

## PROTECTION AGAINST GROUP B MENINGOCOCCAL DISEASE

### I. Comparison of Group-Specific and Type-Specific Protection in the Chick Embryo Model\*

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While effective polysaccharide vaccines have been developed for meningococcal groups A and C (15, 25), no such vaccine exists for group B (26). Group B is now a major cause of meningococcal disease in the United States (17) and abroad. Group B meningococci have been subdivided, as have group C, into several distinct serotypes (9, 12) based upon the presence of protein serotype antigens located in the outer membrane (11). The majority of both group B and group C disease is caused by a single serotype, type 2 (9, 13, 20). The type 2 antigens of groups B and C are chemically and serologically identical (11, 20). Antibodies against the serotype antigens are bactericidal in the presence of complement (7), and the majority of bactericidal antibody in the sera of rabbits immunized with group B is directed against the serotype antigen (18).

Study of meningococcal infection has been hampered by the absence of a useful laboratory model, since most common laboratory animals, including germ-free animals (19), are resistant to meningococcal challenge. The developing chicken embryo is one of the few laboratory animals readily susceptible to meningococcal infection. Following challenge, 9-15-day-old chick embryos develop lesions typical of meningococcal infection in man such as meningitis, sinusitis, and pulmonary infection (3, 4, 23). Ueda et al. (24) have developed a chick embryo model for the study of serum protection against group A meningococci. For intravenous (i.v.) challenge of 12-day-old embryos, they found that best protection was obtained when diluted serum was injected simultaneously with the bacterial challenge. Based upon the work of Ueda et al. (24), we have used the chick embryo model to investigate the role of type-specific and group-specific antibodies in protection against group B meningococcal infection.

#### Materials and Methods

*Bacterial Strains and Growth Conditions.* The group B strains M986, M981, and M136 were characterized by Frascch and Chapman (7, 8). The group B type 2 strain S946 was received from Dr. Harry Feldman, Upstate Medical Center, Syracuse, N. Y. The group C type 2 strain 138I was

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obtained from Dr. Malcolm Artenstein, Walter Reed Army Medical Center, Washington, D. C. Strains A-1 and C-11 have been described previously (14). The organisms were grown on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) and then inoculated into 100 ml trypticase soy broth (TSB,<sup>1</sup> Difco Laboratories) and grown overnight on a gyratory shaker at 37°C. 5 ml of the overnight broth culture was inoculated into a new flask of TSB and grown for 3–4 h to obtain log phase cells for challenge of chick embryos. Preparation of the challenge dose will be described under chick embryo techniques.

*Antigens and Antisera.* Purified group B polysaccharide was prepared as described previously (16), as was the type 2 serotype antigen from strains M986 and 138I (11). Preparation of the type-specific antisera and hyperimmune rabbit sera used in these studies is described elsewhere (7, 11). Hyperimmune goat antisera against group B was prepared as described for hyperimmune rabbit sera. Anti-capsular and serotype antibodies were absorbed from sera using aluminum hydroxide (alum)-precipitated antigens. The sera could not be absorbed using whole bacterial cell preparations since endotoxin, which is highly toxic to the embryos, was released into the sera. Approximately 1 mg of antigen was mixed with 1 ml of resuspended 0.05 M aluminum hydroxide. The alum-precipitated antigen was pelleted and washed once with 0.01 M phosphate-buffered saline, pH 7.5. Undiluted serum was added to the pellet, resuspended, and incubated at 37°C with occasional mixing for 2 h, then for 1 h at 4°C. The adsorbed serum was separated from the alum by centrifugation.

Group B polysaccharide antibody was purified from goat serum by use of an immunoadsorbent column. Sepharose 4B was coupled with 3,3'-diaminodipropylamine as described by Cuatrecasas (5). Group B polysaccharide (50 mg) purified as described by Gotschlich et al. (16) was dissolved in 10 ml of water, and 20 mg of cyanuric chloride (C<sub>3</sub>N<sub>3</sub>Cl<sub>3</sub>) was freshly dissolved in *N,N*-dimethylformamide and then added to the polysaccharide. The pH of the reaction mixture was maintained at pH 9.0 with NaOH for a period of 4 min. This reaction mixture was added to 50 ml of the derivatized Sepharose suspended in 50 ml of 0.1 M NaHCO<sub>3</sub> pH 7.5. The vessel was closed tightly and stirred for 18 h. All reactions were carried out at room temperature. After washing with 0.01 M phosphate-buffered saline pH 7.5, the immunoadsorbent was poured into a 1.6 × 25-cm column. The immunoadsorbent could deplete up to 25 ml of goat serum of its antibody and the latter was eluted with 2 M NaCl at pH 5.5. The column could be used repeatedly.

