Autoantibodies against ganglioside GM3 are associated with narcolepsy-cataplexy developing after Pandemrix vaccination against 2009 pandemic H1N1 type influenza virus

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A B S T R A C T

Following the mass vaccinations against pandemic influenza A/H1N1 virus in 2009, a sudden increase in juvenile onset narcolepsy with cataplexy (NC) was detected in several European countries where AS03-adjuvanted Pandemrix vaccine had been used. NC is a chronic neurological disorder characterized by excessive daytime sleepiness and cataplexy. In human NC, the hypocretin-producing neurons in the hypothalamus or the hypocretin signaling pathway are destroyed by an autoimmune reaction. Both genetic (e.g. HLA-DQB1*0602) and environmental risk factors (e.g. Pandemrix) contribute to the disease development, but the underlying and the mediating immunological mechanisms are largely unknown.

Influenza virus hemagglutinin is known to bind gangliosides, which serve as host cell virus receptors. Anti-ganglioside antibodies have previously been linked to various neurological disorders, like the Guillain-Barre syndrome which may develop after infection or vaccination. Because of these links we screened sera of NC patients and controls for IgG anti-ganglioside antibodies against 11 human brain gangliosides (GM1, GM2, GM3, GM4, GD1a, GD1b, GD2, GD3, GT1a, GT1b, GQ1b) and a sulfatide by using a line blot assay. Samples from 173 children and adolescents were analyzed: 48 with Pandemrix-associated NC, 20 with NC without Pandemrix association, 57 Pandemrix-vaccinated and 48 unvaccinated healthy children. We found that patients with Pandemrix-associated NC had more frequently (14.6%) anti-GM3 antibodies than vaccinated healthy controls (3.5%) (P = 0.047). Anti-GM3 antibodies were significantly associated with HLA-DQB1*0602 (P = 0.016) both in vaccinated NC patients and controls. In general, anti-ganglioside antibodies were more frequent in vaccinated (18.1%) than in unvaccinated (7.3%) individuals (P = 0.035). Our data suggest that autoimmunity against GM3 is a feature of Pandemrix-associated NC and that autoantibodies against gangliosides were induced by Pandemrix vaccination.
1. Introduction

In 2009–2010, after the mass vaccinations against pandemic influenza A/H1N1 virus, a sudden increase in juvenile onset narcolepsy was detected in Finland and in Sweden, where AS03-adjuvanted Pandemrix vaccine (GlaxoSmithKline Biologicals, Wavre, Belgium) was used [1–3]. After an extensive review, The European Medicines Agency confirmed the existence of the association between the Pandemrix vaccination and an increased incidence of narcolepsy in Finland and Sweden [4]. Epidemiological studies made in Ireland, U.K., France and Norway, further supported the association [5–8]. The epidemiological reports of the six countries indicated a 6.5–14.4-fold increased risk of narcolepsy among Pandemrix-vaccinated children and adolescents compared to those not vaccinated [1–3,5–8]. A recent report from Quebec, Canada, indicated an increased risk of narcolepsy (approximately one case per million) also in young individuals (<20 years of age) vaccinated with Aeraspanrix (GlaxoSmithKline Biologicals, Wavre, Belgium) [9]. Pandemrix and Aeraspanrix are very similar H1N1 pandemic vaccines from the same manufacturer, but have been produced in different facilities (Dresden and Quebec, respectively). Recently, they were shown to contain antigenic differences, which may be relevant to their immunogenicity [10,11]. Specifically, the presence of the influenza virus nucleoprotein, which may have antigenic cross-reactivity with the hypocretin/orixin receptor type 2, has been implicated in triggering an autoimmune response [12].

Human narcolepsy with cataplexy (NC) is a disabling chronic neurological disorder characterized by excessive daytime sleepiness, cataplexy and disturbed nocturnal sleep [13,14]. It is usually sporadic and a rare disorder of young adults. The etiology of NC is unknown, but it is considered as an autoimmune disorder [14]. Post-mortem studies of NC patients have shown a selective loss of hypocretin (hcrtr) producing neurons in the dorso-lateral hypothalamus [15]. Characteristic to NC is a low level (<110 pg/ml) or an absence of hcrtr-1 in the cerebrospinal fluid (CSF) [16,17]. Hcrtr-1 and -2, also called orexin A and B, respectively, are excitatory neuropeptides involved in sleep–wake regulation, food intake and pleasure-seeking behavior [18].

