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Immunoelectrophoretic characterization of the molecular weight polydispersion of polysaccharides in multivalent bacterial capsular polysaccharide vaccines*

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The molecular weight polydispersion of single antigens present in multivalent bacterial capsular polysaccharide vaccines has been characterized by an immunoelectrophoretic method. Chromatographic effluents from Sepharose gel of bacterial capsular polysaccharide vaccines were tested by fused-rocket immunoelectrophoresis and the distribution coefficient (Kd) of each polysaccharide present in the mixture was calculated. The method appeared to be efficient and reproducible. However, different Kd values were obtained by immunoelectrophoretic and chemical or physical analysis of the chromatographic effluents of each single polysaccharide component. The use of this immunoelectrophoretic procedure was extended to the potency control of multivalent meningococcal and pneumococcal polysaccharide vaccines in order to detect changes in the molecular weight polydispersion of each antigen with time.

INTRODUCTION

Polysaccharides are known to be very poor antigens in animal models, but their antigenicity in humans, including that of bacterial capsular polysaccharide vaccines, has been shown to be directly related to their molecular size.[1,2,3] Thus, requirements for the control of molecular weight polydispersion (potency control) of the bacterial

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capsular polysaccharides present in meningococcal and pneumococcal vaccines for human use were introduced by the W.H.O.[4,5,6] on the basis of gel chromatography and Sepharose 4B as molecular sieve. These procedures are accurate and suitable for single polysaccharide antigens or for a mixture of antigens which do not interfere one with another when tested by chemical methods in the chromatographic effluents. However, several problems arise when a mixture of antigens with similar chemical moieties in their structures must be tested after molecular sieving. A similar problem occurs with the pneumococcal polyvalent vaccine, which at the present time includes 14 polysaccharide antigens, when potency control of the single components is performed over a period of time. This paper describes the use of an immunoelectrophoretic method to determine the molecular weight polydispersion of each antigen component of the polyvalent pneumococcal vaccine, there being no significant immunochemical cross-reactions between the type specific polysaccharides present in the vaccine when these are tested with the corresponding homologous rabbit antisera.[7] The use of this procedure was extended to the groups A and C meningococcal bivalent vaccine, although in this case a chemical differentiation of two antigens in the chromatographic effluents was possible.

From these experiments, different values of distribution coefficients (Kd) were found with physical and chemical analyses of chromatographic effluents of single polysaccharides after separation on Sepharose gel. Experiments to explain these differences were carried out.

MATERIALS AND METHODS

Polysaccharides

Pneumococcal capsular polysaccharides serotypes 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F and 25 (Danish nomenclature) were prepared in our laboratories and were present in an experimental lot of vaccine for human use (ISVT Sclavo, Siena, Italy). The characteristics of the polysaccharides complied with the proposed requirements of W.H.O.[6] for pneumococcal polysaccharide vaccine.

Meningococcal capsular polysaccharides of serogroups A and C and meningococcal vaccines were commercial products (ISVT Sclavo, Siena, Italy).

Antisera

Rabbit pneumococcal typing antisera were obtained from the Pneumococcal Department of the W.H.O. Centre for Reference and Research, Statens Seruminstitut, Copenhagen, Denmark.

Horse meningococcal group A and C antisera were kindly supplied by Dr C. E. Frasch (Bureau of Biologics, Bethesda, Maryland).

Chromatographic media and gel chromatography

Sepharose 2B (lot 7594), Sepharose 4B (lot 3325) and Sepharose 6B (lot 10457) were obtained from Pharmacia (Uppsala, Sweden). All chromatographic media were equilibrated in 0·2 M sodium chloride solution with pH adjusted to 7·0 ± 0·1 with base as required. Columns of 1·6 × 100 cm were employed.

Gel chromatography of single polysaccharide antigens were performed by loading with about 3 mg of polysaccharide. Column effluents were monitored at 206 nm with

Uvikord S spectrophotometer (LKB, Bromma, Sweden). Fractions of approximately 4·5 ml were collected and chemically assayed for uronic acid (types 1, 3, 8, 9N) by the Gregory method,[8] methyl pentose (types 2, 6A, 7F, 18C, 19F, 23F) by the Kabat method,[9] hexosamines (types 4, 12F, 14, 25) by the Ashwell method,[10] phosphorus (group A) by the method of Chen, Toribara and Warner[11] and sialic acid (group C) according to the method of Svennerholm.[12] These fractions were also tested by fused-rocket immunoelectrophoresis as later described. Gel chromatography of polyvalent pneumococcal vaccine and bivalent meningococcal vaccine was performed with a loading of the equivalent of ten human doses of these vaccines, corresponding to a total of about 500 μg for each antigen. The collected chromatographic fractions were tested by fused-rocket immunoelectrophoresis.

