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Chronic yersiniosis due to defects in the TLR5 and NOD2 recognition pathways

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ABSTRACT

Infection with Yersinia enterocolitica leads to a self-limiting disease, but in a small number of cases a protracted course can develop. The host genetic factors contributing to the advancement of the disease to the chronic phase are not known. We describe a patient suffering from an abdominal inflammatory mass due to chronic yersiniosis. Functional assays revealed defects in the recognition of flagellin by Toll-like receptor 5 (TLR5) and of muramyl dipeptide by NOD2, leading to a defective inflammatory response to Yersinia enterocolitica. Genetic sequencing showed that the patient was compound heterozygous for five different mutations in TLR5, while being homozygous for the 3020insC NOD2 mutation. In conclusion, we describe a patient in whom specific defects in the TLR5 and NOD2 recognition pathways led to chronic yersiniosis.

KEYWORDS

Cytokine, Crohn’s disease, NOD2, TLR5, Yersinia

INTRODUCTION

Yersinia enterocolitica is a flagellated Gram-negative bacterium that is pathogenic for humans. Infection occurs through consumption of contaminated water or food, and when healthy individuals are infected, generally a self-limiting intestinal inflammation ensues. Occasionally, more serious protracted disease develops.1 Chronic mesenteric lymphadenitis, ileitis and hepatitis are major manifestations of more severe yersiniosis, as are extra-intestinal presentations such as reactive arthritis and erythema nodosum.4 Y. enterocolitica infects the Peyer’s patches and other elements of the mucosa-associated lymphoid tissue, and here the organism may persist for long periods of time. In recent years, many of the virulence factors of Y. enterocolitica have been discovered; among these the Yersinia outer proteins (Yop) and LcrV are the most prominent.3 Delivery of Yop into the cytoplasm of host cells through a type III secretion/translocation system is crucial in the pathogenesis of the infection.4 LcrV is a released multifunctional molecule that acts as an immunomodulatory molecule by interaction with CD14 and Toll-like receptor (TLR) 2 and subsequent induction of interleukin-10 (IL-10).5 In addition to recognition by TLR2, other pattern recognition receptors of the innate immune system are likely to play a role in the interaction of Y. enterocolitica with the host. As Yersinia spp are flagellated bacteria, recognition of flagellin by TLR5 also contributes to the recognition by the host;6 while TLR4 is important for induction of apoptosis7 NOD2, the intracellular recognition receptor for peptidoglycans and muramyl dipeptide (MDP)8,9 could also be expected to play a role in the interaction between Y. enterocolitica and host cells.

From experiments in murine models it is known that interferon γ (IFNγ) and the cytokines responsible for its induction, IL-12 and IL-18, are essential for protective immunity against Y. enterocolitica.10-12 In addition, other proinflammatory cytokines such as IL-6 are also involved in the protective anti-Yersinia mechanisms,13 while triggering IL-10 through an LcrV/TLR2-dependent mechanism is essential for virulence.14

In this paper we report the history of a patient with a severe chronic yersiniosis. The persistence of Yersinia was accompanied by a defective cytokine response to the micro-organisms by leucocytes isolated from the patient,
which was very likely responsible for the protracted course of the disease. We also pinpoint the defective cytokine response to defects in TLR5- and NOD2-mediated recognition of Y. enterocolitica in this patient.

