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Specificity of the immune response to the group B polysaccharide of *Neisseria meningitidis*

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SUMMARY

A panel of monoclonal antibodies (mAb) and polyclonal sera of murine, human and equine origin, of IgM isotype and with specificity for *Neisseria meningitidis* group B polysaccharide, an $\alpha(2\rightarrow8)$-linked homopolymer of sialic acid, were examined for their antigenic and biological specificities. The nature of the antigenic determinants on B polysaccharide was investigated using a series of *N*-acyl derivatives of B polysaccharide, two sialic acid polymers containing $\alpha(2\rightarrow9)$-linkages and a series of polynucleotides. The panel of antibodies recognized an array of unrelated antigenic determinants on the B polysaccharide, despite its structural simplicity, and all but one were highly effective in an *in vitro* bactericidal assay and/or in an *in vivo* murine passive protection model. There was no evidence that B polysaccharide induced antibody capable of blocking biological activity (blocking antibody).

INTRODUCTION

Neisseria meningitidis group B/*Escherichia coli* K1 (colominic acid) polysaccharides have been the subject of continual study over the last 20 years due to their unique structural, serological and pathological properties. Both polymers, apparently identical, are homopolymers of $\alpha(2\rightarrow8)$-linked sialic acid,[1] both have been implicated as virulence factors in meningitis and septicaemia,[2,3] and both are strikingly poor immunogens in humans.[4] This is an intriguing situation, especially when one considers the good immunogenicity of meningococcal group C polysaccharide,[5] an $\alpha(2\rightarrow9)$-linked homopolymer of sialic acid, which has for many years been licensed for use in humans. Nevertheless, antibodies against B polysaccharide have been raised in animals and humans, through immunization[6–8] or infection,[9] and are naturally present in the majority of the adult population.[9,10] Evidence that the response is directed against conformational or discontinuous determinants on the B polysaccharide can be summarized as follows: modifications of carboxyl groups abolished antigenicity,[11–13] whereas modification of the *N*-acetyl function was more variable, with some alterations causing complete and others partial loss of antigenicity,[13,14] which was dependent also on the antibodies used; generally, low molecular weight polysaccharide (colominic acid) and oligosaccharides therefrom do not bind well to anti-B antibodies, nor do they effectively inhibit binding of high molecular weight B polysaccharide to specific antibody.[12,14–16]

In addition, Kabat *et al.*[17] have shown that a human monoclonal macroglobulin, IgM^{Nov}, with specificity for B polysaccharide, cross-reacts strongly with the polynucleotides poly A and poly I, and they postulated that this was due to a similar spatial arrangement of negative charges in the antigens. It has been suggested from space-filling models[18] that the carboxylate and acetamido functions are aligned on opposite sides of the molecule, and Kabat *et al.*[19] subsequently extended their results to indicate that IgM^{Nov} binds to an epitope located on the acidic side of the poly $\alpha(2\rightarrow8)$-linked sialic acid.

The purpose of this study has been to probe the fine structure of the determinants present on meningococcal group B polysaccharide using both monoclonal and polyclonal antibodies of murine, equine and human origin, and to subsequently correlate antibody specificity with biological activity. We were also concerned to explore the possibility that antibodies against some antigenic determinants may act to block the biological activity of other antibodies. Although this does not seem to be the case here, such blocking antibodies (anti-protein III from *Neisseria gonorrhoeae*) have been reported previously to block the bactericidal activity of immune human sera against gonococci.[20]

MATERIALS AND METHODS

Materials

Neisseria meningitidis strains CN 7619 (group B, type 2a), CN 7622 (group B, type 6) and CN 7038 (*O*-acetylated group C, type 2a), an *Escherichia coli* K92 strain (CN 7873), and preparation of high molecular weight B, *O*-acetylated C and K92 polysaccharides, have been described previously.[21,22] Polynucleotides were obtained from Sigma Chemical Co., St Louis, MO. Rabbit anti-meningococcus group C/*E. coli* K92 serum was obtained from Wellcome Diagnostics, Dartford, Kent, U.K.

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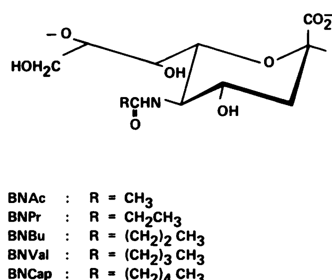

Figure 1. The *N*-acylated series of meningococcal B polysaccharide derivatives.

