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Effect of Aluminum Ions on Chemical and Immunological Properties of Meningococcal Group B Polysaccharide

CARLOS MORENO,* M. ROBERT LIFELY, AND JANE ESDAILE

Department of Experimental Immunobiology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, United Kingdom

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Soluble salts of aluminum were examined for their capacity to complex with purified meningococcal group B polysaccharide. The formation of the complexes resulted, first, in a markedly reduced rate of internal esterification at acid pH and, consequently, prolonged stability of the antigen as measured by its reactivity with antibody at pH 4 and, second, in an increased resistance to neuraminidase. Al^{3+} complexes of B polysaccharides were tested for immunogenicity in mice and found to be no better than the purified polysaccharide in the Na^+ or Ca^{2+} form. However, when *Neisseria meningitidis* type 6 protein (outer membrane) complexed to B polysaccharide was tested, a substantial increase in anti-B titers was detected, whereas antiprotein titers remained unchanged. The possibility of using combinations of metal-polysaccharide-outer membrane protein complexes as vaccines for humans is discussed.

The immune response to *Neisseria meningitidis* group B capsular polysaccharide injected as a complex with outer membrane proteins has been reported before (2, 10, 13, 14). The anti-B response is characterized by the predominance of immunoglobulin M (IgM) antibodies which are completely dependent on the stability of the protein-polysaccharide complex, since the administration of a dissociated complex or the polysaccharide alone, even if combined with adjuvants, does not result in antibody formation (10, 11, 14).

It has been postulated (7) that one of the factors responsible for the poor immunogenicity of B polysaccharide is the intrinsic instability of the polymer due to its tendency to undergo internal esterification under mildly acid conditions, thus resulting in the loss of conformational antigenic determinants that are characteristic of the native polysaccharide (5, 6). It has also been suggested that neuraminidase digestion may reduce immunogenicity (3). It is known that the Ca^{2+} salt of B polysaccharide undergoes internal esterification at a slower rate than the Na^+ salt, a fact that has been ascribed to coordination of Ca^{2+} ions to the 9-OH of sialic acid residues in the polymer (6). Because intramolecular esterification occurs in B polysaccharide through condensation of CO_2H and 9-OH groups of adjacent residues, cations, binding principally to the CO_2H group, but also by coordination to OH groups, were therefore of interest. It is reported here that B polysaccharide formed a stable complex with aluminum (Al^{3+}) ions which was resistant to neuraminidase digestion. Moreover, at pH 4, the antigenicity of the B polysaccharide-Al^{3+} complex was preserved (i.e., internal esterification was inhibited), whereas the native polysaccharide lost the capacity to react with antibody. Furthermore, although Al^{3+} did not increase the poor capacity of purified B polysaccharide to induce antibody formation, the enhancing effect of Al^{3+} on the immunogenicity of outer membrane protein-B polysaccharide complexes was clearly demonstrated.

MATERIALS AND METHODS

Materials. *Vibrio cholerae* neuraminidase was purchased from Koch-Light Laboratories; sodium deoxycholate and methanesulfonic acid were from Fluka AG; 8-hydroxy-7-iodoquinoline-5-sulfonic acid (ferron) was from BDH. *N. meningitidis* group B polysaccharide, non-*O*-acetylated (*O*-Ac^-) C polysaccharide, and *O*-acetylated (*O*-Ac^+) C polysaccharide (*O*-acetyl content, 1.7 μmol/mg) were obtained as previously described (6), and *Escherichia coli* K92 polysaccharide was purified from strain CN7873. The preparation and properties of a type 6 outer membrane protein-B polysaccharide complex have been published previously (10).

Preparation of a protein-*O*-Ac^+ C polysaccharide complex. An outer membrane protein (type 2)-*O*-Ac^+ C polysaccharide complex was prepared essentially as described previously (10) for B polysaccharide complexed with type 6 protein. Briefly, a 1% (wt/vol) cetavlon precipitate from the culture supernatant was dissolved in 1 M $CaCl_2$ and precipitated with 75% (vol/vol) ethanol. The pellet from centrifugation was dissolved in water and passed through a Sepharose CL-2B column. The purified protein-polysaccharide complex eluted at the void volume was made to 1% (wt/vol) deoxycholate and pH 11, sterile filtered through a 0.22-μm membrane, and precipitated with 75% (vol/vol) ethanol, and the pellet from centrifugation was washed with absolute ethanol and dissolved in 0.01 M sodium phosphate buffer (pH 7.3) containing 5% (wt/vol) lactose. Samples were freeze-dried. The composition of the purified complex (MCNT-186D) was 77% sialic acid and 18% protein.

