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Protection Against Group B Meningococcal Disease: Evaluation of Serotype 2 Protein Vaccines in a Mouse Bacteremia Model

DONALD E. CRAVEN* AND CARL E. FRASCH

Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20205

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A mouse bacteremia model was used to evaluate the immunogenicity and protection against challenge provided by five different meningococcal serotype 2 vaccines. Mice vaccinated with serotype 2 protein vaccines had levels of bacteremia reduced by at least 100-fold after challenge with group B serotype 2 meningococci. Mice vaccinated with serotype 2 protein vaccine and challenged with group C serotype 2 meningococci showed 10-fold or less reduction in bacteremia. Vaccines were primarily serotype specific since no increase in protection was observed after challenge with either group B serotype 4 or group C nontypable meningococci. Serotype 2 antibody levels, measured by the bactericidal assay and the enzyme-linked immunosorbent assay 3 weeks after immunization, demonstrated a graded dose-response which correlated with protection up to 40 weeks after vaccination with a single 10- or 25-μg dose of serotype 2 protein vaccine. A 1-μg booster dose of serotype 2 vaccine, given 2 weeks after primary immunization, significantly increased both bactericidal ($P < 0.01$) and enzyme-linked immunosorbent assay ($P < 0.02$) titers. The data obtained from the mouse bacteremia model indicate that serotype 2 protein vaccines are stable and immunogenic, and protect mice against challenge with group B serotype 2 meningococci.

Meningococci causing bacteremia or meningitis, or both, are characterized by immunologically distinct capsular polysaccharides which form the basis for the serogroup designations A, B, C, X, Y, Z, W135, and 29E (Z'). Bactericidal antibody directed against the capsular polysaccharide provides protection against meningococcal disease (18). The purified group A and group C polysaccharide vaccines elicit human bactericidal antibody and have been successfully employed for immunoprophylaxis. The group A vaccine is effective above 3 months of age (26), whereas the group C polysaccharide is most protective in individuals over 2 years of age (15). However, purified group B polysaccharide vaccine has failed to elicit human bactericidal antibody (30). Therefore, other noncapsular surface antigens, such as the major outer membrane proteins, have been investigated as alternative vaccines for protection against group B disease (12-14, 32, 33).

Meningococci are divided into serotypes based on immunologically distinct outer membrane proteins and lipopolysaccharides (8, 17, 31). Epidemiological studies have shown that certain protein serotypes are associated with asymptomatic carriage, whereas others are primarily found among disease isolates (7, 9, 24). Serotype 2 has accounted for more than 50% of group B and group C disease isolates (9, 16, 24), and is also associated with disease in serogroups Y and W135 (10). For this reason, serotype 2 was chosen for initial protein vaccine studies.

Studies on meningococcal infection have been limited by the absence of an animal model simulating human meningococcal infection. The chicken embryo model produces lesions similar to human disease (2, 29), and previous work with this model demonstrated a synergistic protective effect of antibodies directed against the group B polysaccharide and serotype 2 protein (12). However, this model is difficult to use and is limited by an immature complement system. Using the guinea pig chamber model of Arko (1), Frasch and Robbins (13, 14) demonstrated that serotype 2 protein vaccines are immunogenic and protect against challenge with serotype 2 organisms, irrespective of serogroup. Although this model provided an excellent method to study many of the immunological aspects of

group B meningococcal infection, it is time consuming and is based upon a localized subcutaneous infection.

A mouse model, using a mucin-enhanced lethal infection, was described by Miller in 1936 (23) and was later employed to compare meninogococcal strain virulence and the potency of antisera used for therapy of meningococcal infections (27). However, mucin requires standardization and has poorly defined effects upon the mouse immune system. Calver et al. (3) found that iron compounds could replace mucin, but the concentration of iron required was often toxic. Recently, Huet and Suire (20) described a mouse bacteremia model, not requiring the injection of mucin or iron, for evaluating the immunogenicity of meningococcal polysaccharide vaccines. Mice were vaccinated intraperitoneally (i.p.) with 1 μg of polysaccharide and 4 days later challenged i.p. with 10^6 meningococci. Levels of bacteremia were monitored by cardiac blood cultures taken from vaccinated and control mice.

