NOD2 Variants and Antibody Response to Microbial Antigens in Crohn's Disease Patients and Their Unaffected Relatives

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BASIC–ALIMENTARY TRACT

Background & Aims: **The Cdcs1 locus of the C3Bir mouse confers severe colitis associated with a decrease in innate immune function and an increase in adaptive T-cell responses to commensal bacterial products. The aim of our study was to determine if defects in innate immunity are similarly associated with increased adaptive immune responses to microbial antigens in Crohn's disease patients.** ***Methods:*** **Sera from 732 patients, 220 unaffected relatives, and 200 healthy controls were tested for antibodies to oligomannan, the *Pseudomonas fluorescens*–related protein, *Escherichia coli* outer membrane porin C, CBir1 flagellin, and DNA from the same subjects was tested for 3 Crohn's disease–associated variants of the *NOD2* gene, and 5 toll-like receptor (TLR) 2, 2 TLR4, and 2 TLR9 variants. The magnitude of responses to microbial antigens was examined according to variant status.** ***Results:*** ***NOD2*** **variant carriage increased in frequency with increasing number of positive antibodies and increasing cumulative quantitative response as measured by quartile sum (*P* for trend, .0008 and .0003, respectively). Mean antibody and quartile sums were higher for patients carrying any *NOD2* variant versus those carrying none (2.24 vs 1.92 and 10.60 vs 9.72; *P* = .0008 and *P* = 0.0003, respectively). The mean quartile sum was higher for unaffected relatives carrying any *NOD2* variant versus those carrying none (10.67 vs 9.75, respectively; *P* = .02). No association was found between any TLR variant and the magnitude of response.** ***Conclusions:*** **Patients with Crohn's disease and unaffected relatives carrying variants of the *NOD2* gene have increased adaptive immune responses to microbial antigens.**

Crohn's disease (CD) is a complex clinical and genetic disorder believed to relate to aberrant immunologic responses to commensal bacteria.[1,2] Animal models of CD have shown this response to a wide range of bacterial species.[3–8] In the interleukin-10 knockout mouse model, an inflammatory bowel disease–like phenotype can be demonstrated upon exposure to specific bacterial species in an otherwise germ-free environment.[9] Several lines of evidence have implicated enteric bacteria in the pathogenesis of CD in humans. The use of antibiotics has been associated with an inconsistent treatment response in CD.[10–14] Fecal diversion has been shown to decrease the recurrence of CD in the neoterminal ileum after resection, with subsequent instillation of the fecal stream in the excluded ileum leading to inflammatory lesions.[15,16] Moreover, one study has suggested that patients with CD with increased seroreactivity to microbial antigens are more likely to respond to antibiotics.[17]

A hyperresponsive adaptive immunologic response to microbial antigens has been characterized in patients with CD and is believed to be reflective of the underlying immunopathogenesis of this disorder. Measures of this adaptive immunologic response include antibodies to oligomannan (anti–*Saccharomyces cerevisiae* antibody [ASCA]), the *Pseudomonas fluorescens*–related protein (I2), *Escherichia coli* outer membrane porin C (anti-OmpC), and most recently CBir1 flagellin (anti-CBir1).[18–23] Whether this adaptive immunologic response is reflective of acquired characteristics or has an underlying genetically determined influence is an important question. Supporting the latter possibility is the observation that the prevalence of ASCA expression is elevated not only in patients with CD but in 9%–25% of unaffected relatives versus <5% of healthy controls.[24–29] More recently, a study by Mei et al showed that reactivity to anti-OmpC was present in 15.5% of unaffected relatives versus 6% of healthy controls, yielding a highly significant heritability estimate of 39%. Moreover, there were increased levels of serum expression in unaffected relatives compared with healthy controls even in those individuals with expression falling within the normal range, thus underscoring the fact that seroreactivity to microbial antigens is a quantitative trait.[30] These studies thus suggest that the adaptive immunologic response reflects underlying genetic determinants.

Abbreviations used in this paper: **ASCA, Anti–*Saccharomyces cerevisiae* antibody; ELISA, enzyme-linked immunosorbent assay; MDP, muramyl dipeptide; OmpC, outer membrane porin C; TLR, toll-like receptor.**

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0016-5085/07/$32.00
doi:10.1053/j.gastro.2006.11.013

The concept of an underlying genetic determinant mediating the adaptive immunologic response to bacterial antigens has recently been described in an animal model. In the C3H/HeJBir (C3Bir) interleukin-10–deficient mouse model, the presence of a colitogenic cytokine deficiency induced colitis susceptibility (Cdcs1) allele was associated with an impairment of innate responsiveness to bacterial ligands such as CBir1 flagellin, flagellin X, lipoteichoic acid, muramyl dipeptide (MDP), and CpG oligodeoxynucleotides and a compensatory increase in adaptive CD4 T-cell response to CBir1 flagellin and flagellin X.[31]

