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Humoral and B-cell memory responses in children five years after pertussis acellular vaccine priming

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ABSTRACT

The resurgence of pertussis suggests the need for greater efforts in understanding the long-lasting protective responses induced by vaccination. In this paper we dissect the persistence of humoral and B-cell memory responses induced by primary vaccination with two different acellular pertussis (aP) vaccines, hexavalent Hexavac® vaccine (Hexavac) (Sanofi Pasteur MSD) and Infanrix hexa® (Infanrix) (GlaxoSmithKline Biologicals). We evaluated the specific immune responses in the two groups of children, 5 years after primary vaccination by measuring the persistence of IgG and antibody secreting cells (ASC) specific for vaccine antigens. Part of the enrolled children received only primary vaccination, while others had the pre-school boost dose. A similar level of antigen-specific IgG and ASC was found in Infanrix and Hexavac vaccinated children. The mean IgG levels were significantly higher in children that received the pre-school boost as compared with children that did not receive the boost dose. A longer persistence after the pre-school boost of IgG-Pertussis Toxin (PT) and IgG-pertactin levels was observed in Infanrix primed children, but it was not statistically significant. More than 80% of children presented a positive ASC B memory response. Around 50% of children still presented protective IgG-PT levels which are reduced to 36% in no-boosted children. The pre-school booster dose restores the percentage of protected children above 50%. In conclusion our data underline the importance of giving a booster dose 5 years after primary vaccination and suggest the need for a new vaccine able to induce a long lasting protective response.

1. Introduction

The recent resurgence of *Bordetella pertussis* infections, in spite of the high vaccine coverage in the developed countries, suggests the need of major efforts in the understanding of the long lasting protective responses induced by vaccination. The acquisition of this knowledge will help in defining better vaccination strategies [1–4]. Both infection- and vaccine-induced immunity wanes. Gustafsson and colleagues indicate that protection wanes some 5–7 years after acellular pertussis (aP) vaccination in infancy [5]. The recent California outbreak allowed a more precise quantification of the time of protection induced by aP vaccines. The results showed that protection decreases in a period of five years after the fifth dose, at the age of 5–6 years [3].

In most developed countries aP vaccines are administered in infancy and in pre-school age. Consequently, the peak of incidence of whooping cough has shifted to adolescents and adults [6,7]. Although the majority of older age groups suffer from mild symptoms, they are the main source of infection for the not fully vaccinated infants [8] who are at risk for pertussis associated life-threatening complications. During the pertussis-outbreak in

Abbreviations: aP, acellular pertussis; Ab, antibody; ASC, antibody secreting cells; CI, confidence interval; IgG, immunoglobulin G; FBS, foetal bovine serum; FHA, filamentous hemagglutinin; GM, geometric mean; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; Prn, pertactin; PT, pertussis toxin.

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California, 10 unvaccinated infants died from pertussis disease [3].

The classical approach to evaluate the persistence of immune responses after vaccination or infection is based on the measurement of the antibody levels against the vaccine components, namely pertussis toxin (PT), pertactin (Prn), filamentous hemagglutinin (FHA) and eventually fimbriae. However, the protective immune mechanisms for pertussis are still elusive. Specific serum antibody (Ab) titers are important but not sufficient to represent a correlate of protection [9,10]. Since serum Ab levels rapidly wane after vaccination [11–15], cellular immunity such as memory B and T cells determination can be important parameters to evaluate the protection against whooping cough induced by vaccination [16 –21].

In this paper we dissect the B-cell memory responses induced by the primary vaccination with two different aP vaccines, hexavalent Hexavac® vaccine (Hexavac) (Sanofi Pasteur MSD) and Infanrix®-hexa vaccine (Infanrix) (GlaxoSmithKline Biologicals), in the 6–7 years of age children groups. We compare the specific immune responses induced by the priming with the two aP vaccines 5 years after primary vaccination, by assessing the persistence of specific IgG, the frequency of B memory cells and the frequency of Ab secreting cells (ASC) specific to the vaccine antigen components. This age group was chosen because it is close to the supposed waning of aP vaccine induced protection [3] and near to the pre-school boost. Since, some of the children surveyed had already received the preschool booster, we could also analyze the impact of primary vaccination on the booster dose of pertussis vaccine.

