

Evaluation of an Immunoglobulin G Enzyme-Linked Immunosorbent Assay for Pertussis Toxin and Filamentous Hemagglutinin in Diagnosis of Pertussis in Senegal

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The enzyme-linked immunosorbent assay is widely employed for the serological diagnosis of pertussis. It is generally concluded that a significant increase in specific immunoglobulin G (IgG) or IgA against the pertussis toxin (PT) or against filamentous hemagglutinin (FHA) in paired sera correlates with *Bordetella pertussis* infection. However, this type of diagnosis of pertussis has mainly been applied to unvaccinated children, with timely sampling of acute- and convalescent-phase sera. In current practice and in epidemiological studies, such criteria are not always fulfilled. The aim of this study was to analyze the significance of decreases in IgG antibody titers against PT and FHA between paired sera observed in suspected cases of pertussis infection. Serological results from paired sera were available for 460 children experiencing at least 8 days of cough. An anti-PT IgG decrease was observed in 25% of the children, more frequently than the anti-FHA IgG decrease. Fourteen percent of the serologic decreases were observed in children with culture-confirmed infection, and 59% of the decreases were observed in children with confirmation criteria according to World Health Organization recommendations. Most of the decreases were observed when serum samples were collected according to a standard recommended schedule. Serologic decreases were observed more frequently among vaccinated children than among unvaccinated children. This difference, which was highly significant ($P < 0.00001$), was explained by the different kinetics of the antibody responses between vaccinated and unvaccinated children. The importance of the antibody response for the evaluation of vaccine efficacy, namely a bias toward higher absolute vaccine efficacy when this response is not taken into account, is discussed. This study supports an earlier recommendation that a significant decrease in PT or FHA should be added to the diagnostic criteria for pertussis.

Serology is widely employed for the diagnosis of pertussis, and its use has been recommended (15) as part of the case definition in vaccine efficacy trials. Many serological assays have been developed during the last 20 years, but some lack sensitivity and/or specificity. The enzyme-linked immunosorbent assay (ELISA), using purified pertussis factors such as pertussis toxin (PT) or filamentous hemagglutinin (FHA), has recently been used extensively for epidemiological studies (8). This technique has proven to be reasonably sensitive and specific (1, 3, 13). It is generally concluded that a significant increase in specific anti-PT immunoglobulin G (IgG) or IgA and/or anti-FHA IgG or IgA in paired sera correlates with *Bordetella pertussis* infection. However, this type of diagnosis of pertussis has been mainly applied to unvaccinated children, with acute-phase serum collected within 14 days after the onset of illness and convalescent-phase serum collected 4 to 8 weeks later. In current practice and in epidemiological studies, such criteria are not always fulfilled: pertussis may be suspected after it is too late to culture the organism or to obtain an acute-phase serum sample, and convalescent-phase sera may be collected more than 8 weeks after the onset of the cough. Moreover, serological responses after infection may be difficult

to interpret because of previous vaccination (3). Such conditions are observed in pertussis vaccine trials and will probably occur more often with increasing use of pertussis vaccines.

In the present study, we analyzed the serological response of suspected cases of pertussis infection as part of the Senegal Pertussis Trial (11). Increases in anti-PT IgG and anti-FHA IgG titers between paired sera were detected. However, as previously observed (5, 9), we also detected a significant number of anti-PT IgG and anti-FHA IgG decreases between paired sera. The objectives of this study were (i) to undertake a precise analysis of these decreases, compared with increases, in order to determine whether they might also detect children infected with *B. pertussis*; and (ii) to study the possible impact of such findings on the interpretation of vaccine efficacy results.

MATERIALS AND METHODS

Subjects. The study was based on available serological results for children sampled within the pertussis surveillance system of the Senegal Pertussis Trial and included in the main analysis population.

The Senegal Pertussis Trial sought to compare the relative efficacies of three doses of a two-component acellular (AC) vaccine (25 µg of purified detoxified PT and 25 µg of purified FHA) with respect to that of three doses of the French whole-cell (WC) vaccine used by the Expanded Program of Immunization in Senegal. Both vaccines were produced by Pasteur Mérieux. The study and results have been reported elsewhere (11). Briefly, the trial was conducted in Niakhar, Senegal, a rural farming area 150 km from Dakar. The approximately 27,000 inhabitants live in large compounds among scattered hamlets. This population has been monitored since 1983 for demographic and health studies. Pertussis epidemiological surveillance was initiated in 1983, based on annual surveys and

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declarations of the mothers, and has been continued since 1987 by weekly active detection of cough among all children under 15 years of age by field workers triggering physician investigations. During the trial, from 1990 to 1995, all suspect cases (8 days or more of cough) were investigated; nasopharyngeal aspirates and blood samples were taken from children whose parents agreed. Acute-phase serum samples were taken as soon as possible after the onset of the cough, and convalescent-phase sera were mainly sampled 4 to 8 weeks later.