In human studies, several genes or loci have thus far been described that may be associated with CD.[32–38] The best characterized of these susceptibility genes is the innate immune gene *NOD2*.[36–38] *NOD2* is a member of a family of intracellular cytosolic proteins important in mediating the host response to bacterial antigens and is found in epithelial cells of the small and large intestine, as well as monocytes, macrophages, T and B cells, Paneth cells, and dendritic cells.[39–42] *NOD2* senses MDP, a highly conserved component of bacterial peptidoglycan, which leads to the secretion of antibacterial substances such as α-defensins and the activation of nuclear factor κB.[43,44] At least 27 mutations of the *NOD2* gene have been described, but the majority of susceptibility has been attributed to 3 common mutations, including the 2 missense mutations, R702W and G908R, and one frameshift mutation, 1007fs.[37,45–47] Variants in *NOD2* are believed to result in a diminished innate immune response to MDP.[48,49] Moreover, variants in other pattern recognition receptors such as toll-like receptor (TLR) 2, TLR4, and TLR9 have been inconsistently associated with inflammatory bowel disease.[50–54]

In the C3Bir mouse model, a genetic defect in innate immunity accompanying the Cdcs1 allele results in a hyperresponsive adaptive immune response to bacterial ligands. In humans, loss-of-function mutations of the innate immune gene *NOD2* could conceivably result in the same phenomenon, with a compensatory adaptive immunologic response to bacterial antigens. Similarly, defects in other innate immune genes, such as TLR genes, could have the same effect. We thus hypothesized that the presence of mutations in the *NOD2* as well as a variety of TLR genes (ie, defects in innate immunity) would be associated with a greater serologic response to microbial antigens (ie, an increase in adaptive immunity). To approach this question, we used a large sample of patients with CD, their unaffected relatives, and healthy controls, whom we evaluated for serologic and genetic markers. The results of our studies reported herein show that the presence of a *NOD2* variant, although not any TLR variant, is associated with both a greater qualitative and semiquantitative antibody response to microbial antigens in patients with CD. The same relationship was found in unaffected relatives of patients with CD. These results have important implications because they suggest an inherited and, therefore, genetic basis underlying innate and adaptive immune responses in CD and provide a potential pathophysiologic link to similar findings in rodent mucosal inflammation. These findings should facilitate disease-relevant rodent and human crossover genetic and functional studies.

Materials and Methods

Patients

The cohort of 732 unrelated patients was ascertained from patients assessed at Cedars-Sinai Medical Center from 1988 to 2005. This cohort included 303 patients previously reported[55] but also included an additional 429 patients enrolled from the clinic or at the time of surgery. The diagnosis of CD was based on standard endoscopic, histologic, and radiographic features as previously described.[55] In addition, a cohort of 220 unaffected relatives of patients with CD as well as 200 healthy controls were included. Many of these unaffected relatives and healthy controls have been previously described and studied for anti-OmpC status.[30] All research-related activities were approved by the Cedars-Sinai Medical Center Institutional Review Board.

Serologic Analysis and Classification

All blood samples were taken at the time of consent and enrollment. Sera were analyzed for expression of ASCA, anti-I2, and anti-OmpC in a blinded fashion by enzyme-linked immunosorbent assay (ELISA) as previously described.[23,55] Antibody levels were determined and results expressed as ELISA units (EU/mL) that are relative to a Cedars-Sinai laboratory (immunoglobulin [Ig] A-I2, IgA-OmpC) or a Prometheus Laboratory standard (San Diego, CA; IgA and IgG ASCA) derived from a pool of patient sera with well-characterized disease found to have reactivity to these antigens. Quantitation of IgG anti-CBir1 reactivity was expressed in ELISA units derived based on a proportion of reactivity relative to a standardized positive control. Because ASCA can be expressed in both an IgA and an IgG class, positivity to ASCA was determined if either class of antibody was above the reference range. In determining a quantitative measure of ASCA, the reactivity was first log-transformed and standardized. The higher of 2 standardized units was then used to determine the quartile of reactivity. With the exception of determining variance (see Statistical Analysis), the magnitude of reactivity to the other 3 antigens was not standardized because each is represented by a single class of antibody. The magnitude of the serologic response to each antigen was divided into 4 equal quartiles in patients with CD, unaffected relatives, and healthy controls, evaluated as 3 separate cohorts, to determine quartile sum scores as previously described.[22,55] Figure 1 shows the patients with the serologic response to each antigen broken down by quartiles and assigned scores of 1–4 on the basis of their designated quartile. By