NC has an exceptionally strong association with the human major histocompatibility (MHC) class II antigen DQB1*0602. As many as 98% of NC patients carry this MHC allele, whereas in the general population its incidence is in the range of 12–38% [14,19]. Thus, the DQB1*0602 allele appears to be an almost necessary, but not a sufficient factor for the development of narcolepsy. In addition to HLA, recent genome-wide association studies have revealed associations of narcolepsy with several other immunologically important gene loci (e.g. T cell receptor alpha, purinergic receptor P2RY11, cathepsin H and TNFSF4, also called OX40L) [14,19]. All these indicate a strong role for the immune system in the development of NC.

In addition to Pandemrix vaccination, other environmental factors, such as Streptococcus pyogenes or seasonal influenza A infections, have been associated with the onset of narcolepsy [14,19]. In China, after the 2009 H1N1 pandemic, a 3.3-fold increase in juvenile narcolepsy cases was reported among unvaccinated people suggesting an association of NC with the pandemic A/H1N1 virus infection itself [20]. However, in Finland no such association was found. In the study of Melén et al. [21] patients with NC after Pandemrix vaccination did not show influenza A/H1N1 virus infection specific antibody responses to the NS1 protein, unlike individuals who were infected by the virus.

Autoimmune diseases linked to MHC class II antigens imply specific T cell-mediated immunity as the cause of the disease. T cell-mediated autoimmune is frequently accompanied by specific antibody responses against the target antigens. These can be used conveniently for diagnosis and often also for analysis of the pathogenetic mechanisms of the disease. Antibodies against Tribbles homologue 2 (Trib2), a protein expressed by hypocretin producing neurons (and by other neurons, as well), have been detected in 14–26% of NC cases [22–24], especially within few years of the onset of cataplexy [24]. However, a recent Swedish study could not confirm the association of anti-Trib2 antibodies with narcolepsy [25]. In a previous study, no autoantibodies against prepro-hypocretin nor its cleavage products nor hcrtr-receptors were found in narcolepsy patients [26]. A recent study reported autoantibodies to hypocretin receptor type 2, that were cross-reactive with a neuropeptide peptide from the Pandemrix vaccine in 17/20 individuals with narcolepsy [12]. However, antibodies were also found in some healthy individuals (11/20) and in 5/20 persons who had had a pandemic A/H1N1 infection.

Recent immunohistochemical studies with NC patient serum or CSF and rat and mouse brain sections revealed reactivity with brain epitopes in approximately with 1/3 of NC patients [27,28], but also in patients with other sleep disorders, and in some healthy controls [27]. In two studies, a changed sleep behavior, either fragmented sleep [27] or narcolepsy-like episodes [29] were detected in mice and rats when purified IgGs from NC patients’ sera were injected intracerebroventricularly (icv) to mice or rats. No changes were detected with a control sera. Although the results of these two studies differ, they both suggest that antibodies play a role in NC.

To our knowledge, narcolepsy has not been associated with any vaccines before Pandemrix. However, Guillain–Barré syndrome (GBS), which is a post-infectious or post-vaccination neuropathy, has been associated with various vaccinations e.g. with swine flu vaccination in the 70’s. GBS is characterized by neuromuscular paralysis and cross-reactive anti-ganglioside antibodies (AGAs) [30]. In GBS following Campylobacter jejuni infection, ganglioside-like bacterial lipopolysaccharides have induced AGAs cross-reactive with neuronal gangliosides [31]. Various forms of GBS exist with different clinical features, depending on which AGAs are involved and which gangliosides are affected. Some patients with GBS have been reported to have low CSF hcrtr-1 levels, similar to NC [32].