A total of 1,224 children received three doses of the study vaccines (587 in the WC group and 637 in the AC group); they received no other pertussis vaccine dose outside the study, had not had clinical pertussis prior to 28 days after the third dose, and were living in compounds which have been investigated for possible pertussis. A total of 253 children in the WC group and 361 children in the AC group experienced episodes of at least 8 consecutive days of cough and thus were candidates for bacteriology and serology. Serological results from paired sera were available for 153 children (60%) in the WC group and 228 children (63%) in the AC group. A total of 545 children belonging to the same birth cohorts remained unvaccinated because they had not attended any vaccination session for various reasons and were living in a compound under investigation; 249 of these children had had at least 8 days of cough, and serological results were available for 79 (32%) of them.

This analysis was performed with the children with available serological results. Most of them also had results for bacteriology and information on epidemiological linkage. Results are presented for suspected cases of infection in children with 8 days or more of cough and for children with 21 days or more of paroxysmal cough, corresponding to the clinical part of the definition established by the World Health Organization (WHO).

Serology. (i) Handling of serum specimens. Blood was collected by finger prick on microtainer tubes (Becton Dickinson) and centrifuged, and sera were kept at -80°C in 0.05-ml aliquots in 1.8-ml Nunc tubes. A volume of 0.02 ml was sufficient for two different assays of IgG for PT and FHA.

(ii) Antigens. Purified PT and FHA were obtained from Pasteur Mérieux, Lyon, France, in vials containing, respectively, 200 and 500 μg of purified protein per ml. The antigens were stored at -20°C in 50% glycerol. One batch of each antigen was used throughout the study.

(iii) ELISAs. The main ELISA procedures were as follows. Microtiter plates (Nunc Maxisorp certified) were coated with PT and FHA diluted to 2 $\mu\text{g}/\text{ml}$ in bicarbonate buffer (pH 9.6) and phosphate-buffered saline (PBS) (pH 7.4). Coated plates were stored for 1 week maximum.

Sera were diluted in eight twofold dilutions starting with 1:60 with PBS (pH 7.4) containing 0.5% bovine serum albumin, 0.5% Tween 20, and 0.005% polypropylene glycol. Paired sera from the same patient (acute- and convalescent-phase sera) were run on the same plate with a reference human serum containing 217 ELISA units (EU)/ml for anti-PT IgG and 175 EU/ml for anti-FHA IgG (calibrated with the Food and Drug Administration human serum lot 3), a high-positive serum sample, and/or a negative serum sample. The plates were incubated for 2 h at 28°C . After washing, alkaline-phosphatase-conjugated antihuman IgG (Kirkegaard and Perry laboratories) was added, and the mixture was incubated at 28°C overnight.

The system was developed with four nitrophenyl phosphate tablets for 60 min. The enzyme reaction was performed at room temperature. The results were expressed in units by the computer program Unitcalc (Byosis-Inova) developed by R. Möllby and I. Kühn.

The minimum levels of detection were 2 EU/ml for anti-PT IgG and 2.5 EU/ml for anti-FHA IgG. A seropositive serum sample was one that contained at least four times the minimum level of detection. An IgG ELISA titer rise for PT or FHA of 100% or more was considered a significant increase, and an IgG ELISA titer fall of 50% or more was considered a significant decrease. Otherwise, ELISA titer changes were considered not significant. Specifically, high antibody titers of acute- and convalescent-phase serum samples without a rise or decrease were considered nonsignificant.

Sampling techniques for samples with very high antibody concentrations were redone after dilution so that the concentrations would fall within the optimal detection ranges. In these cases, the plates were reexamined with a starting dilution of 1:600 for both acute- and convalescent-phase sera.