In this communication we discuss a mouse bacteremia model similar to that of Huet and Suire. Bacteremia was monitored by serial tail bleeding after i.p. injection of log-phase meningococci into mice vaccinated 3 weeks previously with a serotype 2 protein vaccine. The data suggest that serotype 2 vaccines are immunogenic and effective in preventing or reducing bacteremia in mice challenged with group B serotype 2 meningococci.

MATERIALS AND METHODS

Strains. Meningococcal strains were serogrouped by antiserum-agar (4) and serotyped by the method of Frasch and Chapman (8). Strains M986, serogroup B, serotype 2 (B,2), B16B6 (B,2), M981 (B,4), and M982 (B,9) were previously characterized (14). S-946 (B,2), S-1975 (Y,2), S-1211 (Y,2), and S-3247 (Y,2) were kindly provided by Harry Feldman, Upstate Medical Center, Syracuse, N.Y. Strains 138I (C,2) and C-11 (C, nontypable [NT]) were obtained from Malcolm Artenstein, Walter Reed Army Institute of Research, Washington, D.C. Strain R.K. (C,2) was provided by Melvin Marks, Montreal Children's Hospital, Montreal, Canada. Strains were cultured on brain heart infusion agar (Difco, Detroit, Mich.) containing 1% normal horse serum.

Vaccines. Serotype 2 protein vaccines were prepared from the serotype antigen of strain M986-NCV-1 by treatment with deoxycholate (Sigma Chemical Co., St. Louis, Mo.) or Emulphogene BC-720 (GAF, New York, N.Y.) to solubilize the lipopolysaccharide (14). Each vaccine was analyzed for protein by the Lowry method (22), for 3-deoxy-D-manno-octulosonic acid by the Osborn method (25), for sialic acid with the amino acid analyzer (21), and for nucleic acid by optical density at 260 nm, *Limulus* amoebocyte lysate gelation (19), and rabbit pyrogenicity. Outer membrane protein patterns were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11).

Immunization and challenge. Female NIH general purpose mice (no. 0302), weighing 12 to 15 g, were immunized i.p. with 25, 10, 1, or 0.1 μg of serotype 2 vaccine. Control mice received 10 μg i.p. of meningococcal group C polysaccharide. Three weeks after vaccination, animals were challenged i.p. with 2×10^3 to 4×10^3 log-phase meningococci grown on brain heart infusion agar containing 1% normal horse serum and diluted in Dulbecco phosphate-buffered saline (with Ca^+ and Mg^+) with 1% human serum albumin.

Blood cultures and bacteremia. Bacteremia was monitored by cutting the tip of the tail with sharp scissors and collecting 0.02 ml of blood with a micropipette (Corning Glass, Corning, N.Y) in a Clinac Micropipetter (La Pine Scientific Products, Chicago, Ill.). Each blood sample was inoculated and spread with a sterile glass rod onto brain heart infusion agar with 1% normal horse serum containing vancomycin, colistin, and nystatin (BBL Microbiology Systems, Cockeysville, Md.) to inhibit other flora. Colonies were counted after incubation at 37°C in the presence of 5% CO_2 for 24 h. The level of bacteremia for each group of animals was calculated as the arithmetic mean $\log_{10}$ of the number of colony-forming units per milliliter of blood. The dose of serotype 2 protein vaccine required to protect 50% of the animals from bacteremia was calculated by the probit method (6). For comparison of different serotype 2 vaccine preparations, the protective doses needed to protect 50% of the animals at 3 and 6 h were averaged to the nearest 0.1 μg for each group.