Preparation of an artificial type 6-*O*-Ac^- C polysaccharide complex. A crude type 6 protein-group B polysaccharide complex, prepared as described previously (10), was suspended at 5 mg/ml in 0.05 M sodium acetate buffer (pH 5.5) containing 1% (wt/vol) NaCl, 0.1% (wt/vol) $CaCl_2$, and 0.01% (wt/vol) NaN_3 and was incubated for 65 h at 37°C. The suspension was then centrifuged (10,000 × *g* for 15 min at 4°C), and the pellet, containing predominantly protein, was suspended in water and centrifuged. The pellet was slurried in water and freeze-dried. Analysis showed that <6% sialic acid and 91% protein were present.

The type 6 protein (2 mg) was mixed with *O*-Ac^- C polysaccharide (3 mg) in water (1 ml). The suspension was placed in an ultrasonic bath for 20 min and then vortexed at intervals over 2 h at 0°C. The suspension was further filtered

* Corresponding author.

TABLE 1. Properties of Al^{3+}-bound sialic acid polysaccharides after equilibrium dialysis

Polysaccharide (Ca^{2+} salt)	Molar ratio of sialic acid/Al^{3+}/Ca^{2+}	Molecular size[a]	
		% K_d = 0	% $K_d \leq 0.5$
B	1:0.37:0.01	32	73
O-Ac^+ C	1:0.32:0.01	56	88
O-Ac^- C	1:0.36:0.01	50	86
K92	1:0.37:0.01	20	70

[a] Sepharose CL-4B chromatography: K_d = (Ve−Vo)/(Vt−Vo), where Ve is the elution volume, Vt is the total volume, and Vo is the void volume.

through a 3-μm membrane and chromatographed on a column (1.5 by 90 cm) of Sepharose CL-2B. The void volume fractions exhibiting absorbance at 280 nm and containing sialic acid-positive material were pooled and precipitated with 3 volumes of absolute ethanol. The precipitate was collected by centrifugation at 10,000 × *g* for 15 min at 4°C, washed once with absolute ethanol, dissolved in water (2 ml), and stored at −20°C. Analysis showed that 44 μg of sialic acid and 280 μg of protein per ml were present.

Analytical methods. Polymeric sialic acid was measured by the resorcinol-hydrochloride method (12), free sialic acid was measured by the thiobarbituric acid method (1), and protein was measured by absorbance at 280 nm and by the method of Lowry et al. (8) with bovine serum albumin as a reference. Metals (Ca^{2+} and Al^{3+}) were determined by atomic absorption spectrometry. Al^{3+} was also determined by a colorimetric ferron assay. Briefly, a solution of Al^{3+} (0 to 4 μg) in 0.1 M ammonium acetate buffer (1 ml [pH 7.0]) was mixed with 1 M HCl (0.025 ml), and 0.2% (wt/vol) ferron solution (0.2 ml) was added. The samples were heated in a boiling-water bath for 5 to 10 min and cooled, and absorbance was measured at 370 nm against a reagent blank. Gel filtration was performed at 4°C with a column (1.5 by 90 cm) of Sepharose CL-2B or Sepharose CL-4B, equilibrated and run in 0.1 M ammonium acetate buffer (pH 7.0) at a flow rate of 12 ml/h. Fractions (2 ml) were analyzed for sialic acid, protein by absorbance at 280 nm, and Al^{3+} by the ferron assay.

The degree of internal esterification was determined by gas-liquid chromatography as described previously (4). Briefly, partially esterified B polysaccharides were reduced with KBH_4, methanolysed with 2 M methanesulfonic acid, *N*-reacetylated, and trimethylsilylated. The derivatives were examined by gas-liquid chromatography on 3% OV-225 at 220°C.

