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Long-Term Human Serum Antibody Responses after Immunization with Whole-Cell Pertussis Vaccine in France

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Three hundred sixty children were tested for pertussis serology 0.5 to 158 months after complete whole-cell pertussis vaccination. An immunoblot assay was used to detect serum antibodies to pertussis toxin, filamentous hemagglutinin, adenylate cyclase-hemolysin, and pertactin, and agglutination was used for detection of anti-agglutinogen antibodies. Antibodies against pertussis toxin, pertactin, and agglutinogens decreased rapidly after vaccination but increased secondarily, suggesting exposure to infected persons. In contrast, anti-filamentous hemagglutinin antibodies persisted and anti-adenylate cyclase-hemolysin antibodies increased continuously, suggesting either cross-reaction with non-*Bordetella* antigens or exposure to *Bordetella* isolates expressing these two antigens, including *Bordetella pertussis*. These data suggest that unrecognized pertussis is common in France despite massive and sustained immunization in infants and that vaccinated children become susceptible to infection more than 6 years after their last vaccination.

Resurgence of pertussis was first observed in 1976 in the United States (7) despite widespread and sustained whole-cell pertussis immunization since 1951. This resurgence may be, in part, attributed to waning vaccine-induced immunity (16). The absence of repeated boosters, by either immunization or exposure to infected persons (natural booster), is the main explanation for this phenomenon. The use of new and safe acellular pertussis vaccines for boosters in infancy or later in age may therefore be the answer. The French pertussis vaccine (Tetracoq and DTCP, the same vaccine licensed by Pasteur Mérieux Serum et Vaccins) is a whole-cell type combined with diphtheria and tetanus toxoids and with the three inactivated poliomyelitis strains. This vaccine has been widely used in France since 1966. Thus, the epidemiological situation of France appears to be comparable to that of the United States with regard to the massive and sustained use of whole-cell pertussis vaccine but with a delay of 15 years. As a matter of fact, a similar resurgence of pertussis has been suspected in France since 1991 (12) but still needs to be confirmed before booster vaccination can be recommended.

Pertussis acellular vaccines have been used in Japan since 1981 (3) and have been recommended for boosters in the United States since 1991 (2). Antigens included in these vaccines are pertussis toxin (PT) and adhesins such as filamentous hemagglutinin (FHA), pertactin (PRN), and fimbrial agglutinogens (AG). These antigens have been shown to induce a protective immunity in murine models. However, no correlation has been made between protection and the level of antibody against one of these antigens in previous clinical assays (1). Most of the previously published serological studies of vaccinated children have been limited to analysis of antibody responses to PT, FHA, PRN, and AG shortly after the last immunization. Furthermore, little information is available concerning another *Bordetella pertussis* antigen, adenylate cyclase-hemolysin (AC-Hly), which was shown recently (i) to induce an antibody response after pertussis infection (13) and pertussis vaccination (11), (ii) to be a protective antigen in a murine model (14), and (iii) to play an important role in *B. pertussis* pathogenesis (18–20, 26).

The purpose of our study was to analyze the duration of antibody responses not only to PT, FHA, PRN, and AG but also to AC-Hly after immunization with whole-cell pertussis vaccine and to determine the evolution of these antibodies with time after complete vaccination (primo-vaccination and one booster at 18 months).

MATERIALS AND METHODS

Patients. Informed consent was obtained from parents or guardians. The study protocol followed the guidelines of the Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale according to present French legislation.

Vaccinated patients. Three hundred sixty children aged 20 months to 16 years who had been previously immunized against pertussis (four injections) were tested for pertussis serology 0.5 to 158 months after the last vaccination. All children were hospitalized at the Trousseau children's hospital for various diseases excluding pertussis and pertussis syndrome (prolonged paroxysmal cough or whoops). However, no information concerning past history of pertussis or prolonged cough compatible with an atypical pertussis syndrome or past exposure to a pertussis case was recorded. Vaccination history was confirmed by reference to the child's health record book. Since 1991, the French immunization schedule has comprised three injections of combined diphtheria–tetanus–whole-cell pertussis–inactivated polio vaccine at 2, 3, and 4 months of age and a single booster at 18 months. Before 1991, the recommended age for the first injection was 3 months.