Immunoelectrophoresis

Rocket immunoelectrophoresis of the pneumococcal polysaccharide type 19F was performed according to a modified Weeke procedure:[13] agarose gels (Agarose M-LKB, Bromma, Sweden) at concentrations of 0·5, 1 and 2% w/v in 0·02 M Tris barbiturate buffer pH = 8·6 were employed on plastic plate supports. Each gel contained 0·5% v/v of homologous rabbit antiserum corresponding to 0·75 μl cm^{-2} gel area. In each antibody containing agarose gel, 5 μl aliquots of solutions of polysaccharide (0·1 mg ml^{-1}) at three different Kd values (0·10, 0·47, 0·71 on Sepharose 4B), were loaded. Electrophoresis was performed at 70 v/cm for 2 h in a Multiphor electrophoresis apparatus (LKB—Bromma, Sweden) at constant temperature (15 ± 1°C). Following electrophoresis, gels on plastic plate supports were moistened with distilled water, pressed with filter paper and dried at 37°C for 30 min. Rocket immunoprecipitates were then detected by Coomassie Blue-R 250 stain.

Fused-rocket immunoelectrophoresis of polyvalent vaccines and of the single polysaccharide antigens, following the molecular sieving on different Sepharose gels, was performed according to a modified Svendsen method:[14] 10 μl aliquots of the column fractions were transferred to a row of wells in an antibody-free agarose gel 1% w/v. Samples were allowed to diffuse into the gel for 60 min. Electrophoresis was performed in agarose 1% w/v containing 0·5% v/v of homologous antiserum to a specific polysaccharide antigen under the same conditions described for rocket immunoelectrophoresis.

Quantitative precipitin procedure for nitrogen antibody estimation

The microprecipitin technique of Schiffman, Kabat and Thompson was used as modified by Etzeler.[15] Briefly, aliquots of solutions of type 6A pneumococcal polysaccharide at two different Kd values (0·17 and 0·51) and type 19F pneumococcal polysaccharide at three different Kd values (0·10, 0·47 and 0·71) containing amounts of polysaccharide ranging between 1–10 μg, were incubated for 1 h at 37°C in conical tubes with a constant volume (10 μl) of homologous rabbit antiserum. The final salt concentration was 0·9% sodium chloride and the final volume in each tube was 100 μl Tubes were kept at 4°C for 18 h, then the precipitates were collected by centrifugation at 10 000 r min^{-1} and washed in cold saline. Nitrogen was determined in the washed precipitates by the ninhydrin method of Etzeler,[15] using ammonium sulphate as standard. The calculated quantities of antibody nitrogen in the precipitates collected were plotted against the respective amounts of polysaccharide used to check the

behaviour of the typical immunoprecipitation curve. From this curve, the maximum and average value of the nitrogen antibody quantity precipitated by 1 μg of polysaccharide (at different Kd's) were calculated.

Determination of the distribution coefficient (Kd) of polysaccharides

Calibration of the chromatographic columns was performed by determination of interstitial volume (Vi) using sodium azide and void volume (Vo) using Blue Dextran 2000 (Pharmacia-Uppsala, Sweden).

In each chromatographic run, Vi was determined from the position of the elution peak for sodium azide. The elution volume (Ve) of the polysaccharide antigens was calculated as follows: the chemical amount or the height of the immunoprecipitate rockets determined in each of the chromatographic fractions collected were plotted against the chromatographic effluent volumes. On the maximum peak of the curve obtained, two tangents were drawn and their intersection point represented the value of the elution column. The Kd value corresponding to the Ve value was calculated according to Acker's formula $Kd = (Ve - Vo)/(Vi - Vo)$.[16]

