUCTION

Several procedures have been devised for the preparation of leptospiral antigens: ultrasonication, heat treatment, hot phenol-water extraction, and methanol extraction [1, 15, 21, 25, 26]. SHINAGAWA & YANAGAWA[24] reported the preparation of antigens from Kyoto strain extracted with phenol and precipitated with ethanol.

Many authors have usually not purified single antigens but have reported studies with mixtures of antigens. A leptospiral genus specific protein antigen (G1-Ag) was partially purified from serovars **kremastos** and **canicola** with Triton X-100 followed by fractionation with DEAE-cellulose, column chromatography and ethanol precipitation[22]. A genus specific antigen was obtained by ethanol fractionation of **L. biflexa** with further purification on Sephacryl-300 gel filtration[5]. A glycolipid antigen possessing a serovar specific antigenic determinant was purified from a chloroform-methanol extract of the organism[19]. A protein from serovar **hardjo** was partially purified by Triton X-100, ion-exchange chromatography and sucrose gradient centrifugation[4].

This paper reports on the partial purification and chemical characterization of **canicola** serovar antigen extracted with methanol and on the evaluation of the use of these purified antigen in complement fixation test for diagnosis of human leptospirosis.

MATERIALS AND METHODS

Preparation of the antigens

The **Leptospira interrogans** serovar **canicola** strain Hond Utrecht IV was cultured in chemically defined medium of SHENBERG[23]. Incubation was carried out at 28° C for 8-10 days. The cells were harvested by continuous centrifugation (10,800 x g, 4° C), washed three times with 0.15M NaCl, and lyophilized. One gram of the lyophilized cells was suspended in 100 ml of methanol. After 8-10 days at 25° C in the dark, the suspension was centrifuged at 2,500 x g for 15 min. The supernatant was the methylic antigen.

Purification of methylic antigen

The methylic antigen was purified by precipitation with either pure acetone or acetone and chloroform at -20° C. The sediment obtained after cen-

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trifugation at 4,500 x g for 30 min. was dissolved in methanol to its original volume. They constitute antigens (A, and AC) precipitated with acetone (A) or acetone plus chloroform (AC), respectively. The A and AC antigens were tested for their complement fixation capacity with anti-**canicola** serovar rabbit serum. The specific activity of the antigen was determined by the ratio: complement fixing units/polysaccharide content (mg). One complement fixing unit was defined as the amount of antigen which promote 50% of lysis on a system containing five hemolytic units[17] and anti-**canicola** serovar rabbit serum diluted 1:40.

Sera

Fifteen samples of sera from patients with leptospirosis and sera from eight healthy humans were used.

Anti leptospira sera from serovar **canicola** were obtained by inoculation in adult rabbit of 1 ml of a seven days culture of leptospira grown in synthetic medium[23]. The inoculation was done every day, during 30 days. The animal was bled and sera titers were determined by microscopic agglutination test (MAT).

Antisera for antigen A were obtained by immunization of rabbits with antigen A containing 1 mg/ml of polysaccharide complexed with 1 ml of bovine serum albumin (BSA) containing 20 mg/ml[3]. The preparation mixed with complete Freund adjuvant was inoculated intradermally in rabbits. After 30 days, a booster inoculation was applied. Fifteen days later, the rabbits were bled and the agglutinating and complement fixing antibodies for leptospira were determined.

Solubility tests

The methylic antigens after dialyzed against distilled water were precipitated. This precipitated was suspended in 0.85% NaCl and its solubility in chloroform, acetone, isopropanol, sodium dodecyl sulphate (SDS) and Triton X-100 were tested by mixing slowly equal volumes of the solvents and antigens at room temperature with agitation.

Action of periodic acid and proteolytic enzymes

To 1 ml of methylic antigen A diluted 1:10, 0.1 ml of 0.1 M periodic acid was added. The mixture was incubated at 25° C for 1 h. and then dialyzed against distilled water at 4° C for 24 h. ANSONS's method[2] was used for determination of pepsin and pronase activities. The complement fixing activities of these antigens were tested with anti-**canicola** serovar rabbit serum.

