Gene Polymorphism in Toll-like Receptor 4: Effect on Antibody Production and Persistence After Acellular Pertussis Vaccination During Adolescence

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Background. Toll-like receptors play an important role in the regulation of adaptive immunity. This study aimed to investigate whether Toll-like receptor 4 (TLR4) polymorphisms influence the production and persistence of antibodies after acellular pertussis booster vaccination during adolescence.

Methods. Seventy-five subjects received a single dose of diphtheria and tetanus toxoids and acellular pertussis vaccine 10 years ago, during adolescence. The same cohort was followed up at 3, 5, and 10 years after this booster vaccination. Pyrosequencing was used for detecting polymorphism in TLR4. Concentrations of anti–pertussis vaccine antibodies were measured by standardized enzyme-linked immunosorbent assay and published elsewhere.

Results. The fold increase in antibodies to pertussis tox in after original vaccination 10 years ago was significantly lower in subjects with TLR4 polymorphism than in those without (55% vs 86%; \( P = .028 \)). At the 3-year follow-up evaluation, geometric mean concentrations of anti–pertussis vaccine antibodies were significantly lower in subjects with TLR4 polymorphism, compared with those without the polymorphism (for pertussis toxin, \( P = .028 \); for filamentous hemagglutinin, \( P = .047 \); and for pertactin, \( P = .046 \)).

Conclusions. This study suggests that TLR4 Asp299Gly polymorphism might influence production and persistence of antibodies after pertussis booster vaccination in adolescents. However, the results should be interpreted with caution as the number of subjects included in this study was limited.

Whooping cough, or pertussis, is caused by the gram-negative bacterium Bordetella pertussis. Despite extensive pertussis immunizations among children, pertussis remains one of the world’s leading causes of vaccine-preventable deaths [1]. Childhood vaccinations against pertussis by means of whole-cell pertussis vaccine were started in the 1950s in many developed countries. Because of the reactogenicity of the whole-cell pertussis vaccine, more-tolerated acellular pertussis vaccines containing purified components of B. pertussis have replaced it in many developed countries. Despite the population-wide vaccinations of infants, the immunity induced by vaccination and natural infection wanes with time; this has ensured that pertussis remains endemic, and it is increasingly recognized in adolescents and adults [2, 3]. Vaccine efficacy trials conducted in the 1990s have shown that acellular pertussis vaccines are both safe and able to protect from pertussis to levels comparable to those of whole-cell pertussis vaccines. High levels of immunoglobulin G (IgG) antibodies to pertussis toxin, pertactin, and fimbriae are associated with protection against pertussis [4, 5]. However, the reliable correlates of protection against pertussis have not been defined. Individual variation in antibody response after receipt of diphtheria and tetanus toxoids and acellular pertussis vaccine (dTPa) has been noticed in efficacy
trials [6–8]. However, the role of innate immunity in these effects is not fully understood.

Toll-like receptor 4 (TLR4) is a pathogen-associated molecular pattern receptor known to function as a receptor for lipopolysaccharides of gram-negative bacteria, including *B. pertussis*. TLR4 can also function as a receptor for pertussis toxin [9, 10]. When the role of TLR4 was investigated in the induction of immune response in mice after immunization with a whole-cell pertussis vaccine, TLR4-deficient mice were found to have impaired immunity after the immunization [11].

Several single-nucleotide polymorphisms (SNPs) have been found in the *tlr4* gene. One of them occurs in the Asp299Gly (refSNP ID: rs4986790). The substitution from aspartic acid to glycine causes an impaired response to lipopolysaccharide, resulting in altered susceptibility to certain infectious diseases and inflammatory diseases [12]. An association between polymorphism in the TLR4 promoter and the antibody response to the whole-cell pertussis vaccine has been noticed [11, 13]. However, little is known about the possible association between TLR4 Asp299Gly polymorphism and antibody response to acellular pertussis vaccines.

In this study, we investigated the effect of TLR4 Asp299Gly polymorphism on antibody responses to dTpa and the persistence of the antibody responses to acellular pertussis vaccine in adolescents. We observed that TLR4 polymorphism might affect antibody production and persistence against pertussis antigens.

