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K-1 Antigen Content and the Presence of an Additional Sialic Acid-Containing Antigen Among Bacteremic K-1 *Escherichia coli*: Correlation with Susceptibility to Opsonophagocytosis

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Eighty percent of blood culture isolates of *Escherichia coli* K-1 are resistant to in vitro opsonophagocytosis by normal human granulocytes and fresh serum. To determine the basis for susceptibility to phagocytosis in 20% of bacteremic K-1 *E. coli*, we investigated possible quantitative and qualitative immunochemical differences in the K-1 antigen content among resistant and sensitive isolates. We prepared extracts of blood culture K-1 *E. coli* by sonication and determined the K-1 polysaccharide content per dry weight of bacteria by rocket immunoelectrophoresis using cross-reactive equine anti-group B meningococcal sera. We assessed qualitative differences in the antigen content by crossed immunoelectrophoresis, using an immune globulin fraction and isolated immunoglobulin G (IgG) and IgM from the group B antisera. Three different resistant K-1 isolates contained a mean K-1 content of 48.5 ± 7.6 μg/mg ± standard deviation of dry bacteria, and three sensitive isolates contained 23.2 ± 5.6 μg/mg ($P < 0.005$). Crossed immunoelectrophoresis of extracts from both sensitive and resistant strains revealed a secondary sialic acid-containing antigen that was electrophoretically different from both the major K-1 antigen and a reference group B meningococcal antigen. This negatively charged secondary antigen was susceptible to *Clostridium perfringens* neuraminidase degradation and reacted only with IgG whereas the major K-1 antigen reacted only with IgM. This antigen was detected in the extracts of resistant isolates only at 10^{10} but not at 10^9 colony-forming units per milliliter. This study demonstrates that (i) the degree of phagocytosis of bacteremic *E. coli* K-1 isolates is inversely associated with K-1 content, and (ii) more easily phagocytosed (sensitive) K-1 isolates have greater amounts of an additional sialic acid-containing antigen that appears to be unrelated to the previously described *O* acetyl K-1 antigen.

A total of 84% of strains of *Escherichia coli* causing neonatal meningitis have been shown to contain the K-1 sialic acid capsular polysaccharide (14). Additionally, a recent study of adult bacteremias has shown the *E. coli* K-1 serotype to be associated with a higher morbidity than non-K-1 *E. coli* (22). Using an in vitro opsonophagocytic assay of normal human polymorphonuclear leukocytes (PMN) and serum, we have observed that approximately 80% of bacteremic *E. coli* K-1 are resistant to phagocytosis (R. J. Weinstein and L. S. Young. Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, New York, N.Y., 1977). Since previous studies with *Klebsiella* (10) and *Haemophilus influenzae* (23) have shown an inverse relationship between the amount of capsular antigen and the rate of in vivo clearance, our primary goal was to determine whether the susceptibility of blood isolates of *E. coli* K-1 to in vitro opsonophagocytosis was related to the quantity of K-1 polysaccharide present per bacterial cell population.

In this study several *E. coli* K-1 blood isolates found to be either susceptible or resistant to in vitro phagocytosis were extracted by sonication and assessed for K-1 content by rocket immunoelectrophoresis with cross-reacting equine group B meningococcal antisera. The sonic bacterial antigen extracts were further qualitatively analyzed by crossed immunoelectrophoresis with immunoglobulin M (IgM) and IgG fractions from the anti-group B sera.

Our results indicate that the amount of capsular antigen is inversely related to the susceptibility of *E. coli* blood culture isolates to opsonophagocytosis since resistant strains contain 50% more K-1 than sensitive strains. Furthermore, we observed an additional sialic acid-containing antigen that appears to be present in greater amounts among sensitive isolates and is recognized primarily by IgG. The relationship of

these data to the virulence factors of *E. coli* K-1 is discussed.

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MATERIALS AND METHODS

Preparation of human PMNs and serum. PMN and serum were obtained from normal healthy laboratory personnel after obtaining informed consent. PMN were prepared by dextran sedimentation by the method of Boyum (2) and as previously described (17). Normal human serum was obtained by pooling serum from five normal healthy donors and storing it at −70°C.