RESULTS

Table 1 summarizes the distribution coefficient values of the single polysaccharides investigated, calculated according to Acker's formula. Comparisons of the results of chemical or immunoelectrophoretic analyses for single pneumococcal and meningococcal antigens are shown. Similar values were also found when the multivalent vaccines were subjected to immunoelectrophoretic investigation for each polysaccharide component after molecular sieving on Sepharose 4B. Type 7F and Type 14 pneumococcal polysaccharides were not included because of the absence of anodal migration of these antigens at pH 8·6, when subjected to an electric field.[7] Experiments under similar conditions using a borate-derivative buffer[17] for these polysaccharides are planned in our laboratory. Plate 1 shows some examples of fused-rocket immunoelectrophoresis for pneumococcal antigens after gel chromatography of the multivalent vaccine on Sepharose 4B and Plate 2 shows a comparison between physico-chemical and immunoelectrophoretic analysis of the molecular weight polydispersion of two polysaccharide antigens. Immunoelectrophoretic analysis gave good reproducibility of results with a maximum discrepancy corresponding to 0·02 for a typical Kd of several polysaccharides.

Some single polysaccharides were also tested on different gel matrices to compare the immunoelectrophoretic behaviour of the molecular weight polydispersion of polysaccharides under conditions of different molecular sieving. Plate 3 shows a comparison of molecular polydispersion detected by immunoelectrophoresis, obtained from Sepharose 4B and 6B for the single pneumococcal type 8 polysaccharide. A similar comparison for pneumococcal type 4 polysaccharide from Sepharose 2B and 4B is shown in Plate 4. As recorded in Table 1, differences existed in all Kd values determined by chemical or immunoelectrophoretic procedures and were more marked when the molecular sieving properties of the gels were increased. The reasons for these differences were attributed as follows: the possible molecular sieve effect afforded by agarose gel support in immunoelectrophoresis on different chromatographic polydispersion, and the possibility that a typing antiserum could form different amounts of immunoprecipitates when reacting with different polysaccharide polydispersions. Rocket immunoelectrophoresis

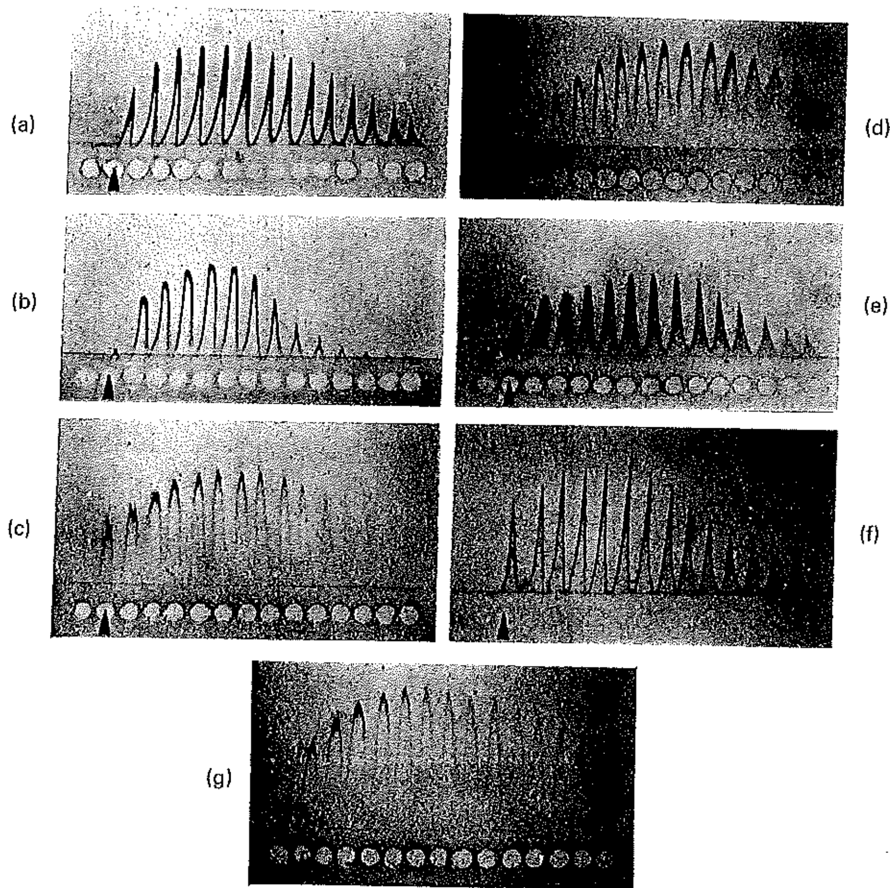

Plate 1. Fused-rocket immunoelectrophoresis of some pneumococcal polysaccharides after gel chromatography of multivalent vaccines (see Methods) on Sepharose 4B. Pneumococcal polysaccharides: (a) type 1, (b) type 6A, (c) type 18C, (d) type 25, (e) type 9N, (f) type 19F, (g) type 12F. Arrows indicate the chromatographic fraction corresponding to the void volume.