2. Materials and methods

2.1. Study population, vaccines' information and sample collection procedures

One hundred and four children were included in the study (Table 1). Sixty-six children received Hexavac and 38 children received Infanrix for all the three doses of the primary vaccination. The vaccine was administered at 3, 5 and 11 months of age [22]. Immune-monitoring was performed when the children were 6–7 year old. All children had a properly completed primary vaccination schedule.

Each 0.5-ml vaccine dose is formulated to contain:

- Hexavac – PT 25 μg; FHA 25 μg (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-Product_Information/human/000298/WC500074586.pdf).
- Infanrix – PT 25 μg; FHA 25 μg, and Prn 8 μg (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000296/WC500032505.pdf).

As indicated in Table 1 part of the children already received the pre-school booster before the immune monitoring; the vaccine used was Boostrix® (GlaxoSmithKline): PT toxoid 8 μg; FHA 8 μg and Prn 2.5 μg.

This study was conducted in accordance with the Declaration of Helsinki (1964). Ethics approval was obtained by the Bambino Gesù paediatric hospital, and the children's parents provided a written informed consent.

Seven milliliters of peripheral blood in sodium heparin were collected for B-cell memory response and serological analysis.

Discrepancies in the number of children enrolled and the number of sample tested in the ASC assay, as indicated in figures, are due to insufficient number of peripheral blood lymphocytes; the pertussis antigen priority was PT > FHA > Prn.

2.2. Reagents, antigens and mitogen

Genetically detoxified PT and FHA antigens were purified starting from crude material obtained from Novartis, Siena, Italy, as described in [23]. Prn was expressed and purified from an *Escherichia coli* construct as described in [24]. Tetanus toxoid (4200 Lf/ml Novartis) was used as positive control of B memory responses.

2.3. Pertussis specific serology

For measurement of plasma IgG levels specific for PT, Prn and FHA antigens the fluorescent bead-based multiplex immunoassay was used as described in Ref. [19,23–25]. In the absence of well-established serological markers for protection against pertussis, arbitrary level of ≥20 EU/ml for the IgG-PT was defined as protective [26–28].

An IgG-PT value above 100 EU/ml was considered indicative of a recent *B. pertussis* infection, unless the children had the booster less than a year before [28,29]. We found one no-boosted child in the Hexavac group and one boosted child in the Infanrix group with IgG-PT levels above 100 EU/ml. Considering that we focused on the persistence of vaccine induced immunity, these children were removed and not considered for the further analysis in all assays performed.

2.4. Pertussis specific B-cell memory responses

Peripheral blood mononuclear cells (PBMC) were isolated and frozen as previously described [19]. On the day of the experiment, the vial was quickly thawed at 37 °C, cells washed in RPMI-1640, counted and suspended in AIM-V® (Gibco) at the final concentration of 2×10^6 cells/ml. Medium was supplemented with 10% FBS (Hyclone), human CpG (3 μg/ml from Eurogentec, Seraing, Belgium), human IL-10 and IL-2 (both 10 ng/ml, Peprotech, Milan, Italy), PT, FHA, and Prn at 10 ng/ml each. Cells were cultured 5 days at 37 °C in a 5% CO_2 incubator.

96-wells high-protein-binding MultiScreen Immobilon-P Membrane plates (MAIPS10, Millipore, UK) were pre-wetted with 35% ethanol (15 μl/well/60 s), washed three times with sterile PBS, coated with 50 μl/well of 20 μg/ml of FHA, 30 μg/ml of PT or Prn, 7 Lf of tetanus toxoid in sterile PBS or with goat anti-human IgG (2 μg/ml, Dako) as a positive control, and left overnight at 4 °C. Plates were blocked (AIM-V® +10% FBS, 2 h, 37 °C) and 2×10^5 cells/well cultured cell suspensions were incubated in duplicate wells overnight. Plates were washed with 0.05% Tween-20 in PBS (PBS-T) and incubated with alkaline phosphatase-conjugated goat anti-human IgG (Southern Biotechnologies, UK) for 2 h at 37 °C. After incubation, plates were washed with PBS-T followed by washing with PBS and developed with 50 μl/well of a ready-to-use substrate solution of 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium (BCIP/NBT, KPL, USA) for 5–10 min at room temperature. Plates were washed with tap water and dry. Spots, representing single ASC, were counted using the ELISPOT Reader (Eli.Expert, the A.EL.VIS Thema, Bologna, Italy) and expressed as ASC specific to the indicated antigen normalized to total IgG ASC. The phenotype of the expanded B cells was analyzed, both at day 0 and after 5 days of culture, by flow cytometry using CD19-PE, CD27-FITC, CD3PcP monoclonal antibodies (all purchased from BD Biosciences, San Jose, CA). A response was defined positive when spots (ASC ≥ 1) were present in the wells coated with antigen as compared to not-coated control wells that were negatives.

