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The biological and physico-chemical characteristics of meningococcus polysaccharide group C prepared by two different methods of purification*

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We wished to evaluate the physico-chemical and biological characteristics of meningococcus polysaccharide group C prepared by two different methods of purification: cold phenol alone and cold phenol with the addition of two extractions with a chloroform–*n*-butanol mixture. With the latter method we obtained a polysaccharide less pyrogenic than that obtained with the procedure using only cold phenol. The physico-chemical characteristics of the polysaccharides obtained by the different procedures were identical.

INTRODUCTION

Two methods have been described by Gotschlich, Liu & Artenstein (1969) and Gotschlich, Rey, Etienne, Sanborn, Triau & Cvjetanovic (1972) for the purification of meningococcal polysaccharides. The method employing cold phenol is rapid and losses are negligible but the removal of pyrogenic material is incomplete. The chloroform–butanol extraction method (Servag, 1934) is laborious, causes unacceptable losses of polysaccharide, but effectively removes pyrogenic lipopolysaccharide. We have tested a combination of these two purification procedures and have defined the physico-chemical characteristics of the products obtained. We undertook this work in order to lower the threshold of pyrogenicity due to the presence of residual endotoxic lipopolysaccharide in the meningococcal polysaccharide, using a simple and rapid method.

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MATERIALS AND METHODS

Preparation of the polysaccharide

Strain used: *Neisseria meningitidis* C11 was obtained from the Walter Reed Institute of Medical Research, Washington, D.C., U.S.A.

Culture medium

Frantz medium (1942) supplemented with the dialyzate from 2 g l^{-1} of Difco yeast extract was used.

Purification of the polysaccharide

Polysaccharide was purified according to the methods summarized in the 27th W.H.O. Report (1976). In our experiments, after cold phenol extraction, a part of a lot was further treated with two extractions with a chloroform–*n*-butanol mixture (5 : 1 v/v) in a volumetric ratio of polysaccharide solution (4 mg ml^{-1}) to organic mixture of 4 : 1. Each extraction was performed in a mixer for 30 min at +4 °C. Following extraction the mixture was centrifuged at 10 000 × **g** for 10 min and the supernatant was drawn off under negative pressure. After two extractions the aqueous phase was dialyzed against cold 0·1 M-$CaCl_2$ for 18 h. The procedure was then continued using the accepted method with ultracentrifugation at $10^5 \times$ **g** for 3 h and reprecipitation of the polysaccharide at a final concentration of EtOH 75% v/v.

Determination of the molecular size of the polysaccharide

The molecular weight of the polysaccharide obtained using two different purification methods was determined by gel filtration on Sepharose 4B (Pharmacia lot no. 3325) on a column (1·6 × 100 cm) with ammonium acetate pH 7·0 at an ionic strength of 0·2 M kg^{-1} as eluent. The flow rate was approximately 20 ml h^{-1}. The calibration of the column for V_0 and V_t was carried out, respectively, with Blue Dextran 2000 (2 mg ml^{-1}, Pharmacia) and sodium azide (3 mg ml^{-1}, Merck). Six mg of polysaccharide was used for the determination of the K_d. The K_d of the volumes collected was calculated from the elution volume according to the formula of Ackers (1964):

$$K_d = (V_e - V_o)/(V_t - V_o)$$

The quantity of polysaccharide recovered before $K_d = 0{\cdot}5$ was determined by measuring the sialic acid content of the eluate emerging before that point.

Determination of sialic acid content

The procedure used was based on the method of Svennerholm (1957) using a 0·015% solution of N-acetyl neuraminic acid (Merck lot no. 7437814) as a standard. The colour developed during the reaction was extracted with isoamyl alcohol and the optical density of the organic phase read at 580 and 450 nm.

Determination of O-acetyl content

O-acetyl content was determined using Hestrin's (1949) procedure with 0·004 M acetylcholine HCl in sodium acetate 0·001 M at pH 4·5 as a reference standard (Sigma Chemical Co., lot no. 83C–0860).

Determination of residual protein and nucleic acids content

Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) utilizing bovine serum albumin as a standard (Armour Pharmaceutical Co., lot no. F–33206). Nucleic acids were assayed by measuring the optical density at 260 nm and assuming that the absorbancy of an aqueous salt free solution containing 50 μg ml^{-1} of nucleic acids was equal to 1·0.

Serological properties

Specificity. Inhibition of haemagglutination (HI) was performed by the method of Cohen & Artenstein (1971). A standard meningococcus group C rabbit antiserum with an HA titer of 1 : 1000, supplied by the Bureau of Biologics, Bethesda, Md., U.S.A., and sheep red blood cells sensitized with homologous antigen were employed. The highest dilution of the serum which gave definite haemagglutination was considered to have one activity unit. The test was considered satisfactory if there was inhibition of haemagglutination in the well containing the vaccine at a concentration of 100 μg ml^{-1} or less.

Purity. The purity of the group C polysaccharide was tested by HI using sheep red blood cells sensitized with meningococcus group A polysaccharides and group A antiserum with an HA titer of 1 : 5000 (supplied by the B.O.B., Bethesda, Md.). The test was considered satisfactory if there was no inhibition of haemagglutination with polysaccharide C at a final concentration of 100 μg ml^{-1} or greater.