Al^{3+} binding to polysaccharides. (i) Equilibrium dialysis. A solution of the Ca^{2+} salt of the polysaccharide (20 μmol of sialic acid) was placed inside a dialysis sac and dialyzed against $AlCl_3$ solution (20 μmol of Al^{3+} in 2 liters of water) for 24 h at 4°C. The contents of the dialysis sac were then mixed with 0.1 volume of 1.1 M ammonium acetate, and the polysaccharide was precipitated with 3 volumes of absolute ethanol. After 1 h at 0°C, the precipitate was collected by centrifugation (10,000 × *g* for 15 min at 4°C), and the pellet was washed once with absolute ethanol, dissolved in water (2 ml), and freeze-dried.

(ii) Direct incubation. The Ca^{2+} salt of the polysaccharide was incubated with various concentrations of $AlCl_3$ solution (molar ratios of sialic acid/Al^{3+}, 1:0.1 to 1:1.33) for 10 min at room temperature, followed by the addition of 0.1 volume of 1.1 M ammonium acetate and 3 volumes of absolute ethanol. After 1 h at 0°C, the precipitated polysaccharide was collected by centrifugation at 10,000 × *g* for 15 min at 4°C, and the pellet was washed once with absolute ethanol, dissolved or suspended in water, and freeze-dried.

The chloride ion was the preferred counterion for both direct incubation and equilibrium dialysis of Al^{3+} ions and sialic acid polysaccharides because $AlCl_3$ is readily soluble in ethanol, thus aiding purification.

Neuraminidase digestion. Samples of B polysaccharide (0.4 mg), bound to various concentrations of Al^{3+} (by the direct-incubation method described above), were incubated with neuraminidase from *V. cholerae* (100 μl) at 37°C in 0.05 M sodium acetate buffer (pH 5.5) containing 1% (wt/vol) NaCl, 0.1% (wt/vol) $CaCl_2$, and 0.01% (wt/vol) NaN_3 (4.9 ml). Samples (40 μl) were removed at timed intervals and analyzed for free sialic acid (1).

Immunological methods. Counterelectrophoresis was used to estimate the antigenic content of B polysaccharide and colominic acid (5) by using a rabbit anti-B serum (Wellcome Diagnostics, Dartford, Kent, United Kingdom), and solid-phase radioimmunoassay was adopted for inhibition of binding (6) with monoclonal antibodies MB34 and MC45.

Serum anti-B, anti-C, and antiprotein (type 6) antibody levels were estimated by solid-phase radioimmunoassay (10) by sensitizing Linbro round-bottom plates with purified polysaccharide for anti-B and anti-C titration and with an artificial type 6 protein-C polysaccharide complex for antiprotein titrations. Monoclonal antibodies MB34, MB85 (anti-B), and MC45 (anti-C) (9) were used as references in the first case, and a mouse anti-type 6 protein pool was used as a reference in the second.

Immunization of mice. Female $CBAT^6T^6$ mice, about 8 weeks old, were injected intraperitoneally (i.p.) with antigen in a total volume of 0.5 ml of 0.01 M sodium phosphate buffer (pH 7.3) containing 5% (wt/vol) lactose given with or without 10^{-3} M $Al_2(SO_4)_3$ or 100 μg of $Al(OH)_3$. $Al_2(SO_4)_3$ was given in preference to $AlCl_3$ due to the greater acidity of the latter in solution at an equimolar concentration. The times at which animals were bled for antibody titrations are indicated separately for each experiment.

RESULTS

Al^{3+} binding to sialic acid polysaccharides. (i) Equilibrium dialysis. Al^{3+} binding to the Ca^{2+} salts of meningococcal B and C and *E. coli* K92 polysaccharides was performed by equilibrium dialysis (equimolar ratios of sialic acid and Al^{3+}). The products were analyzed by atomic absorption spectrometry and by gel filtration on Sepharose CL-4B (Table 1). A molar ratio of sialic acid/Al^{3+}/Ca^{2+} of 1:0.35:0.01 for each polysaccharide indicated that (i) Al^{3+} ions are sequestered from the environment by the polysaccharide, (ii) one Al^{3+} ion binds three $-CO_2^-$ groups, and (iii) equilibrium was truly reached, due to elimination of Ca^{2+} ions. Gel filtration on Sepharose CL-4B showed that Al^{3+} ions emerged in the void volume with tailing to a lower molecular weight only for K92 polysaccharide.