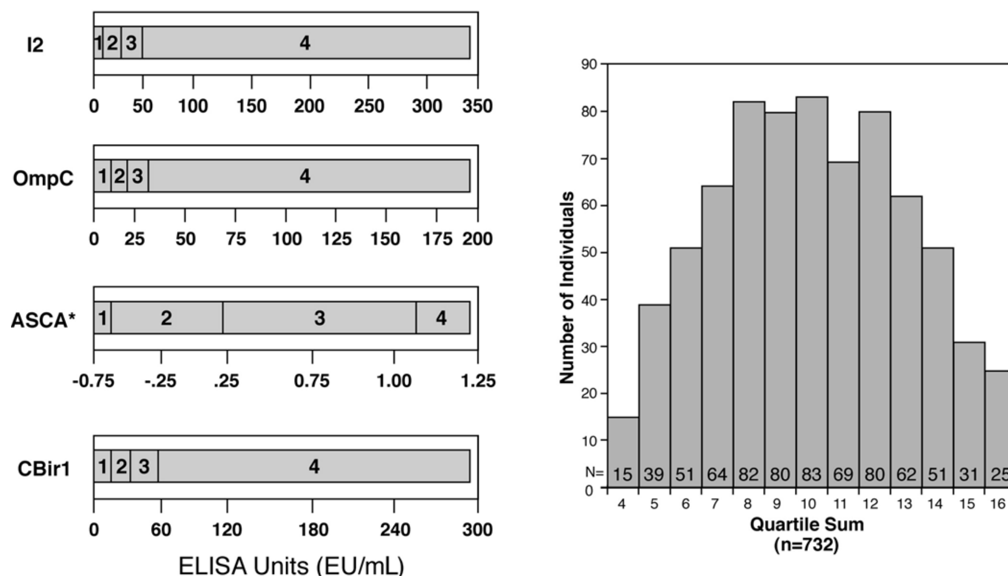

Figure 1. Quartile analysis of the CD cohort for the 4 tested microbial antigens (ASCA, I2, OmpC, and CBir1). Reactivity to each antigen was divided into 4 quartiles and a value ascribed to a given individual based on their quartile of reactivity to each antigen (*left panel*). Quartile sums were calculated by the addition of the quartile value for each antigen (range, 4–16; see Materials and Methods). The distribution of quartile sums is shown (*right panel*). Values for binding levels are in ELISA units except for ASCA, which is presented in standardized format. Quartile sums were calculated similarly for unaffected relatives and healthy controls based on the distribution within each group (the quartile cutoff values and the distribution of quartile sums for the other 2 groups are not represented in this figure).

adding individual quartile scores for each microbial antigen, a quartile sum (range, 4–16) was derived that represents the cumulative semiquantitative immune response toward all 4 antigens. The quartile ranking reflects the pool of individuals under study (ie, patient with CD, unaffected relative, or healthy control) and is not directly comparable between groups.

Genotyping

Three *NOD2* variants that have been previously associated with CD[37] (R702W, G908R, and 1007fs) were adapted to the TaqMan MGB (Applied Biosystems, Foster City, CA) genotyping platform as previously described.[55,56] Five TLR2 variants (intron, N199N, S450S, P631H, 3′-genomic), 2 TLR4 variants (D299G, S360N), and 2 TLR9 variants (5′-genomic, P545P) were similarly adapted to the TaqMan MGB genotyping platform (Table 1). Variants in the TLR genes were selected based on prior evidence of association with inflammatory bowel disease[50–54] or by the use of Haploview and data from the International HapMap Project.[57,58]

Statistical Analysis

We first assessed the relationship between carriage of an *NOD2*, TLR2, TLR4, and TLR9 variant and collective seroreactivity to microbial antigens both qualitatively and semiquantitatively (because no association was found between any TLR variant and seroreactivity, all subsequent analyses were conducted with only *NOD2* variants). We then determined if any particular *NOD2*

Table 1. Genotyped SNPs for TLR2, TLR4, and TLR9

Gene	Designation	Database SNP	Gene position	TaqMan MGB assay reagents
TLR2				
	Intron	rs4696480	540	C_27994607_10
	N199N	rs3804099	29866	C_22274563_10
	S450S	rs3804100	30639	C_25607727_10
	P631H	rs5743704	31181	C_25607736_10
	3′-genomic	rs2405432		C_16230373_10
TLR4				
	D299G	rs4986790	13015	C_11722238_20
	S360N	rs4987233	13315	C_43308516_10
TLR9				
	5′-genomic	rs187084	1656	C_2301954_20
	P545P	rs352140	5991	C_2301952_10

SNP, single nucleotide polymorphism.

Table 2. Serologic and Genetic (*NOD2*) Characteristics of the CD Patient Cohort

Serologic and genetic characteristics	Cohort (n = 732)
Serologic profile (%)	
ASCA positive (n = 369)	50.4
Anti-I2 positive (n = 425)	58.1
Anti-OmpC positive (n = 272)	37.2
Anti-CBir1 positive (n = 413)	56.4
NOD2 genotype for R702W, G908R, 1007fs (%)	
No mutations (n = 499)	68.2
Heterozygous (n = 194)	26.5
Compound heterozygous (n = 23)	3.1
Homozygous (n = 16)	2.2

variant was predominant and examined whether any particular antibody or combinations of antibodies was predominant in determining the relationship between *NOD2* variants and seroreactivity. The contribution of *NOD2* to collective seroreactivity was evaluated by calculating the percent of variance that could be attributed to the presence of *NOD2* variants. Finally, we examined whether the presence of an *NOD2* variant was related to seroreactivity to microbial antigens in unaffected relatives of patients with CD and healthy controls.

Determination of the relationship of *NOD2* variants to seroreactivity. To determine the significance of increasing frequency of carriage of any *NOD2* variants with increasing numbers of qualitatively positive antibodies and with increasing quartile sum (range, 4–16), the Cochran–Armitage trend test was performed.[59] To test for differences in the mean quartile sum between those individuals with no *NOD2* variant and those with any variant, Student *t* test was used because the distribution was approximately a normal distribution.[59] One-way analysis of variance was performed to test the linear trend of mean quartile sum among those with 0, 1, and 2 *NOD2* variants.[59] One-way analysis of variance was used to test for a difference in seroreactivity associated with specific *NOD2* variants and similarly when comparing mean quartile sum between differing TLR genotypes.