Antibodies

Eight IgM murine monoclonal antibodies (mAb) (MB14, MB22, MB32, MB34, MB35, MB36, MB62 and MB85), with specificity for meningococcal B polysaccharide, and mAb MC11 (IgM), with specificity for meningococcal C polysaccharide, were selected as described previously.[12,21] Some of the characteristics of some of these mAb have also been described.[21,23] The human monoclonal macroglobulin IgM^{Nov} was obtained from Dr E. Kabat, Columbia University, NY, and the horse antiserum, H46, raised against group B *N. meningitidis*, was provided by Dr J. B. Robbins, Food and Drug Administration, Bethesda, MD, from which a lyophilized IgM fraction was prepared. A pool of immune mouse sera (IMS), containing <6% IgG compared with IgM anti-B polysaccharide antibody as assessed by ELISA, was obtained by immunizing CBA mice four times with a complex of group B polysaccharide and meningococcal outer membrane proteins (OMP).[24]

N-deacetylation of B polysaccharide

This was performed as described elsewhere.[25] Briefly, to 10 M NaOH (2 ml) was added B polysaccharide (100 mg) in water (6 ml), thiophenol (200 μl) and DMSO (30 ml). The mixture was heated at 80° for 3 hr, dialysed against 0·01 M ammonium carbonate at 4° and lyophilized. A ^{1}H-NMR spectrum of the sample indicated a completely *N*-deacetylated product, as judged by the lack of an *N*-acetyl signal at δ 2·07 and from the large downfield shift ($\sim\delta$ 3·3) of the H-5 proton.

N-reacylation of B polysaccharide

N-deacetylated B polysaccharide (4 mg) was suspended in 1 M $NaHCO_3$ (1 ml). Four aliquots (25 μl) of acetic anhydride at 15-min intervals were added with vigorous stirring. After 2 hr, ethanol (1 ml) was added drop-wise, followed by a further aliquot (50 μl) of acetic anhydride. This was left for a further 2 hr, then dialysed against 0·01 M ammonium carbonate for 3 days at 4° and lyophilized to yield BNAc. Similarly BNPr, BNBu and BNVal were prepared from propionic, butyric and valeric anhydride, respectively (Fig. 1). ^{1}H-NMR spectra of the products were fully consistent with the expected *N*-acylated derivatives. Attempted *N*-caproylation using caproic anhydride only resulted in 10–20% derivatization by the above method, probably due to the insolubility of the anhydride in the aqueous environment. *N*-caproylation was achieved by the following method. *N*-deacetylated B polysaccharide was dissolved in DMSO (1 ml) and treated with three aliquots (25 μl) of caproic anhydride at 30-min intervals with stirring. After 2 hr, the solution was dialysed against 0·01 M ammonium carbonate overnight, the suspension formed made to 0·1 M with NaOH and incubated at 37° for 4 hr (to remove any *O*-caproyl groups). The solution thus formed was extracted twice with ether, lyophilized, redissolved in 0·1 M ammonium acetate (0·5 ml) and desalted on a gel filtration column of Sephadex G-25 (0·9 × 60 cm) to yield the fully *N*-caproylated derivative BNCap (Fig. 1), as confirmed by ^{1}H-NMR.

ELISA

Wells of flat-bottomed 96-well PVC microtitre plates (Dynatech, Billinghurst, Sussex, U.K.) were precoated with 50 μl of poly-L-lysine (260,000 MW; Sigma Chemical Co.) at 1 μg/ml in 5 mM sodium phosphate-buffered saline (0·425%) pH 7·2 (PBS) for 1 hr at room temperature. Wells were emptied and washed five times with 0·05% Tween 20 in PBS (PBS-Tween). The wells were then coated with 60 μl of antigen at 5 μg/ml in PBS for 1 hr at room temperature. After washing as above with PBS-Tween, the wells were filled with 50 μl of serial twofold dilutions of antibody in 1% bovine serum albumen (BSA) (Fraction V; Sigma Chemical Co.), 0·05% Tween in PBS (BSA-PBS-Tween) and incubated for 2 hr at room temperature. After washing seven times in PBS-Tween, 50 μl of a 1:350 dilution in BSA-PBS-Tween of alkaline phosphatase conjugates of goat anti-mouse polyvalent immunoglobulins, goat anti-human IgM or rabbit anti-horse IgG (whole molecule) (Sigma Chemical Co.) were added to the wells and the plates were incubated for 1·5 hr at room temperature. p-Nitrophenyl phosphate was used as the substrate and the optical density read at 405 nm (A_{405}) in a Titertek Multiscan.