Gangliosides are sialic acid-containing glycosphingolipids that are important constituents of all plasma membranes. They are most abundantly present in the central nervous system (CNS) [33]. Gangliosides have essential roles in numerous cellular functions, such as in cell growth and differentiation, proliferation, adhesion, signaling and immune responses [34]. They function as immunosuppressive molecules on the cell surface, but can also serve as receptors for bacterial toxins (e.g. GM1 for the cholera toxin) and viruses (e.g. GM3 for influenza A H1N1). Gangliosides play important roles in neurological diseases, such as GBS, certain type of epilepsies and various gangliosidoses [35].

In 1976, during a mass vaccination campaign in the U.S. an increase in GBS following the receipt of the A/NI/1976/H1N1 swine flu vaccine was observed. The mechanism responsible for this association remained unknown. Nachamkin et al. [36], however, found that immunization with certain influenza vaccine preparations (including the 1976 vaccine) or with recombinant hemagglutinin proteins from different influenza A strains induced antibodies against GM1 ganglioside in mice [36]. They hypothesized that hemagglutinin-sialic acid complexes, could mimic the GM1 ganglioside and induce AGA production in the host. These results, however were not confirmed in other studies, which included the same vaccine preparations [37,38].

Due to the association of Pandemrix vaccination with narcolepsy, the similarity of the 2009 pandemic A/H1N1 strain with the 1976 strain, and the fact that gangliosides serve as receptor molecules for the H1N1 virus in humans, we asked the question whether Pandemrix vaccine could induce the production of AGAs. Thus, our
aim was to study whether AGAs are found in patients with NC associated with Pandemrix vaccination. We therefore screened AGAs against 11 human brain gangliosides and a sulfatide from the sera of NC patients and controls. Our results indicate that Pandemrix vaccine induced the production of antibodies against selected gangliosides. In particular, anti-GM3 antibodies were associated with NC related to Pandemrix vaccination, as well as with the disease predisposing HLA-DQB1*0602 allele.

2. Materials and methods

2.1. Patients, controls, and diagnostics

The present study comprises a total of 173 children/adolescents (Table 1), of whom 68 were narcolepsy patients and 105 healthy controls. Of the 68 narcolepsy patients, 48 were Finnish children who developed NC in 2009–2011 after receiving Pandemrix vaccination. They were recruited to the immunological narcolepsy study at the neurological outpatient clinics in Finnish hospitals during the year 2011. The diagnostics and clinical picture of Pandemrix-associated NC patients have been described in Ref. [3]. Our material is an unselected subgroup of these patients. Twenty patients out of 68 were Italian narcolepsy children/adolescents, who were not vaccinated with Pandemrix or with any other H1N1 vaccine preparation used during the 2009 pandemic. Of the 105 healthy controls, 57 were Pandemrix-vaccinated siblings of children with type 1 diabetes, recruited at the Finnish Diabetes Registry. In addition, 48 randomly selected elementary and junior high school students in Finland [39], not vaccinated with Pandemrix, were used as unvaccinated healthy controls. All Pandemrix-vaccinated individuals had received one dose of Pandemrix (a registered trademark of GlaxoSmithKline Biologicals, manufactured in Dresden, Germany) between October 2009 and August 2010. The study protocol was approved by the Ethics Committee of the participating hospitals and informed consents were given by the children and their families.

2.2. Serum and plasma samples from narcolepsy patients and healthy controls

Heparinized whole blood was drawn. HLA genotyping was performed with a panel of sequence-specific oligonucleotide probes as described previously [21]. Plasmas from Pandemrix-vaccinated narcolepsy patients and healthy controls were separated. The first plasma samples from 48 Pandemrix-vaccinated NC patients were collected during 1/2011–10/2012 and from vaccinated controls during 11/2009–7/2011. The follow-up samples from 14 Pandemrix NC patients were collected 16–26 months after the initial sampling. Sera from NC patients with no Pandemrix association were collected in Italy during 06/2004–09/2012. The sera from unvaccinated elementary and junior high school students in Finland, were collected in April 2004 [39], prior to the 2009 pandemic and stored at −70 °C. The characteristics of the narcolepsy patients, healthy controls and study groups are described in Table 1.

