

MF59 Adjuvant Enhances Antibody Responses of Infant Baboons Immunized with *Haemophilus influenzae* Type b and *Neisseria meningitidis* Group C Oligosaccharide-CRM₁₉₇ Conjugate Vaccine

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Received 17 December 1996/Accepted 20 February 1997

The ability of the adjuvant MF59 to enhance the immunogenicity of polysaccharide-protein conjugate vaccines was investigated in infant baboons. MF59 consists of stable droplets (<250 nm) of the metabolizable oil squalene and two surfactants, polyoxyethylene sorbitan monooleate and sorbitan trioleate, in an oil-in-water emulsion. In humans, MF59 is well tolerated and enhances the immunogenicity of recombinant protein subunit or particle vaccines. Its effect on the immunogenicity of polysaccharide-protein conjugate vaccines is unknown. Baboons 1 to 4 months of age were immunized intramuscularly with *Neisseria meningitidis* group C and *Haemophilus influenzae* type b (Hib) oligosaccharide-CRM₁₉₇ conjugate vaccines. The lyophilized vaccines were reconstituted with phosphate-buffered saline (PBS), Al(OH)₃ (alum), or MF59. Groups of five animals each were given three injections of the respective formulations, with one injection every 4 weeks. Four weeks after each immunization, the MF59 group had up to 7-fold-higher geometric mean anticapsular-antibody titers than the alum group and 5- to 10-fold-higher *N. meningitidis* group C bactericidal-antibody titers. Twenty-one weeks after the third immunization, the MF59 group still showed 5- to 10-fold-higher anticapsular-antibody titers. The antibody responses of the animals given the vaccines reconstituted with PBS were low at all times measured. Both the MF59 and alum groups, but not the PBS group, showed booster antibody responses to unconjugated Hib and *N. meningitidis* group C polysaccharides, results consistent with induction of memory B cells. Thus, MF59 may be useful for accelerating and augmenting immunity to polysaccharide-protein conjugate vaccines in infants.

Once the major cause of bacterial meningitis and other serious infections in infants and children, diseases caused by *Haemophilus influenzae* type b (Hib) have been virtually eliminated by incorporation of Hib polysaccharide (PS)-protein conjugate vaccines into the routine infant and toddler immunization schedules (1, 4, 8, 25).

The success of Hib conjugate vaccines also has stimulated development of new conjugate vaccines for prevention of diseases caused by other encapsulated organisms, such as *Streptococcus pneumoniae* (36) and *Neisseria meningitidis* groups A and C (10, 19, 20, 38). However, in contrast to Hib conjugate vaccines, which contain only a single serotype-specific PS, these new vaccines require the use of multiple serotype- or serogroup-specific PS, each conjugated separately to a carrier protein. Maintaining optimal immunogenicity of each component may be difficult with conjugate vaccines containing multiple PS antigens.

One approach to increasing both the immunogenicity and the effectiveness of PS-protein conjugate vaccines is the use of an adjuvant. An effective adjuvant also may permit the use of lower dosages and fewer injections, thereby decreasing the cost of an immunization program. Currently, the only vaccine adjuvant licensed for human use by the U.S. Food and Drug Administration is alum (several salts of aluminum). While

alum has a good safety record, it is a relatively weak adjuvant. A promising, more potent adjuvant, designated MF59, has been developed at Chiron Vaccines. MF59 consists of small, uniform, stable droplets (<250 nm) of the metabolizable oil squalene with two surfactants, polyoxyethylene sorbitan monooleate (Tween 80) and sorbitan trioleate (Span 85), in an oil-in-water emulsion. When compared to alum or incomplete Freund's adjuvant (39), MF59 has been shown in mice, guinea pigs, rabbits, and nonhuman primates to augment antigen-specific humoral and T-cell responses to a variety of experimental vaccines. In humans, the MF59 adjuvant is generally well tolerated. To date (1996), MF59 has been administered to more than 6,000 humans (estimated total number of injections, >15,000) as part of clinical trials of recombinant glycoprotein subunit vaccines for herpes simplex virus type 2 (gB and gD) (6, 18), cytomegalovirus (gB) (42), human immunodeficiency virus (gp120) (16), or inactivated influenza vaccine (split virion or purified subunits) (22, 23, 27). In these studies, no abscesses or severe local reactions have been reported. Other local reactions were limited largely to transient pain at the injection site (usually mild) and minimal erythema or induration that usually resolved within 2 to 3 days (18). Fever and systemic reactions were infrequent. MF59 also has been investigated as an adjuvant in human newborns immunized with gp120 (human immunodeficiency virus vaccine) (17, 43) and toddlers immunized with gB (cytomegalovirus vaccine) (24a). In both studies, the respective vaccines were well tolerated and elicited high immune responses. Thus, MF59 is a promising candidate

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adjuvant for augmenting immune responses of vaccines intended both for adults and for infants and toddlers.

