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Cross-Protective Antigens of *Neisseria meningitidis* Obtained from Slaterus Group Y[1]

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An extraction of the cells of *Neisseria meningitidis* serogroup Y with an aqueous solution of calcium choride (0.9 M) has been shown to solubilize a number of antigens. By immunodiffusion, this mixture of antigens has been shown to react with its group-specific antiserum and also to cross-react with a number of other group-specific antisera. The cross-reacting antigen appears to be an antigen common to a number of other serogroups of meningococci, and there is some evidence that it is protein in nature. It has been demonstrated further that the calcium chloride extract contains a strong cross-protective antigen, as shown by its ability to provide good, active immunity in mice to both the homologous and heterologous serogroups of meningococci.

The prophylaxis and treatment of meningococcal disease has been the subject of intensive recent research. Sulfadiazine-resistant strains of *Neisseria meningitidis* have evolved which contraindicate the use of this agent for prophylaxis. Many antimicrobial agents are effective in the treatment of a meningococcal infection but are unable to prevent acquisition of the disease or to eradicate the carrier state. These facts have led to a search for an immunoprophylactic system. One vaccine that has been used extensively is that of the purified, high-molecular-weight polysaccharide antigens obtained from serogroups A and C meningococci (7, 8). These antigens have been proved to be immunogenic, but provide protection only to the homologous group-specific organism (1). Although cross-reacting antigens have been detected in *N. meningitidis* strains (6, 13), their role in functioning as cross-protective antigens has not yet been demonstrated. This paper describes a successful demonstration of cross-protectivity in meningococci by using a mouse-challenge system.

MATERIALS AND METHODS

Organism. The strain of *N. meningitidis* chosen for this study was the Slaterus group Y neotype strain (18) obtained from N. Vedros, Neisseria Repository, University of California, Berkeley.

Cultivation of the organism. The medium used for the growth was *Neisseria* chemically defined medium (NCDM) (10) obtained from General Biochemicals Inc., Chagrin Falls, Ohio, which was sterilized by autoclaving at 115 C for 10 min. It was then dispersed into 1-liter Erlenmeyer flasks in 300-ml amounts. Seed plates of Columbia blood-agar base medium (BBL, Clarkson, Ontario) (4) were incubated at 37 C for 24 hr. The inoculum was prepared by flooding the plates with NCDM medium and scraping the growth from the agar surface with the tip of a pipette. The resultant suspensions were pooled and homogenized by pipette trituration. Immediately before inoculation, each flask of NCDM was adjusted to *p*H 7.4 by the addition of 1 ml of a 5% sodium bicarbonate solution. Each flask was then inoculated with 0.5 ml of the cell suspension and was placed in an incubator at 37 C for 18 hr on rotary shakers which were set for 120 rotations per min. Merthiolate was then added to each flask to a final concentration of 1:5,000/ml, and the flasks were incubated at room temperature until sterility was confirmed (24 hr). The contents of the flasks were then pooled in a sterile carboy; the final volume was 15 liters.

Preparation of antigens. The cells from the carboy were harvested by low-speed centrifugation (3,500 × *g*) to yield 20 g of wet cells. The cells were dispersed and stirred slowly in 150 ml of sterile distilled water at room temperature for 30 min; they were then recovered by centrifugation at high speed (20,000 × *g*) for 30 min. The recovered cells then were extracted with 0.9 M calcium chloride (350 ml, *p*H 7) by stirring them in the solution for 10 min at room temperature and then more severely agitating them in an omnimixer at low speed for 25 min at 0 C. The supernatant fluid was freed of cell debris by centrifugation at 20,000 × *g* for 30 min and was dialyzed against distilled water. The dialyzed extract then was lyophylized to yield the crude antigenic mixture (150 mg).

Immunological studies. Immunodiffusion analysis

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was performed by the Ouchterlony method (12) and microimmunoelectrophoresis was carried out by the method of Scheidegger (17) with a barbital buffer (*p*H 8.6) and an applied voltage of 250 v for 45 min.