Quantitative precipitin test

Aliquots of antibody (100 μl in PBS) and increasing concentrations of antigen (0–16 μg) in PBS (100 μl) were mixed in ependorf tubes. The tubes were allowed to stand at 4° for 5 days with mixing twice daily, centrifuged at 4°, and washed with cold PBS (0·5 ml). The pellets were dissolved in 10 mM NaOH (1 ml) and the protein content was determined colorimetrically.

Radioactive antigen-binding inhibition assay

B polysaccharide was extrinsically radiolabelled with ^{125}I following incorporation of fluorescein isothiocyanate (FITC) into the polymer under mild conditions to minimize reduction in chain length, and the radioantigen binding assay of Kuo *et al.*[26] was performed with minor modifications, as described previously.[8]

Slide agglutination

A neat bacterial suspension (15 μl) from cultures of *N. meningitidis* group B (CN7619), *N. meningitidis* group C (CN7038) and *E. coli* K92 (CN7873) was mixed with undiluted antiserum or mAb (15 μl) on a clear glass slide and mixed by rotation for 3 min. Agglutinations were scored visually.

Bacteriolytic assay

N. meningitidis group B (CN7622), *N. meningitidis* group C (CN7038) and *E. coli* K92 (CN7873) were grown to logarithmic phase and suspended at $\sim 4\times10^4$ CFU/ml in 10% FCS in 10 mM PBS (0·85%): 10% FCS-PBS. For the assay, 25 μl of threefold serial dilutions of antibody in 10% FCS-PBS were mixed with 25 μl of bacteria, 15 μl of baby rabbit complement and 85 μl of 10% FCS-PBS in round-bottomed microtitre plates. The plates

were sealed and incubated in an orbital shaker at 100 r.p.m. and 37° for 1 hr. Aliquots of the mixture were transferred to individual wells of 24-well tissue culture plates and mixed with 0·5% agar in growth medium (0·5 ml) held at 45°. The solidified agar was overlaid with 0·5% agar in growth medium (0·5 ml) and incubated for 24–48 hr at 37° prior to counting colonies. Control wells contained bacteria and: (i) antibody alone; (ii) complement alone; (iii) no antibody nor complement; or (iv) irrelevant antibody (MC11) and complement.

Passive protection of mice

Female CBA/T^6T^6 mice, 7–10 weeks old, were given 1, 0·2 or 0·04 μg antibody in 0·2 ml 10% FCS-PBS intraperitoneally (i.p.) 1 hr prior to challenge with 32 LD_{50} *N. meningitidis* group B in iron-dextran, as described previously.[8]

RESULTS

Antibody specificity—quantitative precipitin analysis and ELISA

Figure 2 shows the quantitative precipitin curves of a series of *N*-acyl derivatives of B polysaccharide with mAb, a human monoclonal antibody (IgM^{Nov}) and a horse serum (H46). Two mAb (MB32 and MB35) and IMS were not included because of scarcity of antibody. However, all antibodies were examined by ELISA, with results similar to those for the quantitative precipitin test (Table 1). Three major specificities were observed: those antibodies that reacted with all of the *N*-acyl derivatives of B polysaccharide (BNAc→BNCap), those that reacted only with the *N*-acetyl derivative (BNAc), and those that reacted with some but not all of the *N*-acyl derivatives. It is implied that the four antibodies (MB14, MB32, MB34, IgM^{Nov}) that reacted with all *N*-acyl derivatives do not recognize the *N*-acetyl group as a critical part of the determinant; conversely, MB22 and MB35 reacted mainly with BNAc, suggesting that alterations of the *N*-acetyl function diminish binding, either directly or perhaps through perturbation of the conformation of the entire molecule. Lastly, five antibodies (MB36, MB62, MB85, H46 and IMS) bound to the *N*-acyl derivatives to various extents, although, for each antibody, the binding specificities were significantly different (Fig. 2 and Table 1).