Determination of the relative contribution of specific antibody or combinations of antibody positivity. The nonparametric Mann–Whitney test was used to compare the level of seroreactivity between those individuals who carried and those who did not carry an *NOD2* variant for each antibody.[59] To identify whether there is a significant difference in the frequency of carriage of an *NOD2* variant among groups within each set with single, double, and triple antibody positivity, χ^2 analysis was performed.[59]

Determination of percent variance contribution by *NOD2*. In order to determine what proportion of the variation in the seroreactivity to microbial antigens was attributable to the presence of an *NOD2* variant, a coefficient of determination (R^2), defined as 1 − SS (regression)/SS (total) in analysis of variance, was used.[59] Seroreactivity was defined, for this analysis, as the sum of the 4 standardized antibodies, where anti-OmpC = (log[anti-OmpC] − mean[log{anti-OmpC}])/SD(log[anti-OmpC]), and similarly for the other antibodies.

All analyses were performed using SAS computer software (version 8.2; SAS Institute, Inc, Cary, NC).

Results

Serologic and Genetic Characteristics of the Study Population

Table 2 shows the serologic and genetic (*NOD2*) characteristics of the 732-patient cohort. ASCA was detected in 50.4%, anti-I2 in 58.1%, anti-OmpC in 37.2%, and anti-CBir1 in 56.4%. Simple heterozygosity for a disease-predisposing *NOD2* variant was detected in 194 patients (26.5%), compound heterozygosity for 2 *NOD2* variants was detected in 23 patients (3.1%), and homozygosity for 2 *NOD2* variants was detected in 16 patients (2.2%).

***NOD2* Variants, But Not Variants of *TLR2*, *TLR4*, or *TLR9*, Are Associated With Seroreactivity to Microbial Antigens in Patients With *CD*.** Our first approach was to determine if we could demonstrate an association between the presence of an *NOD2* variant and seroreactivity to microbial antigens. First, the CD patient cohort was divided into 5 groups based on the number of antibodies (from 0 to 4) for which they were qualitatively positive and the proportion of patients with an *NOD2* variant in each group was determined. Figure 2 shows that *NOD2* variants were present with increasing frequency in patients with reactivity to an increasing number of microbial antigens, especially when there is reactivity to 2 or more antibodies. *NOD2* variants were present in those with 0, 1, 2, 3, or 4 positive antibodies at a frequency of 23%, 24%, 36%, 34%, and 42%, respectively (*P* for trend = .0008). We next sought to investigate the association between the presence of *NOD2* variants and semiquantitative seroreactivity by assessing the

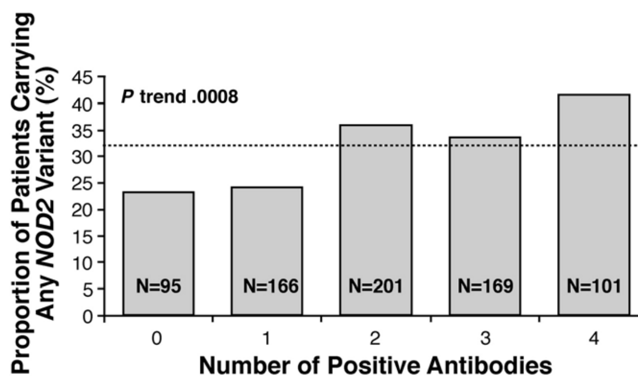

Figure 2. The frequency of carriage of any *NOD2* variant increased with qualitative antibody reactivity, as represented by the antibody sum (number of positive antibodies; range, 0–4). The *dotted line* represents the 31.8% frequency of carriage of at least one *NOD2* variant, across the entire cohort.

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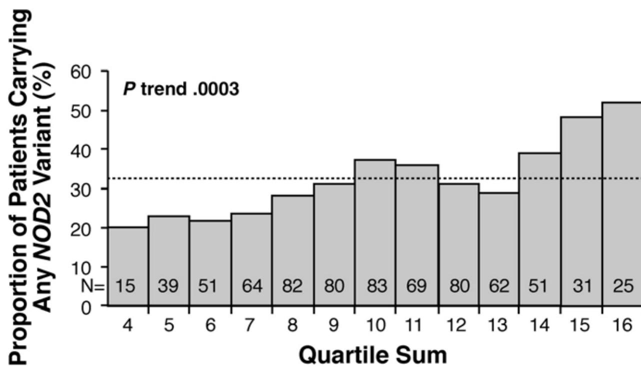

Figure 3. The frequency of carriage of any *NOD2* variant increased with semiquantitative antibody reactivity, as represented by the quartile sum (range, 4–16). The *dotted line* represents the 31.8% frequency of carriage of at least one *NOD2* variant, across the entire cohort.

magnitude of the cumulative serologic response to all 4 antigens using quartile sums as described previously. Figure 3 shows that *NOD2* variants were present at increasing frequency in patients with increasing cumulative semiquantitative immune response as reflected by individual quartile sums (*P* for trend = .0003).