Preparation and K-1 typing of bacterial isolates. All bacteria were blood culture isolates from patients at the UCLA Center for the Health Sciences and identified as *E. coli* by standard clinical laboratory criteria (4). *E. coli* were identified as K-1 with anti-group B meningococcal serum (horse 46) and the antiserum agar halo technique described by Kaijser (8). *E. coli* strains D698 and C94 weak, kindly provided by Mary Glode (Bureau of Biologics, Food and Drug Administration, Bethesda, Md.) and which were recently reported by Ørskov et al. (13) to have weak halos and to represent the OAC^+ variant, were used as controls for this variant. All *E. coli* isolates were grown in brain heart infusion broth to log phase which approximates 4×10^9 to 5×10^9 colony-forming units (CFU) per ml. They were then prepared to the appropriate concentration for each particular procedure and divided into samples for dry weight determinations, sonic extraction, and for the opsonophagocytic assay.

In vitro opsonophagocytic assay. This method is similar to that previously described (17). The assay was performed in a final volume of 1 ml and contained 5×10^6 PMN, 1×10^7 CFU of the various *E. coli* isolates and 0.1 ml of pooled normal human serum. Tubes were mixed for 60 min at 37°C on a tilt table. At 60 min, dilutions of the reaction mixture were made in distilled water to lyse the PMN, and the colony-forming units per milliliter was determined. Reaction mixtures containing bacteria and serum without PMN were included to test for serum killing of the isolates. No isolate was shown to be killed by serum alone after 1 h of incubation at 37°C. The percentage of opsonophagocytosis and killing was determined by dividing the difference in colony-forming units of tubes with and without PMN at the 60-min interval by the colony-forming units in tubes without PMN at 60 min.

Fractionation of antisera. Equine (horse 46) anti-group B meningococcal sera, kindly provided by John B. Robbins (Food and Drug Administration, Bureau of Biologics, Bethesda, Md.), were separated into three different fractions of (i) immune globulins, (ii) IgG, and (iii) IgM, using polyethylene glycol precipitation followed by column chromatography.

The antisera were mixed with an equal volume of 0.1 M borate buffer (pH 8.5). To this serum-buffer mixture we slowly added an equal volume of 22% polyethylene glycol (wt/vol) (6000 M_w) in the same borate buffer, mixed on a tilt table at 4°C for a minimum of 1 h, and centrifuged the precipitate containing the immune globulins at $10,000 \times g$ for 10 min. The supernatant containing albumin was removed, and the precipitate, immune globulin fraction, was reconstituted with an appropriate buffer for column chromatography. To obtain IgG and IgM fractions, we chromatographed the immune globulin fraction on a column (2.5 by 95 cm) using Sephadex G-200.

K-1 rocket immunoelectrophoresis. Equine anti-group B meningococcal serum which cross-reacts with K-1 polysaccharide (9) was used as the antiserum source. To reduce background staining, these antisera were fractionated into an immune globulin fraction free of albumin as described above. Standard K-1 antigen was extracted from a reference strain *E. coli* O7:K1 by a cetavalon extraction procedure as previously described (5, 6).

The principles for quantitating K-1 polysaccharide by rocket electrophoresis were based on the method used for the quantitation of various proteins as originally described by Laurell (11) and reviewed in detail by Weeke (21).

Briefly, glass plates (75 by 50 mm) were prepared and covered with 4.4 ml of 1% molten agarose in barbital buffer (pH 8.6) (I = 0.02) containing 2% (vol/vol) of the fractionated antisera. After the agarose congealed, 2-mm wells were punched on one end of each plate. To the wells we added precisely 5 μl of various concentrations of standard K-1 antigen in barbital buffer or the unknown sample from the sonicated bacterial isolates. Electrophoresis was carried out at 2 mA/plate for 2 h. After drying, the gels were stained with 1% Coomassie brilliant blue for 10 min and destained twice for 10 min with the following mixture: (vol/vol) water-ethanol-glacial acetic acid, 7:5:2.

A standard curve was constructed by a linear-linear plot which correlated the major peak rocket height with the concentration of the standard K-1 antigen. The additional precipitin band above the major rocket was not included in the measurement. The content of unknown sample was determined by interpolation from the standard curve.

CIE. The principle for crossed immunoelectrophoresis (CIE) was similar to that originally described for protein antigens (20).