(facing page 68)

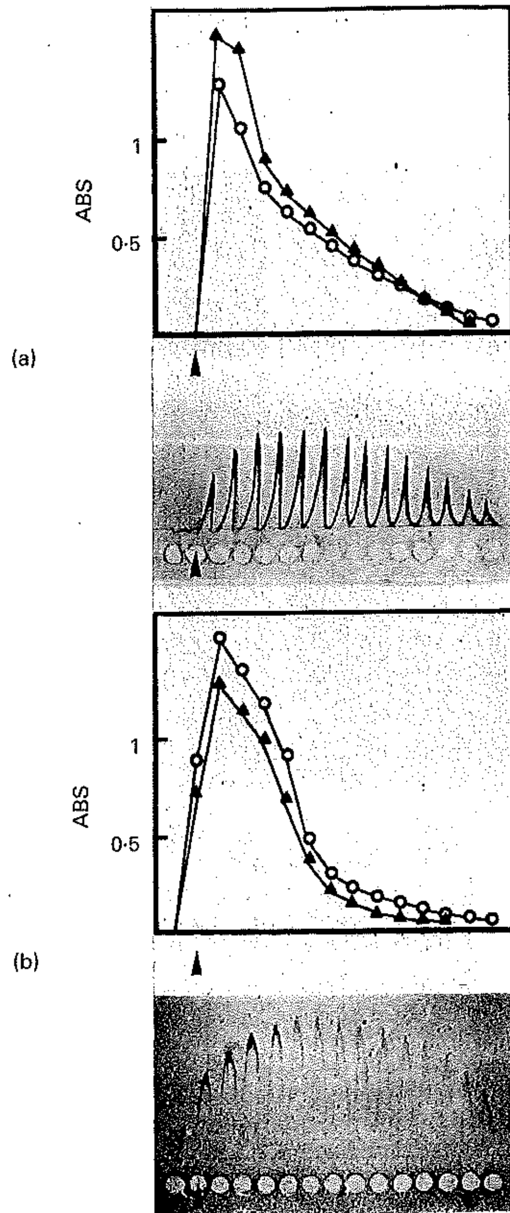

Plate 2. A direct comparison between physico-chemical and immunoelectrophoretic analysis of the molecular weight polydispersion of type 1 (a) and type 12F (b) pneumococcal polysaccharides after gel chromatography on Sepharose 4B. (▲) physical analysis (reading at 206 nm); (○) chemical analysis (uronic acid for type 1 polysaccharide and hexosamines for type 12F polysaccharide). Arrows indicate the chromatographic fraction corresponding to the void volume.

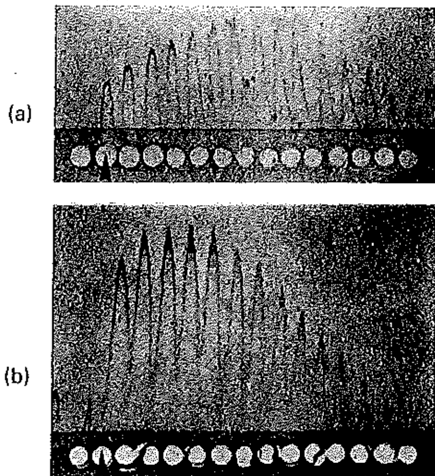

Plate 3. Immunoelectrophoretic comparison of molecular polydispersion of pneumococcal polysaccharide type 8 after molecular sieving on different gel matrices. (a) from Sepharose 4B, (b) from Sepharose 6B. Arrows indicate the chromatographic fraction corresponding to the void volume.

