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# Induction of Systemic Antifimbria and Antitoxin Antibody Responses in Egyptian Children and Adults by an Oral, Killed Enterotoxigenic *Escherichia coli* plus Cholera Toxin B Subunit Vaccine

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**We assessed serologic responses to an oral, killed whole-cell enterotoxigenic *Escherichia coli* plus cholera toxin B-subunit (ETEC-rCTB) vaccine in 73 Egyptian adults, 105 schoolchildren, and 93 preschool children. Each subject received two doses of vaccine or placebo 2 weeks apart, giving blood before immunization and 7 days after each dose. Plasma antibodies to rCTB and four vaccine-shared colonization factors (CFs) were measured by enzyme-linked immunosorbent assay. Immunoglobulin A (IgA) antibodies to rCTB and CFA/I were measured in all subjects, and those against CS1, CS2, and CS4 were measured in all children plus a subset of 33 adults. IgG antibodies to these five antigens were measured in a subset of 30 to 33 subjects in each cohort. Seroconversion was defined as a >2-fold increase in titer after vaccination. IgA and IgG seroconversion to rCTB was observed in 94 to 95% of adult vaccinees, with titer increases as robust as those previously reported for these two pediatric cohorts. The proportion showing IgA seroconversion to each CF antigen among vaccinated children (range, 70 to 96%) and adults (31 to 69%), as well as IgG seroconversion in children (44 to 75%) and adults (25 to 81%), was significantly higher than the corresponding proportion in placebo recipients, except for IgA responses to CS2 in adults. IgA anti-CF titers peaked after one dose in children, whereas in all age groups IgG antibodies rose incrementally after each dose. Independently, both preimmunization IgA titer and age were inversely related to the magnitude of IgA responses. In conclusion, serologic responses to the ETEC-rCTB vaccine may serve as practical immune outcome measures in future pediatric trials in areas where ETEC is endemic.**

Enterotoxigenic *Escherichia coli* (ETEC) strains are the leading cause of childhood diarrhea in developing countries and traveler's diarrhea (4). Advancement of a killed, whole-cell ETEC strain plus recombinant cholera toxin B-subunit (ETEC-rCTB) vaccine into expanded clinical studies has invigorated vaccine development efforts. This vaccine is designed to induce immunity to the commonest ETEC colonization factors (CFs), i.e., CFA/I, CFA/II, and CFA/IV, and cross-immunity to heat-labile (LT) enterotoxin (3, 5, 17, 21). In early-phase trials, intestinal lavage antibodies or circulating antibody-secreting cells (ASCs) served as primary immune endpoints (1, 2, 10, 14). Extrapolating from studies of people with ETEC diarrhea (8, 9, 16, 19, 20), we considered whether serology might offer a more widely applicable measure of vaccine immunogenicity.

In randomized, controlled trials in Egyptian adults, schoolchildren, and preschool children, we found that the ETEC-rCTB vaccine was well tolerated and efficiently stimulated immunoglobulin A (IgA)-ASC responses to CTB and CF antigens (14, 15). In the present study, we assessed the frequency and magnitude of systemic IgA and IgG antibody responses to the vaccine in these same cohorts and examined isotype-specific patterns of response. Our aim was to evaluate the usefulness of serologic measures as indicators of vaccine response in a setting where ETEC is endemic.

## MATERIALS AND METHODS

**Subjects and vaccination.** The Egyptian Ministry of Health and institutional review boards of the U.S. Army and National Institute of Child Health and Human Development approved each protocol. Informed consent was obtained from each subject or a parent before screening, and the human use guidelines of the U.S. Department of Defense were followed in the conduct of these trials. Details of trial design, subjects, and study agents can be found elsewhere (14, 15). In brief, 76 adults (21 to 45 years of age), 107 schoolchildren (6 to 12 years old),