Antisera preparation. The cells of the different serogroups used were obtained from the culture collection of the Canadian Communicable Disease Center and were grown in 18-hr cultures in NCDM medium as previously described. The strains used and their corresponding serogroups were 1027-A, 2092-B, 1628-C, 158-D, Slaterus X, Slaterus Y, and Slaterus Z. The live cells of the culture were suspended in NCDM and diluted to a final count of 10^6 cells/ml. Intravenous injections were made in New Zealand White rabbits three times a week for 3 weeks, with doses increasing from 0.25 to 2.0 ml. Exsanguination was performed 7 days after the final injection.

Ion-exchange chromatography of the calcium chloride extract. A column of equilibrated diethylaminoethyl cellulose (14 by 2.5 cm) was gravity packed in 0.02 M phosphate buffer (*p*H 7.0). The calcium chloride extract was dissolved in 5 ml of the phosphate buffer and was applied to the column which was then eluted with the buffer; the unabsorbed antigens were monitored at 280 nm. The absorbed antigens were then eluted with 1 M sodium chloride made up with the phosphate buffer solution and were also minotored at 280 nm. The basic and acidic fractions were then dialyzed against distilled water and lyophilized.

Proteolytic digest of the calcium chloride extract. The lyophilized calcium chloride extract (9 mg) was dissolved in 3 ml of 0.02 M phosphate buffer (*p*H 8.0). The solution was then divided into three 1-ml fractions (A, B, and C) which were treated in the following way. Fraction A was left at room temperature as a control. Fraction B was treated with 0.1 ml of both solutions of trypsin and chymotrypsin (1 mg/ml of phosphate buffer, *p*H 8.0) and left for 24 hr. Both enzymes were obtained from the Worthington Biochemical Corp., Freehold, N.J. Fraction C was treated in the same way as B, except that it was left for 36 hr. The proteolytic enzymes were deactivated after these periods of time with diisopropyl phosphofluoridate (9).

Mouse challenge procedures. The mice used for the challenge test were a black, C_{57} BL/6-DUB strain obtained from Flow Laboratories, Rockville, Md. All the mice used were males and weighed 14 to 17 g. The vaccine was prepared by dissolving and diluting the lyophilized calcium chloride extract with phosphate-buffered saline to a concentration of 5 μg/ml. Each mouse was injected intraperitoneally with 1 ml of the vaccine; the mice were challenged in two groups, one after 12 days and one after 18 days. The challenge suspensions of meningococci were prepared with 5% gastric mucin. The bacterial dilutions were prepared in NCDM, and counts were obtained with a Coulter counter (model B) by using instrument settings according to a previously described method (10). The mice were injected intraperitoneally with 1 ml of a final suspension, which was comprised of 0.5 ml of 5% gastric mucin and 0.5 ml of the bacteria dilution. The challenge dose was 10 LD_{100}, which was 10^3 cells/ml for strain 604A, 10^6 cells/ml for strain 608B and strain 247X, and 10^7 cells/ml for Slaterus group Y strain. The mortality rate of a group of 20 mice was nil when they were injected intraperitoneally with the mucin and medium alone.

RESULTS

Both the water and the 0.9 M calcium chloride extract of the cells of *N. meningitidis* serogroup Y yielded serologically active material on dialysis and lyophylization. However, the calcium chloride extract, as shown by immunodiffusion analysis, was the more active of the two against the heterologous serogroup antisera used in this study. Therefore, because of its degree of cross-reactivity, it was studied in more detail. Agar-gel diffusion studies indicated that, besides giving a precipitate with the homologous serogroup antiserum, the calcium chloride extract also gave a precipitate with antisera to serogroups A, B, C, D, and Z. No cross-reaction could be detected by this technique with the antiserum to serogroup X, although the presence of a cross-protective antigen was demonstrated in an active immunization experiment (Table 1).

Immunoelectrophoretic analysis of the calcium chloride extract against various group-specific antisera was carried out (Fig. 1). Four antigens were detected when the homologous serogroup antiserum was used, but a simpler pattern was obtained with the heterologous serogroup antisera. The calcium chloride extract, when run against serogroup B antiserum, produced one detectable antigen which was similar in respect to its low migratory properties to one of the antigens detected against the homologous antiserum. A similar antigen was also detected when the extract was run against serogroups D and Z antisera, although in these latter cases another com-

TABLE 1. *Per cent mortality of immunized and nonimmunized mice after the challenge with strains of meningococci (10 LD_{100})*

Strain of meningococcus injected	Per cent mortality of immunized mice after challenge with 10 LD_{100}[c]		Per cent mortality of nonimmunized mice after challenge with 10 LD_{100}[d]	
	12-Day challenge	18-Day challenge	12-Day challenge	18-Day challenge
604A[a]	1	2	100	100
608B[a]	8	10	100	100
247X[a]	39	44	100	100
Slaterus Y[b]	5	0	95	100

[a] Virulent strain from clinical isolate.
[b] Neotype strain of group Y.
[c] Based on 100 mice for each strain.
[d] Based on 20 mice for each strain.

