

Specific IgE to Respiratory Allergens and IgG Antibodies to *Toxoplasma gondii* and *Streptococcus pneumoniae* in Norwegian Military Recruits

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Abstract

Declining exposure to infections has been implicated as a cause for the rising trend of allergy. Gastrointestinal infections, in particular, have been suggested to have a protective effect against allergy development. In contrast, there are only limited data available regarding the effect of respiratory tract infections with encapsulated bacteria on allergy development. We investigated the association between IgG antibodies against the gastrointestinal parasite *Toxoplasma gondii* and the bacterium *Streptococcus pneumoniae* and specific IgE to common allergens in Norwegian military recruits. IgG antibodies to *T. gondii* and to a 23-valent pneumococcal polysaccharide vaccine (Pneumovax®) were determined by ELISA. Specific IgE to common airborne allergens was determined by Phadiatop®. Individuals with Phadiatop values from class 0–2 were classified as non-atopic ($n = 419$), while those with class 3–6 were classified as atopic ($n = 201$). No significant difference was observed in IgG antibody levels to pneumococcal polysaccharides between atopic and non-atopic recruits, whereas seropositivity to *T. gondii* was found to be less frequent among the atopic recruits (odds ratio, 0.37; 95% CI, 0.17–0.81; $P = 0.01$). Hence, in Norwegian recruits, the serological response to a gastrointestinal pathogen, but not to the respiratory encapsulated bacteria, was found to be associated with a lower probability for being sensitized according to the criteria used. The present study conforms to the hypothesis that reduced rates of infection with certain microorganisms may be associated with an increased risk of allergic sensitization.

Introduction

Allergy increased in the Western countries during the last four decades [1, 2]. Western lifestyle has been found to be associated with the increase in the prevalence of allergy. One lifestyle factor implicated is improved hygiene. The hygiene hypothesis was first described by Strachan in 1989 and stated that the increase in allergy was caused by the occurrence of infections [3]. The suggested mechanism has developed from being attributed to an altered Th1/Th2 balance to the reduced induction of regulatory T (Treg) cells [4, 5]. Reduced activity of T regulatory cells because of less stimulation by microorganisms may lead to either Th1- or Th2-dependent inflammatory disorders depending on the individual's

Th1/Th2 predisposition. Inflammatory T_H17 cells may also have implications in relation to the hygiene hypothesis [6]. The hygiene hypothesis has later broadened from the original concept of lack of infections to include exposure to both pathogenic and non-pathogenic microbes and components of these [7]. However, there is still a controversy as to which microbes and microbial loads are necessary for priming for an optimal level of anti-allergic mechanisms including regulatory T cells.

According to the revised hygiene hypothesis, gastrointestinal tract infections play a role in the development of allergy [7]. *Toxoplasma gondii* is one candidate that has been proposed to have a protective effect in this context. However, conflicting results have been reported regarding the association between toxoplasmosis and allergy [8–13].

There are also conflicting findings regarding the link between respiratory infections and allergy, as reviewed in [7, 14, 15]. The studies reviewed mainly compared rates of viral infections and allergy, and little data exist regarding respiratory infections by encapsulated bacteria and allergy [16–18]. In addition to *T. gondii*, we investigated markers of exposure to the common encapsulated bacterium *Streptococcus pneumoniae* in relation to allergic sensitization, which to our knowledge has not been done previously. Pneumococci frequently cause colonization and infection in the airways (acute otitis media, conjunctivitis, sinusitis and pneumonia) [19].

Serological screening was performed in 620 Norwegian military recruits who were divided into two groups depending on their atopy status as defined by Phadiatop (Pharmacia Diagnostics, Uppsala, Sweden) results. The aim of the present study was to investigate the IgG antibody response to *T. gondii* and to 23 pneumococcal serotypes combined in a 23-valent polysaccharide vaccine, in relation to the levels of specific IgE to common airborne allergens.

Material and methods

Study population. The present investigation consists of a retrospective case–control study in which a total of 201 cases and 419 controls were defined according to their atopy status (Table 1). The study population was military recruits from Norway. Sera were collected in 2003. Age and gender were reported from a random group of 329 of the 620 recruits. The mean age ($\pm$ SD) was 19.6 ($\pm$ 1.6) years in the whole group, and similar results were found in both men and women with a mean age of 19.6 ($\pm$ 1.6) years and 19.8 ($\pm$ 0.3) years, respectively. The gender distribution of this random group was 315 men (95.7%) and 14 women (4.3%). The population was allocated to two groups (cases and controls) according to their specific IgE (Phadiatop) results. Participants with Phadiatop class 0–2 (<0.35 – 3.49 kU/l) for the purpose of the study were classified as non-atopic, whereas subjects with Phadiatop class 3–6 (3.50 to >100 kU/l) were classified as atopic.