2.3. Anti-ganglioside antibody assay

Anti-ganglioside antibody reactivity (IgG or IgM) against 11 gangliosides (GM1, GM2, GM3, GM4, GD1a, GD1b, GD2, GD3, GT1a, GT1b, and GQ1b) and sulfatide of each serum and plasma samples (1:100 dilution) was tested with a line blot assay according to the manufacturer’s instructions (Generic Assays GmbH, Germany). In each test series a lot-specific positive control serum sample provided by the manufacturer was included in the analysis.

A sample was considered to be negative for a given specificity, if there was no coloration in the test line. The reactivity of the serum/plasma was considered to be clearly positive (+, ++, +++), if the color of the test line was equal or more intense than the band on the identification/evaluation template. If the test line could be discriminated from the background, but the color of the test line was less intense than the band on the identification/evaluation template it was considered weak positive.

2.4. Statistical analyses

Statistical analyses were performed using Prism 5 software (GraphPad, USA). Fisher’s exact test (one-sided) was used for the comparison of differences between the study groups and to evaluate the statistical significance of the data. The difference was considered significant when P < 0.05.

3. Results

3.1. Anti-ganglioside antibodies in narcolepsy patients and healthy controls

To study whether AGAs are found in patients with Pandemrix-associated NC, we screened the sera of all patients and controls for IgG AGAs against 11 human brain gangliosides and a sulfatide. In general, AGAs were clearly more frequent and diverse in vaccinated than in unvaccinated groups (Fig. 1). Positive reactivity against at

Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Narcolepsy patients</th>
<th>Healthy controls</th>
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<tbody>
<tr>
<td></td>
<td>Pdmx-NC</td>
<td>sNC</td>
</tr>
<tr>
<td>Group size (n)</td>
<td>48</td>
<td>20</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>29/19</td>
<td>6/14</td>
</tr>
<tr>
<td>Age at vaccination in years (mean)</td>
<td>4.5–18.6 (11.6)</td>
<td></td>
</tr>
<tr>
<td>Age at disease onset in years (mean)</td>
<td>4.7–18.6 (11.8)</td>
<td>7.0–20.0 (11.0)</td>
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<tr>
<td>Days between vaccination and disease onset (mean)</td>
<td>0–555 (84.6)</td>
<td></td>
</tr>
<tr>
<td>Days between disease onset and sampling (mean)</td>
<td>306–748 (456)</td>
<td>56–1928 (675)</td>
</tr>
<tr>
<td>Days between vaccination and sampling (mean)</td>
<td>398–760 (540)</td>
<td>13–610 (451)</td>
</tr>
<tr>
<td>HLA DQB1*0602 (%)</td>
<td>48/48 (100%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>Cataplexy</td>
<td>48/48 (100%)</td>
<td>20/20 (100%)</td>
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Pdmx-NC = Pandemrix associated narcolepsy patients, sNC = sporadic narcolepsy patients with no Pandemrix association, Pdmx-HC = Pandemrix vaccinated healthy controls, HC = healthy controls (no Pandemrix vaccination), ND = not determined.
least one (any) of the 11 gangliosides was found in 18.8% (9/48) of Pandemrix-associated NC patients, in 17.5% (10/57) of Pandemrix-vaccinated healthy controls, in 5.0% (1/20) of NC patients with no vaccine association and in 8.3% (4/48) of unvaccinated healthy controls (Table 2). Anti-GM3 antibodies were more frequently 14.6% (7/48) found in Pandemrix-associated NC patients than in other groups, in which the frequency of anti-GM3 antibodies was in the range of 0–3.5% (Fig. 1). Anti-GM4 antibodies were more frequent in vaccinated (10.4–10.5%) than in unvaccinated groups (4.2–5.0%) (Fig. 2A). Anti-GM4 and anti-GM3 antibodies were often found in the same individuals. This may be due to the fact that GM3 and GM4 gangliosides share the terminal NeuAcα2-3Galβ1-4Glc structure. In the case of sulfatide, a certain degree of nonspecific binding was detected in the majority of samples. Thus, only few samples with very strong reactivity against sulfatide were regarded as positive (Fig. 1).

