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Mutations in Toll-Like Receptor 3 Are Associated with Elevated Levels of Rotavirus-Specific IgG Antibodies in IgA-Deficient but Not IgA-Sufficient Individuals

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Double-stranded RNA (dsRNA) triggers immune-mediated responses through toll-like receptor 3 (TLR3), which is involved in innate antiviral defense. Low expression of TLR3 was recently suggested to contribute to susceptibility to rotavirus infection. Thus, we investigated the role of two TLR3 polymorphisms (rs3775291 and rs5743305), both of which resulted in reduced protein function or expression, in healthy blood donors and IgA-deficient (IgAD) individuals. These polymorphisms were associated with elevated rotavirus-specific IgG titers in IgAD individuals but not in healthy individuals. Thus, we propose that TLR3 signaling does not contribute to the rotavirus-specific antibody response in IgA-sufficient individuals, whereas it is associated with elevated antibody titers in IgAD individuals.

Toll-like receptors (TLRs) belong to the pattern recognition receptor (PRR) family, the members of which sense invading microorganisms. Upon stimulation, TLRs induce signaling through different pathways to trigger production of proinflammatory cytokines or type I interferons (IFNs). Double-stranded RNA (dsRNA) induces immune-mediated responses through PRRs such as membrane-associated toll-like receptor 3 (TLR3) or either of the two cytoplasmic retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), MDA5 and RIG1, which work in an orchestrated and collective fashion (1, 2). Activation of TLR3 triggers recruitment of the signaling adaptor molecule TRIF and, subsequently, related adaptor molecules termed tumor necrosis factor receptor-associated factors (TRAFs) (TRAF3 and TRAF6), which induce distinct downstream signaling pathways and stimulate upregulation of several transcription factors in the nucleus, including main mediators of NF- κ B, interferon regulatory factor 3 (IRF3), CREB, and AP1 (3–5). The TLR3-TRIF signaling ultimately leads to production of proinflammatory cytokines and type I interferon (IFN) responses, which are essential for recovery from infections by dsRNA viruses, including rotavirus.

Human infants and mouse pups are susceptible to symptomatic rotavirus infections which could potentially be due to low TLR3 expression at a young age (6). Adult mice with a targeted inactivation of the TLR3-encoding gene (*Tlr3*^{-/-}) also show a markedly elevated degree of viral shedding after infection, thus supporting the notion of a functionally relevant role of TLR3 in the response to rotavirus infection (6). Interestingly, TLR3 activation may in parallel induce the intestinal epithelial cells to produce elevated levels of interleukin 15 (7). The imbalanced cytokine levels cause mucosal homeostasis disruption and severe pathogenic defects limited to the small intestine of mice, including intestinal wall weakening, villous atrophy, and mucosal erosion, which eventually leads to acute rotavirus gastroenteritis (7), suggesting TLR3 involvement in viral pathogenesis.

More than 100 single nucleotide polymorphisms (SNPs) have been identified in the human TLR3 gene, seven of which are located within protein-coding sequences. Among the latter, four SNPs, N284I, Y307D, L412F, and S737T, lead to amino acid sub-

stitutions (8). The L412F (rs3775291) mutation was predicted to be damaging in a PolyPhen/SIFT analysis used for annotating coding nonsynonymous amino acid substitutions and resulted in reduced or absent TLR3 activity (8). Heterozygous L412F mutations are associated with susceptibility to herpes simplex virus 1 (HSV-1) (9), tick-borne encephalitis virus (TBEV) (10), and measles virus (11) infections in humans. Furthermore, the rs5743305 promoter variant results in reduced transcriptional rates of TLR3 (11, 12), and heterozygous carriers produce lower measles-specific antibody titers in response to vaccination (11), suggesting that haploinsufficiency of both the tested mutations may have considerable functional consequences.

Immunoglobulin A (IgA) is the major protective immunoglobulin class at mucosal surfaces, where secreted IgA antibodies block viral entry or primary transcription and promote clearance of the pathogen, thus serving as a first line of defense. In addition, if the virus infects a cell, IgA assists in intracellular neutralization by forming complexes that engage the intracellular immune machinery (13, 14). Even though the correlation of rotavirus-specific IgA antibody titers in serum with protection is not uniformly accepted (15), serum IgA antibody titers are broadly recognized to be a good correlate of protection against rotavirus-induced infections in both infants and children following vaccination (16, 17) and during the course of natural infection (17, 18), based on the neutralizing capacity of IgA antibodies elicited by different rotavirus antigens (19–22).