ELISA. Blood was obtained by cutting the right subclavical artery of mice anesthetized with ether and collected with a sterile Pasteur pipette. The blood was allowed to clot in sterile 1.5-ml plastic tubes, and the serum for enzyme-linked immunosorbent assay (ELISA) was removed after 24 h. The ELISA was performed by the method of Engvall and Perlmann (5) with slight modification (7). The ELISA conjugate was prepared using the immunoglobulin G fraction of a rabbit anti-mouse immunoglobulin (Microbiological Associates, Bethesda, Md.) and alkaline phosphatase (type VII, Sigma Chemical Co., St. Louis, Mo.). The optical density at 400 nm value that would have been attained if the reaction had proceeded to 100 min was calculated, and the results were expressed in ELISA units (optical density at 400 nm, at 100 min). Results of each assay were corrected by an internal standard composed of three sera from mice immunized with vaccine lot D-05.

Bactericidal assay. The microbactericidal assay of Frasch and Robbins (14), employing 4-week-old rabbit serum diluted 1:2 as a source of complement, was used to determine levels of bactericidal antibody on sera obtained as described above. The endpoint was defined as the serum dilution producing greater than 50% reduction in viable count of strain B16B6 (B,2).

RESULTS

Mouse bacteremia model. Challenge experiments were designed to use a 0.02-ml blood

sample and sufficient challenge inoculum to produce bacteremia in all the animals at a mean level of 1,000 to 3,000 colony-forming units per ml. This value was usually 10-fold greater than the number of organisms required to produce bacteremia in 50% of the animals 3 h after challenge. As few as 10 to 40 organisms produced bacteremia in some animals, and a challenge dose of 2,000–4,000 organisms of S-946 (B,2) produced bacteremia in 100% of the animals. Larger challenge inoculum was required to produce similar levels of bacteremia with other serotypes.

Vaccine protection. The mouse bacteremia model was designed to evaluate the relative protection conferred by immunization with five different serotype 2 protein vaccines. The chemical characteristics of the different vaccine lots are shown in Table 1. All vaccines except D-05 passed the present rabbit pyrogenicity guidelines for human administration of meningococcal polysaccharide vaccines.

For protection experiments, groups of 10 mice were immunized i.p. with graded doses of serotype 2 vaccine, and controls received group C meningococcal polysaccharide vaccine. Group C polysaccharide is immunogenic but provides no protection against group B meningococcal challenge. Vaccine E-01 prevented meningococcemia in more than 50% of animals, reducing mean levels of bacteremia more than 100-fold compared to controls (Fig. 1).

Additional experiments were performed to determine the endpoints of serotype 2 vaccine protection. Doses of 10, 1, and 0.1 μg of vaccine were administered i.p., and bacteremia was monitored at 3, 6, and 9 h (Table 2). Fifty percent mean protective dose values demonstrated significant differences between vaccinated animals and controls, and implied that vaccines E-01, VMF-1, and D-05 were superior to E-05 and E-06. Vaccine VMF-1 was effective after storage at 4°C in aqueous solution for 2 years, which suggests that serotype 2 vaccines are stable after long-term storage.

Since serotype 2 strains account for the majority of group C meningococcal disease (9, 16, 24), serotype 2 vaccine E-01 was evaluated for its protection in mice challenged with one of two group C type 2 strains (Fig. 2). Higher doses of vaccine E-01 protected some animals challenged with strain 138I against bacteremia and reduced the levels of bacteremia in others. With strain R.K. none of the mice were completely protected from bacteremia, and mean levels of bacteremia with 25 μg of vaccine were only slightly lower than controls. The data suggest that the level of meningococcal protection provided by noncapsular vaccines may vary considerably with the infecting strain.

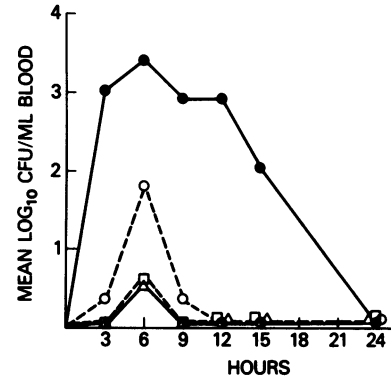

FIG. 1. *Levels of bacteremia in mice after challenge i.p. with meningococcal strain S-946 (group B serotype 2). Groups of 10 animals were vaccinated i.p. 3 weeks before challenge with serotype 2 protein vaccine E-01: 25 μg (□), 10 μg (△), or 1 μg (○). Control animals (●) received 10 μg of meningococcal group C polysaccharide.*