Bacterial strains and growth conditions. The *B. pertussis* strains used in this study were 18323 (ATCC 9797); Pillemer (P134), which was originally obtained from R. Preston (University of Glasgow, Glasgow, Scotland); and vaccine strains 1414 and 1416, obtained from Pasteur Mérieux Serums et Vaccins. Bacteria were grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood at 36°C for 72 h and again for 24 h.

Preparation of the antigens. *B. pertussis* AC-Hly antigens were purified from the bacteria after urea extraction using a calmodulin affinity column as previously described (13). Four different AC-Hly antigens were used for the detection of antibodies in the sera of vaccinated children: AC-Hly-1 was purified from *B. pertussis* P134, AC-Hly-2 was purified from the reference 18323 strain, and AC-Hly-3 and AC-Hly-4 were purified from the vaccine strains 1414 and 1416,

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TABLE 1. DRs of antibodies against different pertussis antigens in the 12 groups of sera classified according to MTPV

Group no.	MTPV (mo)	DR (%) of antibodies against:					
		PT	PRN	AG	FHA	AC-Hly-1	AC-Hly-2
1	3.75	50.00	63.33	90.00	66.67	40.00	8.00
2	8.42	37.93	55.17	86.67	72.41	48.28	12.50
3	14.26	20.69	51.72	70.00	65.52	44.83	14.29
4	20.95	36.67	46.67	76.67	70.00	53.33	34.78
5	27.85	31.03	44.83	63.33	68.97	48.28	20.83
6	34.93	17.86	25.00	46.67	50.00	54.17	18.18
7	46.17	13.64	31.82	40.00	68.18	70.00	75.00
8	57.06	11.54	28.00	46.67	61.54	78.26	66.67
9	68.70	15.38	19.23	51.72	46.15	60.00	63.64
10	86.54	20.69	31.03	60.00	68.97	69.57	60.87
11	104.05	24.14	20.69	60.00	62.07	75.00	72.73
12	137.11	26.67	26.67	56.67	66.67	85.71	73.08

respectively. PT and FHA were purified from the P134 strain (a kind gift of Pasteur Mérieux Serums et Vaccins), and PRN was purified from *B. pertussis* Tohama (a kind gift of SmithKline).

Serological assays. (i) Immunoblot assay. The detection of antibodies directed against purified PT, FHA, PRN, and AC-Hly antigens was performed by immunoblotting as previously described (13). Briefly, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on ready-to-use 8 to 25% polyacrylamide gels with a Phast-system (Pharmacia, Uppsala, Sweden). The separated proteins were transferred to Hybond C-super membranes (Amersham, Little Chalfont, United Kingdom). After blocking, membranes were incubated overnight at 4°C with a 2×10^3 dilution of human sera. The detection of antibodies was performed with peroxidase-labelled anti-human immunoglobulins and the Amersham enhanced chemiluminescence system. Results were graded depending on the duration of exposure to X-ray films: +++, 6 s; ++, 1 min; +, 10 min; and −, no detection. Detection was considered positive when the result was +, ++, or +++.

(ii) Microagglutination assay. Antibodies against AG were detected by the classical microagglutination test (23). Detection was considered positive when the result was superior to 1/20 dilution.

Statistical analysis. The 360 serum specimens were ordered by increasing time elapsed between collection and the last vaccination (fourth injection) and classified into 12 successive groups of 30 serum specimens each. For each group, the mean time postvaccination (MTPV) and the detection rate (DR) of each antibody were calculated. Correlations between DR and MTPV were analyzed by linear and polynomial regression tests (Statview II). The statistical significance was designated at $P < 0.05$.