Agarose gel plates without antisera were prepared as described above. One 2-mm well was punched into the agarose near one of the corners. Into this well we added 5 μl of the sonic extracts of the various bacterial isolates. Electrophoresis was carried out in a manner identical to that described for the rocket electrophoresis. After the first dimension of electrophoresis, the agarose above where the sonic extract was electrophoresed was replaced with 1% agarose containing 2% equine anti-group B meningococcal fractionated sera, and electrophoresis was carried out 90° to the first dimension.

Sonic extraction of K-1 *E. coli* isolates. We extracted K-1 antigen from the various *E. coli* isolates by ultrasound, using a Sonifier cell disruptor (Model W185; Heat-Systems-Ultrasonics Inc., Plainview, N.Y.).

From the identical log-phase *E. coli* isolate suspensions that were used in the opsonophagocytic assay, 10-ml samples (the concentration was appropriately adjusted) were taken for dry weight determinations

and sonic extraction. The sonifier was set at 7, and 10 ml of either 10^9 or 10^{10} CFU of bacteria per ml of water was transferred to a sealed stainless steel chamber with a cooling jacket. During the sonication of 5 min for 10^9 CFU/ml or 50 min for 10^{10} CFU/ml, the chamber was maintained at 5°C by circulating ethanol-ice water. After sonication, the bacterial debris was removed by centrifugation at 3,000 × *g* for 15 min, and the supernatants were assessed for K-1 polysaccharide by rocket immunoelectrophoresis.

For dry weight determinations, 10 ml of 10^{10} bacteria per ml was dried at 110°C to constant weight. K-1 content of each isolate was expressed in micrograms per milligram of dry weight.

Neuraminidase degradation of sonic extracts. Sonic extracts of both resistant and sensitive isolates were tested for their lability to neuraminidase activity by using a method to isolate *N*-acetylneuraminic acid from group B meningococcal polysaccharide as described by Liu et al. (12). An appropriate amount of 0.09 N NaOH was added to 0.5 ml of a sonic extract until the pH remained constant at 11.0. The pH was then brought to 5.0 by the addition of 0.1 N HCl and brought to 2.5 ml with 0.1 M acetate buffer (pH 4.9). To this we added 0.25 ml of 11 U of *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, Mo.; type IX) and allowed the reaction to occur at 37°C for 3 days, after which time a sample was taken for CIE.

Statistics. The data were analyzed by paired Student *t* test.

RESULTS

K-1 typing. All *E. coli* K-1 strains that we studied were of the OAC^- type, i.e., strong halo. Only control strains (94 weak and D698, the OAC^+ variants) formed weak halos.

K-1 polysaccharide quantitation. Figure 1 depicts the standard curve of *E. coli* K-1 polysaccharide using rocket immunoelectrophoresis.

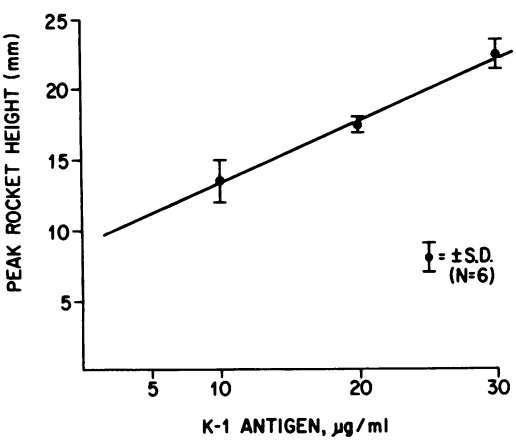

FIG. 1. *Standard curve of K-1 polysaccharide antigen using rocket immunoelectrophoresis in which peak height is correlated with amount of K-1 antigen in micrograms per milliliter.*

Peak rocket height in millimeters was correlated with the linear concentration of K-1 antigen per milliliter of sample. The standard curve range of 10 to 30 µg of K-1 per ml was the range most useful for the assay of K-1 in the supernatants of the bacterial isolates disrupted by ultrasound.

Figure 2 is a photograph of an actual rocket immunoelectrophoresis of the extracts from two sonicated sensitive isolates at 10^9 CFU/ml. Of interest is the additional precipitin band above the major rocket immune complex. This additional band was associated with the three sensitive isolates and not with any of the resistant *E. coli*.