(iv) Culture. Aspirate specimens were obtained with a suction catheter (VYGON). The aspirates were inoculated in the home (primary plates) and in the laboratory (secondary plates) on Reagan-Lowe agar plates containing 10% defibrinated horse blood and 40 mg of cephalexin per liter. Cultures were inoculated at 36°C and observed regularly during a 7-day period after inoculation. Suspected colonies were identified with antisera specific to *B. pertussis* and *Bordetella parapertussis* (Difco Laboratories). Final verification was done with oxidase and urease.

(v) Epidemiological linkage. A suspected case of infection was confirmed as pertussis if the subject was living in the same compound as a child with a culture-confirmed case and if the onset of cough occurred within 28 days either before or after the onset of cough of the reference child.

Data management and statistics. Percentages of antibody changes (increases, decreases, or negatives) were compared by the χ^2 test. Comparisons of means were done by analysis of variance when normal approximation was possible or by the Kruskal-Wallis test, with Epiinfo, version 6 (USD). Significance values were always calculated as two tailed.

TABLE 1. Proportion of increases, seronegative samples, and decreases in anti-PT IgG and anti-FHA IgG titers in paired sera

Anti-PT IgG titer	No. of children with anti-FHA IgG titer ^a			
	Increase	Negative	Decrease	Total
Increase	80	9	1	90
Negative	48	191	14	253
Decrease	18	71	24	113
Total	147 (1)	273 (2)	40 (1)	460 (4)

^a Increase, increase in IgG titer between acute- and convalescent-phase sera; Negative, no IgG detected in either acute- or convalescent-phase sera or no change detected in IgG titer between the two serum samples; Decrease, decrease in IgG titer between acute- and convalescent-phase sera. Values in parentheses represent the number of missing results for PT.

RESULTS

PT and FHA IgG titer changes. As shown in Table 1, an anti-PT IgG increase was detected in 90 children (20%) and an anti-FHA IgG increase was detected in 147 children (32%). An anti-PT IgG decrease was observed in 113 children (25%), more frequently than an increase and more frequently than anti-FHA IgG decreases, which were observed for 40 (9.4%) children.

Decreases in a child whose serologic result was positive for the other antigen were also more frequently observed for PT than for FHA.

Further analysis was restricted to PT IgG because of the small number of decreases observed for FHA IgG.

Serological results in children with culture-verified pertussis. Eighty children were culture positive among the 460 children with suspected cases of infection who had 8 days or more of cough and who had been sampled for both bacteriology and serology. Children with increases in IgG titers (19%) among those with suspected cases of infection had a positive culture 40% (36 of 89) of the time, whereas only 14% (16 of 113) of the children with a decrease in the IgG titer (24% of suspected cases of infection) were culture positive (Table 2). Among responders, defined as those experiencing either an increase or a decrease in antibody titers, 31% (16 of 52) involved decreases in culture-positive children. This rate was 65% (97 of 150) among culture-negative children. When the sample was restricted to children with 21 days or more of paroxysmal cough, the rate of decreases rose to 35% (28 of 80), and it was then close to the rate of increases (37% [30 of 80]).

TABLE 2. Serological results as a function of bacteriological results

Anti-PT IgG titer ^a	No. of children with result by bacteriology ^b					
	8 days of cough			21 days of paroxysmal cough		
	Positive	Negative	Total	Positive	Negative	Total
Increase	36	53	89 [1]	16	14	30
Negative	28	225	253	6	15	21
Decrease	16	97	113	6	22	28
Total	80	379 (4)	460 (4) [1]	28	52 (1)	80 (1)

^a Increase, increase in IgG titer between acute- and convalescent-phase sera; Negative, no IgG detected in either acute- or convalescent-phase sera or no change detected in IgG titer between the two serum samples; Decrease, decrease in IgG titer between acute- and convalescent-phase sera.

^b Values in parentheses represent number of missing results for PT; values in brackets represent number of missing results for bacteriology.

TABLE 3. Serological results as a function of WHO laboratory confirmation criteria

Anti-PT IgG titer ^a	No. of children with result by WHO confirmation criteria ^b					
	8 days of cough			21 days of paroxysmal cough		
	Positive	Negative	Total	Positive	Negative	Total
Positive	90	0	90	30	0	30
Negative	130	123	253	15	6	21
Decrease	67	46	113	23	5	28
Total	288 (1)	172 (3)	460 (4)	49 (1)	11	80 (1)

^a Increase, increase in IgG titer between acute- and convalescent-phase sera; Negative, no IgG detected in either acute- or convalescent-phase sera or no change detected in IgG titer between the two serum samples; Decrease, decrease in IgG titer between acute- and convalescent-phase sera.