RESULTS

Antibody response of children vaccinated four times with a whole-cell pertussis vaccine in France by immunoblot. Between January 1991 and July 1993, 360 vaccinated children aged between 20 months and 16 years had a serum sample collected for pertussis serology. The time elapsed between the collection of serum and the last vaccination ranged from 0.5 to 158 months. All sera were tested for antibodies directed against PT, FHA, PRN, AC-Hly-1, and AC-Hly-2 by immunoblotting and tested for anti-AG antibodies by microagglutination. The mean age for the first injection was 4.4 months (range, 1.8 to 11.8 months), and the mean intervals between the first and second injections, the second and third injections and the third injection and the booster were, respectively, 1.5 (range, 0.7 to 6.9), 1.7 (range, 0.6 to 16), and 13.6 (range, 1.4 to 47.4) months. The sera of vaccinated children were classified by groups of increasing time elapsed between the collection of serum and the last vaccination. The MTPV and the DR of each antibody for each group of sera are indicated in Table 1. The DRs of anti-PT, -FHA, -PRN, -AG, and -AC-Hly-1 antibodies were high shortly after vaccination (group 1, MTPV = 3.75 months). This result indicates that the French pertussis whole-cell vaccine is able to induce the synthesis of antibodies against these factors. Surprisingly, in this same group, the DR of anti-AC-Hly-2 antibodies was very low (8%) in comparison with the DR of anti-AC-Hly-1 antibodies. A similar result was obtained with a subgroup of nine serum samples collected less than 3 months after the last vaccination (mean time = 1.33 months). In this small subgroup, the DRs of the other antibodies were 77.8% for PT and FHA, 88.9% for PRN, 100% for AG, 55.6% for AC-Hly-1, and 0% for AC-Hly-2. To explain this surprising result, the sera from 14 recently vaccinated children (delay, <4 months) were pooled and the ability of this pool to recognize the AC-Hly purified from the two French vaccinal strains and from the reference strain *B. pertussis* 18323 was tested. This pool of sera recognized the AC-Hly purified from the vaccinal strains but not the AC-Hly purified from the 18323 strain (data not shown). This result suggests that AC-Hly from the 18323 strain is immunologically different from that from other *B. pertussis* strains.

Duration of anti-PT, -FHA, -PRN, -AG, and -AC-Hly antibodies in sera from children vaccinated four times with pertussis whole-cell vaccine. As shown in Table 1, a significant decrease of the DRs of anti-PT, -PRN, and -AG antibodies was initially observed and was followed by a secondary increase of the corresponding DR more than 60 months after the last immunization. By linear and polynomial regression tests, the correlations between the MTPV and DR were analyzed, and the results are shown in Fig. 1. For anti-PT antibodies, a rapid initial decrease of the DR with time after immunization was observed and was followed, 60 months after vaccination, by an increase of the DR (Fig. 1A). A significant correlation between the MTPV and DR was found with a polynomial regression model (order 2). This phenomenon was not specific to anti-PT antibodies but was also observed with anti-PRN (Fig. 1B) and anti-AG antibodies (Fig. 1C). The secondary increase of the detection rates of anti-PT, -PRN, and -AG antibodies observed 80 months after the last immunization indicates that *B. pertussis* infection may occur in vaccinated children in our country. For anti-AC-Hly antibodies (Fig. 1E and F) a significant correlation was found between the MTPV and DR, but in this case the DR increased with the MTPV. The constant increase of the DRs of anti-AC-Hly-1 and anti-AC-Hly-2 antibodies supports again the hypothesis that our population was exposed to *B. pertussis* strains. However, it has to be noted that this increase started earlier than that observed with anti-PT, -PRN, and -AG antibodies. In contrast, the DR of anti-FHA antibodies remained high in all groups of vaccinees, and no correlation was found between the MTPV and that DR (Fig. 1D).

DISCUSSION

The absence of data concerning typical or atypical pertussis or exposure to pertussis cases in our patients is a critical point. However, pertussis has been rare for many years in our country, clinical symptoms have been forgotten by physicians and parents, and the disease is nowadays atypical and usually not recognized in France, in contrast to other countries, such as Sweden, where the disease is still endemic. Moreover, a previous attempt to identify previous typical or atypical pertussis or exposure to a pertussis case in adolescents by interview during a similar study in the United States did not succeed (10). This is why we did not include such a questionnaire in our study.

In the present study, we used immunoblot instead of enzyme-linked immunosorbent assay (ELISA), which has been internationally used in clinical trials and epidemiological studies to measure PT and FHA antibodies after whole-cell pertussis vaccination. The reasons for our choice were the following: (i) antibodies specific to PT, FHA, PRN, and AC-Hly can