K-1 content and correlation with resistance to phagocytosis. As shown in Table 1, the mean percentage ± standard deviation (SD) of opsonophagocytosis and killing of the three sensitive isolates is 96.5 ± 2.1 with a mean K-1 content standard deviation of 23.2 ± 5.6 µg of K-1/mg of bacteria. In contrast, the three resistant isolates are opsonized and killed at a significantly lower mean percentage ± SD of 16.5 ± 25.6 ($P < 0.001$) with a mean K-1 content twofold greater than the sensitive isolates 48.5 ± 7.6 µg of K-1 per mg ($P < 0.001$).

CIE of sonic extracts and neuraminidase degradation. To investigate the additional antigen found associated with the sensitive isolates (Fig. 2), sonic extracts from both sensitive and resistant isolates were assessed by CIE.

As shown in Fig. 3, the CIE pattern of an extract from a sensitive isolate demonstrates two distinct peaks and was indicative of all sensitive isolates. In contrast, each of the three resistant

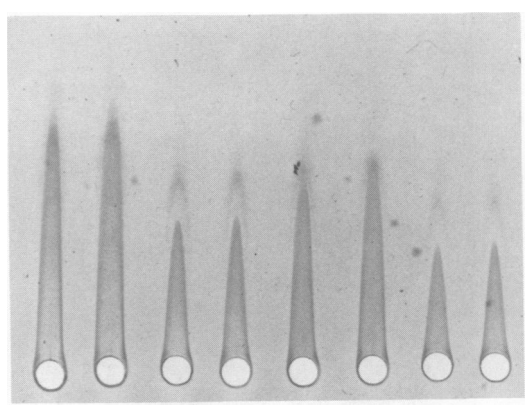

FIG. 2. *Photograph of the rocket immunoelectrophoretic pattern of extracts of two sensitive isolates that were extracted at a concentration of 10^9 CFU/ml. The four rockets to the left are extracts from isolate no. 5704, and the four on the right are from isolate no. 3676.*

isolate extracts had only one peak (Fig. 4). The patterns shown in Fig. 3 and 4 were obtained from extracts of 10^9 bacteria per ml. When other suspensions of 10^{10} bacteria per ml were sonicated and then assessed by CIE, both resistant and sensitive isolate extracts had two peaks similar to that shown in Fig. 3.

To determine the identity of these peaks, both purified K-1 polysaccharide and group B meningococcal polysaccharide (kindly provided by Emil Gotschlich, Rockefeller University, New York, N.Y.) were used in the CIE method as reference antigens. On individual electrophoresis and when combined with the various extracts, the reference K-1 and group B meningococcal antigens had the same electrophoretic mobility as the major antigen peak closest to the anode.

TABLE 1. *Opsonophagocytosis and K-1 content of three sensitive and three resistant E. coli isolates*

E. coli K-1 bacteremic isolate (UCLA no., O type)	Mean K-1 content (μg/mg of dry wt of bacteria) (n = 2) ± SD	% Opsonophagocytosis and killing (n = 2) ± SD
5704, ON	25.0 ± 9.8	98.5 ± 0.7
3438, O1	25.5 ± 2.1	95.5 ± 2.1
3676, O22	19.0 ± 0.0	95.5 ± 2.1
Mean ± SD of sensitive isolates (n = 6)	23.2 ± 5.6	96.5 ± 2.1
3621, O16	53.5 ± 0.7	49.7 ± 0.6
3656, O18	40.0 ± 4.2	0
3011, O18	52.0 ± 7.1	0
Mean ± SD of resistant isolates (n = 6)	48.5 ± 7.6	16.5 ± 25.6

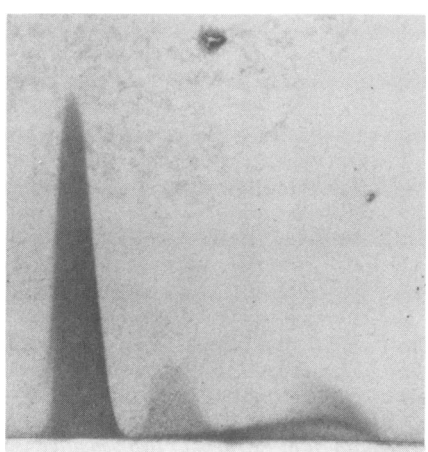

FIG. 3. *Representative photograph of the CIE of an extract from a sensitive isolate at 10^9 CFU/ml using an immune globulin fraction. The anode was on the left, and the application well at the lower right.*