(ii) Direct incubation with Al^{3+} salts. The Ca^{2+} salt of B polysaccharide was incubated with various concentrations of $AlCl_3$ solution (molar ratios of sialic acid/Al^{3+} of 1:1.33, 1:0.67, 1:0.33, 1:0.2, and 1:0.1) at room temperature for 10 min, followed by ethanol precipitation to recover the polysaccharide. At the highest concentrations of Al^{3+} (i.e., molar ratios of 1:1.33 and 1:0.67), the complex became water insoluble after ethanol precipitation, whereas lower concentrations of Al^{3+} (i.e., molar ratios of 1:0.33, 1:0.2, and 1:0.1) resulted in a water-soluble complex. It is likely that saturation of the polysaccharide with Al^{3+} ions causes binding to OH groups with a concomitant decrease in solubility in

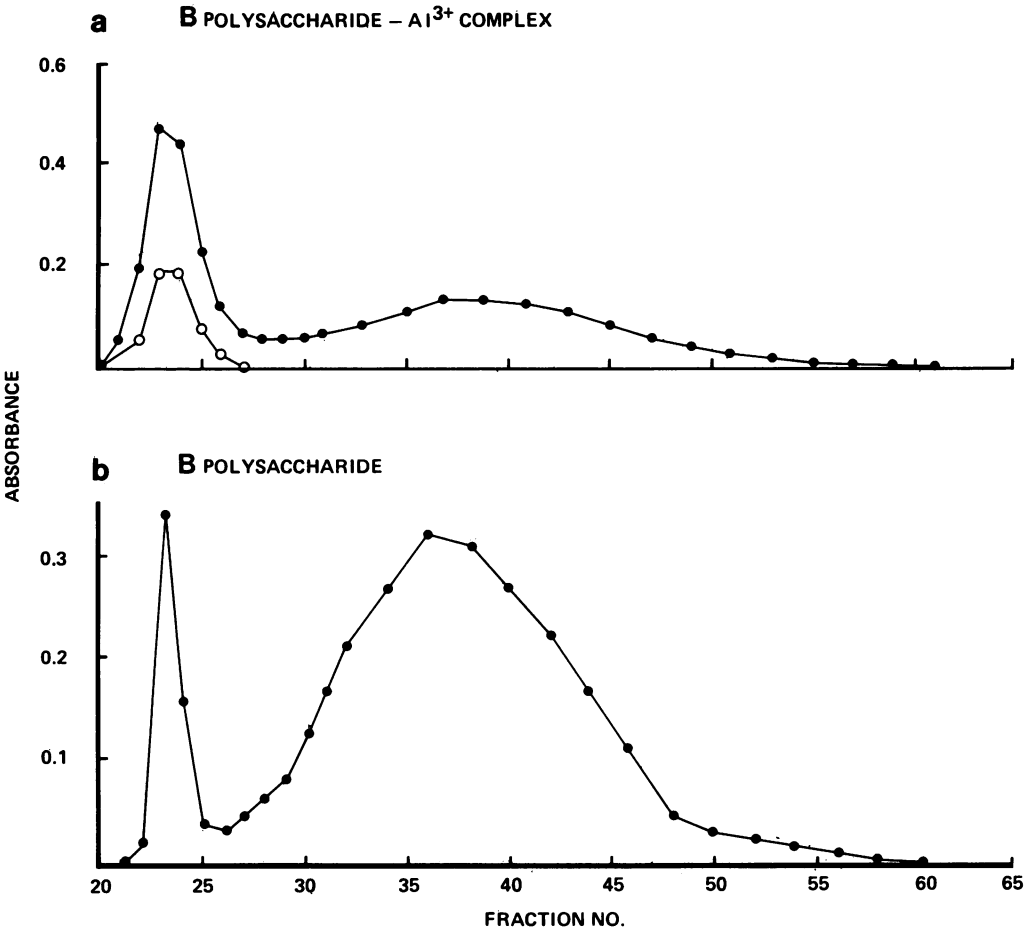

FIG. 1. Sepharose CL-4B chromatography of B polysaccharide (b) and B polysaccharide complexed to Al^{3+} ions (sialic acid/Al^{3+}, 1:0.21 [mol/mol]) (a), as described in Table 2. The column (1.5 × 90 cm) was run as described in the text, and fractions were assayed for sialic acid (●) and Al^{3+} (○).