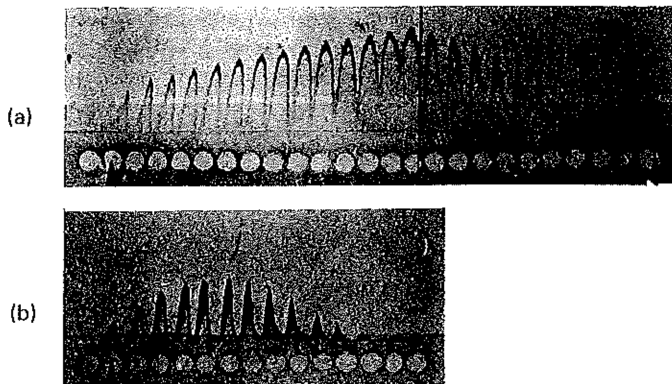

Plate 4. Immunoelectrophoretic comparison of molecular polydispersion of pneumococcal polysaccharide type 4, after molecular sieving on different gel matrices. (a) from Sepharose 2B, (b) from Sepharose 4B. Arrows indicate the chromatographic fraction corresponding to the void volume.

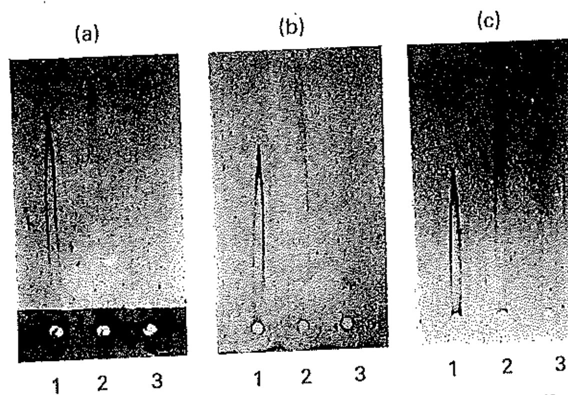

Plate 5. Rocket immunoelectrophoresis on agarose gel supports of three different molecular distributions of the type 19F pneumococcal polysaccharide. 1—polysaccharide with Kd = 0·10 (0·1 mg ml^{-1}), 2—polysaccharide with Kd = 0·47 (0·1 mg ml^{-1}), 3—polysaccharide with Kd = 0·71 (0·1 mg ml^{-1}). Agarose gel supports were at the concentration of: 0·5% w/v (a), 1% w/v (b), and 2% w/v (c).

TABLE 1. A comparison of the Kd values of some bacterial capsular polysaccharide preparations obtained by chemical and immunoelectrophoretic analyses of column effluents from different chromatographic media

Polysaccharide antigen	Kd on Sepharose 6B			Kd on Sepharose 4B			Kd on Sepharose 2B		
	Chemical procedure	Immunoelectro-phoretic procedure	Δ Kd	Chemical procedure	Immunoelectro-phoretic procedure	Δ Kd	Chemical procedure	Immunoelectro-phoretic procedure	Δ Kd
Pneumococcal type									
1				0·05	0·13	0·08			
2				0·05	0·20	0·15	0·28	0·50	0·22
3				0·05	0·09	0·04			
4				0·01	0·20	0·19	0·30	0·54	0·24
6A				0·15	0·19	0·04	0·50	0·68	0·18
8	0·01	0·05	0·04	0·11	0·32	0·21			
9N	0·01	0·09	0·08	0·12	0·25	0·13			
12F				0·05	0·22	0·17			
18C				0·14	0·18	0·04			
19F				0·08	0·12	0·04			
23F				0·09	0·14	0·05			
25				0·30	0·34	0·04			
Meningococcal group									
A				0·37	0·48	0·11			
C				0·26	0·37	0·11			

TABLE 2. The quantitative precipitation of rabbit typing sera by polysaccharides with different values of distribution coefficient

Polysaccharide type	Kd polysaccharide (chemical procedure)	μg NAb* μg^{-1} polysaccharide Maximum	Average
6A	0·16	3·00	2·76
	0·51	1·50	1·31
19F	0·05	3·50	1·15
	0·47	2·85	0·70
	0·71	0·31	0·15

* Nitrogen antibody quantity.