Biological properties

Guinea-pig toxicity. Five healthy guinea-pigs weighing 350 g each were injected intraperitoneally with 500 mg of polysaccharide C in a volume of 0·5 ml. The animals were observed for 7 days and weighed on the first, third and seventh days. A control group was also kept during this period.

Mouse toxicity. Five healthy mice weighing approximately 16 g each were injected with 100 μg of polysaccharide C in a volume of 0·5 ml. The animals were observed for 7 days and weighed on the first, third and seventh days. A control group was also kept during this period.

Pyrogenicity. Groups of three healthy rabbits were inoculated in the merginal vein of the ear with 0·25 and 0·025 μg ml^{-1} of polysaccharide C. The polysaccharide was solubilized in sterile pyrogen-free saline and 1 ml kg^{-1} of body weight was injected into each rabbit. The results of the test were evaluated according to the International Pharmacopoeia (1967).

RESULTS

Table 1 and Fig. 1 show the physico-chemical characteristics of the polysaccharides obtained.

Serological properties

Specificity. Haemagglutination of antigen-sensitized erythrocytes by homologous antibody was specifically inhibited by a concentration of 12·5 μg ml^{-1} of the polysaccharide purified with cold phenol alone and by a concentration of 6·25 μg ml^{-1} of the polysaccharide purified with cold phenol and the chloroform–*n*-butanol mixture.

TABLE 1. Chemico-physical characteristics of the polysaccharide

Properties	Polysaccharide purified with cold phenol	Polysaccaride purified with cold phenol and chloroform–*n*-butanol
Chemical		
Sialic acid (%)	82·5*	83·7*
O-acetyl (μmol mg^{-1})	1·65	1·65
Protein (%)	0·45	0·2
Nucleic acid (%)	0·85	0·85
Physical		
K_d I peak	0	0
K_d II peak	0·24	0·23
Recovered before $K_d = 0·5$ (%)	82·5	82·1

* Values refer to dry weight of polysaccharide.

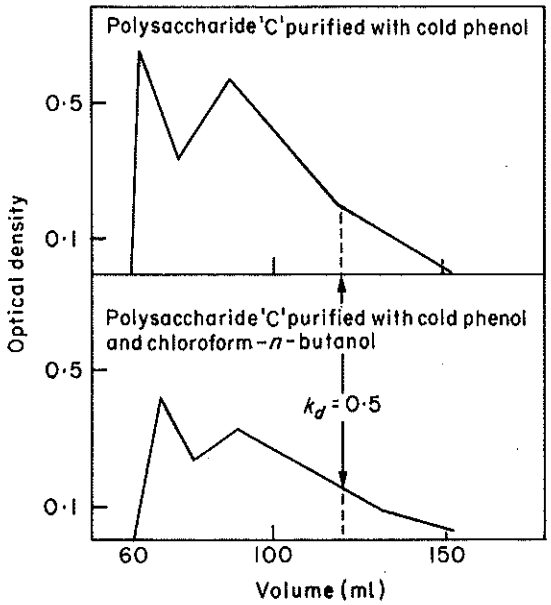

Fig. 1. Chromatographic profile of the polysaccharide on Sepharose 4B.

Purity. There was no inhibition of haemagglutination of erythcocytes sensitized by heterologous antigen in the presence of heterologous antibody by either preparation of polysaccharide C in a minimum final concentration of 100 μg ml^{-1}.

Biological properties

Guinea-pig toxicity. Guinea-pigs were injected with 500 μg of the two polysaccharide preparations and observed for 7 days. There was normal weight gain and none showed significant symptoms of illness or died.

Mouse toxicity. Following injection the mice gained weight normally. None showed significant symptoms of illness and none died.

Pyrogenicity. The capacity to reduce pyrogenicity using two extractions with chloroform–*n*-butanol was investigated on the rabbit with the convenient and reliable biological test. In fact, as already known, the 2-keto-3-deoxyoctulosonic acid assay (to chemically determine LPS) cannot be performed because of the strong interference caused by the group C meningococcal polysaccharide. The results of the pyrogen tests done in groups of rabbits injected with polysaccharide C prepared with the two methods are shown in Fig. 2. Two concentrations of each of the two preparations of polysaccharide were prepared, one 10 times greater than the minimum standard established in the W.H.O. Technical Report Series (1977). This was done in order to observe more clearly the differences in pyrogenicity between the polysaccharides. Four groups of three rabbits received each of the two concentrations of the polysaccharide prepared with the two methods described. The preparation of group C polysaccharide subjected to the additional purification with chloroform–*n*-butanol was less pyrogenic.

An analysis of variance was performed and the values obtained are shown in Table 2.