**MATERIALS AND METHODS**

**Study Design and Subjects**

In the initial study, performed in 1997 in Turku, Finland, 510 adolescents aged 11–13 years were immunized with a single booster dose of acellular pertussis vaccine. A total of 450 children received dTpa (Boostrix; GlaxoSmithKline), and 60 children received diphtheria and tetanus toxoids vaccine followed by acellular pertussis vaccine 1 month later [14]. Follow-up of the same cohort was conducted 3, 5, and 10 years after the booster vaccination [15–17]. At the 10-year follow-up point, the same cohort was invited to receive an additional booster dose of dTpa vaccine, and 82 subjects were enrolled in the ensuing study. The immunogenicity and reactogenicity of the second dTpa booster given at the 10-year follow-up point have been published earlier [17].

Of the 82 study subjects, 75 (64 women and 11 men) had blood samples available for genomic DNA isolation and genotyping.

All subjects had received 4 doses of Finnish diphtheria and tetanus toxoids and whole-cell pertussis vaccine at the ages of 3, 4, 5, and 24 months [14]. During the 10-year follow-up, there was no known clinically confirmed pertussis among study subjects.

The study protocol was approved by the joint commission on ethics of Turku University and Turku University Central Hospital, and written informed consent was obtained from the study subject prior to enrollment. The study was conducted in accordance with good clinical practice guidelines and the Somerset West 1996 revision of the Declaration of Helsinki.

**Laboratory Assays**

**Genotyping**

Genomic DNA was isolated from the blood with Qiagen QiAmp DNA Blood Mini Kit 250 (Qiagen). Genotyping of the *tlr4* gene polymorphism in Arg299Gly was done with pyrosequencing. Before pyrosequencing, polymerase chain reaction (PCR) was performed as described by Ahmad-Nejad [18]. The PCR program was slightly modified: denaturation for 3 minutes at 95°C, followed by 44 cycles for 30 seconds at 95°C, 45 seconds at 60°C, and 1 minute at 72°C and by final extension for 5 minutes at 72°C. The PCR product was verified with agarose gel electrophoresis; the specific band was 102 base pairs. PCR primers were as follows: forward, 5′-TGA CCA TTG AAG AAT TCC GAT TA-3′; reverse, 5′-biotin-CAG GGA AAA TGA AGA AGA AAC A-3′.

Pyrosequencing was performed as described elsewhere [18, 19] with minor modifications. Reaction contained 20 μL of the PCR products, 3 μL of Streptavidin Sepharose High Performance particles (GE Healthcare), 37 μL of binding buffer (10 mmol/L Tris–HCl, 2 mol/L sodium chloride, 1 mmol/L ethylenediaminetetraacetic acid, and 0.1% Tween 20; pH 7.6), and 20 μL of water. After incubation of the mixture for 5 minutes at room temperature with mild shaking, immobilized DNA was treated with 70% ethanol for 10 seconds, 0.2 mol/L sodium hydroxide for 10 seconds, and wash buffer (10 mmol/L Tris; pH 7.6) for 10 seconds. These treatments were done using the vacuum prep tool for the PSQ96MA system (Pyrosequencing Ab). After this, DNA was transferred to wells containing 2 μL of sequencing primer (5′-CTT AGA CTA CCT CGA TG-3′) and 39 μL of annealing buffer (20 mmol/L Tris-acetate and 2 mmol/L magnesium acetate; pH 7.6). After the mixture was incubated at 80°C for 2 minutes and cooled to room temperature, 5 μL of enzyme mixture and 5 μL of substrate mixture (PyroMark Gold Q96 SQA Reagents, Qiagen) were added to the wells. Nucleotides (PyroMark Gold Q96 SQA Reagents, Qiagen) were added to the reagent cartridge. The run was performed with the PSQ96MA machine (Pyrosequencing Ab). Sequential addition of the nucleotides was used in the following order: GATATATGACTATATG.

**Antigen-Specific Antibody Measurement**

IgG antibodies to pertussis toxin, filamentous hemagglutinin (FHA), and pertactin and antibodies to diphtheria and tetanus toxoids were measured earlier by means of enzyme-linked immunosorbant assay, and the results have been published.
elsewhere [14–17]. The detection limits were 5 IU/mL for pertussis antibodies and 0.1 IU/mL for the diphtheria and tetanus antibodies.