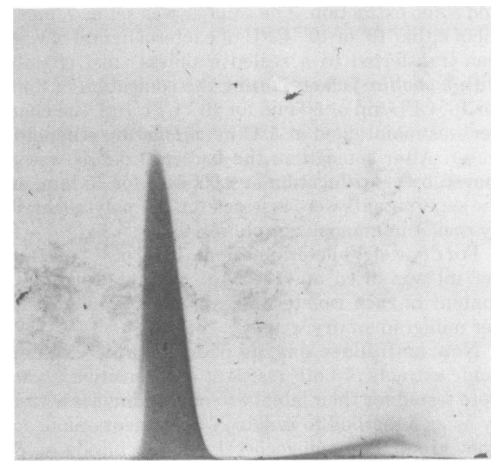

FIG. 4. *Representative photograph of the CIE of the sonic extract from a resistant isolate at 10^9 CFU/ml using an immune globulin fraction. The anode was to the left, and the application was well at the lower right.*

On further investigations, we treated the extracts with neuraminidase as described above. Once treated with the neuraminidase, the extracts were reanalyzed by CIE. In all extracts tested, both antigen peaks were no longer detectable, thereby demonstrating the presence of two sialic acid-containing polysaccharides. Absolute quantitative data on the amount of this second sialic acid polysaccharide per bacterial isolate were not obtainable due to the lack of a reference standard. However, on relative basis it would appear that the sensitive isolates have more of this second sialic acid-containing antigen due to the fact that the second antigenic peak was observed only with the sensitive isolates when using a 10^9-bacteria/ml suspension and was only observed with the resistant isolates when we used a 10^{10}-bacteria-per-ml suspension.

Class-specific antibody reactivity toward K antigens. Equine anti-group B meningococcal sera were separated into IgG and IgM fractions as described above. These fractions were then used in CIE to determine their reactivity toward K-1 and group B meningococcal reference antigens and toward the various sonic extracts.

When we used IgM as the antisera source for the CIE of a source extract from either a sensitive isolate at 10^9 CFU/ml or a resistant isolate at 10^{10} CFU/ml, we observed only one major peak that corresponded electrophoretically to reference K-1 and group B meningococcal polysaccharide. The pattern observed was essentially indistinguishable from that represented in Fig. 4.

Figure 5 illustrates the CIE pattern of the same sonic extract in which only IgG is used as the antisera source. As shown, only the minor, less negatively charged antigen is recognized by the IgG fraction.

DISCUSSION

An important factor in the virulence of different types of bacteria such as *H. influenzae* type B (23), *Klebsiella* species (10), and *Streptococcus pneumoniae* (24) is the presence of capsular polysaccharide envelope-type antigens. *E. coli* is no exception to this phenomenon since the presence of the K-1 serotype capsular antigen has been shown to be a virulence factor not only in coliform neonatal meningitis (14) but also in adult *E. coli* K-1 bacteremia (22). In an earlier report from this laboratory (17th ICAAC, abstr. no. 332), we have found that 6 out of 34 or 17.6% of blood culture K-1 *E. coli* isolates are sensitive to in vitro opsonophagocytosis using normal human PMN and 10% serum. Since work with *H. influenzae* type B and encapsulated *E. coli* (7) has shown that the degree of encapsulation was inversely related to the extent of in vivo clearance, we wanted to determine whether the amount of K-1 antigen could explain why approximately 20% of our bacteremic K-1 *E. coli* isolates were sensitive to in vitro opsonophagocytosis. Based on specific K-1 antigen detection by rocket immunoelectrophoresis, we determined that the extent of opsonophagocytosis of *E. coli* K-1 was inversely related to the amount of K-1 polysaccharide. Although previous work in this area has not exclusively investigated the specific role of K-1 content and opsonophagocytosis among blood isolates of *E. coli*, Van Dijk et al. (18), using hemagglutination inhibition methods for nonspecific capsular antigen quantitation, and Björksten and Kaijser (1), studying *E. coli* from urinary tract infections, recently have correlated the amount of K antigen with resistance to in vitro opsonophagocytosis. The mechanism by which K-1 antigen leads to greater resistance to opsonization and phagocytosis may likely be explained by a combination of factors relating to the hydrophilic, negatively charged character of K-1 on the bacterial cell surface (15). Since PMN more easily phagocytize bacteria that either intrinsically have hydrophobic surfaces or have an acquired hydrophobic surface due to the binding of specific immune globulin (19), any increase in hydrophilicity created by the presence of K-1 polysaccharide will enhance its resistance to phagocytosis. Furthermore, the general physicochemical bacterial surface characteristics of either hydrophilicity, negative charge, or both have recently been shown to correlate with the resistance of various *E. coli* to surface phagocytosis in the absence of serum (15). Because K-1 antigen most likely resides at the outermost layer of the bacteria and can block agglutination by O-antibody (3), it is possible that increased amounts of K-1 could sterically hinder access of potentially opsonic O-antibody or inhibit the possible activation of opsonic complement factors of the alternative pathway by inner O-lipopolysaccharide. Although the latter possibility may apply to other K antigen-containing *E. coli* types, it is not a likely factor with at least the particular bacteremic K-1 *E. coli* we have studied since these same sensitive isolates were not, in spite of their lower K-1 content, opsonized well via the alternative pathway of complement (16). Because these bacteremic K-1 *E. coli* are opsonized by non-K-1-directed antibody via the classical pathway in normal human serum (16), it would appear that those particular sensitive *E. coli* may be more susceptible to opsonophagocytosis because less serum antibody is required to overcome the steric hindrance or to decrease the hydrophilicity of the surface of these isolates or both.