The antibody specificity for the $\alpha(2\rightarrow8)/\alpha(2\rightarrow9)$ linkage position was examined with the B polysaccharide, the *O*-acetylated meningococcal C polysaccharide [an $\alpha(2\rightarrow9)$-linked sialic acid homopolymer], and the *E. coli* K92 polysaccharide [an alternating $\alpha(2\rightarrow8)\alpha(2\rightarrow9)$-linked sialic acid polymer]. Three main specificities were observed: B polysaccharide-specific, $B \approx K92 > C$ and $B > K92$ (Table 1). Examples of each type of specificity found in the quantitative precipitin test are shown in Fig. 3. Only two antibodies (MB22 and MB35) gave binding curves with K92 polysaccharide that were indistinguishable from those with B polysaccharide, suggesting that the $\alpha(2\rightarrow8)$linked monosaccharide unit may be important to the antigenic specificity of these mAb. These antibodies also bound weakly to C polysaccharide. All other antibodies bound only modestly, at best, to K92 polysaccharide and poorly or not at all to C polysaccharide.

These findings were confirmed in a radioactive antigen-binding inhibition assay. The ability of the B, C and K92 polysaccharides, as well as a series of polynucleotides (poly A, poly C, poly G, poly I, poly U), to inhibit binding of ^{125}I-labelled B polysaccharide to the mAb and polyclonal sera was determined. K92 was equally as good an inhibitor as B polysaccharide of binding to MB22 and MB35 (Fig. 4), but had no inhibitory effect on any other antibody. Equally, meningococcal C polysaccharide had no inhibitory effect on any of the mAb or polyclonal sera.

The polynucleotides, poly A and poly I, have been shown to bind to IgM^{Nov} in a quantitative precipitin test, giving identical precipitin curves with B polysaccharide.[17] Denatured but not native DNA, and poly G also gave precipitin curves, although precipitation was not as effective. With the exception that poly A was able to fully inhibit binding of ^{125}I-B polysaccharide to IgM^{Nov} in the radioactive antigen-binding inhibition assay, no polynucleotide showed any inhibitory activity against any of the anti-B antibodies at a level 1000-fold higher than could be achieved with B polysaccharide (data not shown). The fact that poly I binds to IgM^{Nov} in the quantitative precipitin test yet fails to inhibit binding to ^{125}I-B polysaccharide was unexpected, and suggested that poly I may bind to a region on some IgM anti-B molecules which is different from the binding site for B polysaccharide. In support, we (M. R. Lifely and J. Esdaile, unpublished observations) and others[27] have found that cross-reactions with poly I commonly occur with antibodies to bacterial polysaccharides.

Slide agglutination

The ability of the antibodies to agglutinate freshly grown meningococcal group B and group C and *E. coli* K92 bacteria was examined. Only one of the eight mAb (MB32) failed to strongly agglutinate group B meningococci, this giving a very weak reaction (Table 1). No antibody agglutinated group C meningococci and, as expected, MB22 and MB35 alone gave a reaction with *E. coli* K92, although this was not as strong as the agglutination of group B meningococci. As a control, a polyclonal rabbit serum raised against group C meningococci gave a strong reaction against both group C and *E. coli* K92 organisms.

Bacteriolytic activity

All of the antibodies were examined in a bacteriolytic assay for their ability to kill group B meningococci (Table 2). With the exception of MB32 and H46, they were highly effective, killing > 80% of the bacteria at concentrations of 5 ng/ml or lower and killing 99–100% of the bacteria at concentrations between 1·7 ng/ml and 17 ng/ml. MB32 has been shown previously to be poorly able to fix complement,[21] which is consistent with the finding here that it is poorly bacteriolytic. The failure of H46 to effectively kill group B bacteria reflects the inability of immune horse serum to kill meningococci.[28] A control mAb (MC11) with specificity for meningococcal C polysaccharide had no effect on killing at any concentration. None of the antibodies were able to kill either group C meningococci or *E. coli* K92. Since both MB22 and MB35 bind and are inhibitable by K92 polysaccharide, and agglutinate intact *E. coli* K92 organisms, the fact that they are ineffective in a bacteriolytic assay suggests that they may be unable to bind complement or that components present on *E. coli* but not on meningococci (*e.g.* smooth lipopolysaccharide) may prevent effective lysis.