The values represent the maximum and the average amount of antibody nitrogen precipitated by 1 μg of the indicated polysaccharide on the basis of the immuno-precipitation curves obtained as described in the Methods section.

in Plate 5 shows the molecular sieve effect of some agarose gel supports, in addition to a different behaviour of the antiserum, on type 19F polysaccharide with different distribution coefficient values. In fact, the 'rockets' showed a good definition of contours only when a polydispersion of high molecular weight of the polysaccharide was employed and the differences in the heights of the rockets between the different molecular polydispersions of the same amount of polysaccharide were more evident when the concentration of the agarose gel support was increased.

The data shown in Table 2, obtained with types 6A and 19F pneumococcal polysaccharides, quantitatively confirm the finding that different molecular weight polydispersions of a polysaccharide precipitate different amounts of typing antibodies. Although discrepancies existed between the chemical and immunoelectrophoretic determinations of Kd values, it was possible to investigate the behaviour of the molecular polydispersion of bacterial capsular polysaccharides present in a multivalent vaccine by comparing immunoelectrophoretic analysis of chromatographic effluents at the time of vaccine preparation and at later times. Tables 3 and 4 show respectively, a potency control for some antigens present in the polyvalent pneumococcal vaccine and for those present in the meningococcal bivalent vaccine.

TABLE 3. Immunoelectrophoretic determination of Kd values of pneumococcal polysaccharide at different times after manufacture using Sepharose 4B

Polysaccharide antigen	Kd values 0*	12*	18*	Maximum Δ Kd
6A	0·21	0·23	0·23	0·02
18C	0·21	0·21	0·21	—
19F	0·12	0·11	0·11	—
23F	0·14	0·15	0·15	0·01

* Time (months).

TABLE 4. Immunoelectrophoretic and chemical detection of group A and C meningococcal polysaccharides present in bivalent vaccine after separation on Sepharose 4B at different times after manufacture

Polysaccharide antigen	Kd values			
	Chemical procedure		Immunoelectrophoretic procedure	
	0*	18*	0*	18*
Group A	0·37 (75·5%)	0·44 (71·8%)	0·48	0·55
Group C	0·26 (88·9%)	0·26 (88·5%)	0·37	0·37

* Time (months).

The Kd values are calculated on the second major peak of the chromatogram.

The values in parentheses indicated the chemical amount of polysaccharide recovered at Kd = 0·5.

DISCUSSION

Bacterial capsular polysaccharides are found to be polydispersion systems when purification following their isolation is performed. For bacterial capsular polysaccharides present in vaccines for human use, the characterization of the polysaccharide molecular weight polydispersion is expressed as the Kd value calculated after gel chromatography of the single polysaccharide antigens.[4,5,6] Because the Kd value represents a significant potency test for the immunogenicity of the meningococcal polysaccharides in man[2,18] and for polysaccharides in general,[1,19] we examined the molecular weight polydispersion of each polysaccharide antigen present in multivalent vaccines, such as the 14 valent pneumococcal polysaccharide vaccine, in order to control the immunogenic activity of the vaccine with time. Fused-rocket immunoelectrophoresis using rabbit pneumococcal typing antisera appeared to be a suitable method to visualize specifically the chromatographic behaviour of each polysaccharide antigen present in the vaccine. A similar immunoelectrophoretic method was recently adopted as a quantitative assay to verify the amount of polysaccharides in the pneumococcal vaccine in the final container.[7] These authors reported in their experimental conditions, a high specificity between rabbit pneumococcal typing antisera and the respective polysaccharide monovalent preparations.

Similar findings were found in our experiments when both monovalent or multivalent pneumococcal polysaccharide preparations were investigated: only one precipitin line was observed. Meningococcal polysaccharides of group A and C were equally specific when tested with their respective antisera.

The present procedure appeared to be highly sensitive and reproducible. However, differences in the Kd values of single polysaccharides were found from those obtained by chemical or physical analysis of the same chromatographic effluents. These differences were investigated and ascribed to at least two factors: the molecular sieving effect of the agarose gel support on the molecular polydispersion of the polysaccharides, and the different quantity of immunoprecipitates obtained when different molecular sizes of a

polysaccharide polydispersion were precipitated by the same amount of specific antiserum. The latter observation is in good agreement with that published by Lee, Pearson and Robbins,[7] which recorded a change between the molecular size of the pneumococcal capsular polysaccharides and their mass/'rocket' height ratio. However, the discrepancies of Kd values may be considered a minor disadvantage since a relative measure of the Kd values of polysaccharides at the time of vaccine preparation and in the following storage period may be performed.