The purpose of the present study was to determine the ability of MF59 to enhance serum antibody responses of infant baboons to a combined investigational Hib and *N. meningitidis* group C oligosaccharide-protein conjugate vaccine. Infant baboons were selected as the model system because their antibody responses are likely to be more predictive of the anti-PS antibody responses of human infants than are adult primate or rodent models (3, 15, 32, 33, 37, 40, 41).

MATERIALS AND METHODS

Vaccines and adjuvants. *N. meningitidis* group C conjugate vaccine (development lot J35D18L1) and Hib conjugate vaccine (development lot L10HC24) were produced by using selective end-reducing group activation of sized oligosaccharides followed by subsequent coupling to the protein carrier CRM₁₉₇ through a hydrocarbon spacer (7). CRM₁₉₇ is a nontoxic genetic mutant of diphtheria toxin. The respective conjugates were filter sterilized and lyophilized in unit doses. On the day of vaccination, the *N. meningitidis* group C and Hib glycoconjugate vaccines were reconstituted with Dulbecco's modified phosphate-buffered saline without calcium or magnesium (PBS), with aluminum hydroxide (alum; 1 mg per dose; lot 010394; Chiron Vaccines, Siena, Italy), or with MF59 lot KJA342; Chiron Vaccines). The MF59 was prepared under high-pressure homogenization as previously described (26). After reconstitution, each 0.5-ml dose of vaccine contained approximately 10 µg of each saccharide and a total of 40 µg of CRM₁₉₇ protein.

To assess immunologic B-cell priming by the conjugate vaccination (10, 12, 19), animals were given boosters of plain (unconjugated) *N. meningitidis* group C PS vaccine (bulk lot 82) and Hib PS vaccine (bulk lot 25). Both vaccines were prepared at Chiron Vaccines (Siena, Italy). Each 0.5-ml dose contained 5 µg of the respective PS in PBS.

Animals. Infant baboons (*Papio cynocephalus anubis*), estimated on the basis of weight and color to be 1.5 to 4 months of age at study initiation, were assigned to groups of five animals each to ensure an equal distribution of ages and genders (2:3 male to female). The baboons were housed with their mothers at Southwest Foundation for Biological Research, San Antonio, Tex., a registered research facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animal welfare experimentation guidelines as established by the National Institutes of Health, American Association for Laboratory Animal Science, and Food and Drug Administration were followed in the conduct of all animal research throughout the course of this study.

Immunizations and blood samples. There were four study groups. Animals in the first three groups received three immunizations with a combined *N. meningitidis* group C and Hib conjugate vaccine. The immunizations were given intramuscularly (i.m.) 1 month apart. Group 1 received vaccine administered with PBS, group 2 received vaccine with alum, and group 3 received vaccine with MF59. Animals in group 4 (controls) received three monthly 0.5-ml i.m. injections of acellular pertussis vaccine combined with diphtheria and tetanus toxoids adsorbed to aluminum hydroxide (Pertugen; Chiron Vaccines, Siena, Italy; referred to herein as DTaP) (29, 30).

Baboons were bled by venipuncture prior to each immunization and at 4 and 21 weeks after the third immunization. After the week 21 blood collection, all animals were given boosters of 5 µg of plain (unconjugated) *N. meningitidis* group C PS and 5 µg of plain (unconjugated) Hib PS. These vaccines were administered i.m. in separate limbs. Sera were obtained 7, 28, and 90 days after the booster injections.

