Testing the Interaction between NOD-2 Status and Serological Response to *Mycobacterium paratuberculosis* in Cases of Inflammatory Bowel Disease[▽]

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In a population-based case-control study we have previously shown that 14% of healthy Manitobans carry one or two mutations in the NOD-2 locus, a gene highly associated with Crohn's disease (CD). The NOD-2 protein is the receptor responsible for recognition of bacterial peptidoglycans, and it is plausible that NOD-2 is involved in the recognition of mycobacteria. Thirty-seven percent of Manitobans with CD had ≥1 NOD-2 mutation, leading to a threefold increased risk of CD for single-mutant carriers and a 30-fold increased risk for double-mutant carriers. In the same population groups, we assessed the seroprevalence for *Mycobacterium paratuberculosis* and found it to be 35%, with no differences between CD, ulcerative colitis (UC), and controls. Because of high rates of CD and UC in Manitoba, we assessed whether there was an interaction between carrying a NOD-2 mutation and *M. paratuberculosis* seropositivity. An enzyme-linked immunosorbent assay for serum antibodies to *M. paratuberculosis* in cattle was adapted for human use. DNA was purified from whole blood. Subjects were genotyped for three NOD-2 variants, G908R, Cins1007fs, and R702W. Multivariate logistic regression analysis showed that NOD-2 gene mutations significantly associated with CD, but *M. paratuberculosis* serology did not. Furthermore, there was no interaction between NOD-2 mutation status and *M. paratuberculosis* serology status. For those with the NOD-2 mutation, the likelihood of CD subjects having positive *M. paratuberculosis* serology was similar to that of controls (odds ratio, 1.31; 95% confidence interval, 0.55–3.11). No interaction could be proven for UC or by combining CD and UC compared to controls. In conclusion, we could not find an interaction between the NOD-2 genotype and *M. paratuberculosis* serology in relationship to CD or UC.

Parallels have been drawn between Crohn's disease (CD) and mycobacterial diseases of both humans and animals. *Mycobacterium paratuberculosis* causes Johne's disease in cattle, a granulomatous inflammatory enteritis associated with diarrhea, wasting, and a predilection for the ileum, sharing many similarities with human CD (1, 13, 22). There has been controversy as to whether *M. paratuberculosis* is associated with human CD. There have been conflicting reports on the isolation of *M. paratuberculosis* DNA from CD tissues (3, 9), and some have argued that the discrepancies are methodologically based (9). Recently there has been a report of isolating circulating *M. paratuberculosis* in blood from CD patients more often than from controls (18); however, this has yet to be replicated in any other laboratory. We have undertaken a large population-based serological study of *M. paratuberculosis* antibody status and reported that approximately 35% of all Manitobans in our study were seropositive; however, there was no distinction in serological status among subjects with CD, ul-

cerative colitis (UC), and unaffected controls (2). Since a 35% seropositive rate was much higher than that reported from other populations (10, 11), it was hypothesized that *M. paratuberculosis* may still have some relevance for CD if perhaps other important risk factors were also present. Hence, if persons with the genetic predisposition(s) to CD were infected with *M. paratuberculosis*, then that combination might be sufficient to facilitate CD development.

To date there has been one reproducible gene mutation that has been highly associated with CD. Mutations in the nucleotide binding oligomerization domain-2 (NOD-2) gene locus on chromosome 16 have been considered to account for approximately 25% of cases of CD (6, 7). In most, but not all, Western populations, NOD-2 is associated with CD, and in Manitoba, we have recently reported that 14% of healthy controls carried one or two NOD-2 mutations, compared to 37% of the CD group (P, <0.001) (8). Similar to most studies, a gene dosage effect was observed, with a risk of CD for NOD-2 double-mutant (MM) carriers at 30-fold and a risk of threefold for simple heterozygote (MW) carriers. Hence, we considered whether the high seroprevalence of *M. paratuberculosis* in Manitobans might still be relevant in terms of an association with CD, if we found that those with the NOD-2 gene mutation

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were more likely to be *M. paratuberculosis* seropositive among those with CD compared to healthy controls.