*Chick Embryo Techniques.* 10-day- and 12-day-old chick embryos were used in these studies. They were obtained from White Leghorn flocks which had been fed antibiotic-free meal. 10-day-old embryos were used for inoculation of the chorioallantoic membrane (CAM). The CAM was exposed according to the method of Beveridge and Burnet (1). Graded doses of log phase meningococci in 50 μl Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N. Y.) were dropped onto the intact CAM. The opening in the shell was sealed with a cover slip and Histowax (Matheson, Coleman, and Bell, East Rutherford, N. J.). After 24 h, 100 μl of blood was obtained by removing the shell at the air sac end and then nicking a blood vessel with a sterile 27-gauge needle on a 0.5-ml syringe. The blood was diluted 1:10 in HBSS and plated onto Mueller-Hinton agar.

12-day-old chick embryos were inoculated i.v. using a modification of the seroprotection test described by Ueda et al. (24). The embryos were prepared for inoculation using the method of Eichorn (6). Serum dilutions were prepared in HBSS. Log phase organisms were diluted in sterile saline to 78% transmission at 540 nm on a Coleman 44 spectrophotometer (Perkin-Elmer Corp., Maywood, Ill.) in 16 × 125-mm screw cap tubes. The organisms were then diluted 1,000-fold in HBSS to give approximately 4 × 10<sup>4</sup> organisms/ml. To reduce the in vitro interaction of antibody and organisms to a minimum, equal volumes of diluted organisms and serum were mixed immediately before i.v. inoculation of each set. Each embryo received 100 μl of the organism-serum mixture. The embryos were examined 2–3 h after inoculation, and any dead embryos were eliminated from the experiment as having been killed by the injection procedure. The embryos were examined for viability 24 h after challenge. Those embryos that remained alive were bled by venipuncture using a disposable 27-gauge needle on a 0.5-ml syringe. The blood was diluted and cultured as for CAM inoculation.

*Direct Bactericidal Assay.* The microbactericidal assay of Frasch and Chapman (7) was used as described.

<sup>1</sup> Abbreviations used in this paper: CAM, chorioallantoic membrane; HBSS, Hanks' balanced salt solution; TSB, trypticase soy broth.

TABLE I  
*Infection of Chick Embryos Challenged with Different Group B Meningococcal Strains Inoculated via the Chorioallantoic Membrane or Intravenously*

Strain	Serotype	MID <sub>50</sub> CAM*	MLD <sub>50</sub> i.v.‡
M986	2	1 × 10 <sup>5</sup>	<10
S946	2	1 × 10 <sup>5</sup>	<10
M981	4	2 × 10 <sup>5</sup>	1 × 10 <sup>3</sup>
M136	11	2 × 10 <sup>5</sup>	<80

\* MID<sub>50</sub> CAM, minimal dose required to infect 50% of embryos via the chorioallantoic membrane.

‡ MLD<sub>50</sub> i.v., minimal dose able to kill 50% of embryos when organisms given i.v.