When the frequencies of AGAs against at least one of the 11 gangliosides (any) or AGAs against each of the 11 gangliosides (excluding sulfatide) between the study groups were compared, the only significant difference was in the frequency of anti-GM3 antibodies between vaccinated NC and vaccinated healthy individuals (Fig. 1; $P = 0.047$). No anti-GM3 antibodies were detected in the unvaccinated NC group (Fig. 1), indicating that the anti-GM3 antibodies were related to Pandemrix-associated narcolepsy only.

Next, the vaccinated and unvaccinated groups were compared (Fig. 2A). The difference in reactivity against any ganglioside between vaccinated (mean 18.1%) and unvaccinated (mean 7.4%) individuals was significant ($P < 0.035$). When the frequencies of each specific AGA between vaccinated and unvaccinated groups were compared, the only AGA significantly ($P = 0.046$) associated with vaccination was anti-GM3 antibody. Although anti-GM4 antibodies were more frequent in the vaccinated than in the unvaccinated groups, the difference was not significant ($P = 0.125$). When all NC patients and all healthy controls were compared (Fig. 2B) the anti-GM3 antibodies were significantly ($P = 0.045$) associated with narcolepsy, which is essentially explained by the difference in anti-GM3 antibody frequency between vaccinated NC and healthy groups (Fig. 1).

3.2. Association of HLA-DQB1∗0602 with anti-GM3 antibodies

The HLA-DQB1∗0602 allele is strongly associated with narcolepsy. The prevalence of this allele was 100% in our NC patients and 35% (20/57) in Pandemrix-vaccinated controls (Table 1). All vaccinated individuals who had anti-GM3 antibodies (seven in Pandemrix-NC group and two in Pandemrix-control group) carried the DQB1∗0602 allele. None of the vaccinated DQB1∗0602-negative individuals had anti-GM3 antibodies. Thus, to evaluate the association of anti-GM3 antibodies with the DQB1∗0602 allele in vaccinated individuals, all DQB1∗0602-positive individuals ($n = 68$) were combined and compared with DQB1∗0602-negative ($n = 37$) individuals (Fig. 3). The association between DQB1∗0602 positivity and the presence of anti-GM3 antibodies was significant ($P = 0.016$). However, when vaccinated DQB1∗0602-positive and -negative healthy controls were compared for anti-GM3 antibodies, the difference between the groups was not significant ($P = 0.119$). It has to be noted that the number of healthy individuals with anti-GM3 antibodies was only two.

3.3. Correlation between AGAs and patient characteristics and clinical picture

NC patients who were positive for anti-GM3 antibodies did not differ from the anti-GM3-negative patients regarding age, sex or time of the onset of the illness after vaccination or specific symptoms (e.g. hallucinations, sleep paralysis, aggression). All NC patients with Pandemrix-association who had been studied for the CSF hypocretin levels (6/48) had very low hypocretin levels (<110 pg/mL) [3]. There was no association between AGAs and H1N1-virus hemagglutination inhibition titers, or AGAs and H1N1 infection (2/48 of Pandemrix-associated NC patients had antibodies against NS1) [21].

3.4. Persistence of AGAs

To study the persistence of AGAs we screened follow-up samples from 14 Pandemrix-associated NC patients taken 16–26 months after initial sampling and approximately three years after the vaccination. Similar reactivities, with regard to specific ganglioside species and intensity of the reactivity, were detected as in the initial samples, indicating a long-lasting autoimmunity.

4. Discussion

In the present study we found an increased frequency of AGAs, especially anti-GM3 and anti-GM4 antibodies, in Pandemrix-vaccinated vs. unvaccinated individuals. The most striking finding was that anti-GM3 antibodies were significantly increased in patients who developed NC after Pandemrix vaccination. Anti-GM3 antibodies were also associated with the DQB1∗0602 allele in all vaccinated individuals, both in narcolepsy patients and in healthy controls. The results suggest that Pandemrix vaccination has
induced the production of specific AGAs, i.e. autoimmunity against gangliosides, in a subgroup of vaccinated individuals. The AGAs found, at least in patients with Pandemrix-associated NC, were not a transient response to vaccination, because they were still detectable in the follow-up samples after approximately three years from the vaccination. On the other hand, AGAs developed only in a minority of vaccine recipients, since only 18.1% of all vaccinated individuals had AGAs.