TABLE 1. *Characteristics of the serotype 2 protein vaccines tested in the mouse bacteremia model*

Serotype 2 protein vaccine[a]	Protein (μg/ml)	KDO[b] (μg/ml)	LAL (μg of LPS/mg of protein)[c]	Rabbit pyrogenicity (passed at μg/kg)	Identity by SDS-PAGE[d]	Sialic acid (%)	Nucleic acid (%)
E-01	100	3.3	5	ND[e]	Yes	<1	<1
E-05	100	1.5	4	0.025	Yes	<1	<1
E-06	100	1.3	1	0.25	Yes	<1	<1
D-05	100	7.5	400	0.025	Yes	<1	<1
VMF-1	1,360	1.2	1.8	2.7	Yes	<1	<1

[a] Vaccine prepared with Emulphogene BC-720 designated by E and lot number; vaccines prepared with deoxycholate designated by D or VMF and lot number.

[b] KDO, 3-Deoxy-D-manno-octulosonic acid.

[c] LAL, *Limulus* amoebocyte lysate gelation test for endotoxin; LPS, lipopolysaccharide.

[d] Greater than 50% of the vaccine protein consists of the 41,000-dalton band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

[e] ND, Not determined.

TABLE 2. *Protection against bacteremia provided by serotype 2 protein vaccines administered i.p. in the mouse bacteremia model*[a]

Vaccine	Detergent[b]	Dose (μg)	No. bacteremic/no. injected (organisms/ml of blood)[c]			MPD_{50}[d] (μg)
			3 h	6 h	9 h	
E-01	EBC-720	10	0/10	0/10	0/10	0.2
		1	0/10	2/10 (280)	4/10 (270)	
		0.1	2/10 (50)	8/10 (560)	5/10 (180)	
E-05	EBC-720	10	2/10 (120)	1/10 (100)	0/10	2.9
		1	8/10 (540)	8/10 (390)	8/10 (220)	
		0.1	10/10 (1,200)	10/10 (290)	10/10 (280)	
E-06	EBC-720	10	1/10 (100)	3/10 (50)	0/10	0.6
		1	7/10 (121)	2/10 (50)	0/10	
		0.1	7/10 (221)	3/10 (50)	0/10 (240)	
VMF-1	DOC	10	0/10	0/10	0/10	0.1
		1	1/10 (50)	1/10 (70)	1/10 (100)	
		0.1	5/10 (190)	6/10 (240)	6/10 (250)	
D-05	DOC	10	2/10 (100)	2/10 (50)	2/10 (200)	0.2
		1	1/10 (50)	3/10 (70)	1/10 (100)	
		0.1	5/10 (190)	6/10 (240)	6/10 (330)	
C-poly[e]	None	10	30/30 (1,440)	30/30 (1,923)	30/30 (453)	>10

[a] Animals were challenged with S-946, a group B, serotype 2 organism, 2×10^3 to 4×10^3 organisms i.p.
[b] EBC-720, Emulphogene BC-720; DOC, deoxycholate.
[c] Mean level of bacteremia in infected animals.
[d] Mean protective dose of vaccine needed to prevent 50% of the animals from bacteremia at 3 and 6 h.
[e] Control animals received 10 μg of meningococcal group C polysaccharide (C-poly).

Group Y,2 organisms could not be evaluated since i.p. challenge doses of 10^7 organisms were unable to produce bacteremia. Natural bactericidal antibody titers in the mouse sera against the group Y,2 test organism S-1975 were greater than 1:1,280, but no antigens cross-reactive with the group Y capsular polysaccharide could be found by group Y antiserum agar screening of mouse stool flora.

Cross-reactive protein surface antigens are present among meningococcal serotypes. Therefore, mice given 25 μg of serotype 2 protein vaccine lot E-01 i.p. were challenged 3 weeks later with either C,NT, B,4, or B,9 meningococci (Fig. 3). Partial protection with a 25-μg dose of vaccine was noted after B,9 challenge, but no protection was seen after challenge by B,4 or C,NT organisms.