A positive antibody response to pertussis antigens was defined as seroconversion, for initially seronegative subjects (<5 IU/mL), or as a ≥2-fold increase in antibody values, for initially seropositive subjects [14]. This definition has been used throughout the study [14–17].

Statistical Analyses
The Mann-Whitney U test, the unpaired t test, and the Fisher exact test were used for statistical analyses with Prism GraphPad software, version 4.0 (GraphPad Software). Differences were considered statistically significant at $P < .05$ (2-tailed).

RESULTS
Genotyping of TLR4
Genotyping of TLR4 Asp299Gly was successful in DNA samples from 74 study subjects but unsuccessful in a DNA sample from 1 subject. Of these 74 study subjects, 63 (85.1%) were AA homozygotes (referred to as wild type), 10 (13.5%) were AG heterozygotes, and 1 (1.4%) was a GG homozygote. Because the number of study subjects was limited, AG heterozygotes and GG homozygotes were combined into a single group classified as having TLR4 variants. There was no difference in the frequency of TLR4 genotypes between sexes ($P > .99$).

TLR4 Asp299Gly Polymorphism and IgG Antibodies to dTPa Antigens
The differences in the positive antibody responses against pertussis antigens in geometric mean concentrations (GMCs) of these antibodies were compared between the AA homozygotes (wild type) and the AG heterozygotes and GG homozygotes (variants). Before the initial vaccination 10 years ago, 28 (37.8%) of the 74 subjects had undetectable anti–pertussis toxin antibodies. Of the 28 subjects without detectable anti–pertussis toxin antibodies, 22 carried wild-type TLR4 and 6 carried variants of TLR4 ($P = .313$). After the original booster vaccination 10 years before, 60 subjects (81%) developed a positive antibody response to pertussis toxin, whereas 14 (19%) did not. The fold increase in antibody response to pertussis toxin after vaccination was significantly lower in subjects with TLR4 variants, compared with those with wild-type TLR4 ($P = .028$) (Figure 1). No significant difference in the fold increase to antibody responses to FHA and pertactin was observed between the 2 groups.

The antibody GMCs against all 3 pertussis antigens were compared between the subjects with wild-type TLR4 and those with variants (Table 1). Before the first booster dose given 10 years previously, the antibody GMCs to 3 pertussis antigens did not differ between the 2 groups. After the booster dose, however, the subjects with wild-type TLR4 had clearly higher antibody GMCs to all 3 antigens, compared with those with TLR4 variants (Table 1). For subjects having wild-type TLR4, antibody GMCs rose 8-fold for pertussis toxin, 13-fold for FHA, and 26-fold for pertactin, whereas for subjects having TLR4 variants, antibody GMCs rose 6-fold for pertussis toxin, 5-fold for FHA, and 12-fold for pertactin. Despite the limited number of subjects, at the 3-year follow-up evaluation, the antibody GMCs of 3 pertussis antigens were significantly higher in subjects with wild-type TLR4, compared with those with variant TLR4. Furthermore, the antibody GMCs to pertussis toxin and FHA of TLR4 variants had already decreased to the levels observed 3 years before the booster vaccination. For subjects with wild-type TLR4, however, antibody GMCs to pertussis toxin and FHA persisted for 5–10 years after the first booster vaccination. At year 10, a booster response to pertussis toxin after vaccination was noticed in 98.6% of the subjects. The only nonresponder to pertussis toxin had a genotype of AA in the TLR4 gene. There was no difference in the positive antibody responses or the antibody GMCs against tetanus and diphtheria between subjects with wild-type TLR4 and those with variants.

DISCUSSION
Vaccinations have been proven to be the best strategy for control and prevention of pertussis. High concentration of antibodies induced after vaccination is important for long-term protection against this disease. Various factors that are capable of modifying immune responses against pathogens are known. However, the role of these factors in inducing and maintaining antibodies by booster vaccination with
Table 1. Antibody Seropositivity Against Pertussis Antigens After Booster Doses of Diphtheria and Tetanus Toxoids and Acellular Pertussis Vaccine in Subjects With Wild-Type or Variant Toll-like Receptor 4