### Results

For studies on protection of chick embryos against meningococcal infection by antibody, two routes were considered: CAM and i.v. challenge. The minimal infective dose (MID<sub>50</sub>) of four group B meningococcal strains was compared for CAM and i.v. challenge (Table I). The chick embryos were examined 24 h after challenge for either death or bacteremia. The MID<sub>50</sub> for the 10-day-old embryos challenged by the CAM route was considerably higher than that seen for i.v. challenge. After i.v. inoculation, death occurred within 9–12 h, while CAM inoculation rarely caused death by 24 h, even using doses of 10<sup>7</sup> organisms. Ability to cross the CAM was minimal, as compared to *Escherichia coli* K1 infection where as few as 10 organisms placed upon the CAM caused infection that was lethal within 48 h (unpublished observations). Attempts were also made to use the CAM inoculation model to study serum protection whereby serum dilutions and organisms were dropped onto the CAM. Rabbit anti-group B meningococcal sera had little effect upon ability of the group B organisms to invade and cause bacteremia. Furthermore, using CAM inoculation we were unable to demonstrate any difference in virulence between strain M981 and the other strains (Table I), while i.v. challenge showed M981 to be much less virulent. We concluded that CAM inoculation would not be a useful model because large numbers of organisms were required to cause bacteremia, the bacteremia could not be prevented by antisera, and CAM inoculation could not discriminate between strains of differing virulence.

12-day-old chick embryos were quite sensitive to i.v. challenge. As little as 10 organisms of strains M986 and S946 caused death of all embryos within 24 h. In most experiments, between 1,000 and 3,000 organisms were used, and protection was measured as viability at 24 h. In control embryos receiving organisms only, death usually occurred between 9 and 12 h after challenge.

Previous studies suggested that the serotype antibody would be protective against group B infection, and that antibodies to the group B polysaccharide would be less protective (18). Sera prepared against the different group B serotypes were bactericidal only for the homologous serotype (7). Based upon these earlier results, we wanted to determine whether antisera against different immunologically distinct group B serotypes would also show primarily type-specific protection. Antisera against strains M986, M981, and M136 were tested

TABLE II  
*Bactericidal and Protective Effects of Whole Cell Group B Antisera*

Serum	Serotype	Bactericidal titer vs. strain			Protective titer in embryo vs. strain		
		M986	M981	M136	M986	M981	M136
Anti-M986	2,7	256	<20	<20	200	40	100
Anti-M981	4	<20	≥1,280	<20	200	200	ND*
Anti-M136	11	<20	<20	<20	100	ND	200
NRS	—	<20	<20	<20	<40	<40	<40

\* ND, not done.

for bactericidal antibody (Table II). As expected, M986 and M981 were killed only by their homologous antisera. Strain M136 was not killed by its homologous antiserum even though anti-M136 serum contains abundant type-specific antibody. This is characteristic for this particular serotype 11 strain (8). Constant numbers of three different strains mixed with different serum dilutions were injected i.v. into 12-day-old embryos (Table II). In contrast to the bactericidal data, the protective effects were primarily group specific. In this and other experiments, greater homologous protection occurred which could be the result of combined group-specific and type-specific protection. However, the type-specific protection alone was minimal; absorption of the three antisera with alum precipitated group B polysaccharide removed all protective activity. Absorption did not alter the type-specific precipitin activity of the sera. Absorption with alum precipitated group A polysaccharide had no effect upon the protective effects of the sera, thus ruling out nonspecific effects of the alum.

Protection against meningococcal infection has been correlated with the presence of bactericidal antibodies (14, 22). The group A and group C polysaccharide antibodies are strongly bactericidal, whereas the group B polysaccharide antibody is only weakly bactericidal (7, 18). We wanted to know whether quantitative differences existed between the amount of antibody required to protect against the different groups. We, therefore, compared human sera obtained after vaccination with groups A and C polysaccharide vaccines (15) with a group B antibody preparation which was made by affinity chromatography from hyperimmune goat serum, using a purified group B polysaccharide immunoadsorbant (Table III). Three- to sevenfold less human groups A and C antibody (0.03  $\mu\text{g}$ ) was required for protection than goat group B antibody (0.14  $\mu\text{g}$ ). These results were compared with those obtained using rabbit antimeningococcal sera. The group A serum gave almost identical results to that of the human sera, while the group C rabbit antibody showed protection similar to that of group B. We do not know the exact protective level of rabbit anti-group B antibody, since we were unable to accurately quantitate the anti-polysaccharide antibody in rabbit group B sera, however, the estimated protective level was approximately the same as that found using goat antibody.