Selective IgA deficiency (IgAD) is a primary immunodeficiency disorder which is defined as a serum level of IgA lower than

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0.07 g per liter with normal serum levels of IgM and IgG and with a lack of secretory IgA. In symptomatic cases of IgAD, patients often suffer from sinopulmonary and gastrointestinal infections, allergic diseases (allergic rhinoconjunctivitis, urticaria or anaphylaxis, food allergies, asthma, and eczema) and autoimmune disorders (23).

Mice lacking IgA are not protected against infection upon re-exposure to rotavirus and show a significantly longer duration of viral shedding in the stool (24). Protracted virus shedding (poliovirus) is also observed in IgA-deficient individuals (25). In addition, IgAD patients show significantly elevated serum levels of rotavirus-specific IgG (26), suggesting a compensatory role of IgG in systemic rotavirus infection (24) and a protraction of rotaviral persistence/shedding after infection, although the disease is ultimately resolved.

In this study, we investigated the role of TLR3 in antibody responses to rotaviruses in healthy individuals and patients with IgA deficiency.

MATERIALS AND METHODS

Patients. Serum samples from Swedish IgA-deficient individuals ($n = 783$) and anonymous healthy blood donors ($n = 1009$) were collected for the TLR3 SNP analysis. Rotavirus-specific antibody titer evaluations were performed in 180 patients and 198 individuals from the IgAD and IgA-sufficient blood donors (control group), respectively. Ethics permits (Dnr 2011/69-31/3 and Dnr 2013/1176-31/1) were obtained from the ethics review board in Stockholm, Sweden.

Genotyping. SNP genotyping of rs3775291 and rs5743305 was performed at the Mutation Analysis Facility (MAF) at the Karolinska Institutet, Stockholm, Sweden. The method of automated genotyping is based on matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis using the Sequenom platform as described previously (27).

Enzyme-linked immunosorbent assay. The sample selection for rotavirus-specific IgG antibody assessments from a pool of more than 700 individuals was based on availability of serum in consecutive samples. Flat-bottom 96-well sterile plates were coated with rhesus rotavirus (RRV) (virion stock dilution, 1:500) and incubated overnight at 4°C. Enzyme-linked immunosorbent assay (ELISA) to assess the levels of serum IgG antibodies specific to rotavirus was performed as described previously (26). A positive serum sample with a known titer was included in each plate as an internal quality control for between-plate reproducibility.

Statistics. GraphPad software was used in statistical analysis. For comparison of TLR3 polymorphism frequencies, Fisher's exact test with two-tailed significance was used (Table 1). To evaluate the differences in rotavirus-specific IgG titers in serum, multiple comparisons of all groups included in Fig. 1 and Table 2 were performed by use of the Kruskal-Wallis nonparametric analysis of variance with Dunn's *post hoc* test. The significance of the differences in serum-specific IgG responses between all IgAD participants and healthy controls was calculated by use of the Mann-Whitney test.

RESULTS AND DISCUSSION

The frequencies of TLR3 mutations are given in Table 1 and showed no major differences between IgAD individuals and IgA-sufficient blood donors (controls). The minor alleles of rs3775291 and rs5743305 were, however, found to be slightly more common in our cohort, being present in 50 to 51% and 57 to 61% of individuals, respectively (Table 1), compared to cohorts in previous studies in Europe (32 and 38%, respectively) (8, 28). The allele frequency of the rs3775291 variant was markedly lower in African populations (with a frequency of 4%) (29), suggesting a potentially deleterious role in the function of TLR3.