Anti-GM3 antibodies were detected in 14.6% children who developed after Pandemrix vaccination against 2009 pandemic H1N1 type in healthy controls (no Pandemrix vaccination). This suggests that anti-GM3 antibody response was induced by Pandemrix vaccination in susceptible individuals, but also that autoimmunity against gangliosides is not a general feature of NC. The low frequency (3.5%) of anti-GM3 antibodies in healthy vaccinated individuals suggests that in addition to vaccination, other risk factors, such as a distinct genotype, are required for the induction of anti-GM3 antibodies. Interestingly, all vaccinated individuals, both NC patients and healthy individuals, who had anti-GM3 antibodies carried the DQB1*0602 allele. Thus, DQB1*0602 – positivity could be one of the prerequisites for the production of anti-GM3 antibodies. In fact, the frequencies of anti-GM3 antibodies in vaccinated DQB1*0602-positive healthy and DQB1*0602-positive NC children were quite similar, 10.0% and 14.6% respectively (n.s.). This suggests that Pandemrix vaccination induced production of anti-GM3 antibodies primarily in DQB1*0602-positive individuals. An increased frequency of anti-GM3 antibodies in vaccinated NC patients vs. vaccinated healthy controls, therefore, may be partially explained by the 3-fold increase in the HLA-DQB1*0602 carrier status. However, this does not rule out the possibility that autoimmunity against GM3 could contribute to the development of NC in Pandemrix-immunized DQB1*0602 individuals.

IgG AGAs have not been detected earlier in NC patients. We detected anti-GM3 antibodies in vaccinated NC patients only, suggesting that anti-GM3 antibody production is a feature of Pandemrix-associated narcolepsy. In the previous study of Overeem et al. [40] low titer IgM AGAs (against GM1 and GM2) were found. We also found a weak antibody response was induced by Pandemrix vaccination in susceptible individuals, but also that autoimmunity against gangliosides is not a general feature of NC. The low frequency (3.5%) of anti-GM3 antibodies in healthy vaccinated individuals suggests that in addition to vaccination, other risk factors, such as a distinct genotype, are required for the induction of anti-GM3 antibodies. Interestingly, all vaccinated individuals, both NC patients and healthy individuals, who had anti-GM3 antibodies carried the DQB1*0602 allele. Thus, DQB1*0602 – positivity could be one of the prerequisites for the production of anti-GM3 antibodies. In fact, the frequencies of anti-GM3 antibodies in vaccinated DQB1*0602-positive healthy and DQB1*0602-positive NC children were quite similar, 10.0% and 14.6% respectively (n.s.). This suggests that Pandemrix vaccination induced production of anti-GM3 antibodies primarily in DQB1*0602-positive individuals. An increased frequency of anti-GM3 antibodies in vaccinated NC patients vs. vaccinated healthy controls, therefore, may be partially explained by the 3-fold increase in the HLA-DQB1*0602 carrier status. However, this does not rule out the possibility that autoimmunity against GM3 could contribute to the development of NC in Pandemrix-immunized DQB1*0602 individuals.

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LgM reactivity against gangliosides (in most cases against GM2) in 25% (7/28) of vaccinated NC patients (not shown), but not in a small sample of healthy unvaccinated controls (n = 6). Sometimes IgM class AGAs, such as anti-GM2 antibodies can occur in healthy individuals too, e.g. after infection.

The frequency of anti-GM3 antibodies (14.6%) in Pandemrix-associated NC patients and in healthy controls (3.5%), were similar to previously found anti-Trib2 antibody frequencies in patients with sporadic NC (14–26%) and in healthy controls (2–4%) [22–24]. No anti-Trib2 antibodies have been found in Pandemrix-associated NC patients [25,41] suggesting that Pandemrix-associated NC and sporadic NC may differ immunologically. Although serum anti-Trib2 and anti-GM3 antibodies do not indicate them as specific biomarkers for NC these results, together with previous immunohistochemistry results [27,28]), indicate complex disease mechanism and may help to elucidate the pathological mechanisms behind NC. In the study of Bergman et al. different immunohistochemical patterns of antibody reactivity against rat brain tissue were detected in 27% of sera of narcolepsy patients (n = 89) [27, 28]. We screened AGAs from 18 patient sera used in their study. No clear correlation of results for AGA reactivity with immunohistochemical staining patterns were observed (not shown).