Serology. Serum anti-*N. meningitidis* group C PS antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA), using a procedure adapted from that previously described for measuring human serum antibody to group B PS (13). In the present study, the solid-phase test antigen consisted of an adipic dihydrazide-derivatized form of the *N. meningitidis* group C PS (ADH-group C PS) instead of biotinylated ADH-group B PS. The ADH-group C PS (1 µg/ml in PBS) was used directly to coat 96-well U-bottom microtiter plates (Immulon II; Dynatech, Chantilly, Va.) by incubating them for 1 h at 37°C. After washing, plates were blocked with 1% bovine serum albumin (radioimmunoassay grade; fraction V; Sigma Chemical Co., St. Louis, Mo.) in PBS, pH 7.4, for 1 h at room temperature. Serial dilutions of sera were incubated overnight at 4°C. After washing, bound antibody was detected with alkaline phosphatase-labeled, affinity-isolated rabbit anti-monkey immunoglobulin G (IgG) (Sigma). Titers were expressed as the serum dilution giving an optical density at 405 nm of 0.5 after 30 min of incubation with substrate.

Complement-mediated bactericidal activity against *Neisseria meningitidis* group C was assayed as previously described for *N. meningitidis* group B (21) except that Gey's buffer was used instead of barbital buffer. The group C test strain used, 60E, was obtained from Wendell Zollinger (Walter Reed Army Institute of Research, Rockville, Md.). All test serum samples were heat treated (56°C for 30 min) to inactivate endogenous complement activity. For the bactericidal reaction, 20% serum from a healthy human adult whose serum alone showed no killing of the test organism was used as an exogenous complement

source. Bactericidal titers were defined as the reciprocal dilution of baboon serum giving a 50% decrease in CFU after 60 min of incubation at 37°C with bacteria and complement compared to CFU present at time 0. (In the presence of test serum alone or complement alone, CFU counts during the 60 min normally increased by ≥50%.)

Concentrations of serum antibody to Hib PS were measured by a radioantigen binding assay using ¹²⁵I-labeled Hib PS and a procedure adapted from that described by Robbins et al. (31). Immune complexes containing radiolabeled antigen were precipitated with an equal volume of saturated ammonium sulfate. After washing, counts per minute were measured in the precipitate as described elsewhere (2). Anti-Hib antibody concentrations in the baboon sera were assigned from a standard curve generated by using serial twofold dilutions of the U.S. Center of Biologic Evaluation and Research human serum reference pool, lot 1983 (U.S. Food and Drug Administration, Bethesda, Md.).

Statistics. Antibody concentrations were transformed (log₁₀). For these calculations, baboons anti-*N. meningitidis* group C ELISA titers of less than 1:50 were assigned a value of 1:25, and anti-group C bactericidal-antibody titers of less than 1:25 were assigned a value of 1:12.5. Anti-Hib PS anticapsular-antibody concentrations of less than 0.14 µg/ml were assigned a value of 0.07. Geometric means and 95% confidence intervals were computed by using the log transformed means and standard errors computed from a one-way analysis of variance (ANOVA) model. Differences between each pair of groups with respect to geometric means were tested by using the *P* values from the ANOVA model. To evaluate the fold change in titers between different days within each group, the logarithm of the ratio was computed for each animal and then analyzed by using a one-way ANOVA model.

RESULTS

Safety of MF59 when administered with conjugate vaccines to infant baboons. All animals appeared to tolerate the vaccinations without difficulty. On close inspection of the injection sites at the time of reimmunization or obtaining blood samples, there was no evidence of granulomas or abscesses. One animal assigned to the alum group died 3 days after the first immunization from apparent starvation. The animal caretakers attributed this death to insufficient milk production from its mother.

MF59 enhances anti-*N. meningitidis* group C and anti-Hib PS antibody responses of infant baboons. Figure 1 summarizes the anti-PS antibody responses of the infant baboons immunized with Hib and *N. meningitidis* group C conjugate vaccines (Fig. 1B and A, respectively) administered with PBS, alum, or MF59. Although not shown, the preimmunization sera had very low or undetectable antibody levels. Also, with the exception of two animals, none of the control animals immunized with DTaP alone showed more than twofold increases in levels of antibody to *N. meningitidis* group C or Hib PS in serum. The two exceptions had 0.24 and <0.14 µg of anti-Hib PS antibody per ml detected in serum from the first bleed, and these levels increased to 0.57 and 1.46 µg/ml, respectively, in sera obtained 1 month after the third immunization. The reasons for these increases in serum anti-Hib PS antibody concentrations are not known. These two animals showed no detectable increases in titer of serum antibody to *N. meningitidis* group C PS.