The protective effects of group B antisera did appear somewhat unusual, in that the group B organisms were not cleared from the blood as efficiently as either group A or group C organisms. Table IV shows the different protective

TABLE III  
*Protection of Chick Embryos Against Meningococcal Challenge with Antisera Containing Polysaccharide Antibodies and with Purified Antibody to Group B Polysaccharide*

Serum	MPD <sub>50</sub> * against strain			
	A-1	C-11	M986(B)	M136(B)
	$\mu\text{g}$		$\mu\text{g}$	
ECG‡ preimmune	NP	NP	—	—
IG‡ preimmune	NP	NP	—	—
ECG—anti-A	0.05§	—	—	—
IG—anti-A	0.03	—	—	—
3170—rabbit anti-A	0.06	—	—	—
ECG—anti-C	—	0.03	—	—
IG—anti-C	—	0.03	—	—
3189—rabbit anti-C	—	0.40	—	—
B antibody (goat)	—	NP	0.14	0.23

NP, no protection; dashes, not tested.

\* MPD<sub>50</sub>, the minimal protective dose is the amount of antibody required to prevent death at 24 h in 50% of embryos against a challenge of  $1-3 \times 10^8$  organisms.

‡ Laboratory personnel vaccinated with Group A and Group C meningococcal polysaccharides.

§ Specific antibody concentration of sera determined by quantitative precipitation.

TABLE IV  
*Differential Protective Effects of Group A, B, and C Meningococcal Polysaccharide Antisera Against Homologous Challenge*

Serum	Dilution	No. of embryos	At 24 h			
			Dead	Alive	Alive infected	Percent alive infected
Anti-A (ECG)	1:25	8	3	5	0	19
	1:100	9	2	7	1	
	1:200	5	1	4	1	
	1:300	9	4	5	2	
Anti-B (Goat)	1:50	7	0	7	1	50
	1:200	7	0	7	4	
	1:500	7	3	4	3	
	1:1,000	7	5	2	2	
Anti-C (ECG)	1:25	9	2	7	1	16
	1:50	9	0	9	1	
	1:100	8	0	8	1	
	1:500	4	3	1	1	

Number of organisms in challenge dose: Group A:  $5 \times 10^8$ ; B:  $2 \times 10^8$ ; C:  $2 \times 10^8$ .

effects of human group A, goat group B, and human group C antisera against the homologous serogroup. Those embryos that received either group A or C antisera and survived 24 h usually had negative blood cultures. In contrast, the surviving embryos challenged with group B organisms often remained bacteremic. Rabbit sera against group A gave identical results to that of the human group A. Evidence of protection against group B is dependent much more upon the challenge dose than either A or C. With group B, protection was seen with a challenge of approximately 100 LD<sub>100</sub>, while at slightly higher multiplicity no protection could be shown. Although the factors responsible are not clear, group B organisms are cleared by the chick embryo with less efficiency than either group A or group C meningococci.

The serotype antigen is the major outer membrane protein and elicits bactericidal antibodies (11). However, the serotype antibody appeared to afford relatively little protection in the chick embryo (Table II). Since these antisera were not prepared against purified serotype antigens, the role of serotype antibody alone in protection against meningococcal infection of the chick embryo was examined. Antiserum 4588, prepared in rabbits against the purified serotype antigen (11), had a direct bactericidal titer of greater than 1:1,280, and this serum was tested for protection of chick embryos (Table V). There was a much lower level of protection than would have been afforded by an antiserum with an equal titer raised against whole meningococci. The procedure observed was type specific, protecting embryos from infection by both group B and group C type 2 organisms.

The relatively poor protective ability of the serotype 2 antiserum may be due to the absence of active complement in the 12-day-old chick embryo (2) where protection is primarily phagocytic in nature (2). Type-specific antibody is bactericidal only in the presence of complement. As seen in Table V, the fresh serotype 2 antiserum containing complement showed greater protection than heat-inactivated serum.