Serological reaction

Microscopic agglutination test (MAT) was carried out as recommended by WORLD HEALTH ORGANIZATION[29]. The complement fixing test (CF) was performed as described by MAYER et al.[17]. Immunodiffusion was done as reported by OUCHTERLONY[20].

Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-Page)

The antigen A and AC were electrophoresed on 5.6% polyacrylamide gels according to FAIRBANKS et al.[9]. Bovine serum albumin (68 KDa) (Pentex Biochemical), ribonuclease (13 KDa) (Sigma Chemical Co.), H chain (50 KDa) and L chain (25 KDa) of human IgG (150 KDa) were used as standards. The gels were stained with periodic acid Schiff (PAS) reaction and Coomassie blue.

Gel filtration chromatography

Methylic antigen was applied on a Sephadex G-200 column (100 x 1 cm) equilibrated with 0.15 M NaCl at 25° C. Elution was carried out with 0.15 M NaCl at a rate of four drops/min. Samples of 1.8 ml were collected. Absorbance was measured at 280 nm.

Chemical composition analysis of the antigen A

The following analysis were determined: total carbohydrates[8], pentoses[6], 6-deoxyhexose[7], hexose[27], protein[12], deoxyribonucleic acid[13], and phosphate[10].

Sugar analysis

Sugar identification was done by descending paper chromatography on Whatman nº 1 filter paper. The antigen was hydrolyzed with 2M HCl.

The solvents used were: ethyl acetate; pyridine; water (8:2:1) or butanol; acetic acid; water (4:1:5). The chromatography was run for 15 h. Solutions (0.1%) of glucose, galactose, fructose, ribose, arabinose, xylose, mannose, and rhamnose were used as standards. After drying, the sugars were revealed with alkaline silver nitrate[27].

Amino acid and amino sugar analyses

Quantitative analyses of amino acids and amino sugars of antigen A were done according to MOORE & STEIN[18] in an automatic amino acid analyzer.

Lipid analysis

Total lipids in antigen A were determined according to SHINAGAWA & YANAGAWA[24]. Methyl esters were prepared[11] and the identification of the fatty acids was done by gas chromatography on a diethyleneglycol succinate column at 190° C.

RESULTS

The methylic antigen, on dialysis against water, was precipitated. This precipitate was insoluble in acetone and chloroform but soluble in methanol, isopropanol, 1% SDS, and 1% Triton X-100.

Immunodifusion reactions of methylic antigens and antigens A and AC with homologous anti-**canicola** serum revealed an identity pattern.

The antigenicity of methylic antigens was not altered by heating at 100° C for 30 min., 56° C for 60 min. or 37° C for 24 hours as well as complement fixation reaction or immunodifusion carried out with anti-**canicola** rabbit serum. Antigenicity of methylic antigen was not altered either by incubation with 0.1 M HCl for 18 h, at 37° C or by treatment with either 1% pepsin or 1% pronase. However, the antigenicity was lost when the aqueous suspension of antigen was treated with 0.01 M periodic at 25° C for 60 min. The antigen A complexed with bovine serum albumin (BSA) did not elicit precipitating and complement fixing antibodies in rabbits.

Data of specific activity are summarized in Table I. Both methods led to similar purification factors, increasing the antigen specific activity by a factor of 1.6-1.7.

Gel filtration chromatography of methylic antigen showed only one peak appearing in the exclusion volume of the column. The molecular weight of antigens A and AC determined by polyacrylamide gel chromatography was 38 KDa. Only one band was obtained with an Rm=0.60.

The human sera of 15 patients with positive MAT showed positive complement fixation test with antigen A. The CF test exhibited an agreement with the MAT (Table II). The sera from eight healthy human showed negative MAT and negative complement fixation test.

The results of the chemical analysis of antigen A were: 40% carbohydrate (25% pentoses, 22% methylpentose, 28% hexoses) 4.0% protein, 20% lipids, and 2.7% phosphate. Glucose, xylose, arabinose, mannose, and rhamnose were identified after acid hydrolysis of antigen. The results of aminoacid and aminosugar analysis of antigen A

TABLE I

Determination of ratio polysaccharide/protein and specific activity of partial purification of methylic antigen with acetone (Antigen A) or acetone and chloroform (Antigen AC).