The frequency of various AGAs in healthy individuals is not well characterized. In fact, very little information is available about the prevalence of the anti-GM3 and anti-GM4 antibodies in the general population, especially in children, because these antibodies are not routinely studied in clinical laboratories. The frequencies of AGAs in healthy children found in our study (1.7% for anti-GM3 and 3.3% for anti-GM4 antibodies) are in good agreement with few previously published studies [42,43] where frequencies of 2.1–3% for anti-GM3 and 1–4.2% for anti-GM4 IgG antibodies were found in immunologically healthy control groups.

To our knowledge, this is the first report of AGAs associated with pandemic H1N1 influenza vaccination in humans. After the report of Nachamkin et al. 2008 showing that certain H1N1 influenza vaccines induced anti-GM1 antibody production in mice [36], AGAs characteristic to GBS (against GM1, GD1a, GT1b, GQ1b) have been immunogenic as such. However, as shown by Estevez et al. [45], if gangliosides are incorporated into very small size proteoliposomes containing a protein carrier and a strong oil-in-water adjuvant, their immunogenicity is greatly enhanced and AGAs are produced in mice. Pandemrix vaccine contains viral protein antigens, predominantly hemagglutinin and neuraminidase, and oil-in-water adjuvant (AS03) [46]. The H1N1 hemagglutinin (H1) is known to bind GM3 by virtue of its ability to use it as a receptor [47]. We also detected IgM antibodies against GM3 and GM4 may have been missed in the earlier studies.

How could Pandemrix vaccination induce the production of AGAs, especially against GM3? Gangliosides are generally poorly immunogenic as such. However, as shown by Estevez et al. [45], if gangliosides are incorporated into very small size proteoliposomes containing a protein carrier and a strong oil-in-water adjuvant, their immunogenicity is greatly enhanced and AGAs are produced in mice. Pandemrix vaccine contains viral protein antigens, predominantly hemagglutinin and neuraminidase, and oil-in-water adjuvant (AS03) [46]. The H1N1 hemagglutinin (H1) is known to bind GM3 by virtue of its ability to use it as a receptor [47]. We also observed that recombinant hemagglutinin and hemagglutinin of Pandemrix vaccine bound to purified GM3 (not shown). Thus H1 could bind to GM3 and form H1-GM3 complexes. The AS03-adjuvant could have an important role in enhancing the immunogenicity of the gangliosides more specifically. The H1–GM3 complex formation could take place e.g. during the propagation of H1N1 virus in chicken eggs, which are rich in gangliosides GM3, GM4, CD3 [48]. In fact, when the lipid contents of swine influenza and other influenza vaccines were analyzed, trace amounts of GM3 were detected in two out of six (33%) of the analyzed influenza vaccines [49]. Variation in vaccine processing could thus influence the amount of gangliosides in the vaccine preparation, and the adjuvant could potentiate the induction of anti-ganglioside immunity. Alternatively, H1 could form complexes during immunization with tissue derived GM3 which is the most abundant ganglioside in the human extraneural tissue, e.g. in muscles [33]. In both cases, the resulting H1-GM3 complexes could then be taken up and processed by GM3-specific B cells. Some of the resulting peptides could be presented by DQBT1*0602 molecules to CD4-positive T cells via their T cell receptors. While the vaccine induced T cell response against the viral proteins emerges, the activated H1-specific T cells could provide help, i.e. cytokines and a co-receptor-ligand interaction to ganglioside-specific B cells for proliferation and AGA production.

Anti-GM4 antibodies were more frequent in vaccinated than in unvaccinated groups indicating that their production could be induced by vaccination. The AS03-adjuvant contains squalene that is isolated from shark liver [46], which is exceptionally rich source of GM4 gangliosides [50]. Thus, it is possible that residual GM4 in Pandemrix vaccine, either in the adjuvant (derived from squalene) or in the viral component (derived from egg yolk) could induce (together with adjuvant) anti-GM4 antibody production in vaccinated individuals.

