INFECTION AND IMMUNITY, Nov. 1970, p. 640-643
Vol. 2, No. 5
Copyright © 1970 American Society for Microbiology
Printed in U.S.A.

Pilot-Scale Production of Group A and Group C Meningococcal Polysaccharide Immunogens

SANFORD BERMAN, PATRICIA L. ALTIERI, ALBERT GROFFINGER, AND JOSEPH P. LOWENTHAL

Department of Biologics Research, Walter Reed Army Institute of Research, Washington, D.C. 20012

Received for publication 16 June 1970

Methods have been developed for the pilot-scale production of group A and group C meningococcal polysaccharide immunogens based on the meningococcal strains, the medium, and the basic processing procedures used by Gotschlich et al. Physical and chemical assays on the final products obtained on a pilot-scale level indicate that these purified polysaccharides are entirely comparable to the Gotschlich preparations which proved to be immunogenic in man.

Gotschlich, Liu, and Artenstein (4) and Weiss and Long (10) have described methods for producing group-specific high-molecular-weight polysaccharide antigens from the supernatant culture fluids of *Neisseria meningitidis* groups A and C. These investigators have, however, encountered difficulties in growing the organisms in the medium of Watson and Scherp (9) in volumes larger than 1 to 1.5 liters. Thus, the preparation of large volumes of culture filtrates for producing purified polysaccharides would be especially cumbersome in that it would require the processing of a considerable number of individual 2- or 4-liter filasks. In a more recent publication, Robinson and Apicella (7) reported producing meningococcal culture filtrates in 5-liter volumes, growing the organisms in a fermentor. However, the medium used was that of Mueller-Hinton.

Of all of the methods for the preparation of purified meningococcal polysaccharides described by various investigators (5, 7, 9, 10), only that of Gotschlich et al. (4) resulted in a product having demonstrable immunogenicity and protective properties in man (1). Consequently, this study sought to devise practical methods for producing and purifying the group A and C polysaccharides in larger quantities without deviating from the strains, the medium, and the basic processing procedures that have yielded effective products.

MATERIALS AND METHODS

Meningococcal strains. *N. meningitidis* strains A1 and C11 were obtained from the Department of Bacterial Diseases, Walter Reed Army Institute of Research. For each strain, the growth from a series of Mueller-Hinton Agar (Difco) plates, after incubation in a candle jar at 36 C for 18 hr, was harvested in the Watson-Scherp medium, pooled, and distributed in 2-ml amounts per 5-ml screw cap vial. These seed cultures were frozen and stored at −70 C.

Analytical methods. Protein determinations on the meningococcal preparations were performed by the method of Lowry et al. (6) by using bovine serum albumin as a standard. Nucleic acid content was determined by the ultraviolet absorbancy at 260 nm by using $E_{1\ cm}^{0.1\%}$ of 20.0. The method of Fiske and SubbaRow (2) was used for the determination of phosphorus for the "A" polysaccharides. The sialic acid content of the "C" polysaccharides was determined by the method of Svennerholm (8), with a sample of the C polysaccharide used by Gotschlich, Goldschneider, and Artenstein (3) for trials in humans serving as a standard. Molecular size was estimated by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), as described by Gotschlich, Liu, and Artenstein (4).

Toxicity. The presence of biologically active endotoxin was determined, as described by Gotschlich and co-workers (3), by measuring the pyrogenic response of rabbits to a 2.5-μg dose (injected intravenously) of purified polysaccharide.

RESULTS

Preparation of the medium. In preliminary studies, it became apparent that the medium of Watson and Scherp (9) was temperature-sensitive. The C11 strain of *N. meningitidis* grew poorly, if at all, in the medium autoclaved for the length of time required to sterilize 12 liters in a 20-liter carboy. Therefore, it was decided to sterilize, by filtration, concentrates (12×) of those medium components likely to be affected by autoclaving. This procedure consistently resulted in a sterile final medium which supported good growth of both strains of meningococcus. The medium was prepared as follows: 1,440 g of Casamino Acids (Difco, certified), 13.2 g of KCl, 93.6 g of $MgSO_4 \cdot 7H_2O$, 2.9 g of cysteine hydrochloride

(mono), and 720 g of dextrose were dissolved in distilled water and the volume was brought to 12 liters. This solution was passed through six sterile D-8 Hormann filter pads [8 by 6 inches (20.3 by 15.2 cm)] in a Hormann filter press. The sterile filtrate was then dispensed in 100- and 1,000-ml amounts and stored at 22 C. A second solution was prepared consisting of 5.25 g of $Na_2HPO_4 \cdot 7H_2O$ per liter and 3.3 mg of phenol red per liter. For seed broth, 1.1 liters of this second solution was dispensed per 4-liter flask, and, for the production bottle, 9.8 liters was dispensed per 20-liter carboy. These preparations were sterilized by autoclaving at 121 C for 75 min. In addition, 4 ml of UCON Lubricant (LB-625; Union Carbide Corp.) was added to 50 ml of distilled water and sterilized by autoclaving at 121 C for 60 min.