Table 1
Study population: base-line characteristic of the cohort.

Primary vaccination	Number of children enrolled	Sex (F/M)	Age (years)	Number of children no boosted	Number of children boosted [Boostrix]	Suspected pertussis >100 EU/ml >12 months from the boost	
						No boost	Boost
Hexavac	66	26/40	6–7	24	41	1	
Infanrix	38	18/20	6–7	10	27		1

2.5. Statistical analysis

Statistical analysis was performed using the GraphPad Prism® software (San Diego, CA, USA). The results are expressed as geometric mean (GM) with 95% confidence interval (CI). To compare differences between groups two-sided Mann–Whitney test was performed. To compare differences within groups Wilcoxon paired-samples test was performed. To analyze the correlation between variables Spearman rank correlations and linear regression analysis were used. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Pertussis specific serology

Sixty-six children primed with Hexavac and 38 children primed with Infanrix were included in the study (Table 1). IgG-PT and IgG-FHA levels were comparable in the two cohorts. IgG-Prn levels were significantly higher in Infanrix than in Hexavac primed groups (Fig. 1A).

We found a significantly higher level of IgG to all pertussis antigens in children tested after the booster dose as compared to no-boosted in both children groups (Fig. 1B). Moreover, a higher level of IgG-Prn was found in the Infanrix- than in Hexavac-primed group, in the boosted children (Fig. 1B). This result is probably due to the absence of Prn in the Hexavac vaccine and consequently the absence of a recall response due to Prn after the boostrix vaccination in Hexavac primed children.

When the data were analyzed in term of the presence of protective Ab levels, we found that around 50% of children presented levels of IgG-PT higher than 20 EU/ml, an arbitrary protective Ab level [26–28] (Table 2). The percentage of positive responses was lower in children who did not receive a booster dose as only 36.4% of them still had protective IgG-PT levels (44.4% Infanrix vs 33.3% Hexavac). The proportion of positive responses increased after the booster dose to 63.2% (74.1% Infanrix vs 56.1% Hexavac). No significant differences between the two groups were found, even if a higher proportion of Infanrix-vaccinated children had protective IgG-PT levels compared to Hexavac-primed children (Table 2).

In the cohort of boosted children, the time between the booster vaccination and the blood sampling to perform the B response assays was variable from few days to more than one year. The IgG-PT and IgG-Prn concentrations were plotted by six months intervals considering for each child the time elapsed from the booster and the blood sampling (Fig. 2). In Hexavac-primed children group, a significant increase of IgG-PT levels, was found in the 0–6 months ($p = 0.014$) and in the 7–12 months ($p = 0.011$) intervals in the boosted as compared to no-boosted children. A significant decrease of the IgG-PT level was found when comparing the IgG-PT at 13–20 vs 0–6 ($p = 0.024$) and vs 7–12 ($p = 0.009$) months intervals (Fig. 2A). In Infanrix-primed children, the IgG-PT antibody responses significantly increased in the 0–6 months ($p = 0.049$) interval in the boosted as compared to no-boosted children group and tended to persist longer, although not significantly (Fig. 2A).

When the two vaccine-primed groups were compared, a significantly higher IgG-PT level was found at 13–20 months interval ($p = 0.006$) in Infanrix- compared to Hexavac-primed group (Fig. 2A).

The effect of the booster vaccination on the IgG-Prn levels was clear only in Infanrix group (0-6 months group vs no-boosted group $p = 0.020$) (Fig. 2B). This is in line with the fact that Prn is not present in the Hexavac vaccine. The levels of IgG-Prn were higher in Infanrix primed children as compared to Hexavac, statistical significance being reached in the no-boosted ($p = 0.025$) and in 0–6 months interval ($p = 0.036$) in boosted groups. In Hexavac-primed group, a significant increase of IgG-Prn levels was found in 13–20 months group but it did not appear to be linked to the pertussis booster vaccination, being Prn not present in the vaccine. The decrease of IgG-Prn levels respect the time elapsed was not observed.