<table>
<thead>
<tr>
<th>Timing</th>
<th>TLR4 Wild Type</th>
<th>TLR4 Variant</th>
<th>P²</th>
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<tbody>
<tr>
<td></td>
<td>No. of Subjects</td>
<td>GMC (95% CI)</td>
<td>Seropositivity, % (95% CI)</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before initial vaccination 10 years ago</td>
<td>62</td>
<td>11.8 (8.5–17.3)</td>
<td>64.5 (52.6–76.4)</td>
</tr>
<tr>
<td>Month 1</td>
<td>63</td>
<td>100.9 (71.9–141.5)</td>
<td>93.7 (87.7–99.7)</td>
</tr>
<tr>
<td>Year 3</td>
<td>42</td>
<td>21.3 (15.2–29.8)</td>
<td>90.5 (81.6–99.4)</td>
</tr>
<tr>
<td>Year 5</td>
<td>42</td>
<td>14.1 (10.1–19.6)</td>
<td>81 (69.2–92.9)</td>
</tr>
<tr>
<td>Year 10, after booster</td>
<td>61</td>
<td>9.2 (6.9–12.2)</td>
<td>63.5 (51.4–75.6)</td>
</tr>
<tr>
<td>61</td>
<td>96.1 (77.5–119)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Filamentous hemagglutinin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before initial vaccination 10 years ago</td>
<td>61</td>
<td>63.6 (47.9–84.5)</td>
<td>98.4</td>
</tr>
<tr>
<td>Month 1</td>
<td>61</td>
<td>802.4 (605.4–1064.0)</td>
<td>100</td>
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<tr>
<td>Year 3</td>
<td>42</td>
<td>203.8 (163.1–254.8)</td>
<td>100</td>
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<tr>
<td>Year 5</td>
<td>44</td>
<td>130.7 (106.7–160.0)</td>
<td>100</td>
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<td>Year 10, after booster</td>
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<td>66.6 (54.8–80.9)</td>
<td>100</td>
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<td>753.0 (611.5–927.2)</td>
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<tr>
<td>Pertactin</td>
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<tr>
<td>Before initial vaccination 10 years ago</td>
<td>61</td>
<td>15.8 (11.0–22.6)</td>
<td>79.4</td>
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<tr>
<td>Month 1</td>
<td>61</td>
<td>407.1 (260.2–637)</td>
<td>96.8</td>
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<tr>
<td>Year 3</td>
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<td>Year 5</td>
<td>44</td>
<td>84.0 (57.3–123.0)</td>
<td>100</td>
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<td>Year 10, after booster</td>
<td>61</td>
<td>37.2 (27.5–50.3)</td>
<td>96.8</td>
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<tr>
<td>Year 10, after booster</td>
<td>61</td>
<td>600.1 (488.8–736.7)</td>
<td>100</td>
</tr>
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Seropositivity against pertussis antigens is defined as an antibody concentration of $\geq 5$ EU/mL.

Abbreviations: CI, confidence interval; GMC, geometric mean antibody concentration.

* GMC comparison between wild type and variant.
acellul ar pertussis vaccine has not been investigated. We have conducted a 10-year follow-up study on the persistence of pertussis-specific antibodies after booster immunization of adolescents aged 11–13 years with a tricomponent acellular pertussis vaccine [14–17]. Subjects in the same cohort received a second booster dose of the vaccine 10 years after the first booster vaccination, and their antibody response was assessed 1 month later. This study provided us a unique opportunity to evaluate whether TLR4 polymorphism can influence antibody response and persistence after vaccination with acellular vaccines. Our results are the first to suggest that the polymorphism of TLR4 might be associated with altered response to acellular pertussis vaccine in adolescents. There was no difference in the antibody response for tetanus and diphtheria between the subjects with wild-type TLR4 and those with variants.