^b Values in parentheses represent number of missing results for PT.

Serological results in children with WHO laboratory confirmation criteria. By definition, all 90 children with serologic increases were confirmed by WHO criteria (Table 3). However, 67 (59%) of the serologic decreases were also observed in children with laboratory-confirmed pertussis. This rate was 82% in the subsample of children with 21 days or more of paroxysmal cough. Thus, the majority of serologic decreases observed in this sample were from children with pertussis.

Serological results as a function of timing of the samples. In the present study, the decreases in IgG titers between acute- and convalescent-phase sera might have been due to a long interval between collection of sera. The median interval was not significantly longer when decreases were observed (48 days) than when increases were observed (42 days) ($P = 0.07$). For 76% of the children, this interval was between 4 and 8 weeks, the interval generally used in other studies.

The decreases observed might also have been due to a long interval between the beginning of the clinical symptoms and collection of the acute-phase serum. The median delays for the acute-phase sample were 9 days when increases were observed and 12 days when decreases were observed ($P < 0.01$). However, 87 and 96% of acute-phase sera were collected before 21 and 28 days, respectively.

Thus, the occurrence of some serologic decreases was associated with the timing at which the two blood samples were taken, but serologic decreases were also observed when the samples were collected according to a standard recommended schedule.

Serological results as a function of vaccination status. Because of the results presented above, it was anticipated that anti-PT IgG titers might decrease more rapidly in the weeks following infection in vaccinated children compared to that in unvaccinated children because of a more rapid rise in antibodies related to the anamnestic response following vaccination. As seen in Table 4, increases were more frequently observed among unvaccinated children and decreases were more often observed among vaccinated children. This difference was more pronounced in the subsample of children with paroxysmal cough. When only increases and decreases were considered in Table 4 and since they might both have represented positive serological results, it could be calculated from the full sample that decreases represented 64% of the positive results among vaccinees and only 27% of those among unvaccinated children. This difference was highly significant ($P < 0.00001$). A similar analysis of the two groups showed comparable rates of decreases in the AC group (65%) and in the WC group (62%). When the sample was restricted to those children with parox-

TABLE 4. Serological results according to vaccination status

Anti-PT IgG titer ^a	No. (%) of children with result by vaccination status ^b			
	No dose	3 doses		
		AC	WC	Total
Increase				
All coughs	33 (42)	38 (17)	19 (12)	57 (15)
Paroxysmal cough	20 (57)	7 (25)	3 (18)	10 (22)
Negative				
All coughs	32 (40)	120 (53)	101 (66)	221 (58)
Paroxysmal cough	7 (20)	7 (25)	7 (41)	14 (31)
Decrease				
All coughs	12 (15)	70 (31)	31 (20)	101 (27)
Paroxysmal cough	7 (20)	14 (50)	7 (41)	21 (47)
Total				
All coughs	79 [2] (100)	228 (100)	153 [2] (100)	381 [2] (100)
Paroxysmal cough	35 [1] (100)	28 (100)	17 (100)	45 (100)

^a Increase, increase in IgG titer between acute- and convalescent-phase sera; Negative, no IgG detected in either acute- or convalescent-phase sera or no change detected in IgG titer between the two serum samples; Decrease, decrease in IgG titer between acute- and convalescent-phase sera.

^b Values in brackets represent number of missing results for PT.

ysmal cough, comparable rates were observed. When the analysis was restricted to the classical delay between convalescent- and acute-phase sera of more than 27 days and less than 57 days, similar rates of decreases were observed: 62 and 59% in the AC and WC groups, respectively, compared to 31% among unvaccinated children (data not shown).

These results suggest that the kinetics of IgG antibodies may vary between unvaccinated and vaccinated children, who may express a very rapid rise and decrease in IgG titers.

This is illustrated in Fig. 1, in which anti-PT IgG titers were plotted against time of sampling after onset of cough. Both acute- and convalescent-phase samples were plotted. Absolute levels, which are related to laboratory procedures, were not interpreted as such. Only relative differences were interpreted; these showed different responses for vaccinated and unvaccinated children. An anti-PT IgG rise occurred earlier and was briefer and lower in vaccinated children than in unvaccinated children. The same patterns were observed when the sample was restricted to positive bacteriological results or to titers higher than the minimum detectable limit (data not shown).