The IgG-FHA levels analysis indicated a trend for higher IgG levels in Hexavac- as compared to Infanrix-primed children in the group of 0–6 months, even if the differences were not significant (not shown). Moreover the effect of the booster vaccination was limited and the decrease of IgG-FHA levels respect the time elapsed was not observed (not shown).

Table 2
Percentage of children with anti PT titers considered protective IgG-PT > 20 EU/ml.

Primary vaccination	All children	No boosted	Boosted [Boostrix]
All children	54.5	36.4	63.2
Hexavac	47.7	33.3	56.1
Infanrix	66.7	44.4	74.1

3.2. Pertussis specific B-cell memory

We analyzed the frequency of memory B cells, defined as CD3−, CD19+, CD27+ in the two groups of children primed with the two different aP vaccines. The memory B-cell population increased in average 4-fold during the five days of culture. We did not find significant differences in the two groups of children respect to the type of vaccine used in the primary vaccination (not shown).

In order to explore the specificities of the B-cell memory pool, the frequency of ASC for pertussis antigens was evaluated.

The frequency of total IgG ASC and tetanus ASC, internal control, were similar, irrespective of the vaccine used, indicating that the two groups were comparable (not shown).

The number of ASC specific for PT, Prn and FHA, normalized to total IgG ASC, is shown in Fig. 3A. No significant differences were found in the two groups of children. No other differences were found also when the children were grouped considering whether they received the pre-school booster or not (Fig. 3B), even when analyzed by 6 months interval (data not shown). Almost all children presented a positive response (ASC ≥ 1) to PT (88.6% Infanrix vs 83.7% Hexavac). We were not able to see an increase of specific ASC because the post boost assay was not planned in an optimal time-interval after the vaccine boost dose [17].

When the correlation analysis between IgG levels and ASC was performed no correlation was found when PT or FHA antigens were considered, while a low correlation was found for Prn antigen both in all children and in the boosted group in