The baboons given the conjugate vaccines with PBS showed minimal anti-Hib PS antibody responses after one, two, or three immunizations (geometric mean antibody concentrations of <0.14, <0.14, and 0.15 µg/ml, respectively). The corresponding values in the alum group were 0.32, 5.0, and 5.2 µg/ml (*P* ≤ 0.03 compared to the respective values of the PBS group). After doses 1 and 2, the MF59 group showed geometric mean antibody concentrations of 0.75 and 15.1 µg/ml, respectively. After dose 3, the geometric mean serum antibody concentration was 37 µg/ml, which is sevenfold higher than that in the alum group (*P* < 0.03).

The immunogenicity of the MenC conjugate showed a pattern similar to that of the respective anti-Hib PS antibody responses. When the conjugate vaccine was administered without adjuvant (i.e., with PBS), the geometric mean titer (GMT) of IgG anti-group C PS as measured by ELISA was below detectable levels (<1:50) after one and two immunizations and

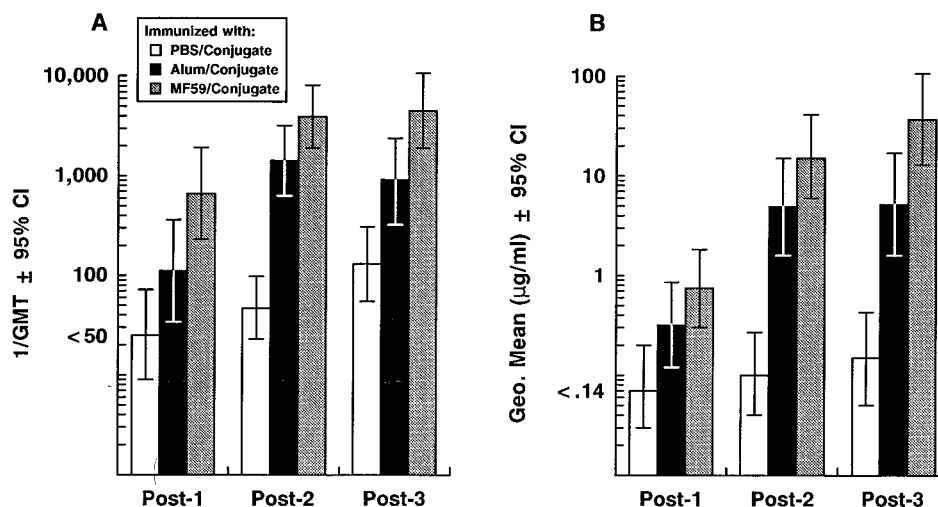


FIG. 1. Effect of MF59 adjuvant on serum anticapsular antibody responses of infant baboons immunized at 1-month intervals with *N. meningitidis* group C and Hib oligosaccharide-CRM₁₉₇ conjugate vaccines. The lyophilized vaccines were administered with PBS alum, or MF59. (A) IgG anti-group C PS antibody responses. Animals administered vaccine with alum had higher GMTs than those given vaccine with PBS ($P \leq 0.06$ for each injection). Animals administered vaccine with MF59 had higher GMTs than those given vaccine with alum ($P < 0.03$ for each injection). (B) Anti-Hib PS antibody responses. Animals administered vaccine with alum had higher geometric mean total antibody concentrations than those given vaccine with PBS ($P < 0.03$ for each injection). Animals administered vaccine with MF59 had higher geometric mean concentrations than those given vaccine with alum ($P > 0.10$ after the first and second injections and $P < 0.03$ for the 4 weeks post-third injection). CI, confidence interval.

rose to a GMT of 1:131 4 weeks following the third immunization. With alum, the antibody responses peaked after dose 2. The corresponding GMTs after doses 1, 2, and 3 were 1:111, 1:1,432, and 1:924 ($P < 0.06$, 0.001, and 0.01 compared to the respective titers in the PBS group). The highest IgG anti-group C PS antibody responses were observed in the group of animals receiving the conjugate vaccines with MF59: GMTs of 1:669, 1:3,957, and 1:4,548 after one, two, and three injections, respectively ($P < 0.03$ for each injection compared to the respective GMTs of the alum group).