These analyses showed that as the serologic response increased, either qualitatively (by number of positive antibodies) or semiquantitatively (by magnitude of the cumulative serologic response), the likelihood of a patient carrying an *NOD2* variant increased. Another approach to test this relationship was to compare the serologic response of those patients carrying an *NOD2* variant with those carrying no variant. Table 3 shows that, in those patients carrying any *NOD2* variant, the mean number of positive antibodies was higher than in those carrying no variant (2.24 ± 1.21 vs 1.92 ± 1.24, respectively; *P* = .0008). Moreover, those patients carrying any *NOD2* variant had a higher mean quartile sum than those carrying no variant (10.60 ± 3.03 vs 9.72 ± 3.01, respectively; *P* = .0003). The mean quartile sum in individuals with and without any of the TLR2, TLR4, and TLR9 variants under study was compared in a similar fashion. Table 4 shows that there was no association between seroreactivity to microbial antigens and the TLR variants listed.

Because our data showed that the presence of a defective innate immune gene (*NOD2*) was associated with a hyperresponsive adaptive immunologic response, we next sought to determine if having 2 defective alleles would be associated with a greater response than having only one. Figure 4 shows that the mean quartile sum increased in parallel with increasing number of *NOD2* variants (*P* for trend = .002).

The Relationship of Specific *NOD2* Variants to Seroreactivity to Microbial Antigens. Different *NOD2* variants are associated with differential degrees of altered sensing of MDP. The frameshift mutation 1007fs is associated with a more significant decrease in nuclear factor κB activity than the 2 missense mutations, R702W and G908R.[38,60] Therefore, we sought to determine if seroreactivity to microbial antigens varied according to which *NOD2* variant was present in an individual. There was no significant difference in the cohort-specific mean quartile sum in individuals with CD with one or 2 1007fs, G908R, and R702W variants, respectively (10.11 ± 3.12, 10.63 ± 3.18, and 11.06 ± 2.78, respectively; *P* = .16).

Increasing Cumulative Seroreactivity Rather Than Specific Antibody Combinations Are Associated With the Presence of an *NOD2* Variant. Our data thus indicated that the presence of an *NOD2* variant was associated with an increased serologic response to microbial antigens both in terms of the number of positive antibodies and the cumulative response as measured by quartile sum. Our next question was whether any particular antibody or combinations of antibodies was the predominant factor in determining this relationship. We first examined the absolute level of response to each antibody individually rather than collectively to determine if the presence of any *NOD2* variant was associated with higher individual reactivity. Table 5 shows that for each of the 4 antibodies, the magnitude of seroreactivity was higher when an *NOD2* variant was present.

Because there is a significant correlation among the expression of these antibodies in patients with CD,[22,55] we then divided the patients with CD into 16 mutually exclusive groups (Figure 5) based on all possible permutations of antibody positivity: no positive antibodies, single antibody positivity (4 groups in set 1), double antibody positivity (6 groups in set 2), triple antibody positivity (4 groups in set 3), and all antibodies positive. We then tested whether there was a significant difference among groups within each set where the groups had the same number of antibody positivity. There was no statistically significant difference in the frequency of *NOD2* variants among groups within each set, implying that no single antibody or combination of antibody positivity was wholly responsible for the association between seroreactivity and variant status (Figure 5). If, for example, a

Table 3. Cumulative Qualitative and Semiquantitative Seroreactivity to Microbial Antigens According to *NOD2* Variant Status in Patients With CD

	No *NOD2* variant (n = 499)	Any *NOD2* variant (n = 233)	*P* value
Mean no. of antibody positivity	1.92 ± 1.24	2.24 ± 1.21	.0008
Mean quartile sum (mean ± SD)	9.72 ± 3.01	10.60 ± 3.03	.0003

Table 4. Cumulative Semiquantitative Seroreactivity to Microbial Antigens According to TLR2, TLR4, and TLR9 Variant Status in Patients With CD

Gene	Variant	Genotype[a]	n	Mean quartile sum	*P* value
TLR2	Intron	11	208	9.98	.53
		12	359	10.11	
		22	164	9.79	
	N199N	11	237	10.30	.16
		12	364	9.85	
		22	129	9.82	
	S450S	11	628	10.10	.06
		12	101	9.36	
		22	3	11.00	
	P631H	11	677	9.99	.50
		12	52	10.06	
		22	2	12.50	
	3′-genomic	11	717	9.99	.88
		12	10	10.20	
		22	2	9.00	
TLR4	D299G	11	650	10.01	.89
		12	76	9.84	
		22	4	10.25	
	S360N	11	654	9.99	.26
		12	74	9.97	
		22	4	12.50	
TLR9	5′-genomic	11	269	9.99	
		12	350	9.98	
		22	110	10.15	
	P545P	11	203	10.18	.19
		12	348	9.78	
		22	179	10.21	

[a]1 denotes the major allele, 2 denotes the minor allele.

given antibody or combination of antibody positivity was responsible for the association, it would be anticipated that the frequency of *NOD2* carriage would be significantly greater in individuals with positivity to that antibody or combination. Therefore, these data indicate that the relationship between *NOD2* variants and serologic response to microbial antigens reflects a cumulative effect rather than being driven by any particular antibody or antibody combination.

After determining that the presence of an *NOD2* variant was associated with both a qualitatively and semiquantitatively increased seroreactivity to microbial antigens, a calculation of variance was performed to determine what proportion of the variability in seroreactivity was attributable to the presence of an *NOD2* variant. This calculation showed that 2.7% of the variability in the sum of the semiquantitative antibody levels was attributable to the presence of an *NOD2* variant.