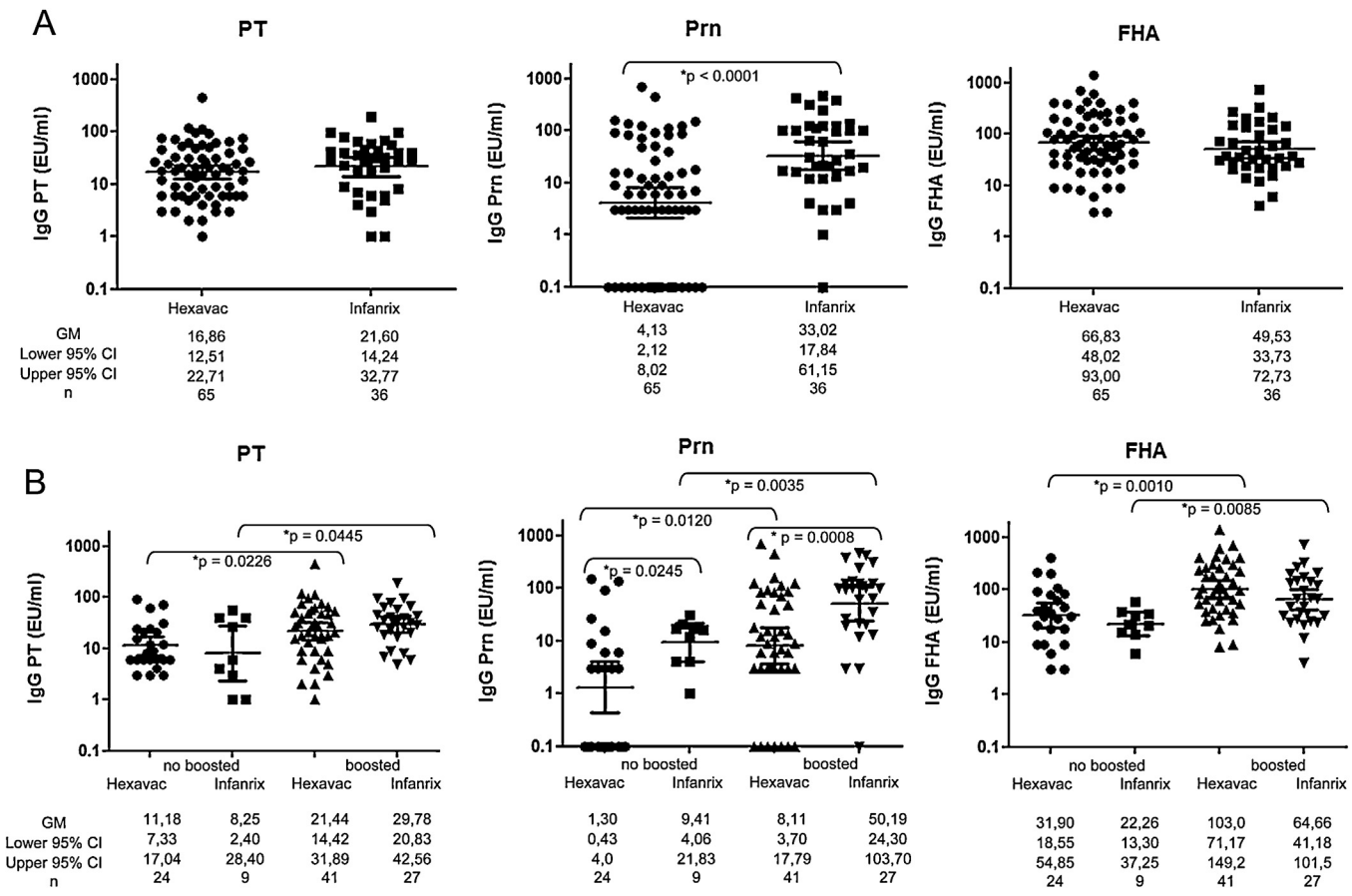

Fig. 1. Pertussis specific serology. IgG levels to the indicated pertussis antigens given in EU/ml in (A) groups in children primed 5 years before with Hexavac or Infanrix or in (B) the same groups of children considering if they had or not received the pre-school boost dose before the performance of the assay. The geometric mean (GM) with 95% confidence intervals (CI) of the specific IgG levels and number (*n*) of children in each group are indicated in the figure and below the *x*-axis. *Significant difference between the groups is indicated.

Hexavac (all children $r = 0.4556$, $p = 0.0017$; boosted children $r = 0.5075$, $p = 0.005$) and Infanrix (all children $r = 0.45$, $p = 0.0086$; boosted children $r = 0.5489$, $p = 0.0045$) primed children.

4. Discussion

Pertussis resurgence, especially among adolescents and adults, is due to the decrease of the protection induced by vaccination with aP vaccines [1,30,31]. In our settings we found, that primary vaccination, performed five years before with the two aP vaccines, results in similar responses respect to Ab levels, expansion of memory B cells and ASC specific for pertussis vaccine antigens in the two cohorts of children. More than 80% of children presented a positive ASC B memory response, indicating the existence of pertussis specific responses induced by the primary vaccination in the great majority of children. We found that around 50% of children still presented IgG-PT levels higher than 20 EU/ml, which is assumed to be protective. This percentage was reduced to 36.4% in the no-boosted children, and reached 63.2% in the cohort of boosted children, thus showing the importance of giving a booster dose 5 years after primary vaccination.

However it is not yet known if the levels higher than 20 EU/ml are truly protective. In recent epidemiological study, Witt and colleagues found that the current schedule of aP vaccine doses is insufficient to prevent outbreaks of pertussis and the authors noted a markedly increased rate of disease from the ages 8 to 12 years [31]. Thus presumably the levels of IgG-PT at six years can be still considered protective. From the Italian epidemiological data no indication of a major incidence of pertussis in this class of age was found [32].

We observed that the levels of Ab specific for the PT and Prn in children primed with Infanrix persist longer after the boost than in children primed with Hexavac. In the case of IgG-Prn levels, a significant difference in the post-boost between Infanrix- and Hexavac-priming was observed (Fig. 1). These results are not surprising, considering that in the Hexavac vaccine the Prn antigen is not present and this may explain the lower power of recall made by the Boostrix vaccine in Hexavac primed children.