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TABLE 1. Percentage of subjects exhibiting IgA and IgG seroconversion against CTB and vaccine-shared CF antigens after any dose in each of three age cohorts of Egyptians[a]

| Ig type | Cohort (age [yr]) | $n^b$ | % Exibiting seroconversion | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CTB | | CFA/I | | CS1 | | CS2 | | CS4 | |
| | | | Vaccine | Placebo | Vaccine | Placebo | Vaccine | Placebo | Vaccine | Placebo | Vaccine | Placebo |
| IgA | 2–5 | 47/46[c] | 96[g] | 9 | 87[g] | 11 | 91[g] | 2 | 79[g] | 12 | 70[g] | 7 |
| | 6–12 | 51/54 | 100[g] | 15 | 96[g] | 11 | 92[g] | 7 | 78[g] | 9 | 84[g] | 6 |
| | 21–45 | 38/35[d] | 95[g] | 3 | 68[g] | 6 | 56[g] | 0 | 31 | 6 | 69[g] | 12 |
| IgG | 2–5 | 20/10 | 90[g] | 0 | 70[g] | 0 | 70[g] | 0 | 50[f] | 0 | 60[f] | 9 |
| | 6–12 | 16/16 | 100[g] | 0 | 75[g] | 0 | 62[g] | 0 | 44[f] | 0 | 44[f] | 0 |
| | 21–45 | 16/17 | 94[g] | 6 | 31[e] | 0 | 44[f] | 0 | 25[e] | 0 | 81[g] | 6 |

[a] Positive seroconversion was defined as a ≥2-fold titer increase over preimmunization level after any dose. Antitoxin seroresponse rates in children have been previously reported (15) and are shown here for comparison.
[b] Number in vaccine group/number in placebo group.
[c] Forty-three and 42 in vaccine and placebo groups, respectively, had responses to CS1, CS2, and CS4 assessed due to insufficient plasma in the remainder.
[d] Responses to CS1, CS2, and CS4 were assessed in 16 vaccine and 17 placebo recipients.
[e] $P < 0.05$, comparing proportion of responders in vaccine group with those in the corresponding placebo group.
[f] $P < 0.01$, comparing proportion of responders in vaccine group with those in the corresponding placebo group.
[g] $P < 0.001$, comparing proportion of responders in vaccine group with those in the corresponding placebo group.

and 106 preschool children (2 to 5 years old) from Benha, Qalyubia Governorate, Egypt, were enrolled into three serial trials. Each 4-ml vaccine dose (lot E003) contained 1 mg of rCTB plus a mixture of $\sim 2 \times 10^{10}$ bacteria each of five strains individually expressing CFA/I, CS1, CS2 plus CS3, CS4, and CS5 (14). Each 4-ml placebo dose contained $\sim 10^{11}$ heat-killed *E. coli* K-12 cells. Each dose was added to 150 ml of water containing 4 g of sodium bicarbonate plus 1.45 g of citric acid (Recip AB, Stockholm, Sweden) for adult administration. Schoolchildren and preschoolers received the same full dose of study agent added to one-half and one-fourth, respectively, of the antacid solution. Subjects were randomly assigned to receive two doses of vaccine or placebo 2 weeks apart.

**Sample collection and processing.** Subjects gave venous blood samples preimmunization and 7 days after each dose. Plasma was derived as previously described (14, 15) and stored at −70°C until tested. All assays were done in a blinded fashion at Naval Medical Research Unit No. Three.

Availability of purified antigen preparations dictated the choice of vaccine components included in serologic assessments. According to the protocols, plasma IgA titers against CTB, CFA/I, CS1, CS2, and CS4 were measured in paired samples from as many as 93 preschoolers and 105 schoolchildren; those against CTB and CFA/I were measured in paired samples from 73 adults. Responses to CS1, CS2, and CS4 were assessed in 85 of the 93 preschoolers due to limited plasma amounts. In subsets of 33 adults, 32 schoolchildren, and 30 preschoolers for whom IgA-ASC responses had been assessed and thus for whom more plasma was available (14, 15), we later decided to measure IgG titers to all specified antigens and IgA titers against CS1, CS2, and CS4 in the adults to obtain a more complete profile of serologic responses.

**Plasma antibody measurements.** IgA and IgG antibody titers against rCTB were measured by GM1 enzyme-linked immunosorbent assay (ELISA) (10, 19), and those against CF antigens were measured by ELISA methods as previously described (2, 16). The same lots of purified rCTB, CFA/I, CS1, CS2, and CS4 were used as solid-phase antigens throughout. Plasma samples were threefold serially diluted (initial dilution, 1:5) in microtiter plates. Endpoint titer was assigned as the interpolated dilution giving an absorbance value at 450 nm of 0.4 above background. Titers were adjusted in relation to a reference specimen included in each test to compensate for day-to-day interassay variation. Paired samples from each subject were always tested side by side. The antibody titer ascribed to each sample represented the geometric mean of duplicate measurements done on different days. Reciprocal endpoint titers <5 were assigned a value of 2.5 for computations.