Allergy markers analyzed by UniCAP. Total IgE (Art. No. 10-9251-01) and specific IgE to the most common inhalant allergens (Phadiatop, Art. No. 14-4405-35) were

analyzed in all sera by UniCAP 100 (Pharmacia Diagnostics). Total IgE and specific IgE are expressed in kU/l.

Infection markers. All the sera were analyzed for IgG antibodies to *T. gondii* and to the 23-valent pneumococcal polysaccharide vaccine by ELISA.

The kit for analyzing IgG antibodies to *T. gondii* was purchased from Bio-Rad (Platelia Toxo IgG TMB, Product No. 72741, France). The ELISA kit was used according to the manufacturer's protocol. Shortly described, the diluted sera (in duplicates) and calibrators were added ($200\text{ }\mu\text{l}$ /well) and incubated for 1 h at $37\text{ }^{\circ}\text{C}$. Next, after washing, $200\text{ }\mu\text{l}$ of conjugate (murine monoclonal antibody to human gamma chains coupled with horseradish peroxidase) was distributed to each well and incubated for 1 h at $37\text{ }^{\circ}\text{C}$. Enzyme development solution was dispensed into each well after washing ($200\text{ }\mu\text{l}$) and developed in the dark for 30 min at room temperature. Stopping solution was added ($100\text{ }\mu\text{l}$ /well) and optical density (OD) was measured at $450/650\text{ nm}$ on a MRX Microplate Reader connected to a PC using Revelation software (Dynatech Laboratories, Chantilly, VA, USA) for instrument operation and calculations. The results are expressed as positive or negative. *T. gondii* values $>9\text{ IU/ml}$ represent, according to the manufacturer, a significant level of antibodies, and were considered positive. The continuous variables from *T. gondii* test were used for statistical correlation analysis.

The protocol to analyze IgG antibodies to the 23 pneumococcal polysaccharide serotypes combined in the pneumococcal vaccine has been described in detail previously [20]. In short, polystyrene plates (High Binding Polystyrene Microtiter Plates, Thermo Labsystems, Franklin, MA, USA) were coated with the vaccine (Pneumovax[®]; Aventis Pasteur MSD A/S, Copenhagen, Denmark) diluted to $2.3\text{ }\mu\text{g/ml}$ in PBS pH 7.4 ($100\text{ }\mu\text{l}$ /well). The plates were incubated for 2 h at $37\text{ }^{\circ}\text{C}$ and afterwards stored in a refrigerator ($+4\text{ }^{\circ}\text{C}$) until usage (no more than 2 months). Next, the test sera, the standard sera and the positive control were neutralized with pneumococcal C-polysaccharide (PnC) (diluted 1:10 in $10\text{ }\mu\text{g/ml}$ PnC) and left for incubation for 1 h. Thereafter, the test sera and the standard sera were further diluted. The test sera were diluted 1:100 or 1:400 and distributed onto the plates in duplicates. Serial twofold dilutions of the standard (from 1:400 to 1:51,200) similarly were added in duplicates ($100\text{ }\mu\text{l}$ /well). The standard and the positive control were two separate pools of sera from employees at the Norwegian Institute of Public Health. The incubation period was 2 h at $37\text{ }^{\circ}\text{C}$. As secondary antibody, goat anti-human IgG (Product No. A9544; Sigma, St Louis, MO, USA) was diluted 1:30,000 ($100\text{ }\mu\text{l}$ /well) and used. The plates were incubated overnight at $18\text{ }^{\circ}\text{C}$. The next day, the plates were developed at room temperature for 20–40 min with p-nitrophenyl phosphate (Sigma 104[®] Phosphatase substrate; Sigma)

Table 1 Characterization of the study population.

Groups	Atopy, $n = 201$	No atopy, $n = 419$
Age, year $\pm$ SD	19.86 (± 0.36)	19.62 (± 1.67)
Total IgE (kU/l)		
Median (25, 75 percentiles)	142.00 (60.95, 350.5)	22.30 (9.88, 48.00)
Specific IgE (Phadiatop [®] , kU/l)		
Median (25, 75 percentiles)	25.67 (12.64–54.56)	0.35 (0.35–0.35)

diluted in 10% of diethanolamine buffer (pH 9.8, 37 °C) to 1 mg/ml. OD was measured at 405 nm and the results were expressed in AU (arbitrary units)/ml.