The decrease over the time of the Ab responses was quite limited in the case of Prn and FHA antigens. Moreover a clear response to Prn was also observed in not boosted Hexavac primed children. All this can be an indication of a natural booster due to cross-reactive bacteria or to the encounter with other *Bordetellea* species.

Less clear is the higher persistence of IgG-PT response in children primed with Infanrix compared with Hexavac (Fig. 2A). These data may indicate that the booster response is more efficient in children originally vaccinated with Infanrix and this observation reveals potential differences in long-term protection between the two vaccines, perhaps due to differences in their vaccine composition or preparation procedures.

In both vaccines PT is present and is chemically inactivated. In a recent study [33] the immune responses against *B. pertussis* after immunization of mice with Infanrix and Pediacel (Sanofi-Pasteur) showed similar protection but the levels of Ab to vaccinal antigens were different. The authors concentrated their explanation on the differences in the two different adjuvants, with lower Ab levels

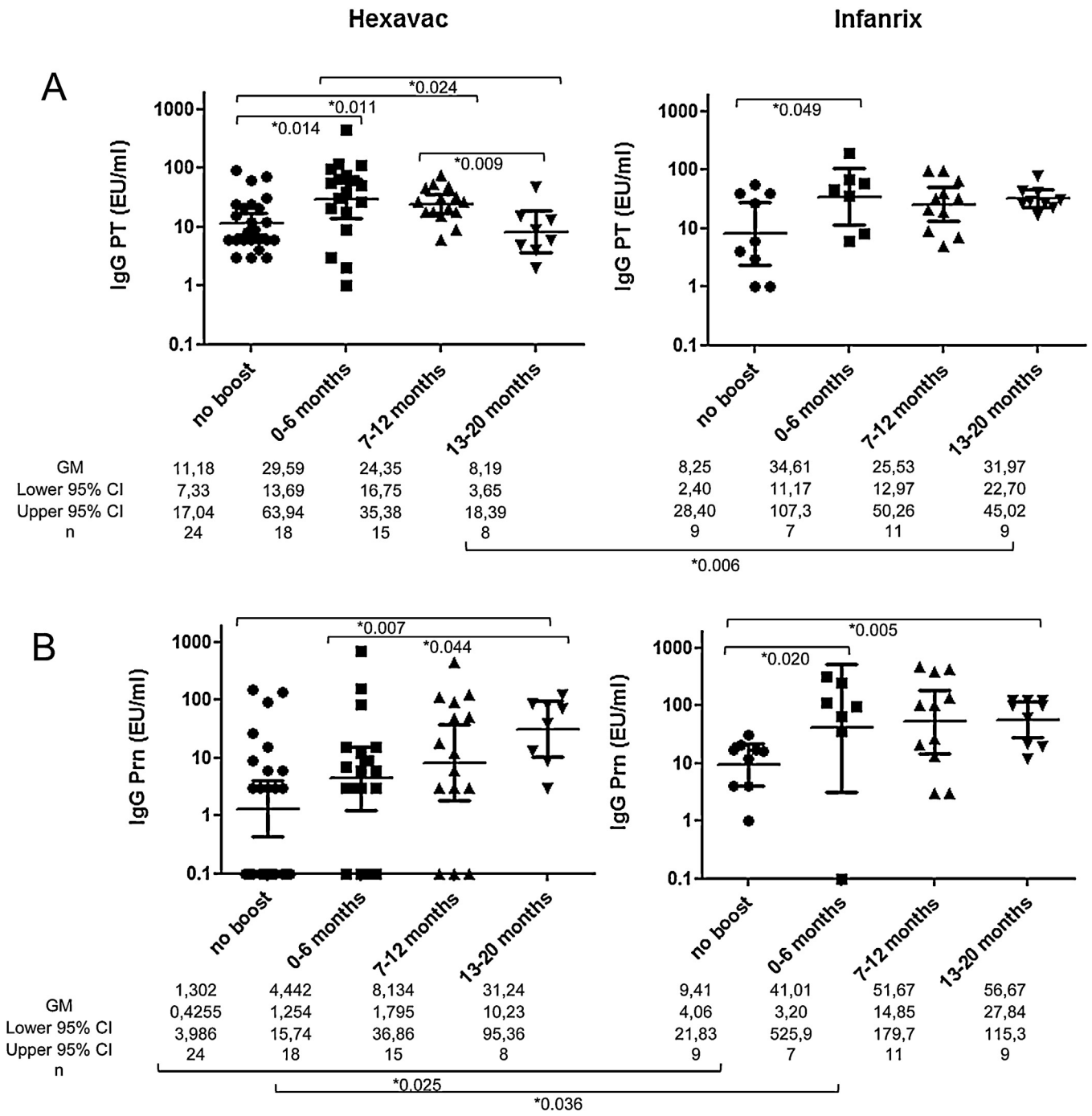

Fig. 2. Analysis of persistence of the level of IgG-PT (A) and IgG-Prn (B) after the pre-school boost groups in children primed 5 years before with Hexavac or Infanrix. IgG to the indicated pertussis antigens (given in EU/ml) were plotted taking in consideration the time frame elapsed from the boost (6 months interval) (*x*-axis). The geometric mean (GM) with 95% confidence intervals (CI) of the specific IgG levels and number (*n*) of children in each group are indicated in the figure, below the *x*-axis. *Significant difference between the groups is indicated.

induced by aluminum phosphate-adsorbed compared to aluminum hydroxide-adsorbed PT, FHA and Prn [34]. In our case, this explanation is not appropriate because both vaccines are formulated using antigens adsorbed onto aluminum hydroxide, even if in Infanrix the content of aluminum hydroxide is higher than in the Hexavac (0.5 vs 0.3 mg).

We cannot exclude a role of other excipients in the vaccine preparations or in the chemically inactivation procedures. In Infanrix, PT is inactivated with treatment with glutaraldehyde and formaldehyde while in Hexavac only with glutaraldehyde. It is not clear if these differences may affect the antigenic power of the vaccines, however, in recent studies, Infanrix vaccine priming demonstrated a greater ability to induce an antibody response for the hepatitis component, greater than the Hexavac vaccine [22] even if the memory B and T cell responses were fully comparable [35,36].

When the levels of responses after the pre-school booster vaccination were considered, our data are only partially in agreement with those from previous authors [16–18], however we need to take into account that, following the Italian National vaccine calendar (http://www.salute.gov.it/imgs/C_17_pubblicazioni_1721_allegato.pdf), the pre-school booster is the fourth dose of vaccine in Italy