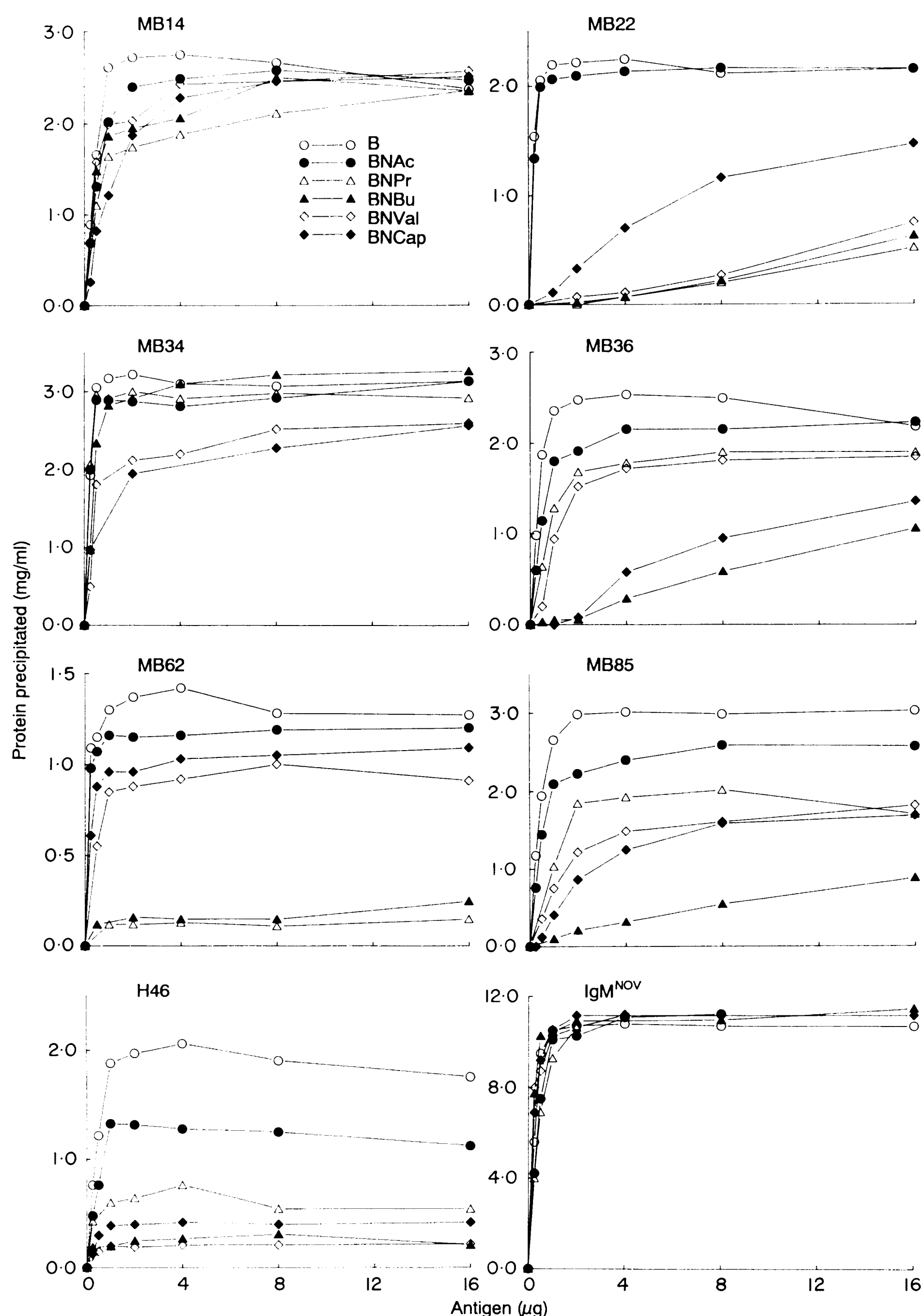

Figure 2. Quantitative precipitin curves for murine mAb (MB14, MB22, MB34, MB36, MB62 and MB85), horse sera (H46) and a human mAb (IgM^{Nov}) with the *N*-acylated series of B polysaccharide derivatives.

Murine passive protection

The ability of the antibodies to passively protect mice against challenge with group B meningococci was examined (Table 2). With the exception of MB32, complete protection of animals was obtained with between 0·2 μg and 1 μg antibody injected 1 hr prior to challenge with 32 LD_{50} bacteria. The control antibody, MC11, afforded no protection. Thus, there is an excellent correlation between the bacteriolytic assay and the murine passive protection model for all antibodies apart from H46, which suggests a non-bacteriolytic mode of action for this antiserum.