Statistical analysis. Serum IgG antibody levels to the pneumococcal vaccine were analyzed with Mann–Whitney *U*-test for comparison between Phadiatop non-atopic and atopic recruits. Fisher's exact test was used for analyzing IgG antibodies to *T. gondii* in the different groups, and the results are described by odds ratio (OR). Spearman rank order correlation (coefficient ρ) was used to correlate allergy and infection markers. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; <http://www.graphpad.com>). *P*-values below 0.05 were considered statistically significant and all tests were two-sided.

Results

Allergy markers

The median level (25th and 75th percentiles) of total IgE in the atopic recruits was 197.0 kU/l (91.4–393.5), whereas in non-atopic recruits it was 27.1 kU/l (12.7–67.5), giving a significant difference between the groups ($P < 0.0001$). Atopy was defined by Phadiatop class 3–6, and a positive correlation was found between total IgE and specific IgE levels by Phadiatop in the atopic recruits ($\rho = 0.681$, $P < 0.0001$). A weak correlation was observed between total IgE and Phadiatop in the non-atopic recruits ($\rho = 0.366$, $P < 0.0001$).

Infection markers

The prevalence of IgG positivity against *T. gondii* was higher in non-atopic recruits than in atopic recruits ($P = 0.01$) (Table 2). The OR of belonging to an atopic group with a positive *T. gondii* test was 0.37 (95% CI, 0.17–0.81).

There was no statistically significant difference in IgG antibody response to the pneumococcal vaccine between the atopic and non-atopic groups ($P = 0.752$) (Fig. 1). The median (25th and 75th percentiles) of IgG antibodies' response to the pneumococcal vaccine in the atopic

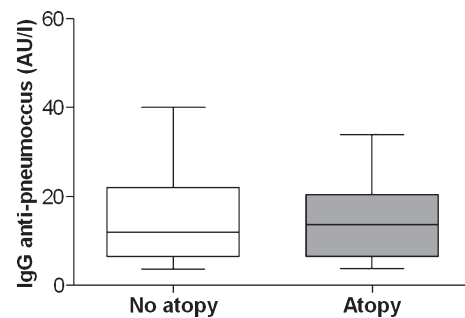


Figure 1 IgG antibody response to 23-valent pneumococcal vaccine (Pneumovax®). No difference was observed in IgG antibody levels to the pneumococcal vaccine between atopic and non-atopic recruits.

recruits was 13.6 AU/ml (6.5–20.4), while for non-atopic recruits it was 12.0 AU/ml (6.5–22.1).

There was no statistically significant correlation of atopy marker levels (total IgE and specific IgE) with infection marker levels in any individual or the combined groups of recruits assuming the variables are continuous.

Discussion

We found that while the presence of sensitization according to our definition (Phadiatop class 3–6) was inversely related to a serological marker of past infection with the gastrointestinal pathogen *T. gondii* in Norwegian military recruits, there was no association between sensitization and antibody levels against the airway pathogen *S. pneumoniae*.

The findings in prior studies on the relationship between *T. gondii* and atopy have been contradictory [8–13]. In accordance with our findings, Matricardi *et al.* observed a lower prevalence of *T. gondii* infection in atopic patients as compared with healthy controls in two separate studies [9, 10]. The first study was performed in Italian military cadets, which represented a population similar to the one in our study [10]. Atopic participants were defined according to their Phadiatop values. The second study examined patients living in USA and registered in a public database. These participants answered questionnaires about asthma and hay fever, both of which were found to be less frequent in subjects seropositive for hepatitis A virus (HAV), *T. gondii* and herpes simplex virus 1 as compared with seronegative subjects. Linneberg *et al.* also found that seropositivity to two to three markers of poor hygiene (hepatitis A virus, *Helicobacter pylori* and *T. gondii*) was associated with lower prevalence of atopy in a Danish material [8]. Atopy was defined by a positive test result for specific IgE to at least one of six inhalant allergens [8]. Taken together, these three studies and our study support the hypothesis that a higher exposure to *T. gondii* reduces sensitization on the population level. In contrast, no association was found between

Table 2 The relationship between *T. gondii* seropositivity and atopy. Atopy is defined as Phadiatop® values > 3.49 (class 3–6). No atopy is defined as Phadiatop values ≤ 3.49 (class 0–2).