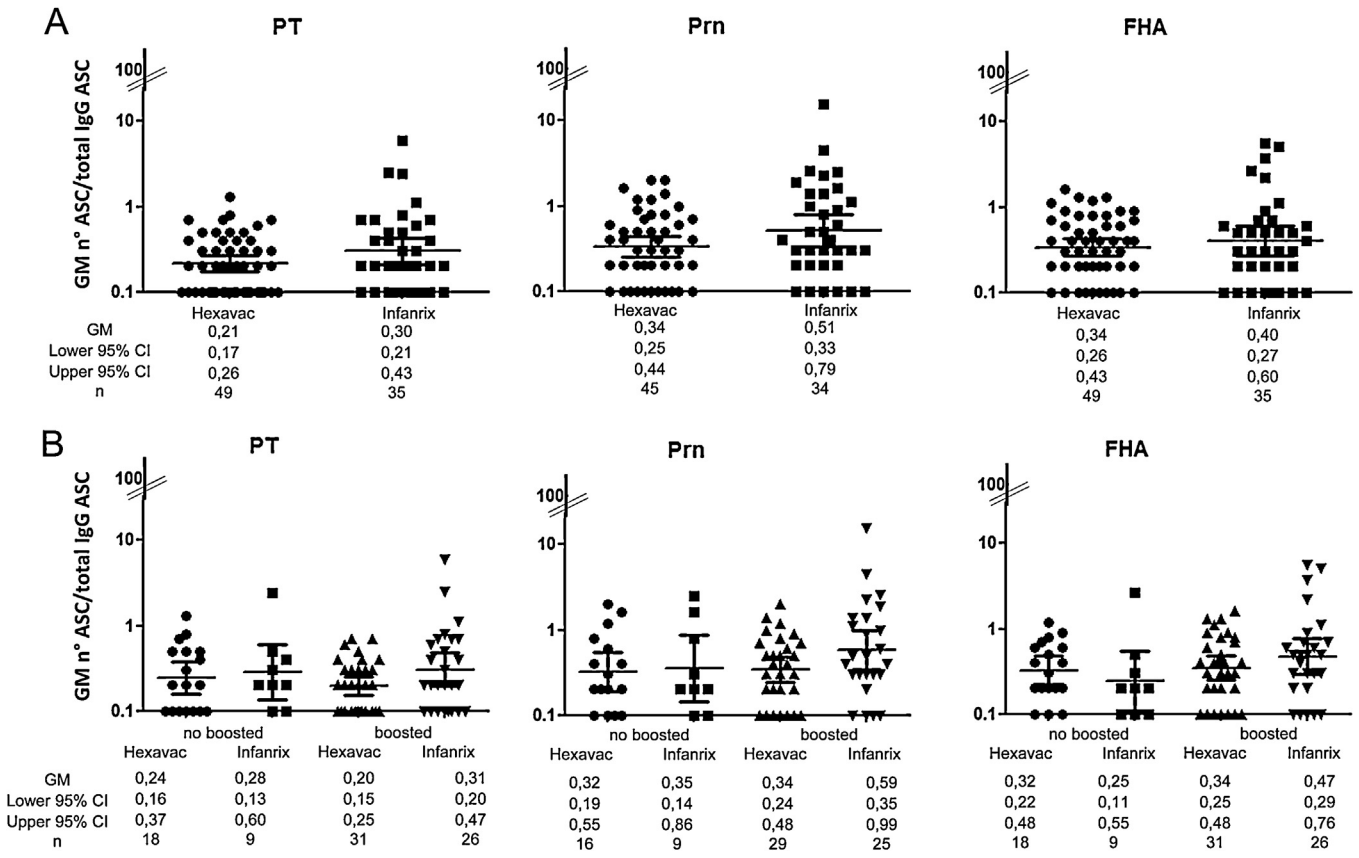

Fig. 3. Pertussis specific B memory cells. Frequency of B memory secreting cells (ASC) specific to the indicated antigen normalized to total IgG ASC in (A) groups in children primed 5 years before with Hexavac or Infanrix or in (B) the same groups of children considering if they had or not received the pre-school boost dose before the performance of the assay. Geometric mean (GM) of ASC with 95% confidence intervals (CI) and number (*n*) of children in each group are indicated below the *x*-axis.

and not the fifth dose as in several other developed countries [37]. Schure and colleagues [18] evaluated the boost response at 28 days and 2 years after boost in a population of 4-year-old children. On average, the Ab response measured in our study is lower compared to that measured in the Shure's study at 28 days but is higher to levels recorded after 2 years. Furthermore, in our study the time elapsed since the boost to the Ab test is greatly variable, ranging between a few days up to almost 2 years, while in Schure's study Ab determination is scheduled at well-fixed time.

In recent papers [16,17] the authors found that the number of PT- and Prn-specific ASC at one month post-booster significantly correlate with the corresponding IgG level. We found a correlation only for Prn-specific ASC and the corresponding IgG level. We did not find a correlation with the PT response. We have no specific explanation, but again the time after the booster and the measurement of the B response is variable and, in some cases, the time between the booster and the test may be too long, when the Ab levels are already vanished. Furthermore, the number of children tested after the booster in the 0–6 months interval group is probably not sufficient to have a significant correlation between the IgG-PT and the ASC PT data.

In conclusion our data underline that (i) the percentage of subjects with anti PT antibody titers above the level considered to be protective, after five years from the primary vaccination, is below 50%, (ii) the pre-school booster restores the percentage of protected children above 50% (Table 2), indicating the importance of the booster vaccination, and (iii) post booster persistence may be different depending on the type of vaccine used for the primary vaccination.

Due to limitation of our study we cannot give conclusions on the persistence of protective responses. Boosting by natural infection could be a major consideration, and it will be very difficult to differentiate between what is due to vaccination, and what is due to infection unless carefully monitored. Our data support the need of sponsoring an ad hoc planned study to answer these specific limitations. Moreover these data support the need to give greater insight in memory immunity studies, important for future pertussis vaccine design as well as for future decisions concerning pertussis vaccination policies to sustain long-lasting protective levels of immunity, still not reached by the vaccine on the market nowadays.

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