**Statistical analyses.** Subjects were included in serologic analyses if both assigned doses were taken and all three blood samples were obtained. Based on our calculations of the methodological error of each ELISA, we defined a significant response as a ≥2-fold increase in endpoint titer between pre- and postimmunization specimens (2, 11), with the added criterion that the postimmunization reciprocal titer be ≥10. Seroconversion after any dose was defined as a positive response. Intergroup comparisons of the proportion of responders were made using the $\chi^2$ test (Fisher's exact test for sparse outcomes).

Geometric mean titers (GMT) were calculated as the antilog of the mean of individual log-transformed titers. Geometric mean fold titer increases (GMFI) were calculated as the antilog of the mean of individual differences between pre- to postimmunization log-transformed titers. The Wilcoxon rank sum test was used to compare titers between treatment groups at each time point, as log-transformed data did not meet the assumption of normality. The Wilcoxon signed-rank test was used to compare within-group titer rises from after the first to after the second vaccine dose.

Age and preimmunization IgA titers were tested as potential predictors of the magnitude of IgA serologic responses to each vaccine antigen using multiple linear regression, pooling data from all cohorts. Age, log preimmunization titer, treatment group, and the two-way interactions between the three terms were used to assess the relationship between log fold responses and age within treatment groups and the association between log fold responses and log preimmunization titers within treatment groups. Since observations were obtained from three separate studies, we adjusted for study-to-study variation by including two dummy variables to model this effect. All statistical tests were interpreted in a two-tailed fashion.

## RESULTS

Three Egyptian cohorts received two oral doses of study agent: 97 preschool children (49 vaccine; 48 placebo), 105 schoolchildren (51 vaccine; 54 placebo), and 74 adults (38 vaccine; 36 placebo). Three blood samples were obtained from all but four preschoolers (two vaccine; two placebo) and one adult placebo recipient. Among the subjects included in serologic analyses, there were 18 preschoolers (10 vaccine; 8 placebo) and 6 schoolchildren (2 vaccine; 4 placebo) who ingested one or two partial doses.

**Serologic responses to CTB.** In the two pediatric cohorts, vigorous serologic responses to CTB were observed among vaccine recipients, as previously reported (15). Egyptian adult vaccinees also exhibited pronounced antitoxin antibody responses (Table 1). Antitoxin titers rose successively after each dose, with maximal titers apparent after the second dose (Table 2).

**Serologic responses to CF antigens in children.** The proportion of vaccinees showing IgA seroconversion to each of the four CF antigens ranged from 70 to 91% in preschool children and 78 to 96% in schoolchildren (Table 1). IgG responses against CF antigens were also common in vaccinated children, but the proportion and magnitude of responses were generally less than those of IgA isotype (Table 1). Seroconversion rates of ≥50% were seen against all four CFs in the younger group and against CFA/I and CS1 in the older group.

TABLE 2. Plasma antibody titers to CTB and vaccine-shared CF antigens after each of two doses of ETEC-CTB vaccine or placebo[a]