DISCUSSION

Most or all of the mAb or polyclonal sera tested recognized a unique fine antigenic specificity, which could be classified into a number of groups based on particular characteristics (Table 1). With regard to the *N*-acylated derivatives of B polysaccharide, four antibodies (MB14, MB32, MB34 and IgM^{Nov}) bound well to all of the antigens in the quantitative precipitin and ELISA tests, suggesting binding to the carboxylate side of the molecule in the model of Kabat *et al.*[19]; two (MB22, MB35) bound mainly to the BNAc derivative, suggesting binding to the acetamido

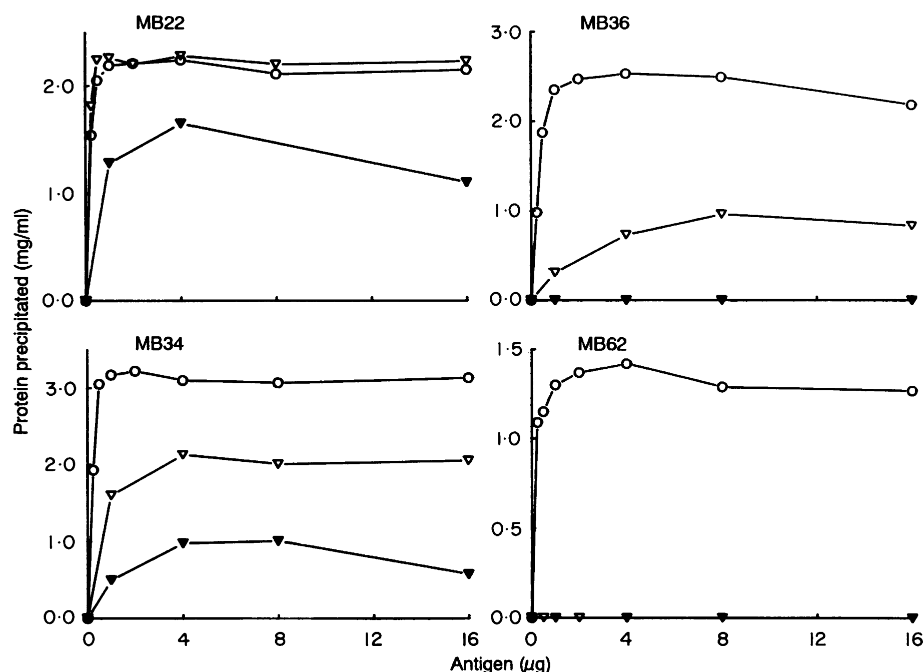

Figure 3. Quantitative precipitin curves for murine mAb (MB22, MB34, MB36, and MB62) with B (○), C (▼) and K92 (▽) polysaccharides.

Table 1. Summary of the antigenic specificity of mAb and polyclonal sera

	Specificity by QP/ELISA*		Slide agglutination		
Antibody	*N*-acyl series†	K92/C‡	B	K92	C
MB14	Ac, Pr, Bu, Val, Cap	B > K92	+	−	−
MB32	Ac, Pr, Bu, Val, Cap	B	±	−	−
MB34	Ac, Pr, Bu, Val, Cap	B > K92 > C	+	−	−
IgM^{Nov}	Ac, Pr, Bu, Val, Cap	B	+	−	−
MB22	Ac	B, K92 > C	+	+	−
MB35	Ac	B, K92 > C	+	+	−
MB36	Ac > Pr, Val	B ≫ K92	+	−	−
MB62	Ac, Val, Cap	B	+	−	−
MB85	Ac > Pr > Val, Cap	B ≫ K92	+	−	−
H46	Ac > Pr > Bu, Val, Cap	B ≫ K92	+	−	−
IMS	Ac > Pr, Bu, Val, Cap	B > K92	+	−	−

* Specificity by quantitative precipitin test and by ELISA (see Figs 2 and 3).

† Specificity for the *N*-acylated series of B polysaccharide derivatives (see Fig. 1).

‡ Specificity for K92 and C polysaccharide.