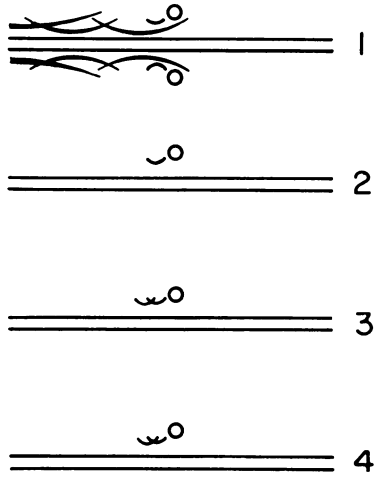

FIG. 1. *Immunoelectrophoresis of the calcium chloride extract (contained in all wells) against serogroup Y antiserum (trough 1), serogroup B antiserum (trough 2), serogroup D antiserum (trough 3), and serogroup Z antiserum (trough 4). The anode is to the left of the diagram.*

mon antigen was also detected. The cross-reacting antigens could not be detected by this technique when serogroup A and C antisera were used.

Evidence that this common antigen was protein in nature was provided by submitting the calcium chloride extract to trypsin and chymotrypsin digestion. The precipitate to the homologous serogroup Y antiserum was not eliminated by this treatment (Fig. 2), probably owing to the presence of the group-specific polysaccharide antigen (15), whereas the precipitate against the serogroup B antiserum disappears completely after this treatment (Fig. 3). A similar disappearance of the precipitate formed against serogroup A antiserum was also observed.

Due to the slow migration, and thus basic nature of the common antigen, a separation of the calcium chloride extract into basic and acidic fractions was effected on diethylaminoethyl cellulose to determine whether all the cross-reactivity would reside in the basic fraction. Immunodiffusion analysis proved this not to be the case, for the basic fraction gave precipitates against serogroups A, B, D, Y, and Z antisera, whereas the acidic fraction gave precipitates against serogroups C, Y, and Z antisera.

In view of the presence of cross-reacting antigens in both fractions and as a preliminary experiment, the whole calcium chloride extract was used in a mouse-protection study to determine whether it could infer active immunity to a broad spectrum of serogroups of *N. meningitidis*. The extract was used to vaccinate mice which were subsequently challenged with live cells of serogroups A, B, X, and Y. The results of the challenge test are shown in Table 1. They indicate that extremely good protection is provided by the calcium chloride extract obtained from the cells of serogroup Y to both the homologous and heterologous serogroups. In fact, the high level of protection afforded against serogroups A and B compares well with that to the homologous serogroup Y, although the level of protection to serogroup X was somewhat lower; nevertheless, the results adequately demonstrate that the extract is still effective in providing some degree of protection. A comparison of the results obtained from the 12-day challenge and the 18-day challenge indicates that prctection is maintained

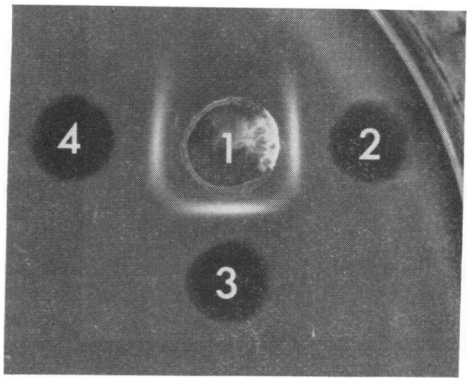

FIG. 2. *Immunodiffusion of serogroup Y antiserum (well 1) against the 24-hr proteolytic digest of the calcium chloride extract (well 2), the 36-hr proteolytic digest (well 4), and the untreated extract (well 3).*