The Presence of *NOD2* Variants Is Significantly Related to Seroreactivity to Microbial Antigens in Unaffected Relatives of Patients With *CD*. Both ASCA and anti-OmpC expression have been noted to be elevated in unaffected relatives of patients with CD, suggesting an underlying genetic determination of seroreactivity.[24–30] To explore this concept further, our final approach was to determine if the presence of an *NOD2* variant in unaffected relatives of patients with CD, and in a separate cohort of healthy controls, would be associated with a similarly greater adaptive immunologic response to microbial antigens. A quartile sum was again derived as previously described for patients with CD. The quartile sums in patients with CD, unaffected relatives, and healthy controls were based on the distribution of the magnitude of seroreactivity within each cohort; thus, the same quartile sum in a patient with CD or in a relative or healthy control is not representative of the same absolute magnitude of response and thus is not directly comparable. The magnitude of serologic response was signifi-

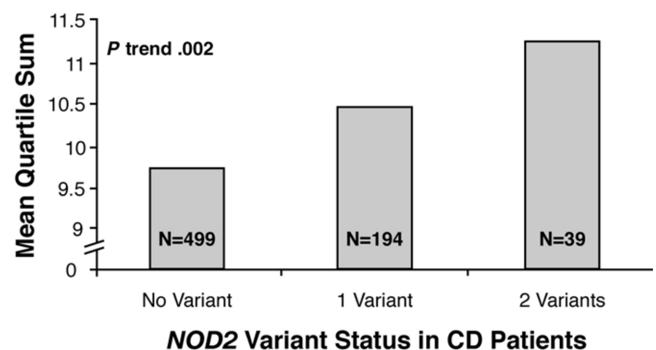

Figure 4. The cumulative semiquantitative antibody reactivity, as represented by mean quartile sum, increased with increasing number of *NOD2* variants by trend analysis ($P = .002$).

Table 5. Median Seroreactivity to Individual Microbial Antigens According to *NOD2* Variant Status in Patients With CD

Antibody	Median seroreactivity in EU/mL (range)		*P* value
	No *NOD2* variant	Any *NOD2* variant	
ASCA[a]	0.032 (−1.40 to 2.31)	0.620 (−1.26 to 2.57)	<.0001
Anti-I2	25.00 (0–248)	27.56 (0–324)	.04
Anti-OmpC	16.32 (0–147)	20.14 (0–203)	.03
Anti-CBir1	28.36 (3.01–257)	33.83 (0–280)	.01

[a]Seroreactivity toward ASCA is expressed in standardized units with a mean of zero and a standard deviation of ±1; thus, a standardized unit may have a negative value.

cantly lower, as expected, in unaffected relatives and healthy controls compared with cases and generally fell within the normal range (data not shown). We utilized sera from 220 unaffected relatives of patients with CD (92% first-degree). Figure 6 shows that in the unaffected relatives, the mean quartile sum in those individuals carrying any *NOD2* variant was higher than in those carrying no variant (10.67 ± 2.73 vs 9.75 ± 2.52; $P = .02$).

The same analysis was undertaken using sera from 200 healthy controls. Again, the magnitude of seroreactivity was divided into quartiles based on the distribution specifically within this cohort. Cohort-specific quartile sums were again derived as previously described. Figure 7 shows that the mean quartile sum in healthy controls carrying any *NOD2* variant (n = 24) showed a trend toward being higher than in healthy controls carrying no variant (n = 176) (10.79 ± 2.95 vs 9.69 ± 2.71; $P = .07$).

Discussion

The major etiologic hypothesis regarding CD is that it is likely related to a dysregulated immunologic response to enteric microorganisms. One manifestation of this dysregulated immunologic response is the expression of antibodies to microbial antigens. High levels of ASCA, anti-I2, and anti-OmpC have been associated with fibrostenosing and internal penetrating disease as well as the need for small bowel surgery.[55] More recently, anti-CBir1 has been found to be independently associated with severe small bowel disease such as internal perforating and fibrostenosing disease.[23] Indeed, defects in the innate immune gene *NOD2* have also been found to be associated with a fibrostenosing clinical phenotype, suggesting a complex interaction between genetic susceptibility, the adaptive immunologic response, and clinical disease behavior.[46,55,56]

A suggestion of a link between the adaptive immunologic response and genetic susceptibility is supported by the finding of increased ASCA and anti-OmpC expression, both qualitatively and quantitatively, in unaffected relatives of patients with CD.[24–30] Moreover, the recent finding that the presence of a defective innate immune gene locus (Cdcs1) that renders the host less responsive to microbial products and confers severe colitis in the interleukin-10–deficient C3Bir mouse in association with a hyperresponsive adaptive immunologic response to these same products supports the concept of a genetic link between innate and adaptive immunity and susceptibility to mucosal inflammation.[31]

We hypothesized that the presence of defective innate immune genes that render the host less responsive to bacterial products would be associated with a compensatory hyperresponsive adaptive immunologic response to microbial antigens. A previous study by Abreu et al found a borderline association between ASCA expression and the 1007fs *NOD2* variant, while a study by Walker et al found no association between *NOD2* variants and ASCA expression.[56,61] Similarly, a study by Arnott et al failed to show an association between ASCA, anti-I2, and anti-OmpC expression and *NOD2* variants.[62] However, this latter study included only 142 patients with CD and, thus, may have been underpowered to detect a relevant association. Moreover, the rate of *NOD2* mutations in the Scottish population in this study was only 23.9%, lower than the 37.3% rate found in our previously studied North American cohort.[55] Finally, recent studies by Annese et al and Cruyssen et al did demonstrate an association between *NOD2* variant status and ASCA expression;