One consequence of this observation might be a misclassification bias in trials of vaccine efficacy when serologic decreases are not included in the case definition. Table 5 gives an illustration of this aspect. With reference to the first line, where children with 21 days of paroxysmal cough were confirmed as having pertussis according to WHO recommendations, the consideration of serologic decreases as a confirmation criterion lowered the absolute efficacy in the AC group. This modification was more marked when the epidemiological link was not included in the confirmation criteria.

DISCUSSION

This study was based on data from the Senegal Pertussis Trial, and therefore the data were not collected specifically to address its objectives. Therefore, the design was not optimized

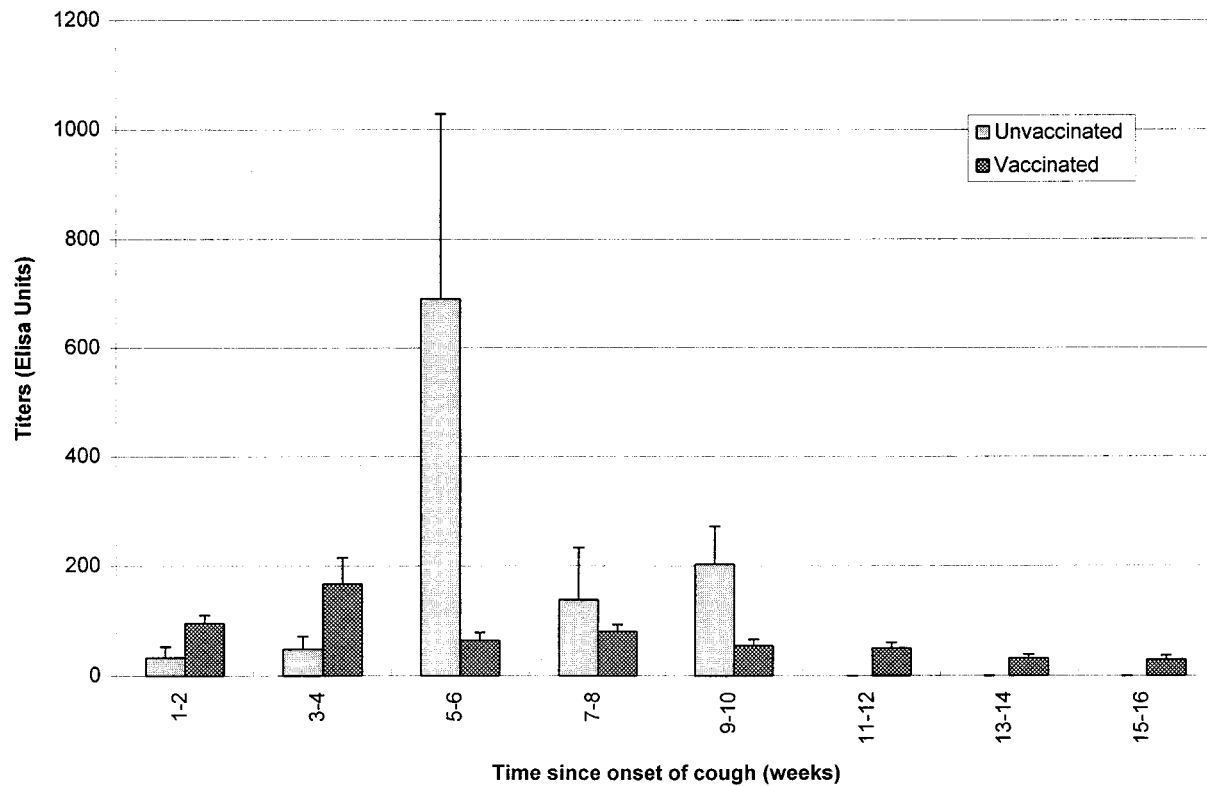


FIG. 1. Evolution of anti-PT IgG antibody titers after exposure to pertussis according to vaccination status. Values are shown as means ± standard errors.