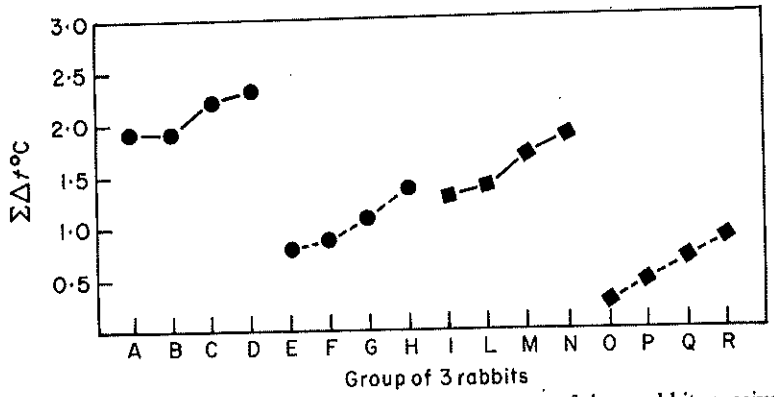

Fig. 2. Comparative pyrogen tests in rabbits. Four groups of three rabbits received each of the two concentrations of polysaccharide prepared with the two different methods of purification. ●——●, purification with cold phenol 0·25 μg kg^{-1}. ● - - - - ●, purification with cold phenol 0·025 μg kg^{-1}. ■——■, purification with cold phenol + chloroform–*n*-butanol 0·25 μg kg^{-1}. ■ - - - - ■, purification with cold phenol + chloroform–*n*-butanol 0·025 μg kg^{-1}.

TABLE 2. Analysis of variance of pyrogen test

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F* ratio
(1) Between groups	0·135	3	0·045	1·77
(2) Between doses	4·000	1	4·000	157·74*
(3) Interaction 1 and 2	0·455	3	0·151	5·98
(4) Between purification methods	0·902	1	0·902	35·59*
Error	0·177	7	0·025	
Total	5·669	15		

* Statistically significant, $P = 0{\cdot}05$.

DISCUSSION

A comparison of the properties of the group C meningococcal polysaccharide prepared using the two methods permits the following conclusions to be drawn. Two extractions

with the chloroform–*n*-butanol mixture after treatment with cold phenol resulted in a product having significantly less pyrogenicity than the polysaccharide obtained by purification with cold phenol alone. The polysaccharide purified using the latter method passed the pyrogen test at the dose of 0·025 μg kg^{-1} but not at a dose 10 times greater. The polysaccharide first purified with cold phenol and subsequently with chloroform–*n*-butanol extraction easily passed the pyrogen test at the recommended dosage (0·025 μg kg^{-1}) and at a 10-fold greater dosage there was a marked decrease in pyrogenicity in comparison with the polysaccharide purified solely with cold phenol. The analysis of variance showed a statistically significant difference, $p < 0{\cdot}05$, in the pyrogenicity of the polysaccharide prepared by the two methods.

In addition it must be pointed out that the two extractions with the chloroform–*n*-butanol mixture after treatment with cold phenol did not have any effect on the molecular weight of the product (Table 1, Fig. 1). The polysaccharide exhibited an elution profile identical to that of the polysaccharide obtained with the cold phenol procedure alone. This is in contrast to the findings of Wong, Barrera, Sutton, May, Hochstein, Robbins, Robbins, Parkman & Seligman (1977) in respect of the meningococcus polysaccharide group A which, when purified only with continuous extraction with a chloroform–*n*-butanol mixture, lost its characteristic molecular profile on gel chromatography due to depolymerization during purification.

With the latter procedure our experiments showed losses up to 50% in polysaccharide in respect to the optimal yield (40 mg l^{-1} culture) for the group C polysaccharide published by Gotschlich (1975). This is due to the need for nine continuous extractions with chloroform–*n*-butanol to obtain a protein residue content in the final product less than 1% (Fig. 3).

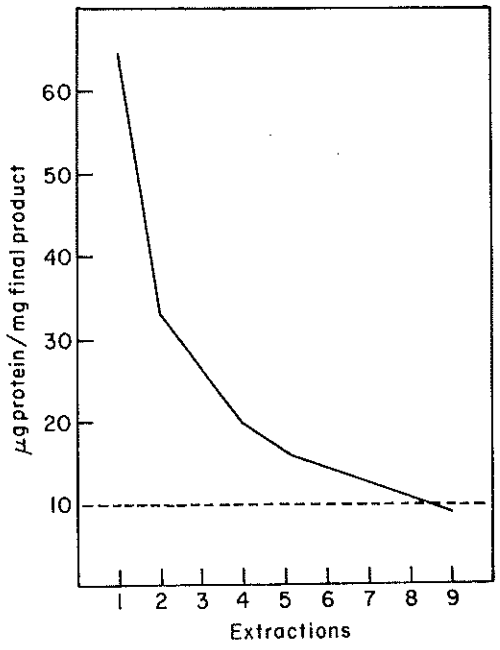

Fig. 3. Deproteinization of group C meningococcal polysaccharide using the Sevag method.

On the other hand, using cold phenol and only two extractions of a chloroform–*n*-butanol mixture there was a final product yield in agreement to the value of the 40 mg l^{-1} culture. Significant losses of purified bacterial polysaccharide using the Sevag method for the removal of proteins is also described by Staub (1965).

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