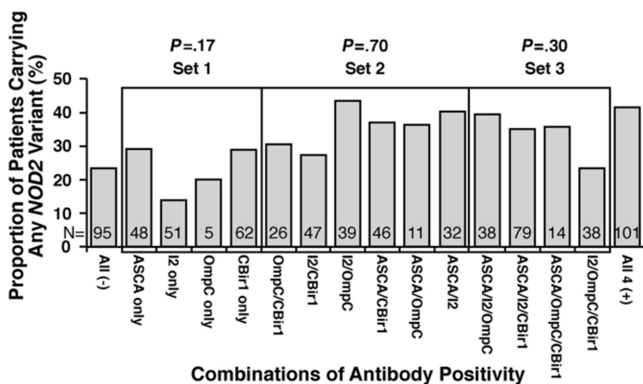

Figure 5. The cohort of patients with CD was divided into mutually exclusive groups based on all possible permutations of antibody positivity: no positive antibodies, single antibody positivity (*4 groups in set 1*), double antibody positivity (*6 groups in set 2*), triple antibody positivity (*4 groups in set 3*), and all antibodies positive. Within each of the 3 sets where the groups had the same number of antibody positivity, there was no statistically significant difference in the frequency of *NOD2* variants among sets 1, 2, and 3, respectively.

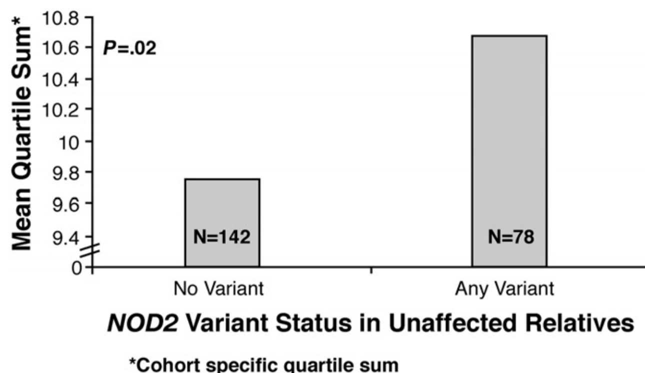

Figure 6. The cumulative semiquantitative antibody reactivity in unaffected relatives of patients with CD, as represented by mean quartile sum, was higher in individuals carrying any *NOD2* variant than in those carrying no variant (P = .02). *The quartile sum in unaffected relatives is based on quartiles of seroreactivity within this cohort specifically and is not representative of the same magnitude of reactivity as an equivalent quartile sum value in a patient with CD or a healthy control. No individuals carried 2 variants.

however, they did not study the reactivity to other microbial antigens as in the present study.[63,64] In our study herein, we showed that patients with CD with a predominant qualitative (number of positive antibodies) and semiquantitative (absolute magnitude of response) serologic response to microbial antigens were more likely to carry an *NOD2* variant (Figures 2 and 3). Moreover, we showed that patients with CD carrying an *NOD2* variant had a higher qualitative and semiquantitative serologic response than patients carrying no variants (Table 3). This relationship was seen not only with the cumulative response to all 4 antibodies, but also with each antibody individually (Table 5). Because this had not been shown previously, we sought to explore whether the finding was reflective of a relationship between a specific antibody, particularly anti-Cbir1, because it had not been studied in this context previously, and *NOD2* variant status. We were able to show that the association of seroreactivity to microbial antigens to *NOD2* variant status was more a reflection of the cumulative semiquantitative response than any particular antibody or combination of antibodies. *E coli*, *P fluorescens*, and most flagellated bacterial species will express MDP as components of their bacterial cell walls. However, the increased expression of antibodies directed against bacterial and yeast antigens is likely a function of increased exposure of the mucosal immune system to a range of microbial antigens owing to diminished initial clearance, perhaps due to impaired secretion of defensins. Hence, a defect in MDP signaling via *NOD2* variants could result in impaired defense against microbial species, with the subsequent development of antibodies to microbial antigens being a secondary phenomenon due to bacterial invasion and increased exposure of the mucosal immune system to a range of microbial antigens.

The finding that only 2.7% of the variance in seroreactivity is attributable to the presence of an *NOD2* variant is not surprising and does not detract from the relevance of our finding. This is in keeping with other complex genetic disorders such as insulin resistance and hypercholesterolemia. Approximately 6% of the variability in insulin clearance is due to variation in the gene for muscle-specific AMP deaminase, and as little as 6% of the variability in serum cholesterol is ascribable to different apolipoprotein E polymorphisms.[65,66] Furthermore, the variance we found in our study is likely the lower end of the true association between *NOD2* variants and adaptive immunologic response, because we were only testing for the 3 most common variants (R702W, G908R, and 1007fs). More than 27 variants have been described, and in one large cohort more than 19% of disease-associated variants were not of the 3 most common mutations for which we tested.[46]