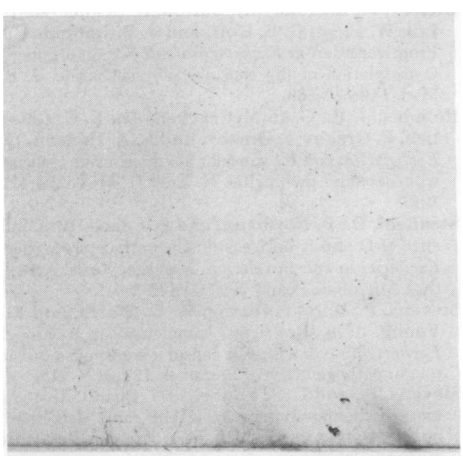

FIG. 5. *Photograph of the CIE of a sonic extract of an isolate at 10^{10} CFU/ml using IgG as the antisera source. The anode was to the left, and the application well was at the lower right.*

Of additional interest in this study was the recognition of an additional sialic acid-containing polysaccharide. This negatively charged secondary antigen does not appear to be the O-acetyl positive (OAC^+) antigen recently described by Ørskov et al. (13) for the following reasons: (i) unlike the OAC^+ antigen it has a different electrophoretic mobility than the OAC^- antigen as represented by the OAC^- ref-

erence antigen from group B meningococcus; (ii) unlike the OAC^+ variant it is degraded by *C. perfringens* neuraminidase; and (iii) use of the halo technique did not identify an OAC^+ form variant among any of the *E. coli* we isolated from blood. Because we used equine antisera it is not known whether normal pooled human serum contains antibody directed towards this antigen. However, if human serum does contain a similarly directed antibody, increased susceptibility to phagocytosis of *E. coli* isolates with greater amounts of this antigen may be explained, in part, by a greater binding of such antibody.

Since ultrasonic disruption of bacteria can result in the release of a number of various antigens and the possible breakdown of various molecules by intensive vibrational forces, it is possible that the secondary sialic acid antigen is an artifact of the extraction process and is a degradation product of the primary antigen. Two lines of evidence against that possibility are that (i) IgG only reacted with secondary antigen and not with the primary antigen that corresponds, at least electrophoretically, to reference group B meningococcal polysaccharide, and (ii) IgM only reacted with the primary antigen and not with the secondary antigen. Such data would lend support to the proposition that the observed secondary antigen does not appear to be a breakdown product of the primary antigen.

As shown, the primary antibody present in the equine anti-meningococcal group B sera that recognizes K-1 and group B meningococcal polysaccharide is of the IgM class. In contrast, the less negatively charged sialic acid-containing secondary antigen is recognized only by IgG. Although the possible implications of these findings, if any, are presently unknown, such differences in antibody recognition could greatly aid in establishing affinity chromatography for the isolation and identification of this secondary sialic acid-containing antigen. Since recent work with the OAC^+ variant suggests that it may be more immunogenic than the OAC^- variant (13), similar further work in identification of this additional secondary antigen and investigations into its potential as an immunogen may aid in finding an *E. coli* sialic acid antigen preparation that can elicit a good immune response.

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