Long-term protection was noted after a single 25-μg dose of serotype 2 protein vaccine (Table 3). This protection correlated with the presence of bactericidal antibody.

Antibody response to serotype 2 vaccines. Groups of four mice received a single i.p. injection of serotype 2 protein vaccine, and bactericidal antibody responses were measured 3 weeks later (Table 4). Except for vaccine E-05, 10- and 1-μg doses of serotype 2 vaccines produced bactericidal levels significantly above those of control animals. These results correlated with the challenge studies (Table 2).

ELISA antibody values (Table 4) showed a graded dose-response similar to bactericidal titers. Bactericidal titers and ELISA results indicated that vaccines D-05 and E-01 were more immunogenic in mice.

The mean antibody responses to a second 1-μg dose of serotype 2 vaccine at week 2 are shown in Fig. 4. Taking the log of the geometric mean of four mice, statistical analyses of the booster response were determined by the *t* test (28). Bactericidal antibody levels at 3 weeks were significantly increased ($P < 0.01$) in both the 10-μg and 1-μg groups with a second dose of vaccine. A significant booster effect in ELISA titers was also noted for both the 10-μg ($P < 0.02$) and 1-μg ($P < 0.01$) groups at 3 weeks.

DISCUSSION

Two alterations were made to the mouse bacteremia model described by Huet and Suire (20). Firstly, blood cultures were obtained by sequentially cutting the tip of the tail. In our experience this method was more rapid than cardiac puncture and also allowed serial quantitation of bacteremia in the same mouse. Secondly, mice were challenged 3 weeks after vaccination instead of 4 days. This decreased the chances of "nonspecific" protective effects, and allowed correlation of challenge results with bactericidal and ELISA antibody determinations.

The mouse bacteremia model demonstrated significant differences among serotype 2 vaccine

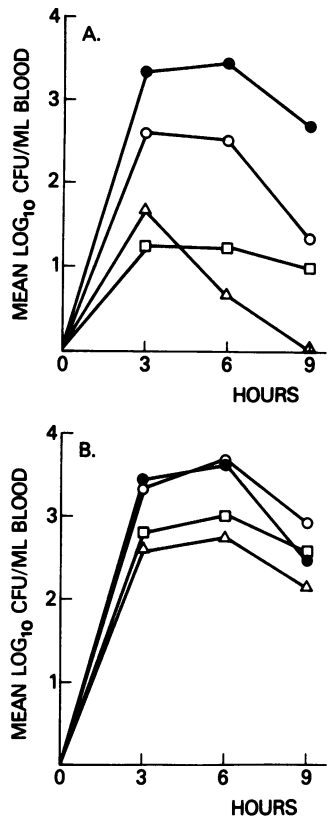

FIG. 2. *Protection against challenge with group C serotype 2 strains: (A) 138I, (B) R.K. Groups of 10 mice vaccinated with 25 μg (□), 10 μg (△), or 1 μg (○) of serotype 2 protein vaccine E-01. Control animals (●) received 10 μg i.p. of meningococcal group A polysaccharide.*

preparations, emphasizing the usefulness of in vivo assays of potency. Except for lot E-01, vaccines prepared with the detergent deoxycholate appeared to give better protection and higher bactericidal and ELISA antibody levels than vaccines prepared with Emulphogene BC-720. Effective serotype 2 vaccines such as E-01, D-05, and VMF-1 reduced levels of bacteremia 100-fold in mice challenged with S-946 (B,2). One vaccine, E-05, gave poor protection and elicited low bactericidal and ELISA antibody responses, which would not have been anticipated from the chemical analysis shown in Table 1. The potency data suggest that the antigenic composition of the protein in vaccine E-05 may have been altered during preparation.