Before use, the 100-ml volume of the Casamino Acids-dextrose-salt solution was added to the 1.1 liters of seed solution, and the 1,000-ml volume of the Casamino Acids-dextrose-salt solution, together with the UCON solution, was added to the 20-liter carboy. The final *p*H of the medium was 7.3 to 7.4. The bottles of the final medium were incubated at 36 C for 48 to 72 hr before inoculation, to check for contamination which may have occurred during the transfer process.

Inoculation and growth of the organism. The procedure described by Gotschlich et al. (4) was modified in that more concentrated inocula were employed and three transfers of the organisms were involved rather than four. A vial of frozen *N. meningitidis* seed culture was thawed, and the culture was streaked onto the surface of a series of Mueller-Hinton Agar (Difco) plates which were then incubated in a candle jar at 36 C for 18 hr. The plates were examined visually for purity, and the entire growth on each plate was harvested in 10 ml of the complete Watson-Scherp medium (9). The harvests from the series of plates were pooled. A 15-ml amount of the suspension was then transferred to each 1.2 liters of seed broth, and the seed flasks were incubated at 36 C while on a reciprocal shaker operating at 110 to 120 strokes/min. After 4 to 5 hr, when good growth was established, the entire contents of a flask were transferred to the 10.8 liters of medium in the 20-liter carboy. A sterilized asparger, attached to a tube through a cotton plug, was then placed in the carboy and the carboy was incubated at 36 C, for 16 to 18 hr for the C strain and 14 to 15 hr for the A strain. Aeration was accomplished by bubbling sterile air into the medium at a flow rate of 2 to 3 liters/min while the carboys were on a reciprocal shaker operating at 120 3-inch strokes/min.

Preparation of the polysaccharides. For each of the two meningococcal strains, 36 liters of culture (three 20-liter carboys each containing 12 liters of culture) was grown. The cultures were then treated with Cetavlon (hexadecyltrimethylammonium bromide, technical; Eastman Organic Chemicals, Rochester, N.Y.), the resulting precipitates were collected by centrifugation (in a Sharples supercentrifuge), and the "wet" crude polysaccharide was obtained by the method described by Gotschlich et al. (4).

At this point, a series of steps, which included (i) converting the "wet" crude material to dryness, (ii) redissolving the dry material, and (iii) centrifuging the crude solution twice at 100,000 $\times$ *g*, was eliminated. Analysis of fluid materials for polysaccharide content after the subsequent homogenization procedure with and without the above steps indicated that, in terms of yield, there was no advantage to their inclusion. It was also found that a significant amount of polysaccharide could be extracted from the emulsions formed during the homogenization procedure [this step was used by Watson and Scherp (9) in their method for preparing purified meningococcal polysaccharides], and, although these materials were relatively high in protein and nucleic acid content, these contaminants were readily removed in the subsequent purification steps. Accordingly, the "wet" material was dissolved in 100 ml of distilled water and was processed through four to five cycles of homogenization with chloroform (30 min/cycle) to remove the bulk of the contaminating protein and nucleic acid from the aqueous material. The chloroform emulsions formed at each cycle were pooled and extracted twice with 50 ml of distilled water, and the extracts were homogenized once more with chloroform. The clear supernatant obtained after centrifugation was added to the first aqueous material. The aqueous pools were then treated with either 3 volumes of ethanol to precipitate the C polysaccharide or 10 volumes to precipitate the A polysaccharide. The precipitates were collected by centrifugation and were redissolved in 100 ml of distilled water. Final purification was then attained [to bring the protein, nucleic acid, and endotoxin content of the final purified polysaccharides to the levels found in the standard preparations (3)] by first adding 2 ml of 1 M $CaCl_2$ and then adding cold ethanol until a slight opalescence developed. This solution was placed at 4 C for 30 to 60 min. (If the opalescence became too pronounced, distilled water was added back until the condition of slight opalescence stabilized.) The opalescence was removed by centrifugation at 78,000 $\times$ *g* for 2 hr at 4 C. The clear supernatant fluid was collected, and treatment with ethanol was repeated twice more. After the third

TABLE 1. *Processing of meningococcal polysaccharides*

Stages of the procedure	Group A polysaccharide				Group C polysaccharide			
	Yield[a]	Protein	Nucleic acid	Phosphorus	Yield[a]	Protein	Nucleic acid	Sialic acid
	g/liter	%	%	%	*g/liter*	%	%	%
Culture fluid[b]								
Sharples sediment	4.3				2.17			
Crude precipitate	0.11	27.2	3.0	4.83	0.089	11.1	1.7	91.0
Chloroform extraction								
5th chloroform extract	0.011	0.93	0.62	6.70	0.033	3.0	0.74	99.0
emulsion extract	0.013	2.28	1.60	6.30	0.022	6.4	1.45	99.0
pool of 5th chloroform and emulsion extracts	0.021	1.90	1.23	6.35	0.058	3.9	1.02	99.0
Final purification								
1st alcohol treatment	0.017	0.91	0.53	4.99	0.046	3.0	0.76	99.0
2nd alcohol treatment	0.013	0.83	0.59	5.08	0.031	1.2	0.34	99.0
3rd alcohol treatment (final product)	0.016	0.50	0.36	7.06	0.016	0.15	0.23	99.0

[a] For the Sharples sediment and the final product, the figures given are the actual weights of the materials obtained. The figures given at the other stages are calculated from the weight of the polysaccharide precipitated from a small sample of the fluid obtained at that stage.