for all aspects. For example, the group of nonvaccinated children was not as comparable to the vaccinated children as were the two groups of vaccine recipients, which had been randomized. Unvaccinated children were younger, before the age at vaccination, or their parents refused vaccination. Also, the study of the kinetics of antibodies lacked balance in terms of the sizes of the classes, as reflected by the standard deviation. However, the design of the trial was unique in providing serologic results within an active surveillance system, with a large number of suspected and confirmed cases of infection determined by other techniques, including the use of a large number of vaccinated patients. Also, serologic testing was performed in the same laboratory, according to standard procedures, and was blind to vaccination status. External quality assurance was performed through an international collaborative initiative, and our results fit well with those of the reference laboratory (7). Therefore, these results provide some insight into the high

proportion of decreases in antibodies in paired sera to PT and/or FHA. These decreases were observed over a wide span of time between the onset of cough and when the acute-phase sample was obtained, but with a marked positive trend over time of acute-phase sampling, associated with an inverse trend for increases in paired sera. These decreases were observed in children with pertussis confirmed by other laboratory-based methods or with typical clinical whooping cough. Furthermore, this observation is in agreement with that of a study reported recently from Sweden (5), in which the authors recommended considering decreases in antibodies for the diagnosis of pertussis. They stressed the importance of the timing of blood sampling with respect to the onset of cough, which may be insidious and reported late by parents. However, while this is also observed in our study, another important independent factor is the different kinetics of titer rises observed between vaccinated and unvaccinated children.

TABLE 5. Influence of interpretation of serological results on vaccine efficacy in children with 21 days of paroxysmal cough

Confirmation criteria ^a	Result for vaccine group:								
	No dose		AC			WC			Relative risk of pertussis in AC vs WC vaccine group
	No. of children	Incidence density	No. of children	Incidence density	AVE (%) ^b	No. of children	Incidence density	AVE (%)	
B+ or E+ or Si+ (WHO) ^c	15	0.039	41	0.013	67	16	0.005	87	2.6
B+ or E+ or Si+ or Sd+	15	0.039	47	0.015	62	16	0.005	87	2.9
B+ or Si+	13	0.033	23	0.007	78	10	0.003	90	2.4
B+ or Si+ or Sd+	14	0.036	35	0.011	69	13	0.004	89	2.7

^a B+, bacteriology positive; E+, epidemiological link with a culture-confirmed case of infection; Si+, serologic increase; Sd+, serologic decrease.
^b AVE, absolute vaccine efficacy.
^c WHO recommended confirmation criteria.

Those results were observed in a rural population of a developing country, and they could be thought to express a particular antibody response due to such factors as low nutritional status, high general infectious background, and intense exposure to the diseases. This might well be true. However, study vaccines have been found to induce immunogenicity comparable to that in other settings (12), and a recent comprehensive study of the same population failed to show an impaired immune response among children (10). Decreases have also been observed in vaccine failures for measles (14). Thus, it is likely that our observation strengthened earlier ones (9, 13) and has general relevance.

These results suggest that decreases as well as increases might be taken as serologically positive diagnoses. This could have a major impact, since, in the present study, depending on whether or not decreases were taken into consideration for positive pertussis diagnoses for either PT or FHA (Table 1), the final positive results rose from 157 (34%) to 266 (58%) cases of infection.

In recent vaccine trials, judgments were based on cases of infection including not only bacteriology but also serology and epidemiological contact with a culture-confirmed case. Vaccine efficacy may be biased if the antibody response differs between vaccinated and unvaccinated children. Even when preacute-phase sera are available in specific settings or as part of research protocols in vaccine trials (2), this bias can be observed because of the rapid decline in the antibody response in vaccinated children. Thus, nonuse of decreases when absolute efficacy is being measured (i.e., when unvaccinated children are part of a trial) will cause an underestimation of cases of infection among vaccinated children and an overestimation of vaccine efficacy. Similarly, if two vaccines are to be compared, the relative vaccine efficacy could also be biased if the serologic responses between vaccine groups are not similar. This is not supported by our results for the vaccines under study. The magnitude of the bias depended on and was minimized by the proportion of cases confirmed by bacteriology or by epidemiological contact.

Our data suggest that vaccinated children have an earlier, lower, and briefer antibody response when the disease occurs. If the response to exposure among unvaccinated children is considered a primary immune response and the response among vaccinated children is considered a secondary immune response, the lower and briefer antibody response observed among vaccinated children is atypical. Comparable observations were reported for tetanus (4) and leishmaniasis (6). This "negative phase" may be related to an increase in the avidity of the antitoxin produced during maturation of the immune response. It may also reflect less-severe disease among vaccinated children, rather than temporary immunodeficiency associated with vaccine failure, and deserves further investigation.

In conclusion, this study supports an earlier recommendation that a significant decrease in PT or FHA should be added

to the diagnostic criteria. A differential antibody response in vaccinated and unvaccinated children was established, which should be considered in vaccine efficacy studies so as to avoid an overestimation of absolute vaccine efficacy.

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