MF59 augments serum antibody functional activity against *N. meningitidis* group C. Table 1 summarizes the serum anti-group C bactericidal antibody responses of the baboons immunized with the conjugate vaccines administered with PBS, alum, or MF59. There were no detectable bactericidal responses in the group administered the vaccines with PBS (GMTs of $<1:25$ after all three doses). The group administered the conjugate vaccines with alum showed no detectable bactericidal antibody response after dose 1 but had a >18 -fold increase in GMT after dose 2 ($P < 0.001$) and no significant further change after dose 3 ($P > 0.3$). The highest antibody responses were observed in the group given vaccine with MF59 (4-fold higher than that with alum after the first immunization [$P > 0.10$], approximately 6-fold higher after the second immunization [$P < 0.01$], and 11-fold higher after the third immunization [$P < 0.01$]). Thus, MF59 not only augments IgG anticapsular-antibody responses measured by an ELISA (Fig. 1A) but also augments *N. meningitidis* group C bactericidal titers, a measure of functional antibody that correlates in humans with protection against disease caused by *N. meningitidis* (5, 9, 24).

Serum antibody response to plain Hib and *N. meningitidis* group C PS booster. Twenty-one weeks after the third immunization, concentrations of antibody to both Hib PS and MenC PS in serum had declined, on average, approximately 10- to 20-fold in the MF59 and alum groups compared to the respective peak GMTs present 1 month after the third injection (compare respective GMTs at time zero of the booster injection

[Fig. 2B and D] to GMTs after the third conjugate vaccine injection [Fig. 1A and B]). However, 21 weeks after the third conjugate vaccination, the group administered the conjugate vaccines with MF59 still had 5- and 12-fold-higher GMTs to group C and Hib than the respective titers of the group administered the vaccines with alum ($P < 0.001$ for each comparison).

To assess whether the conjugate vaccination had primed the animals for the ability to respond to unconjugated PS (i.e., induction of B-cell memory), all animals were boosted at the 21-week follow-up visit with 5 μ g of plain *N. meningitidis* group C PS vaccine and 5 μ g of Hib PS vaccine, both given i.m. in separate hind limbs. At the time of this booster, the animals ranged from 8 to 10 months of age. The groups previously primed with the conjugate vaccines administered with alum or MF59 showed increases in the respective geometric mean anti-Hib PS antibody concentrations, which peaked at 7 days and returned to prebooster levels by 28 days (Fig. 2B). These booster responses appeared to parallel those of the unprimed control group that had previously received only DTaP (Fig. 2A). The lowest responses were observed in the group previ-

TABLE 1. Serum anti-group C bactericidal antibody responses of infant baboons^a

Adjuvant	1/GMT (95% confidence interval)		
	1 mo p.i. 1	1 mo p.i. 2	1 mo p.i. 3
PBS	$<25^b$ (9-23)	$<25^c$ (9-28)	41^h (17-100)
Alum	$<25^c$ (7-75)	427^f (100-1,816)	277^i (73-1,052)
MF59	102^d (9-1,190)	$2,390^g$ (833-6,860)	$3,091^j$ (490-19,479)

^a Infant baboons (five per group) were immunized monthly for 3 months with *N. meningitidis* group C and Hib vaccines reconstituted with PBS, alum, or MF59 at the time of use. Bactericidal titers were measured in sera collected prior to each immunization and 4 weeks after the third immunization. For calculation of GMTs, titers of $<1:25$ were assigned a value of 1:12.5. p.i. 1, post-injection 1.

^b versus ^c and ^c versus ^d, not significant ($P > 0.05$); ^e versus ^f and ^f versus ^g, $P < 0.01$; ^h versus ⁱ, and ⁱ versus ^j, $P < 0.01$.