Serotype 2 protein vaccines provided lower

TABLE 3. *Duration of serotype 2 vaccine protection against i.p. challenge with group B serotype 2 meningococci*[a]

Vaccine	Weeks post-vaccination	No. bacteremic/no. injected (organisms/ml of blood)[b]	
		3 h	6 h
E-01, 25 μg	20	0/10	1/10 (100)
Saline	20	10/10 (890)	10/10 (1100)
E-01, 25 μg	20	0/4	0/4
E-01, 10 μg	20	0/4	0/4
C-poly[c], 10 μg	20	4/4 (290)	4/4 (670)
E-01, 25 μg	40	0/4	0/4
Saline	40	4/4 (330)	4/4 (230)

[a] Mice were challenged i.p. with S-946 (B,2) at 2×10^3 to 4×10^3 organisms.

[b] Mean level of bacteremia in infected animals.

[c] Meningococcal group C polysaccharide (C-poly) used as a control.

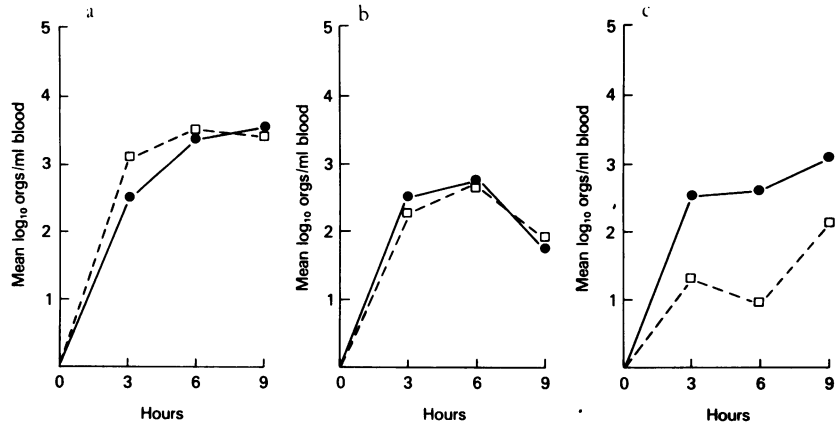

FIG. 3. *To determine the serotype specificity, groups of four mice were immunized i.p. with 25 μg of serotype 2 protein vaccine E-01 (□) and challenged i.p. 3 weeks later with meningococcal strains: (a) C-11 (C, NT), (b) M-981 (B,4), (c) M-982 (B,9). Control animals (●) received 10 μg of meningococcal group A polysaccharide (a) or meningococcal group C polysaccharide (b and c).*

TABLE 4. *Mean antibody responses in mice 3 weeks after vaccination with serotype 2 protein vaccine*

Vaccine	Dose (μg)	Bactericidal assay[a]		ELISA assay[b]	
		Titer	Range	U	Range
E-01	25	175	160–340	1.34	1.16–1.43
	10	340	340	1.26	0.87–1.73
	1	48	20–160	1.04	0.35–1.05
E-05	25	<8	<8	0.50	0.18–0.69
	10	<8	<8	0.43	0.21–0.69
	1	<8	<8	0.21	0.12–0.34
E-06	10	200	160–320	0.86	0.55–1.18
	1	85	20–160	0.17	0.06–0.49
	0.1	20	20	0.07	0.06–0.09
D-05	25	340	80–640	1.68	1.14–2.53
	10	240	160–320	1.21	1.05–1.26
	1	46	20–160	1.04	0.18–1.81
	0.1	<8	<8	0.81	0.70–0.90
C-poly[c]	25	<8	<8	0.02	0.06–0.09

[a] Mean value of four animals expressed as reciprocal of serum dilution producing greater than 50% killing of strain B16B6 (B,2).

[b] ELISA units equal to optical density at 400 nm at 100 min. Values are the mean of four animals (sera diluted 1:200).

[c] Meningococcal group C polysaccharide (C-poly) vaccine control.