water. The three soluble complexes were analyzed as shown in Table 2, giving molar ratios of sialic acid/Al^{3+} of 1:0.21, 1:0.14, and 1:0.07. Gel filtration on Sepharose CL-4B (Fig. 1; Table 2) showed that the apparent molecular weight increased in the B polysaccharide-Al^{3+} complexes in comparison to the native polysaccharide, and the Al^{3+} ions were bound to polysaccharide predominantly in the void volume peak, as found after equilibrium dialysis.

TABLE 2. Properties of Al^{3+}-bound sialic acid polysaccharides after incubation with $AlCl_3$

Polysaccharide (Ca^{2+} salt)	Molar ratio of sialic acid/Al^{3+}		Molecular size[a]	
	During incubation	In complex	% K_d = 0	% $K_d \leq 0.5$
B	1:0	1:0	12	80
B	1:0.1	1:0.07	51	91
B	1:0.2	1:0.14	41	81
B	1:0.33	1:0.21	42	80
O-Ac^+ C	1:0.2	ND[b]	62[c]	85[c]
O-Ac^- C	1:0.2	ND	44[c]	70[c]
K92	1:0.2	ND	27	80

[a] Molecular size by Sepharose CL-4B chromatography. K_d is as explained in footnote *a* of Table 1.
[b] ND, Not determined.
[c] Molecular size by Sepharose CL-2B chromatography.

Incubation of meningococcal C polysaccharide, either *O*-Ac^+ or *O*-Ac^-, and *E. coli* K92 polysaccharide with $AlCl_3$ solution (molar ratio of sialic acid/Al^{3+}, 1:0.2) resulted in complexes similar to B polysaccharide (see Table 2).

Inhibition of intramolecular esterification. (i) Low pH. B polysaccharide was incubated with various concentrations of $AlCl_3$ solution (molar ratios of sialic acid/Al^{3+} of 1:0.3, 1:0.2, 1:0.1, and 1:0), and the products were treated at room temperature for 24 h with various concentrations of HCl (molar ratio of sialic acid/H^+ of 1:0.8, 1:0.4, and 1:0.2). The degree of esterification of the polymers was determined by gas-liquid chromatography (Table 3), and the results indicated a direct correlation between the concentration of Al^{3+} ions bound to B polysaccharide and the resulting protection from internal esterification.

(ii) Counterimmunoelectrophoresis. Group B polysaccharide solutions (0.1 mg/ml in 0.01 M sodium acetate buffer [pH 4.0]), to which $Al_2(SO_4)_3$ had been added up to final concentrations of 2×10^{-3}, 2×10^{-4}, and 2×10^{-5} M Al^{3+}, were prepared, and one solution to which no Al^{3+} was added was left as a control. All tubes were incubated at 4°C, and samples were withdrawn at 0, 3, 6, 24, and 48 h, neutralized with 0.1 M Na_2HPO_4, and diluted twofold for examination of precipitating capacity by counterimmunoelectrophoresis against a rabbit anti-B serum. Results (Fig. 2) indicated that

TABLE 3. Effect of Al^{3+} binding on intramolecular esterification in B polysaccharide at low pH

Molar ratio of sialic acid/Al^{3+}	Esterification (%) after incubation for 24 h with molar ratio of sialic acid/H^+ of:		
	1:0.2	1:0.4	1:0.8
1:0	12.9	27.1	48.9
1:0.1	3.6	15.0	41.0
1:0.2	<0.5	7.4	27.7
1:0.3	<0.5	2.3	15.6

in the absence of Al^{3+}, the polysaccharide had no detectable precipitating capacity after 3 h of incubation, whereas those solutions containing 2×10^{-4} and 2×10^{-5} M Al^{3+} retained their precipitating capacity for 24 h, and, at the highest concentration tested (2×10^{-3} M), full precipitating capacity was retained for at least 48 h.