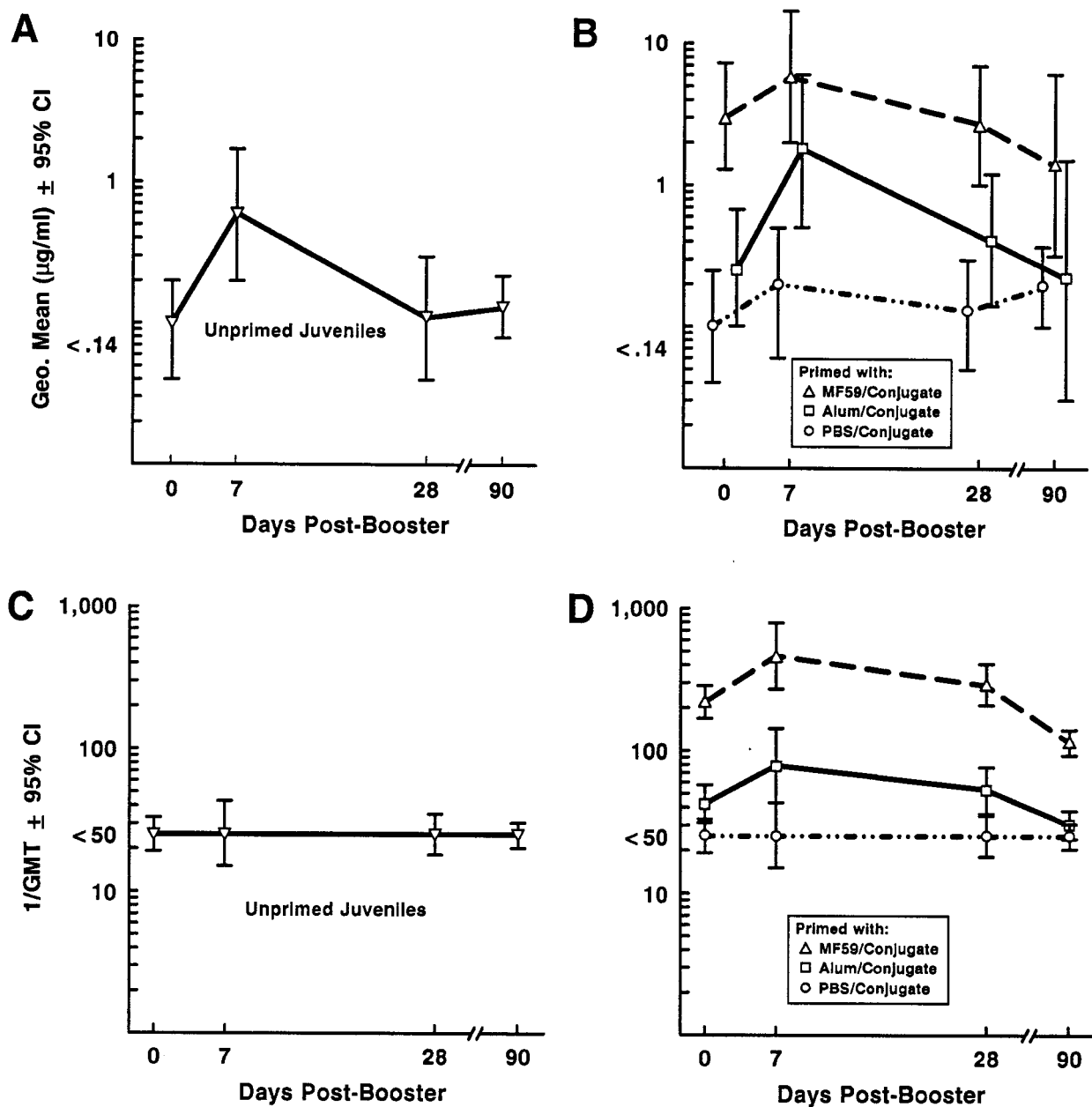


FIG. 2. Serum anticapsular antibody responses of juvenile baboons administered booster injections of unconjugated Hib PS vaccine and *N. meningitidis* group C PS vaccine administered as separate injections 21 weeks (time zero) after the three-dose primary conjugate vaccination schedule had been completed. (A) Anti-Hib PS antibody responses of unprimed control baboons previously vaccinated with DTaP only. (B) Anti-Hib PS antibody responses of baboons previously primed with the conjugate vaccines administered with PBS, alum, or MF59. (C and D) Respective IgG antibody responses to *N. meningitidis* group C PS for the same groups as shown in panels A and B.

ously vaccinated with the conjugate vaccines given with PBS (less than a twofold increase in anti-Hib PS antibody concentration at day 7 versus day 0).

With one exception, the IgG anti-group C PS antibody responses of the respective groups to the plain PS vaccine booster immunization (Fig. 2C and D) paralleled the anti-Hib PS antibody responses. The exception was that the unprimed juvenile baboons (i.e., the DTaP control group) showed no detectable serum antibody responses to group C PS vaccine (Fig. 2C), whereas this group had responded to the plain Hib PS vaccination (Fig. 2A).