| Antigen | Cohort (age [yr]) | Ig type | *n* | | Geometric mean titer | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Preimmunization | | After 1st dose | | After 2nd dose | |
| | | | Vaccine | Placebo | Vaccine | Placebo | Vaccine | Placebo | Vaccine | Placebo |
| CTB | 2–5 | IgA | 47 | 46 | 6 | 7 | 48[d] | 7 | 202[d,g] | 8 |
| | 6–12 | IgA | 51 | 54 | 5 | 6 | 54[d] | 7 | 212[d,g] | 7 |
| | 21–45 | IgA | 38 | 35 | 7 | 8 | 33[d] | 9 | 89[d,g] | 8 |
| | 2–5 | IgG | 20 | 10 | 926 | 746 | 2,943[c] | 706 | 12,175[d,g] | 765 |
| | 6–12 | IgG | 16 | 16 | 319 | 380 | 2,046[c] | 365 | 6,188[d,g] | 389 |
| | 21–45 | IgG | 16 | 17 | 184 | 222 | 1,117[d] | 249 | 3,171[d,g] | 235 |
| CFA/I | 2–5 | IgA | 47 | 46 | 16 | 19 | 165[d,g] | 21 | 94[d] | 20 |
| | 6–12 | IgA | 51 | 54 | 14 | 17 | 105[d,g] | 18 | 61[d] | 14 |
| | 21–45 | IgA | 38 | 35 | 45 | 34 | 95[d] | 31 | 98[d] | 30 |
| | 2–5 | IgG | 20 | 10 | 450 | 357 | 1,091[c] | 342 | 1,283[d,e] | 359 |
| | 6–12 | IgG | 16 | 16 | 356 | 361 | 784[d] | 372 | 925[d,e] | 367 |
| | 21–45 | IgG | 16 | 17 | 422 | 444 | 588 | 419 | 738[f] | 392 |
| CS1 | 2–5 | IgA | 43 | 42 | 4 | 5 | 32[d,f] | 5 | 23[d] | 5 |
| | 6–12 | IgA | 51 | 54 | 5 | 6 | 31[d,g] | 6 | 23[d] | 5 |
| | 21–45 | IgA | 16 | 17 | 7 | 7 | 12[b] | 6 | 12[b] | 6 |
| | 2–5 | IgG | 20 | 10 | 62 | 88 | 210 | 84 | 242[b] | 87 |
| | 6–12 | IgG | 16 | 16 | 106 | 106 | 261[b] | 106 | 287[c] | 100 |
| | 21–45 | IgG | 16 | 17 | 104 | 173 | 163 | 169 | 196[e] | 177 |
| CS2 | 2–5 | IgA | 43 | 42 | 7 | 7 | 39[d,f] | 7 | 29[d] | 7 |
| | 6–12 | IgA | 51 | 54 | 13 | 12 | 54[d,g] | 12 | 40[d] | 10 |
| | 21–45 | IgA | 16 | 17 | 20 | 20 | 33 | 22 | 31 | 20 |
| | 2–5 | IgG | 20 | 10 | 132 | 198 | 257 | 166 | 296[e] | 173 |
| | 6–12 | IgG | 16 | 16 | 352 | 296 | 559 | 270 | 614[b] | 274 |
| | 21–45 | IgG | 16 | 17 | 552 | 606 | 737 | 600 | 776 | 554 |
| CS4 | 2–5 | IgA | 43 | 42 | 4 | 6 | 13[d,f] | 5 | 10[c] | 5 |
| | 6–12 | IgA | 51 | 54 | 7 | 7 | 33[d,e] | 6 | 27[d] | 6 |
| | 21–45 | IgA | 16 | 17 | 14 | 11 | 31[d] | 12 | 28[c] | 12 |
| | 2–5 | IgG | 20 | 10 | 41 | 47 | 73 | 45 | 89[e] | 47 |
| | 6–12 | IgG | 16 | 16 | 159 | 134 | 250[b] | 129 | 278[b] | 127 |
| | 21–45 | IgG | 16 | 17 | 73 | 128 | 198 | 130 | 282[b,f] | 129 |

[a] Antitoxin titers in children reported previously included all subjects receiving one or two doses of vaccine or placebo (15). Here, the pediatric antitoxin titers (shown for comparison) are for only subjects who received both doses of study agent.
[b] $P \leq 0.05$, Wilcoxon rank sum test, comparing contemporaneous titers between vaccine and placebo groups.
[c] $P \leq 0.01$, Wilcoxon rank sum test, comparing contemporaneous titers between vaccine and placebo groups.
[d] $P \leq 0.001$, Wilcoxon rank sum test, comparing contemporaneous titers between vaccine and placebo groups.
[e] $P \leq 0.05$, Wilcoxon signed rank test, comparing antibody titers after first dose to after second dose in the vaccine group.
[f] $P \leq 0.01$, Wilcoxon signed rank test, comparing antibody titers after first dose to after second dose in the vaccine group.
[g] $P \leq 0.001$, Wilcoxon signed rank test, comparing antibody titers after first dose to after second dose in the vaccine group.