[b] For group A polysaccharide, the *p*H of the culture fluid was 5.4. For group C polysaccharide, the *p*H of the culture fluid was 5.2.

treatment, the centrifugation time was extended to 18 hr. The polysaccharide was then precipitated by the addition of either 3 volumes of ethanol to the supernatant fluid in the preparation of the C antigen or 10 volumes in the preparation of the A materials. The precipitates were converted to the sodium salt by dissolving them in saturated sodium acetate at *p*H 7.0 and reprecipitating them with ethanol. The precipitates were washed twice with ethanol and twice with acetone and dried.

The results of measurements and assays of samples taken at convenient steps in the processing of the group-specific polysaccharides, from crude to final purified form, are shown in Table 1.

When assayed in this laboratory, the purified polysaccharides prepared by the methods of Gotschlich et al. showed a molecular weight of over 100,000; a sialic acid content of at least 85% for the C polysaccharide; a phosphorus content of at least 7.0% for the A polysaccharide; and protein and nucleic acid contents each below 1%. Therefore, by these criteria, the final products obtained in this study were considered equivalent to the preparations of Gotschlich et al.

In addition, data obtained from M. S. Artenstein (*personal communication*) indicated that the purified group A and C materials prepared by the methods described in this study performed in in vitro systems and the C materials performed in man, similarly to the polysaccharides prepared by Gotschlich et al.

DISCUSSION

The results of these studies indicate that group A and C purified polysaccharides can be produced on a pilot-scale level by using the medium and essentially the methods described by Gotschlich and co-workers (4). Although some modifications were made to simplify and shorten the procedure, no new reagents were introduced.

Although the yield of Sharples sediment after Cetavlon precipitation of the A preparation was twice that of the C, this was not reflected in the amount of crude precipitate or in yield of final product which, in both instances, were approximately the same. At the crude precipitate step, there appeared to be approximately twice as much contaminating protein and nucleic acid in the A preparation than in the C, but the five repeated treatments with chloroform appeared to remove these contaminants more efficiently from the A material. The A product at this point had approximately the same nucleic acid content but considerably lower protein than the C preparation. With both preparations, the amount of polysaccharide extracted from the chloroform emulsions added considerably to the final product yield, and, although the level of contaminants with the polysaccharides available in the extract pools was increased, the three subsequent alcohol and centrifuge treatments reduced the protein

and nucleic acid contents of the final purified product to a satisfactory level.

Both the A and C purified antigens were found, by Sephadex G-200 gel filtration, to have molecular weights exceeding 100,000, and 2.5 μg of either material injected intravenously into rabbits did not cause any perceptible pyrogenic response.

The yields of purified A and C polysaccharides obtained from 36-liter cultures (16 mg/liter) were approximately twice those reported by Gotschlich et al. (4), obtained from 1- to 1.5-liter cultures (A = 8.6 mg/liter; C = 9 mg/liter), and were comparable in per cent protein, nucleic acid, sialic acid, phosphorus, molecular weight, and freedom from endotoxin to those preparations of A and C polysaccharides used in humans (1, 3). Thus, a single pilot run yielded a total of 576 mg of purified polysaccharide or the equivalent of 11,500 human immunizing doses (50 μg each).

LITERATURE CITED

1. Artenstein, M. S., R. Gold, J. G. Zimmerly, F. A. Wyle, H. Schneider, and C. Harkins. 1970. Prevention of meningococcal disease by group C polysaccharide vaccine. New Engl. J. Med. 282:417–420.
2. Fiske, C. H., and Y. SubbaRow. 1925. The colorimeter determination of phosphorus. J. Biol. Chem. 66:375–400.
3. Gotschlich, E. C., I. Goldschneider, and M. S. Artenstein. 1969. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. J. Exp. Med. 129:1367–1384.
4. Gotschlich, E. C., T. Y. Liu, and M. S. Artenstein. 1969. Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B and group C meningococcal polysaccharides. J. Exp. Med. 129:1349–1365.
5. Kabat, E. A., H. Kaiser, and H. Sikorski. 1944. Preparation of the type-specific polysaccharide of the type I meningococcus and a study of its effectiveness as an antigen in human beings. J. Exp. Med. 80:299–307.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
7. Robinson, J. A., and M. A. Apicella. 1970. Isolation and characterization of *Neisseria meningitidis* groups A, C, X, and Y polysaccharide antigens. Infec. Immun. 1:8-14.
8. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol hydrochloric acid method. Biochim. Biophys. Acta 24:604–611.
9. Watson, R. G., and H. W. Scherp. 1958. The specific hapten of group C (group IIα) meningococcus. I. Preparation and immunological behavior. J. Immunol. 81:331–336.
10. Weiss, E., and J. L. Long. 1969. Simplified method for the production of carbohydrate antigen from *Neisseria men ingitidis*. Appl. Microbiol. 18:843–847.