TABLE 1 Allele frequencies of TLR3 variants in IgA-deficient individuals and IgA-competent controls

TLR3 variant and allele	Frequency (n) of TLR3 variants	
	Individuals with IgA deficiency ($n = 783$)	Control individuals ($n = 1,009$)
rs3775291		
C ^a	0.92 (719)	0.94 (950)
T	0.50 (393)	0.51 (510)
CC	0.50 (390)	0.50 (501)
CT	0.42 (326)	0.41 (417)
TT	0.08 (67)	0.09 (91)
rs5743305		
T ^a	0.86 (675)	0.90 (908)
A	0.61 (480)	0.57 (571)
TT	0.39 (304)	0.44 (438)
AT	0.47 (369)	0.43 (437)
AA	0.14 (110)	0.13 (134)

^a Wild-type allele.

The IgG antibody levels against rotavirus in IgA-sufficient individuals were not affected by either of the two TLR3 mutations (neither in a homozygous nor in a heterozygous form) (Fig. 1A). It has been previously suggested that low expression of TLR3 on epithelial cells contributes to rotavirus susceptibility (6). However, in this study, we found no correlations between the deleterious TLR3 polymorphisms (rs3775291 and rs5743305), which cause nonfunctional and reduced levels of TLR3, respectively, and rotavirus-specific antibody titers in healthy individuals.

IgAD individuals display higher rotavirus IgG antibody titers than IgA-sufficient controls (26). To determine the immunological basis for the increased rotavirus-specific titers in IgAD individuals, intragroup differences in individuals with IgAD were analyzed. Serum rotavirus-specific IgG antibody titers of individuals with IgAD in our cohort were significantly higher than those in the IgA-sufficient group ($P < 0.01$) (Fig. 1A). However, no differences in IgG antibody titers between IgAD individuals and IgA-sufficient individuals (controls) were observed for individuals not carrying any of the two investigated TLR3 mutations ($P = 0.42$). In contrast, carriers of either of these TLR3 mutations in the IgAD group showed markedly elevated antibody titers ($P < 0.01$) (Fig. 1A and Table 2), with no differences between those carrying rs3775291 and those carrying rs5743305. More than 30 individuals, belonging to either of the two groups, carried homozygous mutations and a few ($n = 4$) are carriers of combined homozygous mutations of both rs3775291 and rs5743305. However, the antibody titers against rotavirus from the carriers with either homozygous or heterozygous mutations did not show any major differences ($P > 0.05$) (Fig. 1B).

Both serum IgA and IgG antibodies are involved in resistance to rotavirus infection, but their dynamics *in situ* might be different (24). IgA confers immunity to several invading mucosal pathogens, including rotavirus, by preventing attachment to cells and by facilitating their intracellular neutralization. During early infection, lack of IgA allows viral entry followed by intracellular virus replication. Furthermore, in IgA-deficient individuals, rotaviral particles released from infected cells may hypothetically spread more easily to neighboring cells than in IgA-sufficient individuals, thus potentially contributing to protracted viral shedding. How-