The combined effects of antibodies against the serotype antigen and the group B polysaccharide were examined using a group B type 2 whole cell hyperimmune rabbit serum (no. 881). This serum was examined for its protective effects before and after absorption with either alum-precipitated group B polysaccharide or alum-precipitated group C type 2 serotype antigen (Table V). Serum 881 had a direct bactericidal titer against strain M986 of 1:1,280 and protected the embryos much better than the pure type 2 serum no. 4588, which had a similar direct bactericidal titer. As seen previously, the hyperimmune serum provided greater protection against the homologous serotype, suggesting the possible additive effects of protection by serotype and polysaccharide antibodies. Absorption of the serum with the serotype antigen reduced the MPD<sub>50</sub>; however, absorption with the group B polysaccharide removed all protective effects of the serum, even though we could show that the serotype antibody was undiminished after polysaccharide adsorption. Therefore, it was apparent that serotype antibody was maximally effective only in the presence of group-specific antibody, that is, the two antibody specificities were synergistic in their effects.

To study the synergistic effect more directly, purified group B polysaccharide antibody was obtained by affinity chromatography, and this was used together

TABLE V  
*Protection of Chick Embryos with Antisera Against the Type 2 Serotype Antigen*

Serum	Heat 56°C 30 min	Adsorb with	MPD <sub>50</sub> vs. strain		
			M986 (B,T2)*	138I (C,T2)*	M136 (B,T11)*
Anti-T2 STA (4,588)	No	—	1:200	—	—
	Yes	—	1:75	1:75	<1:20
Anti-T2 whole cell (881)	Yes	—	1:500	— ‡	1:200
	Yes	B-CHO	<1:50	—	—
	Yes	A-CHO	1:500	—	—
	Yes	T2 STA	1:100	—	—

\* B,T2, Group B, type 2; C,T2, Group C, type 2; B,T11, Group B, type 11.

‡ Another Group B anti-T2 whole cell serum had a MPD<sub>50</sub> titer of 1:500 against M986 and 1:350 against 138I.

with antisera made against the purified type 2 serotype antigen. We chose to add a constant amount of one of the antibodies at a concentration below which any significant protection occurred and determine whether this low amount of antibody would increase the MPD<sub>50</sub> of the other antibody (Table VI). There was a significant cooperative effect between the two antibodies. Presence of a low level of serotype antibody increased the MPD<sub>50</sub> of the polysaccharide antibody fourfold, while the polysaccharide antibody increased the MPD<sub>50</sub> of the type 2 serum approximately 14-fold. Thus, minute amounts of group B polysaccharide antibody have a very significant effect upon the protective ability of the type-specific serum.

Observation of a strong synergistic effect between group B polysaccharide antibody and serotype 2 antibody suggested that a similar phenomenon may also exist with the other meningococcal groups. To test this possibility we chose to examine the group C system because both group and type antigens are known (12). A group C whole cell serum (anti-C-11) containing antibody against the group C polysaccharide and the homologous serotype antigen was tested for protection against infection by the homologous strain and another group C strain of a noncross-reactive serotype (Table VII). The serum gave an approximately twofold greater homologous protection which is what we had observed for the group B whole cell sera. When we compared ability of the chick embryos to clear their infections, those embryos that were challenged with the homologous C-11 strain cleared their infection much better than those challenged with the other strain. Thus, the effects of group-specific and type-specific antibody are probably synergistic for the other meningococcal groups as well.

### Discussion

We wanted to demonstrate the protective effects of serotype antibody in an *vivo* model, and we chose the chick embryo because it is susceptible to meningococcal infection and develops lesions typical of meningococcal infection in man. The 12-day-old chick embryo does not possess an active complement system (2),

TABLE VI  
*Synergistic Effects of Antibodies to the Group B Polysaccharide  
 and to the Serotype 2 Antigen*

Serum dilution	No. embryos	At 24 h			MPD <sub>50</sub> *	
		Dead	Alive	Percent survival		
CHO‡	1:500	29	5	24	83	1:2,200
	1:1,000	26	8	18	69	
	1:2,000	26	5	21	81	
	1:6,000	9	9	0	0	
CHO & STA (1:150)	1:1,000	30	4	26	83	1:8,900
	1:2,000	21	4	17	81	
	1:5,000	25	5	20	80	
	1:10,000	18	11	7	39	
	1:20,000	18	15	3	17	
STA§	1:50	15	5	10	66	1:70
	1:100	15	12	3	20	
	1:150	12	10	2	17	
	1:200	12	12	0	0	
STA & CHO (1:6,000)	1:100	11	3	8	73	>1:1,000
	1:200	12	4	8	66	
	1:500	12	4	8	66	
	1:1,000	10	4	6	60	

\* MPD<sub>50</sub>, minimal serum dilution protecting 50% of the embryos as determined by method of Reed-Muench.