	Atopy	No atopy	Total
IgG anti- <i>T. gondii</i>			
Yes (%)	8 (4.0)	42 (10.0)	50 (8.1)
No (%)	193 (96.0)	377 (90)	570 (91.9)
Total	201	419	620

seropositivity to *T. gondii* and atopy by Birgisdottir *et al.*, Radon *et al.* and Bodner *et al.* [11–13]. However, these studies used a less strict definition for atopy than the present study. Atopy was defined by IgE levels >0.35 kU/l [11, 13] or >0.7 kU/l [12]. In the present study, atopy was defined by Phadiatop values >3.49 kU/l (class 3–6). Previously, it has been shown that the rate of respiratory allergic disease increases linearly with the increase in specific IgE (Phadiatop) [21, 22]. We therefore wanted to use a higher cut-off value associated with a high probability of clinical allergy symptoms. However, the cut-off value used represents an operational definition only, and certainly some individuals having been defined as non-atopic in our study may have clinical symptoms of allergy, and likewise some individuals having been defined as atopic may not have clinical symptoms. Moreover, in our study, no statistically significant (inverse) relationship between atopy and infection marker was found if a lower cut-off value of 0.35 kU/l was used in the analysis (data not shown). This suggests that infections, rather than preventing sensitization entirely, reduce the strength to levels less associated with clinical disease.

Another difference between the studies was the distribution of gender and age. While the present study and the Italian study of recruits [10] build on data from around 20-year-old young adults, mainly men, both genders were included and the mean age was higher in the three studies that did not find any association between *T. gondii* and atopy. Waning of the antibody response to *T. gondii* with increasing age might, therefore, hypothetically be an explanation for the discrepancy between the findings in different studies. Similarly, the absence of a relationship between atopy and IgG antibodies to *S. pneumoniae* might be due to the short duration of T-cell independent, non-boosterable serological response to pneumococcal polysaccharides. In contrast, we were able to observe a difference in serological response to *T. gondii* between our population groups, perhaps because this was a T-cell dependent response that induced more long-lasting immunity. It was then possible that we may have seen a reduction of atopy associated with anti-pneumococcal antibodies if we had been able to measure the response to pneumococcal protein antigens instead of polysaccharides.

There are limited data on respiratory tract infections with encapsulated bacteria in relation to the prevalence of sensitization and allergy. Previously, no association has been observed between *Haemophilus influenzae* type B vaccination and atopy development [16–18], but vaccination may not represent an appropriate stimulation of the immune system in our context. To our knowledge, the present study was the first to investigate IgG antibodies against *S. pneumoniae* in relation to allergy markers. *S. pneumoniae* is a Gram-positive diplococcus with a

polysaccharide capsule and causes infections like otitis media, conjunctivitis, sinusitis and pneumonia. The polysaccharide capsule is the most important virulence factor of the pneumococcus. Accordingly, the pneumococci are classified into serotypes based on the polysaccharide capsule. The ELISA applied [20], based on the 23-valent pneumococcal polysaccharide vaccine as antigen, has been in routine use at the Norwegian Institute of Public Health. The 23-valent pneumococcal polysaccharide vaccine covers 80–90% of antigens of the serotypes responsible for systemic pneumococcal disease in Europe and United States [23], and we used antibody levels against the vaccine as an exposure marker. In the present study, we did not observe a difference in anti-pneumococcal IgG antibodies between atopic and non-atopic populations, suggesting that both groups had similar exposure to *S. pneumoniae*. One weakness of our study was the inability to distinguish between *S. pneumoniae* infection and non-infectious exposure, because colonization without an infection may elicit an antibody response [24]. However, according to the revised hygiene hypothesis, both infectious and non-infectious microbial exposure can have a protective effect on the development of allergy [7].

Our study was conducted in a relatively homogeneous group of people. Although this is generally a strong aspect of a study design, it could, at the same time, raise the question whether generalization of the conclusions is justified. Another limitation of the present study was the possibility that men who were strongly allergic may have been rejected as military recruits. It was uncertain whether the difference in microbial exposure would be greater or lesser if severely allergic individuals were included. Our findings with regard to the effect of different cut-off levels for specific IgE against *T. gondii* suggest that the difference in anti-microbial IgG would be clearer among the strongly allergic subjects. However, one might also reason that the influence from genetic factors would be very strong in the highly allergic individuals and might overrun the putative protective effect from infection.

An alternative explanation to the apparent protective effect of *T. gondii* in relation to atopy is that atopic individuals have a defect in their immune response to T cell-dependent microbial antigens [25]. This would result in weaker and more short-living immune responses against microbial protein antigens in atopic subjects, creating the impression that allergic individuals have been less exposed to microbes. This explanation would also be in agreement with the observed absence of a difference in T-cell independent anti-pneumococcal polysaccharide antibodies between atopic and non-atopic subjects.

In the present study, we found an inverse association between seropositivity to *T. gondii* and allergic sensitization in Norwegian military recruits. In contrast, no association was found between IgG antibodies to

S. pneumoniae and allergic sensitization. The findings support the notion that gastrointestinal infections may protect against allergic sensitization.

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