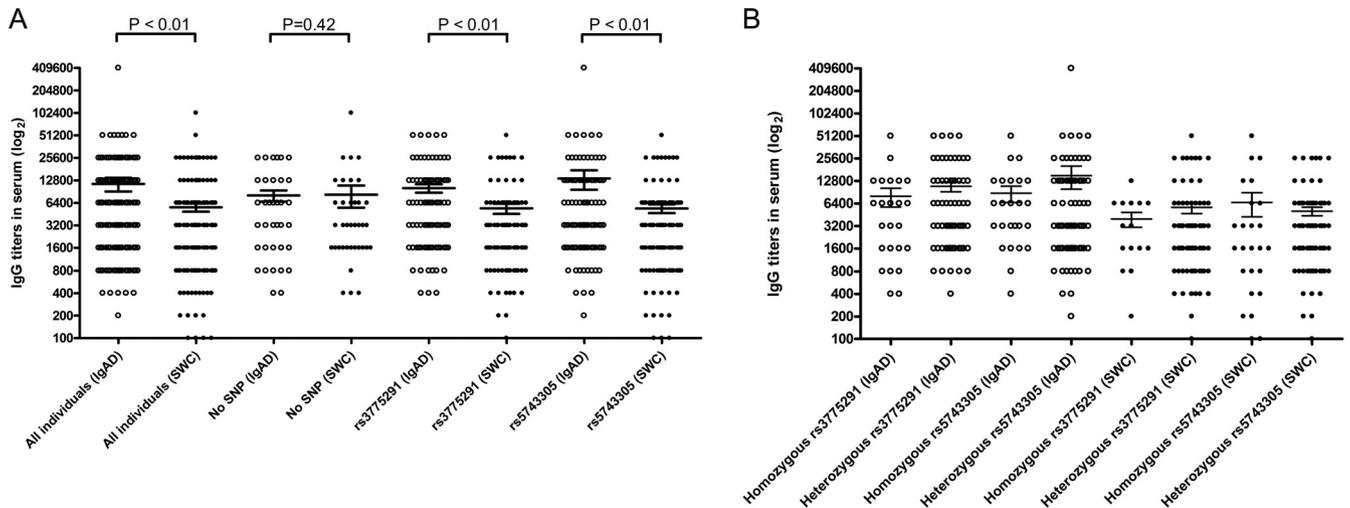


FIG 1 IgG antibody titers against rotavirus in individuals with IgA deficiency (IgAD) and IgA-sufficient controls (SWC). (A) There was a significant difference in rotavirus-specific IgG titers between all individuals with IgAD and controls ($P < 0.01$) (Dunn’s multiple comparison test). The elevations of the antibody titers of individuals with IgAD carrying TLR3 SNPs rs3775291 and rs5743305 were significant compared to the elevations in the SWC ($P < 0.01$ in both comparisons). In contrast, no changes were detected in serum IgG levels of individuals with no TLR3 SNPs ($P = 0.42$). In addition, there were no significant differences in individuals with IgAD carrying the TLR3 SNP rs3775291 versus those carrying rs5743305 and in SWC individuals carrying the TLR3 SNP rs3775291 versus those carrying rs5743305 ($P > 0.05$) and rs5743305 ($P > 0.05$) and in individuals with IgAD carrying homozygous and heterozygous SNPs in rs3775291 ($P > 0.05$) and rs5743305 ($P > 0.05$). Horizontal bars represent means with standard error of the mean (SEM). Statistical analyses were performed with one-way analysis of variance (ANOVA), Dunn’s multiple comparison test, using the Prism 4 program (GraphPad Software).

ever, protection does not always correlate with levels of specific IgA antibodies (30), and IgG antibodies can also help neutralize rotavirus (30, 31), especially during a systemic infection (24). Rotavirus replication, or production of the viral enterotoxin NSP4, lyses the intestinal epithelial cells and causes paracellular leakage and deformations in the gut epithelium through which anti-rotaviral IgG can enter the gut lumen and clear the virus (32). Additional intracellular mechanisms assisting in viral clearance include cytosolic helicases (RIG1/MDA5) and the membrane-associated (TLR3) receptor. Polymorphisms in MDA5, however, do not appear to cause a change in the rotavirus-specific IgG titers (N. Wang and L. Hammarström, unpublished data). In this study, TLR3 was therefore identified as the major defense mechanisms in IgAD individuals.

We thus propose that the reduced TLR3-TRIF signaling does not affect innate immune responses in terms of antibody production in IgA-sufficient individuals but leads to an elevation in IgG

antibody titers, potentially caused by protracted viral shedding, in IgAD individuals. These findings suggest that recognition of rotavirus is mediated through distinct sensors or mechanisms in individuals with different genetic backgrounds even within the same ethnic group.

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We declare no conflicts of interest.

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TABLE 2 TLR3 mutation status and rotavirus-specific IgG antibody titers in Swedish individuals with IgA deficiency and IgA-competent controls

Study participant IgA status	TLR3 SNP	n	Antibody titer			
			Mean	SEM	Mean – SEM	Mean + SEM
IgAD	All	180	11,508	2,383	9,125	13,891
	No SNP	39	8,041	1,369	6,672	9,410
	rs3775291	90	10,076	1,327	8,749	11,403
	rs5743305	106	13,553	3,949	9,604	17,502
SWC	All	198	5,592	697.5	4,894.5	6,289.5
	No SNP	39	8,215	2,706	5,509	10,921
	rs3775291	94	5,401	840.3	4,560.7	6,241.3
	rs5743305	114	5,396	727.1	4,668.9	6,123.1

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