Neuraminidase digestion. Complexes of B polysaccharide and Al^{3+} (sialic acid/Al^{3+} molar ratios of 1:0.31, 1:0.14, and 1:0) were incubated with neuraminidase from *V. cholerae* at 37°C for various times. Sialic acid was extensively released from native B polysaccharide (Ca^{2+} salt), whereas binding of Al^{3+} to the polymer markedly inhibited digestion (Fig. 3).

Effect on the immune response to protein-polysaccharide complexes. To test whether Al^{3+} did have an effect upon the anti-B response of CBA mice, groups of animals were immunized i.p. with 10-μg doses of group B-type 6 protein complex (batch MB6-180D) given in buffer alone (0.5 ml of 5% lactose and 0.01 M sodium phosphate buffer [pH 7.3]), buffer plus 10^{-3} M $Al_2(SO_4)_3$, or buffer plus 100 μg of $Al(OH)_3$. The anti-B response was examined 7 days later, and the results (Table 4) clearly indicate that the response to antigen increased two- to threefold when $Al_2(SO_4)_3$ was added and about ninefold with $Al(OH)_3$, both results being highly significant statistically. This potentiation was not apparent when the same experiment was performed with group C-protein complex (MCNT-186D) administered in identical fashion to group B complex. A follow up of these mice for 3 weeks revealed no enhancement of the response (Table 5).

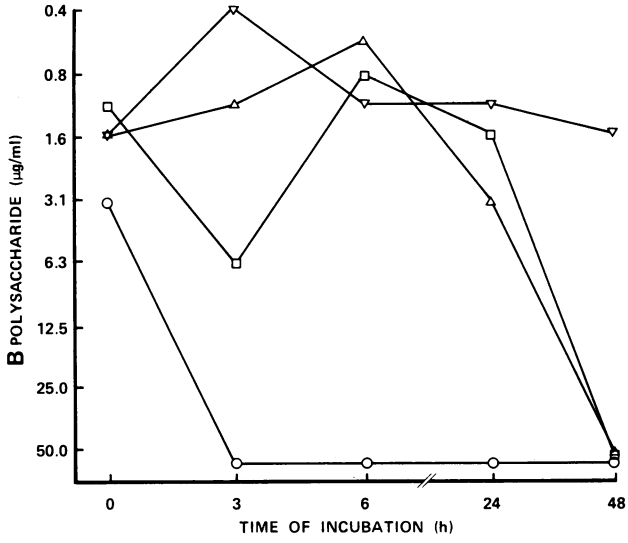

FIG. 2. Rate of loss of B polysaccharide precipitating capacity at pH 4.0 in the presence of 10^{-3} (▽), 10^{-4} (△) and 10^{-5} (□) M $Al_2(SO_4)_3$ and in the absence (○) of $Al_2(SO_4)_3$, by counterimmunoelectrophoresis with a rabbit anti-B antiserum. Numbers along the vertical axis represent the minimal amounts of B polysaccharide required to give a visible precipitate with the antiserum.

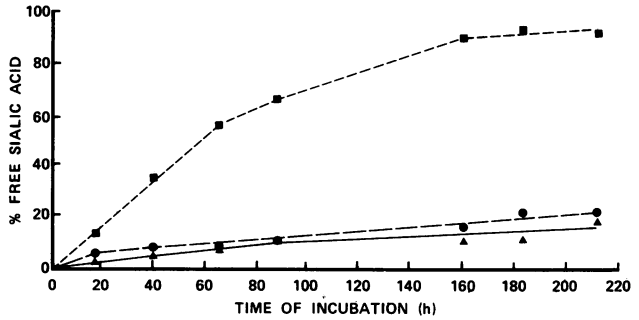

FIG. 3. Rate of release of sialic acid by neuraminidase digestion of B polysaccharides complexed to Al^{3+} ions; the polymers had molar ratios of sialic acid/Al^{3+} of 1:0 (■), 1:0.14 (●), and 1:0.31 (▲), as described in the text.

Al^{3+} ions were also capable of enhancing secondary responses. Mice were immunized as described for groups 1 and 3 in Table 4, i.e., 10 μg of group B polysaccharide-protein (type 6) complex with and without 10^{-3} M $Al_2(SO_4)_3$, respectively, and given on days 0 and 34. The results (Table 6) show once more a significant increase in the anti-B titer, but no effect on the anti-type 6 protein response.