The magnitude of IgA anti-CF responses was considerable, particularly after the first dose (Table 2). For both pediatric cohorts, a second vaccine dose was followed by a decrease in IgA titers to each of the four CF antigens. In contrast, IgG titers were higher after the second than after the first dose for each CF antigen, the difference being statistically significant for CFA/I in both cohorts and for CS2 and CS4 in preschoolers.

To assess the possible effect of fractional dosing on serologic responses, we compared vaccinees in the preschool group who received one or two partial doses to those receiving two full doses. The former group, which took a mean of 78% (by volume) of the total two-dose vaccine-antacid drink, showed no diminution in either the proportion of children showing seroconversion or in the magnitude of response to any individual antigen (data not shown).

**Serologic responses to CF antigens in adults.** The proportion of adult vaccinees exhibiting seroconversion against each CF ranged from 31 to 69% for IgA and 25 to 81% for IgG isotype (Table 1). These were significantly greater than the corresponding rates in the placebo group except in the case of IgA responses to CS2. The magnitude of IgA and IgG responses against CF antigens was more modest than that of the children. Similar to the pattern observed in children, the second vaccine dose was associated with a boost in IgG responses against all CFs, the incremental titer rise being significant for CFA/I, CS2, and CS4.

**Predictors of serologic responses.** In simple stratified analyses, the magnitude of IgA serologic responses to each vaccine-shared antigen appeared to decrease with age and with elevation in preimmunization titer (Table 3). In multivariate models that simultaneously examined age and preimmunization titers as determinants of vaccine-induced plasma IgA antibody responses, increasing age was significantly associated with reduced log-fold plasma antibody responses for CFA/I ($P < 0.001$), CS1 ($P < 0.001$), and CS2 ($P < 0.01$). Similarly, there was a significant inverse relationship between increasing preimmunization titers and log-fold antibody responses for CTB ($P < 0.001$), CFA/I ($P < 0.001$), CS2 ($P < 0.01$), and CS4 ($P < 0.001$).

## DISCUSSION

This is the first comprehensive report of serologic responses to the oral ETEC-rCTB vaccine in subjects living in an area

TABLE 3. Relationship between age and preimmunization titer and magnitude of IgA serologic response against rCTB and vaccine-shared CF antigens among Egyptian vaccinees 7 days after the first dose of oral ETEC-CTB vaccine

| Age (yr) | GMFI[a] (no. of subjects/cell) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | rCTB | | | CFA/I | | | CS1 | | | CS2 | | | CS4 | | |
| | LT, 2.6 | MT, — | UT, ≥8.9 | LT, ≤13.3 | MT, — | UT, ≥34.7 | LT, 2.5 | MT, — | UT, ≥5.1 | LT, ≤6.0 | MT, — | UT, ≥15.5 | LT, 2.5 | MT, — | UT, ≥8.6 |
| 2–5 | 9.5 (13) | 10.7 (18) | 4.3 (16) | 17.0 (22) | 10.9 (11) | 4.2 (14) | 5.5 (18) | 13.1 (13) | 5.4 (12) | 5.9 (24) | 6.4 (10) | 3.6 (9) | 4.1 (28) | 3.5 (9) | 1.6 (6) |
| 6–12 | 16.1 (20) | 13.5 (20) | 5.5 (11) | 14.3 (21) | 5.3 (21) | 4.4 (9) | 8.3 (22) | 6.7 (10) | 4.5 (19) | 6.3 (12) | 5.9 (20) | 2.4 (19) | 10.8 (15) | 3.5 (15) | 3.2 (21) |
| 21–45 | 9.6 (12) | 5.8 (11) | 2.2 (15) | 4.9 (2) | 2.3 (13) | 1.9 (23) | 2.1 (3) | 2.2 (6) | 1.4 (7) | 5.6 (2) | 2.0 (4) | 1.2 (10) | 6.4 (2) | 2.5 (2) | 1.9 (12) |

[a] GMFI, geometric mean of fold increase in titer from pre- to postdose 1. LT, UT, and MT denote lower tertile, middle tertile, and upper tertile for preimmunization titers in all vaccinees, where cutoff values are expressed as the reciprocal of the preimmunization titer. —, greater than the cutoff for the LT but less than the cutoff for the UT.