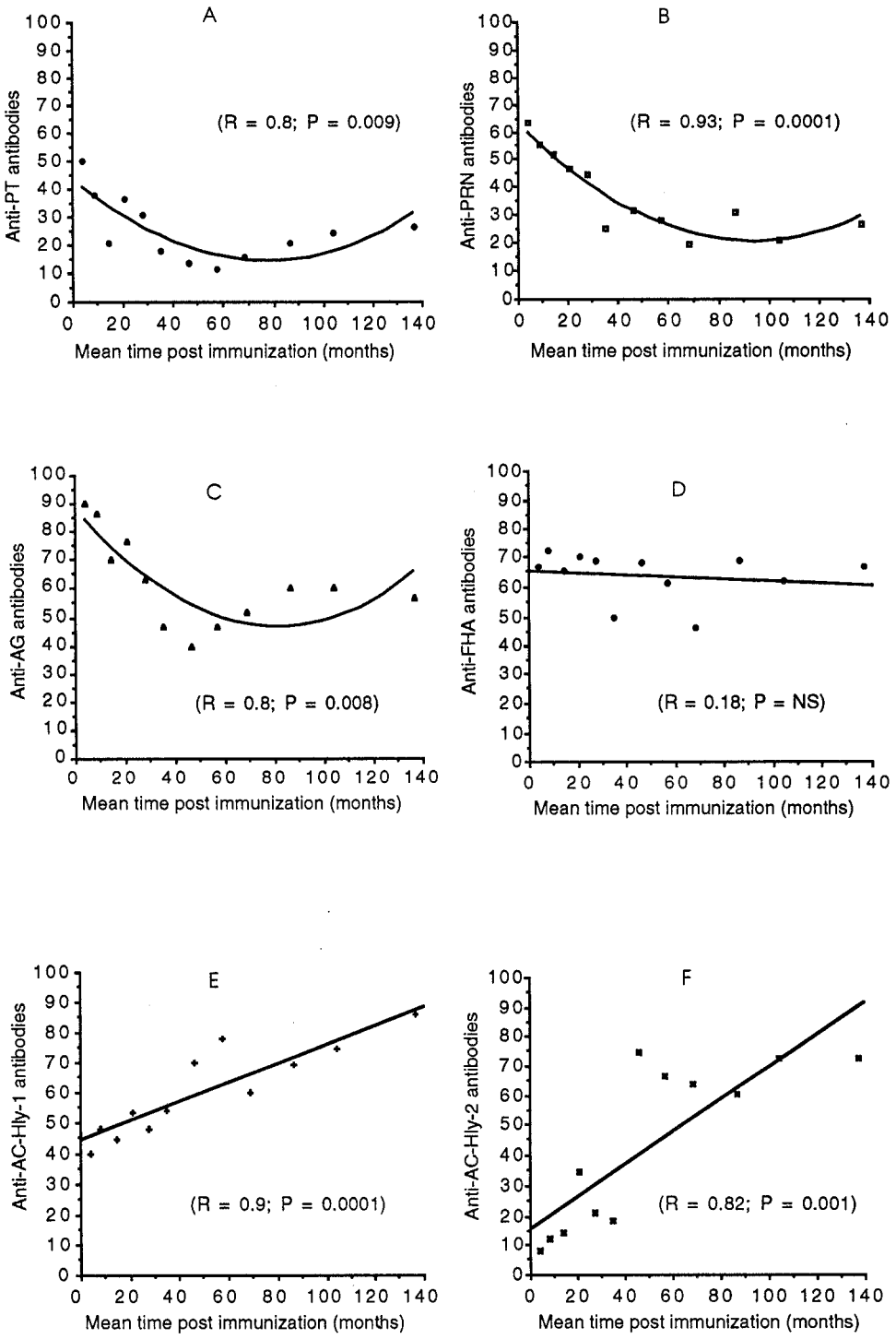

FIG. 1. Correlations between the mean time postimmunization and the DRs (percent) of antibodies directed against pertussis antigens: PT (A), PRN (B), AG (C), FHA (D), AC-Hly-1 (E), and AC-Hly-2 (F).

readily be detected by immunoblot (13), (ii) the amount of purified antigen required for ELISA is larger than that required for immunoblot, (iii) immunoblot is now adapted for routine diagnosis (with ready-to-use gels), and (iv) immunoblot is sensitive and specific and allows the control of the stability of the antigen for each experiment. ELISA and immunoblot cannot be compared since ELISA detects antibodies which recognize proteins in a native conformation and immunoblot detects antibodies which recognize mostly denatured proteins. Thus, our study described the evolution of a certain type of pertussis antibodies after immunization which might have been different if we had used ELISA.