MATERIALS AND METHODS

Study design. This study was based on a nested sample of the case-control study previously published (5). In brief, cases were drawn from the University of Manitoba Inflammatory Bowel Disease Research Registry, which has been described previously (4). We accessed the Registry to enroll subjects under the age of 50 years and mailed them information sheets and questionnaires for the case-control study. The purpose of limiting the study to subjects under 50 years old was to enhance recall for questionnaire completion regarding events of early life as well as to enhance the likelihood of maintenance of antibody responses on serological testing. All subjects completed questionnaires and consent forms for participation and were contacted for venipuncture. Diagnoses of CD and UC were verified by chart review for clinical data including but not limited to endoscopic, histological, and radiological findings.

Control selection. A population-based set of controls was selected from the Manitoba Health population registry. The Manitoba Health registry contains demographic information on all persons registered with the Manitoba Health public health insurance system. The registry is regularly updated with vital registrations and information from medical and hospital transactions and closely matches population estimates derived from the Canadian census (Statistics Canada) (19). A random sample of registered persons was selected, with restriction to ages 18 to 50 years and stratification by gender and 5-year age groups to approximate the age and gender distribution of the combined inflammatory bowel disease (IBD) case group in the research registry. Manitoba Health's Information Services generated a mailing list of eligible controls and sent an information package prepared by the investigators that explained the study and requested participation. The investigators did not know the identity of the controls unless they received a mailed response. Like cases, controls completed a questionnaire and provided a venous blood sample.

The questionnaire included demographic and historical questions, including questions about childhood water sources and the frequency of consumption of various meats and of unpasteurized milk, all potential sources of *M. paratuberculosis* (questionnaires are available on request from the authors). The results of the questionnaire study have been published elsewhere (5).

Enzyme-linked immunosorbent assay for *M. paratuberculosis*. The enzyme-linked immunosorbent assay (ELISA) for serum antibodies to *M. paratuberculosis* in cattle (*M. paratuberculosis* antibody test kit; IDEXX Laboratories, Inc. Westbrook, ME) was adapted for human use. *M. paratuberculosis* antigen-coated 96-well microtiter plates and *Mycobacterium phlei*-containing serum diluent (used to absorb cross-reactivities antibodies) supplied with the kit were used. The kit employs a horseradish peroxidase-labeled protein G conjugate to detect binding of antibodies to the solid-phase *M. paratuberculosis* antigens. On every ELISA plate, we ran bovine and human serum controls. All sera were tested in duplicate, including controls. Sample-to-positive (S/P) ratios based on bovine controls were used to interpret the assay. The *M. paratuberculosis* ELISA for humans was not validated against proof of *M. paratuberculosis* infection because there are no established (validated or gold standard) methods to do so. ELISA were run blinded to subject status (IBD or healthy controls [HC]).

Sera were diluted 1:20 in a kit serum diluent (identical to the kit protocol for testing bovine sera) and incubated at room temperature for 30 min prior to transfer to the microtiter plate. One hundred microliters of each diluted serum sample and of control serum were dispensed into duplicate microtiter wells and incubated for 30 min at room temperature. Unbound serum components were then removed by washing wells six times with phosphate buffer provided with the kit. After removal of residual wash fluid, 100 µl of conjugate was added, and the plate was incubated for another 30 min. After washing the wells as before, 100 µl of TMB (3,3′,5,5′-tetramethylbenzidine dihydrochloride) substrate solution was added to each well and incubated for 15 min. The enzymatic reaction was stopped after 15 min by the addition of 100 µl of stop solution provided with the kit. The optical density (OD) for each well was measured at 650 nm (model EL312; BioTek Instruments, Inc., Winooski, VT). Assays were considered valid based on the kit manufacturer's interpretation guidelines.