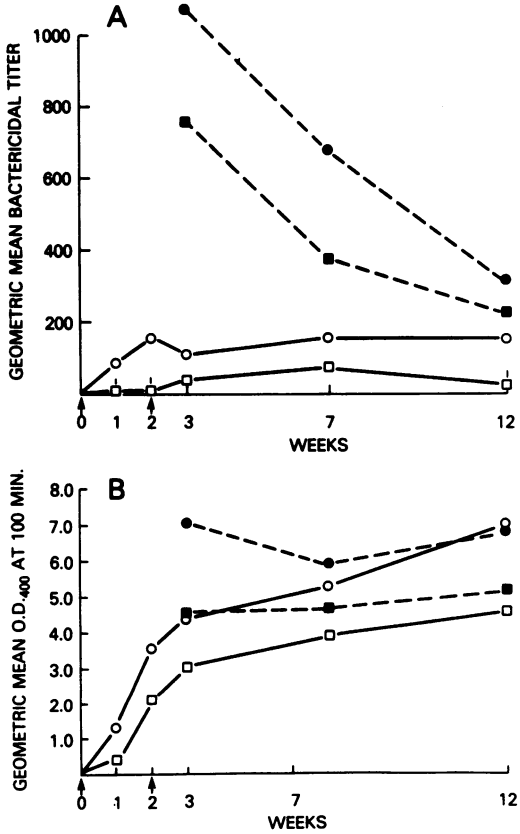

FIG. 4. *Bactericidal antibody (A) and ELISA antibody (B) levels in mice immunized with serotype 2 vaccine E-01 at 10 μg (○) or 1 μg (□). Booster responses, after 1 μg i.p. given to each group at 2 weeks (↑), are indicated by dotted lines with ● and ■.*

levels of protection against challenge with group C serotype 2 meningococci. Greater protection was observed with strain 138I than strain R.K. Since antibodies elicited by serotype vaccines are directed against noncapsular surface antigens, the presence of a large capsule on an infecting strain may result in differences in protection among strains. Compared to strain 138I, strain R.K. produces a large halo on antiserum agar, suggesting that strain R.K. produces larger amounts of capsular polysaccharide, which may impair immunological clearance in the absence of specific anticapsular antibody. These data suggest that for optimal protection a two-dose serotype vaccine regimen or a combination polysaccharide-protein vaccine may be required. In this respect, mice vaccinated with a combination of serotype 2 protein and group B polysaccharide showed increased protection against bacteremia compared to mice immunized with either meningococcal group B polysaccharide or serotype 2 protein vaccines alone (D. E. Craven and C. E. Frasch, unpublished observations).

The protection provided by serotype 2 vaccines appears to be primarily serotype specific. Partial protection against challenge was evident for only one of three heterologous group B strains examined. This protection could be the result of other cross-reactive determinants present in the serotype 2 vaccine and suggests that future serotype vaccines may require a combination of two or three carefully chosen disease-associated serotypes to maximize the range of vaccine protection.

ELISA and bactericidal antibody titers were measured up to 12 weeks after either a single or

two-dose vaccine regimen. Both assays showed a statistically significant booster effect and sustained antibody levels greater than controls over the 12-week period.

These studies demonstrate that serotype 2 protein vaccines are immunogenic and protect mice against group B serotype 2 meningococcemia. Similar observations regarding the immunogenicity and protection were made in the guinea pig chamber model (14). However, the degree of correlation between the protection observed in the mouse bacteremia model and protection in humans remains to be determined. Studies by Zollenger et al. (32) and from this laboratory (Frasch and Craven, unpublished observations) on small numbers of volunteers suggest that serotype 2 vaccines alone are poor immunogens in humans. Further studies in larger numbers of adult volunteers with lower levels of serotype 2 antibody before vaccination are presently in progress. Studies of serum from children convalescing from meningococcal disease have demonstrated increased serotype 2 antibody levels (7). Therefore, serotype 2 vaccines presented in proper form may provide important additional protection. Since both capsular and noncapsular antigens elicit host antibody responses, serotype vaccines in combination with polysaccharides may provide synergistic protection for groups at high risk for group B and group C meningococcal disease. Recent studies by Zollenger et al. with a group B polysaccharide-serotype 2 protein vaccine have demonstrated increased levels of bactericidal antibody in adult volunteers, but this preparation had significant reactogenicity and induced short-term elevations in immunoglobulin M antibody (33).

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