Are the anti-GM3 antibodies found in NC patients involved in the pathogenetic processes of narcolepsy? Although GM3 is a minor ganglioside of the human adult brain it has been shown to be involved in many important regulatory functions of the nervous system, including regulation of neuronal cell death [51]. Ganglioside species specific for the hypocretin neurons or for the hypothalamus have not been described, but at least in the mouse brain, GM3 gangliosides are found in the hypothalamus [52]. We also detected GM3 gangliosides in rat hypothalamus by immunofluorescence with mouse monoclonal anti-GM3 IgM antibody (not shown). Thus, if anti-GM3 antibodies can cross the blood–brain barrier (BBB) they could (i) cause, or at least participate in neuronal damage in the hypothalamus by binding to cross-reactive neuronal gangliosides alone or in a complex with protein antigens (e.g. the hypocretin receptor). Alternatively (ii) circulating anti-GM3 antibodies could contribute to the development of autoimmune condition by binding to GM3 gangliosides present on brain microvascular endothelial cells (BMECs) or astrocytes which are both important for the proper function of the BBB [53]. The damage in the cell—cell attachments of BMECs could disturb the integrity of the BBB and allow the entry of pathogenic T cells and antibodies to the central nervous system. Finally, it is possible, that (iii) the found AGAs are produced as a result of ongoing autoimmune reactions and neuronal destruction.

Although Pandemrix-vaccine increased the incidence of autoimmune NC, induction of autoimmunity by vaccines e.g. by molecular mimicry, is a rare phenomenon. In addition to Pandemrix-NC association only few other associations between vaccination and autoimmune diseases have been reliably demonstrated [54–56]. One of them is the association of A/J/NJ/1976/H1N1 swine flu vaccine and GBS. However, in the case of GBS, in addition to C. jejuni, also viral infections such as influenza A have been described to precede GBS. In fact, more cases of autoimmune diseases have been reported after natural infection, than after vaccination indicating that vaccination can prevent not only infectious diseases, but also post-infectious autoimmune diseases. Further, as vaccines differ in their composition, even the same vaccine may induce variable immunological reactions in physiologically, genetically and immunologically different individuals. Thus, development of an autoimmune disease is a multifaceted process affected by individual factors and possibly several environmental factors/triggers. In some cases, vaccination can be one factor that disturbs the balance.
between the normal immunity and autoimmunity.

The pathogenicity of the Pandemrix-vaccine might be tested in a humanized mouse model. We have immunized groups of HLA-DQB1*0602, human CD4 transgenic Ab0 NOD mice, with various antigenic stimuli, including Pandemrix vaccine, in order to study immune-mediated narcolepsy in mice. However, we have not been able to induce narcolepsy in these mice. Pandemrix vaccination did not induce mouse IgG AGAs either (results not shown). Thus, both the emergence of NC and the appearance of AGAs, especially against GM3 and GM4, after Pandemrix vaccination appears to be specific to humans, or may require an additional factor not present in laboratory mice. More studies are required to resolve the potential pathologcal role of anti-GM3 antibodies.

As a conclusion, our results suggest that Pandemrix-vaccine induced a humoral immune response against selected gangliosides, particularly against GM3, in a proportion of individuals who carried the HLA-DQB1*0602 allele. The anti-GM3 antibodies were significantly associated with the vaccination, the DQB1*0602 allele and NC developing after Pandemrix. Although no association of anti-GM3 antibodies was found with NC in unvaccinated patients, the anti-GM3 antibodies may have had a contributory role in autoimmunity and in the pathogenesis of Pandemrix-associated NC. Most importantly, our results indicate that AS03-adjuvanted H1N1 vaccination induced humoral autoimmunity against GM3, a receptor for the hemagglutinin of H1N1 in virus, in individuals who carry the HLA-DQB1*0602 risk allele of narcolepsy. In future studies, the possible pathogenetic role of AGAs in narcolepsy should be further explored. It will also be important to characterize the distinct roles of the critical components of the Pandemrix vaccine responsible for the autoimmune reactions, including the induction of AGA production.

Conflict of interest statement

OV is an employee of AstraZeneca R&D, Sweden. The other authors declare no conflicts of interest.

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