Mean ELISA OD readings for patient sera were transformed and expressed as the S/P ratio (as done for the cattle assay), in which S/P = (sample OD − negative control OD)/(positive control OD − negative control OD). Results of testing 252 sera from Red Cross blood donors (Red Cross, Madison WI) using this prototype ELISA for human serum antibodies to *M. paratuberculosis* were used to establish the S/P cutoff for a positive test. A positive test with human serum was defined based on Red Cross donor sera as the mean (0.065) plus 2 standard deviations (2 × 0.123), i.e., S/P = 0.312, which by definition gave the

TABLE 1. Multivariate logistic regression analysis of risk factors for subjects with Crohn's disease versus those of healthy controls

Risk factors	Adjusted (multivariate)		
	OR	*P* value	95% CI
NOD-2 mutant carrier (yes vs. no)	3.52	<0.001	2.19–5.66
M. paratuberculosis (seropositive vs. seronegative)[a]	1.08	0.74	0.69–1.66
Smoking (ever or never)	1.99	0.001	1.30–3.02
Age (yr)	0.94	<0.001	0.91–0.97
Residency (rural vs. city)	0.93	0.73	0.61–1.41
Family history of IBD (yes vs. no)	2.27	0.01	1.20–4.28

[a] In this analysis, *M. paratuberculosis* was treated as a categorical variable.

assay a 95% specificity. The relationship between the ELISA S/P ratio and the likelihood of *M. paratuberculosis* infection in cattle was recently demonstrated (12).

NOD-2 gene testing. DNA was purified from whole blood using a Puregene kit according to the manufacturer's protocol. Two NOD-2 variants, G908R and Cins1007fs, were genotyped using a TaqMan method of allelic discrimination for single nucleotide polymorphisms; and R702W was genotyped by allele-specific PCR with internal controls, as previously reported (8). For all genotyping, two sets of control homozygotes, heterozygotes, wild-type homozygotes, and water controls were included in each 96-well plate analyzed. Genotype determinations were performed blinded to the status of IBD or HC.

Analysis. Analyses were done using Stata statistical software, release 8 (23). Multiple logistic regression analyses were run for each CD versus healthy control, each UC versus healthy control, and all CD and UC subjects combined (IBD) versus healthy controls. Adjusted odds ratios were estimated with 95% confidence intervals. *M. paratuberculosis* serology was considered categorically (positive versus negative according to established cutoffs) as well as continuously (by S/P ratios based on bovine controls). A Wald test of homogeneity of odds ratios across different strata of NOD-2 mutation status through two-by-two table and likelihood ratio test statistics for the added interaction term in the multivariate logistic regression model were both undertaken to evaluate the interaction between NOD-2 mutation status and *M. paratuberculosis* serology status (14, 17, 20). The analyses of NOD-2 genotyping and *M. paratuberculosis* serology were performed independently using blinded subject study numbers, and a separate investigative site collated the results from each laboratory.

RESULTS

There were 182 subjects with CD, 105 with UC, and 294 controls with complete data on NOD-2 genotyping and *M. paratuberculosis* serology. On multivariate logistic regression analysis for all CD or UC or all IBD combined, there was a significantly decreased risk of having either CD or UC compared with that of controls, by gender and age. This reflects conditions in our original sample in which there were significantly more female controls and controls were slightly older than patients with either form of IBD (5). We did not interpret these as risks for either disease but rather a manifestation of subject enrollment, and the multivariate analysis controlled for this in assessing the impact of other variables. The multivariate logistic regression analysis for CD is presented in Table 1. Being a carrier of at least one NOD-2 mutation and a smoker at any time and having a family history of IBD were factors significantly associated with CD, but *M. paratuberculosis* serology was not. Being a smoker ever or having a family history of IBD was significantly associated with UC, but neither NOD-2 mutation carriage nor *M. paratuberculosis* serology status was associated with UC (Table 2). The results of the multivariate analyses for both CD and UC were unchanged if the natural logarithm of *M. paratuberculosis* serology results was entered

TABLE 2. Multivariate logistic regression analysis of risk factors
for subjects with UC versus those of healthy controls

Risk factors	Adjusted (multivariate)		
	OR	P value	95% CI
NOD-2 mutant carrier (yes vs. no)	1.66	0.10	0.90–3.08
M. paratuberculosis (seropositive vs. seronegative)[a]	0.78	0.37	0.46–1.33
Smoking (ever or never)	1.68	0.04	1.04–2.72
Age (yr)	0.94	0.001	0.90–0.97
Residency (rural vs. city)	0.72	0.19	0.44–1.18
Family history of IBD (yes vs. no)	2.26	0.03	1.08–4.76

[a] In this analysis, M. paratuberculosis was treated as a categorical variable.