‡ CHO, purified goat Group B polysaccharide antibody containing 2.8 mg specific antibody per ml.

§ STA, serum no. 4588 prepared against purified type 2 serotype antigen.

TABLE VII  
*Protection of a Group C Antiserum, Anti-C-11, Against the Homologous  
 and a Heterologous Group C Strain*

Strain	Serotype	MPD <sub>50</sub> *	Surviving embryos			Percent infected‡
			No.	Infected	Uninfected	
C-11	NT§	1/519	51	8	43	16
138I	2	1/292	44	18	26	41

\* The MPD<sub>50</sub> serum dilution was calculated by method of Reed and Muench.

‡ For difference in survival— $P < 0.01$  with  $\chi^2$  of 7.56.

§ NT, nontypable.

therefore protective effects observed must be primarily opsonic and phagocytic. Specific antibody was required for uptake by the reticuloendothelial system because 10 organisms or less proved lethal in the absence of specific antibody. In contrast to our expectations, we found that the different group B serotype antisera prepared against whole cells showed primarily group-specific protection in the chick embryo. The serotype antisera contained both anti-polysaccha-



ride and anti-serotype antibodies (10). These findings indicate that antibody against the serotype antigen, although highly bactericidal in presence of complement, is only weakly opsonic in the absence of complement, which is in agreement with the observations of Roberts (21). In contrast, while the group B polysaccharide antibody is only weakly bactericidal (7, 18), it appears to be an effective opsonin. The protective effects of the group B antibody were several fold less than those for group A antibody, which may be related to the fact that group A antibody is both strongly bactericidal and an effective opsonin (15, 22).

It should be noted that there were two differences in the response of chick embryos to group B infection as compared to group A or group C infection. Firstly, the chick embryos cleared their blood streams of both group A and group C organisms with greater efficiency than they did group B organisms in the presence of immune rabbit sera. Secondly, demonstration of protection by anti-group B sera was much more dependent upon challenge dose than was the case with either group A or C sera. Evidently, the group B antibody was less efficient in promoting clearance of the organisms. A possible interpretation of these differences is that the group B organisms are cleared more slowly, therefore there is a lower critical challenge dose for group B above which multiplication of the organisms exceeds the ability of the reticuloendothelial system to take up and kill the organisms.

A deficiency of bactericidal antibody has been related to susceptibility to meningococcal disease (14). Failure to demonstrate significant bactericidal antibodies against the group B polysaccharide does not rule out the possible protective effects of a group B polysaccharide vaccine. Our results indicate that group B polysaccharide antibody is primarily opsonic in nature. Very low levels of this antibody are strongly synergistic when combined with the serotype antibody. The protective effects of the serotype antibody were increased 14-fold with 0.02  $\mu$ g of group B polysaccharide antibody, an antibody concentration below detection by indirect hemagglutination and by assay in the chick embryo. In a more general sense, these findings indicate that undetectable amounts of one antibody may have very significant effects upon assessment of the effects of another antibody.

In light of the poor immunogenicity of the group B polysaccharide, the protein serotype antigen appears to be the most suitable choice for an effective group B vaccine. However, these results indicate that the best vaccine may be one that includes both the protein and polysaccharide antigens.

### Summary

Protection against group B meningococcal infection was examined using the chick embryo. 12-day-old embryos were challenged intravenously with various meningococcal strains. The chick embryo has an active reticuloendothelial system but lacks functional complement. In this model we found that protection against group B infection was primarily group specific. The group B polysaccharide antibody is an effective opsonin, but is a very poor bactericidal antibody. In contrast, the serotype antibody was bactericidal but only slightly protective in the chick embryo where protection is primarily phagocytic in nature. The group-specific and type-specific antibodies are strongly synergistic. Minute amounts of

group B polysaccharide antibody caused a very significant increase in the protective effects of the serotype antibody.

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