where ETEC is endemic. We consider the pediatric data presented herein especially relevant, since children and infants in Egypt and other developing countries represent important targets for ETEC immunization. Several of our findings bear importance for future evaluations of the ETEC-rCTB vaccine. First, the vaccine elicited pronounced antitoxin antibody responses of both IgA and IgG isotypes in all three age groups as reported here and elsewhere (15). Second, a high proportion of vaccinated children achieved seroconversion to each of the four vaccine-shared CF antigens studied, and the magnitude of antibody elevations, particularly of IgA isotype, was substantial. Third, both age and preimmunization titer were independently and inversely associated with the magnitude of IgA antibody responses to the vaccine. Last, IgG antibodies to CF antigens appeared to reflect a dose-to-dose boosting effect. Our findings differ somewhat from those of a small, uncontrolled study of this vaccine in Bangladesh, inasmuch as a paucity of IgG serological responses against CFA/I (the only CF examined) was observed in Bangladeshi adults and children (13).

Similar to what we have reported for these Egyptian children (15), Egyptian adults given the ETEC-rCTB vaccine mounted vigorous systemic anti-CTB antibody responses. These findings agree with a large body of data on the whole-cell cholera-plus-CTB vaccine (7, 18) and studies of the ETEC-rCTB vaccine in Swedish adults (2, 10). The robustness of these serologic responses has encouraging implications for the prospects of vaccine-induced protection against LT-producing ETEC (LT-ETEC) disease, considering that (i) clinical cholera induces cross-reactive anti-LT antibodies (19), (ii) the whole-cell cholera-plus-CTB vaccine cross-protects against LT-ETEC diarrhea (6, 12), and (iii) serum antitoxin responses appear to be reliable indicators of gut mucosal responses (1, 11).

Our findings with respect to antifimbrial responses contrast with those observed in Swedish adults given the ETEC-rCTB vaccine (10). One-third or fewer of Swedish vaccinees had detectable serum IgA antibody elevations against all CFs except CFA/I, to which as many as two-thirds of subjects responded. IgG anti-CF responses were less frequently observed. Several factors may have contributed to the discrepancies. Technical factors are not invoked since the same vaccination schedule, assay methods, and reagents were used in both settings. Priming of CF immune responses by past infection may have contributed to the higher seroconversion rates in Egyptians. Age may also be important, as we have shown here that the magnitude of IgA serologic response is inversely associated with age, even after controlling for preimmunization titer. Whatever the determinants, our observation that Egyptian children exhibit prominent serological responses against vaccine-shared CF antigens suggests the usefulness of these outcome measures for future vaccine evaluations in at-risk pediatric populations.

Systemic antibody responses to the CF antigens exhibited distinct isotype-specific patterns most evident in the two cohorts of Egyptian children. IgA titer elevations appeared to be transitory. Fold rises over preimmunization levels for each antigen were maximal after the first dose and decreased after the second dose though remaining above preimmunization levels. In contrast, IgG titers generally rose successively after each dose. This is consistent with studies of patients following naturally acquired ETEC diarrhea, in whom evanescent increases in IgA anti-CF serum titers differed from more sustained IgG antibody elevations (16). That the ETEC-rCTB vaccine appears to exert a dose-to-dose booster effect on anti-CF IgG antibody levels suggests that IgG antibodies may be a suitable marker for assessing the incremental response to multiple vaccine doses as well as the persistence of immune responses in populations with endemic ETEC disease.

In summary, we have shown that the oral ETEC-rCTB vaccine clearly induces systemic antibody responses to vaccine antigens in subjects living in an area where ETEC is endemic and that these responses were more pronounced in children than in adults. Considering the weight of favorable findings reported here and elsewhere (14, 15), we have undertaken studies to determine the safety, immunogenicity, and efficacy of this vaccine in Egyptian infants. In these trials, serologic responses against ETEC vaccine antigens have been adopted as the primary determinant of immunogenicity. Since the degree of past exposure to ETEC will vary with age, it remains to be seen how vaccine-induced serologic responses in children under 2 years of age will compare to those observed in 2- to 12-year-old children. Moreover, the clinical significance of vaccine-induced serologic responses as correlates of protection remains to be determined.

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