Lack of effect on immunization with purified group B polysaccharide. Groups of five mice were immunized i.p. with 1-, 10-, and 100-μg doses of group B polysaccharide given together with 10^{-3} M $Al_2(SO_4)_3$. This resulted in no significant increase in the anti-B titers at days 7 and 15 when compared with preimmunization levels or with antibody levels in mice immunized with the same doses but in the absence of Al^{3+}.

DISCUSSION

The work presented in this communication was guided by the following four basic questions. (i) Is there a stable interaction between some cations and B polysaccharide? (ii) Does this interaction result in any inhibition of the spontaneous process of internal esterification or neuraminidase susceptibility? (iii) As a result of this inhibition, is there any detectable stabilization of the main epitope(s) of native B polysaccharide? (iv) Does stabilization of the antigen result in any improvement in the immunogenicity of the polysac-

TABLE 4. Immunization of CBA mice with type 6 protein-group B polysaccharide complex[a] administered in combination with $Al_2(SO_4)_3$ or $Al(OH)_3$

Group	Antigen at day 0	Additive to antigen	Anti-B response at day 7[b]	*P* value[c]
1	+	None	1.6 (1.38)	
2	−	None	0.49 (1.04)	
3	+	10^{-3} M $Al_2(SO_4)_3$	3.92 (1.16)	<0.025
4	−	10^{-3} M $Al_2(SO_4)_3$	0.65 (1.20)	
5	+	100 μg of $Al(OH)_3$	14.7 (1.83)	<0.01
6	−	100 μg of $Al(OH)_3$	0.46 (1.04)	

[a] In each group, five mice received i.p. injections (10 μg per mouse) of batch MB6-180D in 0.5 ml of 5% lactose–0.01 M sodium phosphate buffer (pH 7.3). Control mice (groups 2, 4, and 6) received 0.5 ml of lactose–0.01 M sodium phosphate buffer (pH 7.3).

[b] Antibody (micrograms per milliliter) in the serum (geometric average, with the standard error in parentheses).

[c] Calculated by the Student's *t* test when comparing groups 3 and 5 with group 1.

charide? Evidently, each of these questions is meaningful only if the previous ones have already been answered positively and we attend to them seriatim.

Binding occurred spontaneously between B polysaccharide and Al^{3+} ions, either by equilibrium dialysis or by direct incubation. It seemed clear that sequestration of Al^{3+} by the polysaccharide occurred in preference to Ca^{2+} binding and that this complex was dissociated by neither ethanol precipitation nor gel filtration. The nature of the binding is still unclear, but nuclear magnetic resonance data, although preliminary, suggest that, apart from the obvious interaction with the carboxylic groups, additional interactions take place involving OH groups at C-9 (J. C. Lindon, C. Moreno, and M. R. Lifely, unpublished data). More work is in progress to determine the detailed structure of the complex.

Binding between Al^{3+} and B polysaccharide resulted in a clear resistance to neuraminidase digestion (Fig. 3). Moreover, at acid pH, Al^{3+} forms of B polysaccharide, as compared with the Ca^{2+} and Na^{+} salts, showed markedly reduced rates of esterification. This, in itself, resulted in a persistent antigenicity of B polysaccharide measured both by immunoprecipitation (counterimmunoelectrophoresis) and by radioimmunoassay. Lindon et al. (7) postulated, on the basis of nuclear magnetic resonance spectroscopy and molecular-modeling studies, that a transition between the most energetically stable conformation of B polysaccharide and its intramolecular ester is likely to occur. Al^{3+} may therefore act by increasing the energy barrier between the two states, by complexing with the COOH and possibly the OH groups at C-9 of the neighboring sialic acid residue, or by both processes.