Antigen preparations	Polysaccharide (mg/ml)	Ratio polysacc/protein	Specific activity *(CFU/mg polyssacc.)	Purification Factor
Methylic	1.10	5.0	4.0	1
A	1.10	9.2	6.9	1.7
AC	0.97	9.7	6.5	1.6

*CFU: complement fixing units. 1 CFU defined as the amount promoting 50% of lysis of system containing five hemolytic units and anti-**canicola** serovar rabbit serum diluted at 1:40.

TABLE II
Sera of patients with positive MAT and positive CF antigen A of serovar **canicola**

Patients nº	CF test Antigen A Titers*	MAT				
		icterohaemorrhagiae	**javanica** Titers*	**bataviae**	**pomona**	**wolffi**
1	320		800			
2	160		400		100	
3	320				400	
4	640	800				
5	1280	1600				
6	640					800
7	640	800				
8	1280	1600				
9	1280			3200		
10	1280	3200				
11	640					400
12	1280			1600		
13	640	800				
14	320	400				
15	640	1600				

* reciprocal of the dilution

TABLE III
Determination of antigen A aminoacid and aminosugar composition by hydrolyse.

Aminoacid/ aminosugars	Hydrolysed antigen for 20 hs (umols)
Lysine	0.309
Histidine	0.120
Arginine	0.033
Aspartic acid	0.525
Threonine	0.015
Serine	0.051
Glutamic acid	0.165
Proline	0.015
Glycine	0.102
Alanine	0.066
Valine	0.036
Isoleucine	0.018
Leucine	0.024
Glucosamine	1.365

are shown in Table III. Lysine, aspartic and glutamic acids, and glycine were found in higher concentration than the other aminoacid, and galactosamine was not found. Gas chromatography analysis revealed the presence of lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:0), and linoleic acids (C18:2).

DISCUSSION

The methylic antigen after dialysis were slightly soluble in aqueous solutions and insoluble in acetone and chloroform and very soluble in methanol, isopropanol, and sodium dodecyl sulphate. This suggests the presence of a high percentage of hydrophobic groups in the antigen. This was confirmed by analysis of purified antigen which contained 20% of lipids. The methylic antigen showed a strong tendency to aggregation in aqueous solutions as revealed by the immobility in agarose gel electrophoresis and the elution in the void volume on Sephadex G-200 chromatography.

The non-immunogenicity of antigen A, when inoculated in rabbits, may be due to its chemical structure. The antigen A seems to be constituted of a lipopeptide phosphoglycan complex with a molecular weight of 38 KDa. The polysaccharide residues, apparently, have an important role in its antigenicity which is not altered by treatment with pepsin or pronase but is completely destroyed by periodic acid.

This antigen is chemically similar to the endotoxins from Gram-negative bacteria[16]. SHINAGAWA & YANAGAWA[24] also reported a type-specific leptospira antigen from Kyoto strain showing a similar chemical composition.

The antigen A contained 40% of carbohydrate. Glucose, galactose, xylose, arabinose, mannose, and rhamnose were identified. These results agree with the one reported by KASAI & YANAGAWA[14]. The aminoacid composition shows a predominance of aspartic acid and lysine. However the detection of several aminoacids in small concentrations suggests either that the antigen is not pure or that it is constituted of several small peptides.

The CF test with sera from patients with leptospirosis using the antigen A exhibit an agreement with the MAT. The methylic antigen purified by acetone could be used in diagnosis of human leptospirosis.

RESUMO

Isolamento e caracterização de antígenos parcialmente purificados de Leptospira.

Extrato metílico de **Leptospira interrogans** sorovar **canicola** foi purificado por precipitação com acetona ou acetona e clorofórmio. A antigenicidade não foi alterada por aquecimento ou tratamento com pepsina e pronase, entretanto foi perdida quando o antígeno foi tratado com ácido periódico. Análise química revelou a presença de 40% de carboidrato (22% de metilpentose, 28% de hexose), 4% de proteína, 20% de lípide e 2,7% de fosfato. Reação de fixação de complemento realizada com soros de pacientes com leptospirose apresentou concordância com a reação de aglutinação microscópica.

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