TABLE 3. Assessing the interaction between the NOD-2 genotype
and M. paratuberculosis serology in Crohn's disease
and ulcerative colitis

IBD form	M. paratuberculosis serology result					
	Seronegative			Seropositive		
	OR	P value	95% CI	OR	P value	95% CI
Crohn's disease						
NOD-2 carrier−	1.0[a]			1.03	0.9	0.64–1.65
NOD-2 carrier+	2.63	0.001	1.51–4.59	4.86	<0.001	2.45–9.65
Ulcerative colitis						
NOD-2 carrier−	1.0[a]			0.57	0.06	0.32–1.02
NOD-2 carrier+	1.02	0.95	0.49–2.11	1.65	0.27	0.68–3.99

[a] Reference values.

instead of using a positive or negative nominal variable for *M. paratuberculosis* (data not shown).

For CD there was no interaction between NOD-2 mutation status and *M. paratuberculosis* serology status. For those with the NOD-2 mutation, the likelihood of CD subjects having positive *M. paratuberculosis* serology was similar to that of controls (odds ratio [OR], 1.31; 95% confidence interval [CI], 0.55–3.11); and for those who were NOD-2 wild-type homozygous (no mutations), the likelihood of subjects with CD being *M. paratuberculosis* seropositive was similar to that of controls (OR, 1.02; 95% CI, 0.62–1.67). The interaction between NOD-2 status and *M. paratuberculosis* seropositivity in CD versus controls was assessed by two methods, the Wald test of homogeneity with 1 degree of freedom, and the likelihood ratio test for the added interaction term in the multivariate logistic regression model. The results showed no significant interaction between NOD-2 mutation status and *M. paratuberculosis* serology status; however, a trend existed toward a subject being more likely to have CD if the subject was both NOD-2 mutation positive and *M. paratuberculosis* seropositive (Table 3).

For UC there was some suggestion of an interaction between NOD-2 status and *M. paratuberculosis* seropositivity. In comparing only UC cases and controls, for those with at least one NOD-2 mutation, the likelihood of being *M. paratuberculosis* seropositive was not significantly different than that of controls (OR, 2.55; 95% CI, 0.7–9.34; *P* = 0.16); and for those with no NOD-2 mutations, there was no significant difference in *M. paratuberculosis* seropositivity (OR, 0.63; 95% CI, 0.34–1.16; *P* = 0.14). Because of these trends toward an interaction between NOD-2 mutations and *M. paratuberculosis* seropositivity, we assessed the likelihood of harboring at least one NOD-2 mutation in those who were *M. paratuberculosis* seropositive, and this likelihood was increased in UC subjects compared to that in controls (OR, 3.02; 95% CI, 1.1–8.2; *P* = 0.03). For those who were *M. paratuberculosis* seronegative, the likelihood of having a NOD-2 mutation was no different between UC subjects and controls (OR, 1.25; 95% CI, 0.56–2.76). The interaction between NOD-2 status and *M. paratuberculosis* seropositivity in UC versus controls was assessed by two methods (described above), which showed no significant association between NOD-2 mutation status and *M. paratuberculosis* serology status in UC; however, an evident trend was that *M. paratuberculosis* seropositivity in the presence of NOD-2 mutations was more likely to be associated with UC (Table 3).

We tested for a relationship between NOD-2 mutation status and *M. paratuberculosis* serology status among all subjects (*n* = 581). Subjects with IBD who harbored at least one NOD-2 mutation (*n* = 88) were not significantly more likely to be *M. paratuberculosis* seropositive (*n* = 40) than controls (*n* = 14 of 44; OR, 1.38; 95% CI 0.6–3.18; *P* = 0.45). Subjects with IBD and with wild-type NOD-2 were also not significantly more likely to be *M. paratuberculosis* seropositive than controls with wild-type NOD-2 (56 of 199 compared with 81 of 250; OR, 0.85; 95% CI, 0.55–1.31; *P* = 0.47).