The fact that antigen stability is increased by Al^{3+} complexing led us to try immunization of mice with Al^{3+} forms of B polysaccharide, with the negative results that have been reported here. The poor immunogenicity of B polysaccharide was characterized by two features: the incapacity to elicit antibody responses with purified polysaccharide and the preponderance of IgM antibodies when the polysaccharide was administered in a complex with protein. As we have reported before (10), the lack of antibody production does not necessarily reflect a lack of effective interaction with the immune system, since immunization with purified B polysaccharide leads to immune memory. The interaction of native B polysaccharide with the immune system was apparently transient and of an abortive nature,

TABLE 5. Ineffectiveness of $Al_2(SO_4)_3$ and $Al(OH)_3$ upon the anti-C polysaccharide response of mice injected with group C-protein complex[a]

Group	Immunization at day 0	Anti-C response at day:[b]		
		7	14	21
1	10 μg of antigen in buffer	0.40 (1.23)	0.38 (1.26)	0.38 (1.45)
2	10 μg of antigen + 10^{-3} M $Al_2(SO_4)_3$	0.20 (1.26)	0.33 (1.34)	0.23 (1.24)
3	10 μg of antigen + 100 μg of $Al(OH)_3$	0.19 (1.08)	0.27 (1.40)	0.22 (1.14)
4	Buffer + 10^{-3} M $Al_2(SO_4)_3$	0.17 (1.11)	0.18 (1.06)	0.14 (1.04)

[a] CBA mice (five per group) were injected i.p. with C polysaccharide-outer membrane protein complex (MCNT-186D) in 0.5 ml of 5% lactose–0.01 M sodium phosphate buffer (pH 7.3) (group 1) to which $Al_2(SO_4)_3$ (group 2) and $Al(OH)_3$ (group 3) were added.

[b] Immunoglobulin (micrograms per milliliter of serum [geometric average with the standard error in parentheses]).

TABLE 6. Anti-group B and anti-protein (type 6) secondary responses in mice immunized with group B-outer membrane protein complex given with and without $Al_2(SO_4)_3$[a]

Antigen at days 0 and 34 given in:	Day 41 response[b] to:	
	Anti-B	Antiprotein
Buffer	2.40 (2.06)	0.85 (3.25)
Buffer + 10^{-3} M $Al_2(SO_4)_3$	8.40 (1.51)	1.74 (1.84)
Nonimmune	0.03 (1.02)	0.05 (1.18)

[a] Immunization procedures were exactly as described in Table 4, footnote a.

[b] Anti-B response (micrograms per milliliter); anti-protein response is expressed in reference to an antiserum pool which equals 1. Data given are geometric averages for five mice, with standard errors in parentheses.

and complexing with Al^{3+} did not alter this situation. However, when Al^{3+} forms of protein-B polysaccharide complexes were injected, they induced at least twice as much anti-B antibody as the Na^{+}-Ca^{2+} forms of the same complexes. We interpret these results in terms of stabilization of B polysaccharide in its native form rather than an adjuvant effect, based on the following observations: (i) administration of protein-B polysaccharide complexes in alhydrogel (as an adjuvant) boosted both anti-B and antiprotein responses, whereas Al^{3+} as a salt stimulated the production of anti-B antibodies alone, and (ii) Al^{3+} forms of protein-C polysaccharide complexes did not show this enhancing effect.

Why is it, then, that purified B polysaccharide, as the Al^{3+} salt, did not seem to stimulate antibody production? The answer to this question probably lies in the way phagocytic cells handle, process, and present antigens to B cells. Unpublished experiments in this laboratory have indicated that B polysaccharide (or colominic acid) is rapidly eliminated from liver and spleen after intravenous injection, when compared with the elimination rate for B512 dextran of a similar molecular weight. This relatively short-lived antigen can stimulate the production of memory cells (10), but not antibody, which requires B polysaccharide stabilization to preserve the conformational determinants (7) and, possibly, B and T cell proliferation factors together with T helper function. Probably none of these activities are stimulated by polysaccharide alone, but protein-polysaccharide complexes did so.

Al^{3+} is not the only cation capable of enhancing the anti-B response in protein-B polysaccharide complexes. Results obtained in our laboratory with ruthenium red as a complexing agent have indicated similar chemical stability and antigenic stimulation. It cannot be established at the moment whether reduction of internal esterification or increased resistance to neuraminidases is more important in promoting the immunogenicity of B polysaccharide, and, although we favor the former as being the most significant, it is possible that both phenomena play a role. Whatever the explanation may be, it is important that the findings reported here suggest the interesting possibility of using Al^{3+} to enhance the immunogenicity of *N. meningitidis* group B vaccines for immunization of humans.

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