However, our results are in agreement with those of other studies which used ELISA or immunoblot (4, 6, 22, 24, 25) and

showed that pertussis whole-cell vaccine induces antibodies directed against PT, FHA, PRN, and AG. In a Swedish serological study using ELISA and conducted with children primed with whole-cell pertussis vaccine and boosted with an acellular vaccine (8), a rapid decrease of anti-PT antibodies was observed in the months following either primo-vaccination or booster. Using immunoblot, we confirmed these data and showed that a rapid decrease in detection is also observed with anti-PRN and anti-AG antibodies 2 years after vaccination. This result is a confirmation that whole-cell pertussis vaccine-induced protection is limited in time. Since the introduction of whole-cell pertussis vaccine in France, pertussis morbidity decreased and, as a consequence, natural boosters due to exposure to pertussis cases became rare. The present suspected resurgence of pertussis in France may be explained, as in the United States, by the increasing number of vaccinated adults who are susceptible to pertussis.

The secondary increase of the DRs of anti-PT, -PRN, and -AG antibodies observed 80 months after the last vaccination suggests that *B. pertussis* infection occurs in vaccinated children in our country and that the whole-cell vaccine does not protect for more than 6 years in countries with low pertussis morbidity. In a recent publication (15), using culture and PCR to diagnose *B. pertussis* infection during a community outbreak in Finland, He et al. showed that protection against clinical pertussis decreased with time and was significantly reduced 6 years after the last vaccination in that country, using whole-cell pertussis vaccine with a high coverage. Our study, using serology in vaccinated children, tends to confirm this estimation.

The constant increase in the DR of anti-AC-Hly antibodies supports again the hypothesis that our population was exposed to *B. pertussis* strains. However, it should be noted that this increase was observed earlier (24 months) than that of anti-PT, -PRN, and -AG antibodies (80 months). The reason for this observation is still unclear. It could be attributed to differences in the levels of sensitivity for the detection of these antibodies by immunoblot. The other possibility is that AC-Hly is not specific to *B. pertussis* and cross-reacts with other antigens such as other RTX toxins expressed by *Escherichia coli* or other *Bordetella* species (9).

The fact that sera from recently vaccinated children did not recognize AC-Hly-2 purified from the reference strain *B. pertussis* 18323 in contrast with AC-Hly antigens purified from the two vaccine strains suggests that AC-Hly-2 is immunologically different. It has already been shown that *B. pertussis* 18323 expresses low levels of PT with immunological properties that differ from those of other *B. pertussis* isolates (5). Furthermore, it was recently shown that the DNA restriction fragment pattern of *B. pertussis* 18323 by pulse-field gel electrophoresis was distinct from those of other *B. pertussis* clinical isolates (17). All these data taken together are in agreement with the fact that *B. pertussis* 18323 may not be suitable as a reference strain, as it may be considered atypical.

The DRs of anti-FHA antibodies remained high in all groups of vaccinees, and no correlation was found between the MTPV and DR. Again, different hypotheses may explain this observation. (i) Anti-FHA antibodies persist longer than other *B. pertussis*-specific antibodies. In this hypothesis, the initial decrease and the secondary increase observed in the case of anti-PT, -PRN, and -AG antibodies after exposure of our population to *B. pertussis* might not be detectable with anti-FHA antibodies. (ii) FHA is not specific for *B. pertussis* and may cross-react with other antigens such as gram-negative pili or fimbriae (21). (iii) Our population was exposed previously to other *Bordetella* species such as *B. parapertussis* and *B. bronchiseptica*. This last hypothesis seems unlikely to us because *B. parapertussis* is only rarely isolated in our country in patients with pertussis syndrome and since *B. bronchiseptica* is isolated mainly in immunocompromised patients (27). None of our patients was immunocompromised.

In conclusion, our study demonstrates that the French pertussis whole-cell vaccine induces the synthesis of antibodies against PT, FHA, PRN, AG, and AC-Hly. In the absence of late booster vaccination, anti-PT, -PRN, and -AG antibodies decrease rapidly after the fourth injection (at 18 months of age) but increase later, more than 80 months after vaccination, suggesting persistent circulation of *B. pertussis* strains and exposure to infected persons. The consequence is that unrecognized pertussis is common in France despite massive and sustained immunization in infants and that vaccinated children become susceptible to infection more than 6 years after their last vaccination. A national epidemiological study was recently performed in France to confirm this hypothesis in order to define the future booster strategy for pertussis vaccination.

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