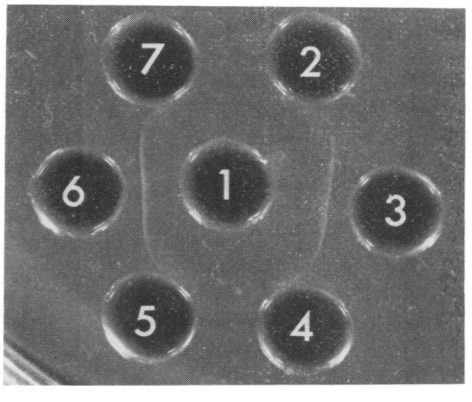

FIG. 3. *Immunodiffusion of the serogroup B antiserum (well 1) against the 24-hr proteolytic digest of the calcium chloride extract (wells 4 and 7), the 36-hr proteolytic digest (wells 2 and 5), and the untreated extract (wells 3 and 6).*

at a fairly constant level; however, there are indications of a slight decline in the level of protection to the heterologous serogroups.

DISCUSSION

This work has demonstrated that a number of antigens of *N. meningitidis* group Y can be solubilized by the extraction of the cells with a calcium chloride solution of high ionic strength. These antigens are both serogroup-specific and cross-reactive in nature, and this cross-reactivity extends over a wide range of serogroups of meningococci. Cross-reactive, meningococcal antigens have been recognized for some time and probably are responsible for difficulties encountered in the serological classification of meningococci (3, 11, 14). Goldschneider, Gotschlich, and Artenstein demonstrated by using cross-absorption techniques that the immune response to meningococci in humans is in part due to the presence of cross-reactive antigens (6). Prost, Vandekerkove, and Nicoli recently demonstrated by immunoelectrophoresis the presence of a common cross-reactive antigen in all the serogroups of meningococci (13). They were able to distinguish this common protein antigen (amido black positive) from the faster-migrating group-specific polysaccharide antigens (alcacian blue positive) by this technique. Our results are comparable, for we have also detected a common protein antigen to a number of serogroups of meningococci in the calcium chloride extract by using this technique, together with an additional common antigen in serogroups D, Z, and Y. It is possible that the common antigen of Prost et al. is identical to the one we have detected, although theirs underwent cathodic migration and ours exhibited anodic migration; however, this could be attributed to the different immunoelectrophoresis performance conditions. We have also provided evidence that the antigenic determinants in the cross-reactive antigens are protein in nature, for proteolysis of the antigenic mixture destroyed the precipitin reactions to the antisera of both serogroups A and B.

As a preliminary to studying the possible immunogenicity of the common protein antigen, an attempt was made to separate the calcium chloride extract into an acidic and basic fraction. However, it was found that all of the cross-reactivity of some serogroups was not confined to the basic fraction. Because our primary objectives in this study were, first, to establish whether cross-protective antigens were present in the extract and, second, to determine the widest range of cross-protection possible, a preliminary mouse-challenge experiment was carried out with the whole extract. Previous work on the immunogenicity of the meningococcal antigens has dealt mainly with the high-molecular-weight polysaccharide antigens which have been shown to be highly group-specific (1, 7, 8, 15). We have now provided evidence that cross-protective antigens can be extracted from meningococci, for immunized black mice were protected against a number of heterologous meningococcal challenges.

To what degree the common protein antigen is responsible for this cross-protection in mice cannot be assessed from our results because the antigen was detected by means of group-specific antisera produced in rabbits. A definitive answer about its function would require its isolation and purification. It could then be ascertained also whether it is a common antigen on the basis of structural identity or whether common structural features are shared with similar antigens derived from meningococci of other serogroups. The possibility that this cross-protection was due to nonspecific resistance induced with meningococcal endotoxin was discounted on the evidence of the greatly prolonged period of protection afforded by the calcium chloride extract, in contrast to the much shorter periods obtained with endotoxins of other gram-negative bacteria (2). Also it is likely that only a minimal amount of endotoxin could be present in the calcium chloride extract for it has been demonstrated that the lipopolysaccharides of gram-negative bacteria are insolubilized by divalent cations (5, 16).

Cross-protection has been clearly demonstrated in mice, and a study of the immunogenicity of the common protein antigen in man may lead to the development of effective meningococcal vaccines for human use.

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