The gradual depolymerization of the molecular polydispersion of pneumococcal polysaccharides is reported to be more rapid when polysaccharides are stored in aqueous solutions than when kept at −20°C as dried powder.[7] A potency control for pneumococcal vaccine relative to time may be performed by investigation of the immunoelectrophoretic molecular weight polydispersion of all or some of the polysaccharides such as types 6A, 18C, 19F and 23F, which contain in their molecular structure the potential acid-base labile and temperature sensitive phosphodiester bond.[20] Alternatively, a method to check the Kd shifts of pneumococcal polysaccharides could be done by storing the individual solutions of the polysaccharide components for the same time and under the same conditions as the vaccine preparation. This procedure became laborious and differences in the stability of the stored antigens in individual solutions or mixed with the other polysaccharides could exist. Using the present procedure, the absence of significant shifts in the distribution coefficient values of types 6A, 18C, 19F and 23F polysaccharides present in the pneumococcal vaccine were determined 18 months from the date of the vaccine preparation (Table 3).

In the case of multivalent meningococcal polysaccharide vaccine, requirements for the determination of distribution coefficients of the antigens in the final container exist.[5,21] For meningococcal group A and C combined vaccine a chemical differentiation of the antigens is possible and easy, although a problem similar to that of the pneumococcal polyvalent vaccine is encountered when a tetravalent meningococcal vaccine containing groups Y and W135 needs to be investigated for the distribution coefficient values of the single antigens. For this purpose it must be pointed out that, as shown in Table 4, the immunoelectrophoretic detection of Kd values for group A and C polysaccharide gave discrepancies with the chemical determination of Kd values which, for a potency control with time, provided unsatisfactory results of stability of the molecular polydispersion of the polysaccharides when compared with the polydispersions detected by chemical analysis. However, corrections in these values could be made by considering the difference in the Kd values shown by the two methods of analysis of the single polysaccharide antigens. The problem of the detection of the amount of antigen recovered after gel chromatography, although not required for meningococcal polysaccharides combined in the vaccine, deserves some consideration. Meningococcal polysaccharides showed a larger molecular weight polydispersion, after molecular sieving on Sepharose gel, than pneumococcal polysaccharides. These properties of the polysaccharide antigens, as mentioned above, are responsible for a change in immunoelectrophoresis in the corresponding polysaccharide concentration/'rocket' height ratio. Thus, a quantitative estimation of a polysaccharide may become very inexact if rocket immunoelectrophoresis is performed on different molecular sizes of a molecular polydispersion unless as many selected molecular sizes of the same polysaccharide are available as there are standards.

The nature of the different rocket immunoprecipitates obtained using the same amount of antiserum against a polysaccharide with different polydispersions was not

investigated, but reasonable explanations could be found in the heterogeneity of the antibody populations present in the rabbit antisera with respect to the polysaccharide determinant(s) recognized and/or in the heterogeneity of the affinity of antibody populations reacting with the polysaccharide determinant(s).

Because these bacterial capsular polysaccharides are composed of repeating mono or oligosaccharide units,[20,22,23] the second hypothesis could be more convincing in the light of the observations of Krause[24] and Haber and Strosberg[25] on the homogeneity of the antibodies to certain polysaccharide antigens in several animal models such as rabbits and mice, and the views of Berzofsky and Schechter[26] on the concepts of cross-reactivity and specificity of the antigens.

From the above mentioned findings, another point can be raised. The immunochemical methods employed in the antibody titration of human sera to meningococcal and pneumococcal polysaccharide vaccine, such as Radioimmunoassay[27,28] and ELISA,[29,30] need purified meningococcal and pneumococcal polysaccharides homologous to those present in the vaccines. Although in each titration test performed, the polysaccharide antigen employed is the same for 'pre' and 'post' immunization sera, differences in the sera titres using polysaccharides with different Kd values can exist, and this appears to constitute a fundamental variable when estimations of human sera titres are made in several laboratories, even when the same immunochemical procedure is used. To avoid significant discrepancies the titration of human antibodies to pneumococcus or meningococcus by immunochemical methods should make use of purified bacterial capsular polysaccharides with Kd values corresponding to those required by W.H.O. for the polysaccharides present in vaccines for human use.

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