The innate immune system is complex and involves the sensing of bacterial products via many mechanisms, including not only *NOD2* but TLRs, which act as pattern-recognition receptors serving to regulate the immunologic response of the host to enteric bacteria.[2] There are 11 known mammalian TLRs, including TLR2, TLR4, TLR5, and TLR9, that sense bacterial lipoproteins, lipopolysaccharide, flagellin, and bacterial and viral CpG DNA, respectively.[67] Defects in TLR signaling could also lead to diminished sensing and subsequent clearance of bacteria, leading to invasion and a compensatory adaptive immunologic response. Indeed, the TLR4 D299G polymorphism has been associated with both ulcerative colitis and CD in a Belgian study, whereas no association was found in a Scottish and Irish cohort.[53,68] In one Greek study, the presence of both TLR4 and *NOD2* mutations was associated with increased susceptibility to inflammatory bowel disease, suggesting a synergistic ef-

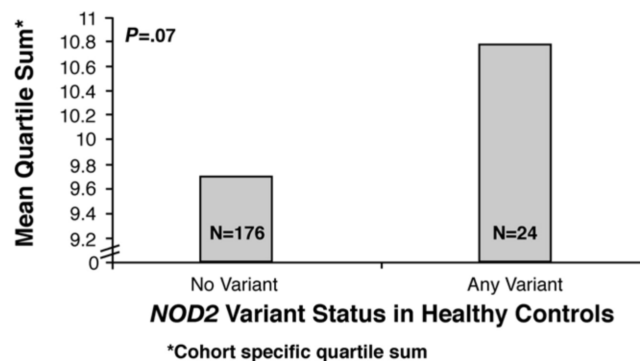

Figure 7. The cumulative semiquantitative antibody reactivity in healthy controls, as represented by mean quartile sum, was numerically higher (although not achieving statistical significance) in individuals carrying any *NOD2* variant than in those carrying no variant (P = .07). *The quartile sum in healthy controls is based on quartiles of seroreactivity within this cohort specifically and is not representative of the same magnitude of reactivity as an equivalent quartile sum value in a patient with CD or unaffected relative. No individuals carried 2 variants.

fect.[69] As previously discussed, variants of the genes for both TLR2 and TLR9 have also been associated with CD.[50,54]

In this study, we were not able to show an association between variants in these TLR genes and seroreactivity to microbial antigens. This is not necessarily surprising because the association between variants in TLR genes and inflammatory bowel disease has been less consistent than the association between CD and functional variants of the *NOD2* gene. Indeed, it has been suggested in a study by Oostenbrug et al that the D299G polymorphism is not causal but is in linkage with the true susceptibility variants of the TLR4 gene.[51] This could serve as a potential explanation for the lack of association of seroreactivity to the D299G variant in our study. We would hypothesize that as we advance our understanding of defects in innate and adaptive immune response in CD, new gene defects will be characterized and new associations will be found with seroreactivity to microbial antigens paralleling our finding with *NOD2* variants.

Our finding that unaffected relatives carrying an *NOD2* variant had a greater serologic response to microbial antigens than those carrying no variants further strengthens our conclusion. Because both seroreactivity and *NOD2* variant status have been linked to disease severity, one argument could be that the association between *NOD2* variant status and seroreactivity is a function of a common end point, and the relationship is only a surrogate. Arguing against this is the fact that in a study by Cruyssen et al,[64] the association between ASCA and *NOD2* variant status was independent of disease phenotype and that the unaffected relatives in our study have no apparent disease activity. However, it has been shown in a study by Thjodleifsson et al that 49% of unaffected relatives of patients with CD have elevated levels of fecal calprotectin, thus implying an element of subclinical intestinal inflammation.[70] Subclinical intestinal inflammation could lead to increased mucosal permeability and subsequent exposure of the mucosal immune system to microbial antigens. However, if altered gut permeability is etiologic in determining the seroreactivity of unaffected relatives, then there would be no difference between those with and without *NOD2* variants unless the presence of a variant itself was a determining factor. Therefore, this argues that the association between innate immune defects (*NOD2*) and adaptive immunologic response as measured by seroreactivity to microbial antigens is direct.

The same relationship between *NOD2* variant status and seroreactivity to microbial antigens was not statistically significant in healthy controls. However, only 12% of these individuals carried a variant; therefore, the sample size may have been too small to detect a significant difference (type II error). There was a trend toward healthy controls carrying an *NOD2* variant having higher seroreactivity than those carrying no variant (Figure 7). This further supports the supposition that CD has a complex genetic basis and that a single innate immune defect is insufficient to cause disease but is nevertheless sufficient to be associated with an aberrant adaptive immunologic response.

In summary, this study has shown that a significant degree of the variability in the adaptive immunologic response to CD-associated microbial antigens is due to the presence of a defective innate immune gene (*NOD2*). This relationship can be found in unaffected relatives of patients with CD and even perhaps in healthy controls as well. This supports the concept of a genetic basis for a link between innate immune defects and dysregulated, hyperresponsive adaptive immunity to microbial antigens in human CD, a link that parallels findings in rodent mucosal inflammation. Further studies can now explore this relationship between multiple innate or perhaps adaptive immune defects and adaptive immunologic response as new variants in innate and adaptive immune genes are described. Finally, this cumulative quantitative response could be used as a basis for targeting individuals in whom to search for novel genes associated with CD.

BASIC–ALIMENTARY TRACT

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Received May 4, 2006. Accepted October 26, 2006.

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Supported by National Institutes of Health grant P01 DK46763.