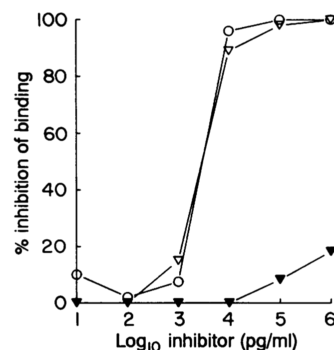

Figure 4. Inhibition of binding of ^{125}I-B polysaccharide to MB35 in a radioactive antigen-binding inhibition assay using B (○), C (▼) and K92 (▽) polysaccharides.

side of the molecule, although substitutions of the *N*-acetyl group which cause distortions to the conformation of the entire molecule cannot be ruled out. This leaves a third group of antibodies (MB36, MB62, MB85, H46 and IMS) which reacted with some but not all of the antigens. MB62 binds to neither the BNPr nor the BNBu derivative but regains binding ability with the BNVal and BNCap antigens, possibly reflecting steric constraints imposed on the molecule which affect its antigenic specificity.

Both direct binding (Fig. 3 and Table 1) and inhibition studies (Fig. 4), as well as slide agglutination, show that MB22 and MB35 bind indistinguishably to B and K92 polysaccharides. Taken together with their requirement for the *N*-acetyl derivative (BNAc), this suggests that MB22 and MB35 have the same or similar antigenic specificities.

Although there are similarities between IgM^{Nov} and MB34 in their specificity for the *N*-acyl derivatives of B polysaccharide, they recognize different antigenic epitopes based on the ability of IgM^{Nov}, but not MB34, to bind to polynucleotides, in particular poly A.

Table 2. Biological activity (bacteriolytic activity and passive protection of mice) of anti-B antibodies

Antibody	Ab conc.* (ng/ml) > 50% killing	Ab conc.* (ng/ml) > 80% killing	% maximum killing	Ab (μg) giving 100% passive protection of mice†
MB14	5	5	100 (17)‡	0·2
MB22	0·5	1·7	100 (1·7)	0·2
MB32	167	167	83 (> 167)	> 10
MB34	5	5	100 (17)	0·2
MB35	1·7	5	100 (5)	0·2
MB36	1·7	1·7	100 (5)	1
MB62	5	5	100 (17)	1
MB85	1·7	1·7	100 (5)	1
IgM^{Nov}	1·7	5	100 (5)	1
H46	50	> 167	79 (> 167)	0·2
IMS	5	5	100 (17)	1
MC11	> 167	> 167	0 (> 167)	> 10

* Bacteriolytic killing of *N. meningitidis* group B (~4 × 10^4 CFU/ml) with antibody and baby rabbit complement.

† Groups of five mice were challenged with 32 LD_{50} *N. meningitidis* group B.

‡ Figure in parentheses is antibody concentration (ng/ml) required to give maximum percentage killing.

There was a good correlation between bacteriolytic activity and passive protection of mice with the anti-B antibodies, except for H46, which was effective in the latter but not the former assay, suggesting that phagocytosis rather than bacteriolysis is a more important defence mechanism in these assays with this antiserum. Only MB32 was ineffective in both assays, which reflects its inability to fix complement.[21] This may be due to the low affinity of MB32, which is poorly able to agglutinate group B meningococci, rather than to its antigenic specificity. All of the other antibodies, which recognize diverse antigenic epitopes on B polysaccharide, function well in both biological assays. This leads to the suggestion that antibody affinity or antibody isotype rather than antigenic specificity *per se* may determine the protective immune status of the host.

In summary, our results, like those of Rubinstein & Stein,[29] who used a panel of murine mAb against meningococcal group C polysaccharide, show that antibodies with a diversity in their fine specificities may be formed against structurally simple polysaccharides. All of the antibodies, apart from MB32, which was of low affinity and failed to fix complement, were functionally effective in biological assays of killing of meningococci. Since IgM is the predominant isotype raised against meningococcal B polysaccharide, it has not been possible to compare different antibody isotypes with respect to their antigenic specificity or biological activity. In order to better understand and explain some of the unusual antibody-antigen reactivities seen in this study, it will probably be necessary to probe further the three-dimensional structure of B polysaccharide, a task which began several years ago through the use of NMR spectroscopy.[18,30] This, in turn, may lead to the stabilization, or perhaps synthesis, of important determinants on the polysaccharide as potential immunogens.

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