Ninety days after the plain group C PS and Hib PS booster

injections, serum anti-group C and anti-Hib PS antibody concentrations still remained higher in the animals previously administered conjugate vaccine with MF59 compared to the respective titers in the group given the conjugate vaccines with alum ($P < 0.01$ for anti-Hib and $P < 0.001$ for anti-group C) or the conjugate vaccines with PBS ($P < 0.01$ for both comparisons) (Fig. 2B and D, respectively).

DISCUSSION

The serum anticapsular antibody responses of the infant baboons observed in this study paralleled those observed in

human infants previously administered an *N. meningitidis* group C oligosaccharide-CRM₁₉₇ conjugate vaccine with alum at 2, 3, and 4 months of age (35). Specifically, both the immunized human infants and the immunized infant baboons showed modest serum anti-group C PS antibody responses after the first injection, very high booster antibody responses to the second injection, and minimal or no further increases in serum antibody titer after a third injection. The baboons also showed evidence of induction of immunologic priming by the *N. meningitidis* group C conjugate vaccination, as measured by booster IgG antibody responses to plain *N. meningitidis* group C PS vaccination administered 21 weeks after a series of three doses of conjugate had been completed. In contrast, unprimed (control) juvenile baboons vaccinated with plain group C PS for the first time showed no detectable antibody response (Fig. 2C). In human infants, priming to *N. meningitidis* group C PS also has been observed with an earlier, prototype version of this conjugate vaccine (a combined group A/group C conjugate vaccine administered with alum [19]). Thus, the pattern of the antibody responses of the infant baboons to group C conjugate administered with alum appears to be predictive of the primary and booster responses of human infants and toddlers to this glycoconjugate vaccine. The only notable difference is the very short duration of the antibody responses to plain group C PS booster injection in the baboons (<28 days).

The most important finding of this study is that MF59, an adjuvant that has been widely evaluated in humans with a variety of glycoprotein subunit vaccines and inactivated influenza vaccines, both accelerates and augments serum anti-PS antibody responses of infant baboons immunized with Hib and *N. meningitidis* group C PS-protein conjugate vaccines. In addition, the animals vaccinated with the MF59-adjuvanted vaccines showed higher titers during the extended follow-up period. These animals also were capable of mounting memory serum antibody responses to unconjugated *N. meningitidis* group C and Hib PS booster immunizations. The immunologic priming appeared to be similar in the animals primed with the conjugate vaccine administered either with alum or MF59 (Fig. 2C).

Because only small numbers of infant baboons could be investigated, many important questions such as the optimal conjugate vaccine dose when given with MF59 or the optimal immunization schedule could not be addressed. Also, we did not examine the influence, if any, of simultaneous separate injections of other vaccines, such as DTaP or diphtheria-tetanus-pertussis, that contain diphtheria toxoid, which might influence serum anticapsular antibody responses to conjugate vaccines containing CRM₁₉₇, a nontoxic mutant of diphtheria toxin. This question will be important to investigate in the future, since in previous studies, immunity to the carrier protein was shown to affect (positively or negatively) the ability to mount anti-PS antibody responses to conjugate vaccines (11, 14, 28, 34).

The present study provides only limited information on the safety of administration of PS-protein conjugate vaccines with MF59. First, only a small number of infant baboons were used (five per group). Second, the observations on vaccine tolerability were limited, because any handling of the infant animals by the staff required separation of the infants from their mothers and the use of a general anesthesia on the mothers. Nevertheless, on general daily inspection, the animals appeared to tolerate the vaccinations without difficulty, as judged by the presence of normal activity and feeding patterns. Also, there was no gross evidence of severe local reactions such as granulomas or abscesses at the injection site; however, close inspection of the injection sites was performed only at the time of the

blood draws and booster immunizations, usually 30 days after each immunization.

In summary, the excellent immunogenicity of *N. meningitidis* group C and Hib conjugate vaccines when administered with MF59 to infant baboons and the lack of apparent toxicity are consistent with the excellent immunogenicity and safety record of MF59 used with other vaccines in clinical trials in humans (summarized in the introduction). These data, together with the potential of an adjuvant either to permit the use of lower conjugate vaccine dosages and/or fewer injections or to enhance the immunogenicity of multicomponent PS-protein conjugate vaccines given alone or in combination with other vaccines, support the initiation of phase I safety and immunogenicity trials of MF59 and glycoconjugate vaccines in humans.

ACKNOWLEDGMENTS

Paula Traquina, George Santos, and Venita Boelloeni provided expert technical assistance. Carol Suennen provided editorial assistance.

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