The frequency of TLR4 Asp299Gly polymorphism was 15% in this study, which is consistent with findings in other studies carried out in Finland [20, 21]. Banus et al [22] examined the association between haplotype tagging of SNPs in TLR4 and the pertussis toxin-specific IgG response after whole-cell vaccination in 1-year-old Dutch children. They found that a lower titer of pertussis toxin IgG antibodies was associated with the minor allele of the TLR4 promoter, indicating a role for TLR4 in the antibody response to whole-cell pertussis vaccination. The same group also observed in mice that TLR4 affected the antibody response after vaccination with both the lipopolysaccharide-containing whole-cell pertussis vaccine and the lipopolysaccharide-free acellular vaccine [23]. Another recent study also observed that TLR4-deficient mice had a lower concentration of IgG antibodies after immunization with the whole-cell pertussis vaccine, compared with wild-type mice [11]. TLR4 is the receptor for lipopolysaccharide and can thus influence innate immune response to B. pertussis present in the whole-cell vaccine. However, dTpa does not contain lipopolysaccharide. Interestingly, we found a significant difference between subjects with wild-type TLR4 and those with variants when comparing the response and persistence of antibodies against all pertussis antigens. It has been shown that TLR4 is also a receptor for pertussis toxin [9, 10]. Our finding suggests that TLR4 might also interact with pertactin and FHA of B. pertussis. The substitution in TLR4 Asp299Gly is located in the extracellular domain of the receptor, causing impaired recognition of its ligand [12]. Further studies are needed to clarify whether there is a physical recognition by TLR4 of pertactin and FHA. Moreover, use of aluminum as an adjuvant is known to affect antibody responses, especially to pertussis toxin [24]. Therefore, the possible involvement of aluminum in modulating the antibody response by TLR4 after vaccination with acellular pertussis vaccine cannot be excluded.

Individual variations in antibody responses are recognized after both primary and booster vaccinations with dTpa [6–8]. From a previous dTpa study performed in our laboratory, we found that subjects with detectable level of antibodies to pertussis toxin and pertactin before vaccination had higher antibody concentrations 1 month and 5 years after vaccination than did subjects with undetectable amounts of antibodies before the first booster vaccination [15]. Ten years after the initial booster vaccination, the concentrations of antibodies to pertussis antigens have returned to almost the same levels as those before the first booster [17]. Although antibody GMCs to pertussis toxin were similar before the first and second boosters, a significant difference in the fold increase of antibodies in subjects with wild-type TLR4 and those with variant TLR4 was observed only after the first booster. One explanation for the difference observed might be the difference in memory for pertussis antigens. It has been shown that pertussis antigen–specific memory B cells can be detected even many years after vaccination, despite the low level of antibodies in the plasma [25, 26]. These specific B cells provide a rapid booster response when pertussis antigens are encountered again. Toll-like receptors are also known to be involved in the initiation of adaptive immune responses, such as induction of cytokine expression and maturation of dendritic cells. It remains to be shown how the TLR4 mutation affects cytokine production after acellular pertussis vaccination in humans.

There are certain limitations in this study. The first was small number of subjects: 75. Second, the sex distribution was not balanced, with 11 male and 64 female subjects. Most of the male subjects who participated in the original study were ineligible for this study, because they had received diphtheria and tetanus vaccine during national military service [17]. This disequilibrium, however, had no effect on the frequencies of different SNPs in TLR4. In addition, there was no difference in antibody levels between sexes. Third, in this study we did not examine the TLR4 Thr399Ile polymorphism. It is known that Asp299Gly and Thr399Ile TLR4 polymorphisms have generally cosegregated in white individuals [12]. In addition to TLR4 polymorphisms, it has been shown that SNPs in a variety of immune response genes, such as those encoding HLA and mannose-binding lectin, can modulate antibody production and persistence after immunizations with viral and bacterial vaccines [13, 27–31]. Therefore, the effect of polymorphisms in other immune response genes cannot be excluded. Furthermore, factors such as age or race of the vaccine recipients, quantity of vaccine antigens, and natural (silent) boosters can also contribute to antibody responses and persistence after vaccination [26, 32].

Because individuals with the TLR4 polymorphism may have reduced antibody responses and short-term persistence of antibodies, and because the prevalence of this polymorphism is not uncommon, the frequency of TLR4 polymorphism in a specific population might in the future become an additional parameter when designing pertussis vaccination policies for
that population. Moreover, it remains to be shown whether individuals with the TLR4 polymorphism in this study have an increased incidence of pertussis.

In conclusion, this study showed that TLR4 Asp299Gly polymorphism might be capable of modulating the production and persistence of antibodies after pertussis booster vaccination in adolescents. Our results warrant further studies of the effect of TLR4 polymorphism on antibody production and persistence after acellular vaccinations in different age groups and populations.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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