Another way to test for an association between the NOD-2 genotype and *M. paratuberculosis* serology was to determine if an association existed just in the 294 healthy controls. As these controls were drawn from the general population, they should be representative. Assessing the outcome of being *M. paratuberculosis* seropositive in a multiple logistic regression analysis including NOD-2 genotype, age, sex, ever having smoked, rural versus urban living, and a family history of IBD, the likelihood of being *M. paratuberculosis* seropositive if harboring a NOD-2 mutation was 1.03 (95% CI, 0.52–2.08; *P* = 0.92).

DISCUSSION

The NOD-2 protein is the receptor responsible for the recognition of bacterial peptidoglycans through interaction with muramyl dipeptide (16). Since the cell walls of mycobacteria contain peptidoglycans, it is plausible that NOD-2 is involved in the recognition of mycobacteria. Hence, defective NOD-2 function might lead to impaired responses to mycobacteria, facilitating intracellular incorporation of mycobacteria and the failure to clear the organism. A recent study has confirmed that NOD-2 and other Toll-like receptors are recognition systems for mycobacteria (15). Peritoneal macrophages from NOD-2 knockout mice produced significantly less tumor necrosis factor than control cells, suggesting the possibility of a role for NOD-2 in recognizing mycobacteria. Similarly, mononuclear cells from CD patients with NOD-2 mutations secreted fewer cytokines in response to *Mycobacteria tuberculosis*. Therefore, it is plausible that in a setting with potentially high *M. paratuberculosis* infection rates (such as in Manitoba), if *M. paratuberculosis* is an important pathogen in CD, then analyzing subjects with NOD-2 gene defects, who may not handle *M. paratuberculosis* similarly to those with wild-type NOD-2, will provide some basis of association between *M. paratuberculosis* and CD. Unfortunately, we could not find an association between *M. paratuberculosis* seropositivity based on NOD-2 sta-

tus. We could not find an interaction between the NOD-2 genotype and *M. paratuberculosis* serology status in predicting either form of IBD or an association with *M. paratuberculosis* seropositivity if harboring a NOD-2 mutation in the general population.

Elsewhere, an interaction between *M. paratuberculosis* seropositivity and NOD-2 gene mutations has been suggested. In a small Sardinian study, colon tissue from 25 of 37 (68%) patients with CD and 7 of 34 (21%) without CD were PCR positive for *M. paratuberculosis*; and 19 of 37 (51%) CD subjects and 7 of 34 (21%) carried at least one mutation for NOD-2 (21). Of the 19 CD subjects who carried a NOD-2 mutation, 14 (73%) were positive for *M. paratuberculosis* compared with 11 of 18 (61%) who were NOD-2 homozygous for wild-type alleles. In the control group, of the seven subjects with NOD-2 mutations only, two were positive for *M. paratuberculosis*. While the authors suggested that there was a trend for an association between having NOD-2 mutations and being *M. paratuberculosis* positive, this association was only present when comparing CD subjects with controls who had NOD-2 mutations, but having NOD-2 mutations did not impact on *M. paratuberculosis* status among the CD subjects.

The advantage of our study is mainly that all subjects with CD and UC and healthy controls were enrolled from population-based registries and hence there were no selection biases. One major caveat, however, is the assumption that the *M. paratuberculosis* assay designed and validated for cattle, using fecal culture of *M. paratuberculosis* as the reference test, is accurate for the detection of *M. paratuberculosis* infections in humans. It is possible that humans respond to different antigens or are infected with different strains of *M. paratuberculosis*, calling into question the validity of the *M. paratuberculosis* ELISA used in this study. Serological studies will require further refinement, preferably with assays to *M. paratuberculosis* strains which have proven to be human pathogens.

Does our study rule out an association of *M. paratuberculosis* with any form of IBD? It does not absolutely, but if the trends suggesting the interaction of *M. paratuberculosis* with NOD-2 mutations for UC are considered, then a very large sample size would be required to prove an effect if one exists. If a very large sample size is required, then how likely is it that the effect would be meaningful? Furthermore, other CD candidate genes would need to be assessed in relation to *M. paratuberculosis* to help determine if infection with *M. paratuberculosis* requires some other permissive factor that will lead to disease. Without this type of evidence there is little hard evidence for an association between *M. paratuberculosis* and CD in Manitobans, particularly in relation to NOD-2 genotype status.

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