**Patients and Methods**

**Case report**

A 28-year-old male was referred to the outpatient clinic for infectious diseases at the Radboud University Nijmegen Medical Centre. In February 2003, the patient developed right-sided abdominal pain followed by chills and high fever. He was admitted 14 days after the start of the illness. A CT scan revealed multiple enlarged mesenterial lymph nodes close to the aortic bifurcation. The immunoblot for the Y. enterocolitica antibodies was positive, showing specific IgG and IgA reactivity against Yop1, 3, 3A, 4 and 5. A presumptive diagnosis of Y. enterocolitica mesenterial adenitis was made, and subsequently the patient was treated with ciprofloxacin for a total of six months. Despite this treatment, the patient’s fever, malaise and elevated C-reactive protein persisted. An extensive work-up search for alternative protein diagnoses such as Whipple’s disease was fruitless. Crohn’s disease was unlikely as a small bowel X-ray series and colonoscopy revealed no abnormalities. A biopsy taken from a mesenterial lymph node mass yielded granulomatous inflammation with epitheloid cells and perilymphadenitis. No micro-organisms were seen and further microbiological investigations remained negative. As the immunoblot against Yersinia epitopes remained positive and his abdominal complaints persisted, we decided to perform positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET), which revealed an FDG accumulation in the lower abdomen consistent with a mass with a diameter of 7 cm.

The patient started treatment with minocyclin (100 mg/day) for additional six months. During this treatment the fever and complaints subsided and a subsequent FDG-PET showed a decrease of abdominal mass. No side effects of minocyclin treatment were reported.

**Materials**

Muramyl dipeptide, poly I:C and flagellin were purchased from Sigma Chemical Co (St. Louis, MO), and synthetic Pam3Cys from EMC Microcollections (Tübingen, Germany). LPS (Escherichia coli 055:B5) was purchased from Sigma, and repurified as previously described. LcrV was kindly provided by Prof. J. Heesemann (Munchen, Germany).

**Isolation of peripheral blood mononuclear cells and cytokine stimulation**

After informed consent, venous blood was drawn from the cubital vein of the patient (collected after minocyclin treatment, during a stable phase of the disease, without acute signs of inflammation), healthy family members and five healthy volunteers (all male, age 22-35 years old) into three 10 ml EDTA tubes (Monoject, s-Hertogenbosch, the Netherlands). In addition, cells isolated from four patients with Crohn’s disease homozygous for 3020insC NOD2 mutation were also used as an additional control group. Isolation of mononuclear cells (MNC) was performed as described elsewhere, with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mm and pyruvate 10 mm. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, the Netherlands) and the number was adjusted to 5 x 10⁶ cells/ml.

5 x 10⁶ MNC in a 100 μl volume were added to round-bottom 96-wells plates (Greiner, Alphen a/d Rijn, the Netherlands) and incubated with either 100 μl of culture medium (negative control), various concentrations of heat-killed Y. enterocolitica, or LcrV (5 μg/ml). In addition, stimulation experiments with various TLR and NOD2 stimuli were performed in separate wells: MDP (10 μg/ml), Pam3Cys lipopeptides (1 μg/ml), poly I-C (50 μg/ml), purified E. coli LPS (1 ng/ml), flagellin (10 ng/ml). After 24 hours incubating at 37°C, the supernatants were stored at -80°C. Cytokine concentrations were measured by specific commercial ELISA kits from R&D Systems (Minneapolis, MN).

**Genotyping of TLR5 gene**

Amplification of the six exons of the TLR5 gene was performed using 11 polymerase chain reactions (PCR). After DNA purification, the sequencing was performed at the Clinical Genetic Centre Nijmegen, Radboud University Nijmegen Medical Centre, using the Big Dye terminator version 2 Chemich (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The sequence was checked using Biology Workbench 3.2.

**Genotyping of 3020insC NOD2 gene variant**

Blood was collected from the patient and his family (mother, father, and three sisters). PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50 μl reaction volumes containing 100 to 200 ng genomic DNA, as previously described. The 3020insC polymorphism was analysed by Genescan analysis on an ABI Prism 3100 Genetic Analyser according to the protocol of the manufacturer (Applied Biosystems).

**Patient consent**

Written consent was given by the patient and family. The analysis performed in the study took place within the diagnostic work-up of the disease, and therefore no ethics commission approval was necessary.
RESULTS

Cytokine production induced by Y. enterocolitica and LcrV in PBMC of healthy volunteers

In a first series of experiments we measured the cytokine responses of peripheral blood mononuclear cells (PBMC) of five human volunteers exposed to Y. enterocolitica. With 5 x 10^3 and 5 x 10^4 cfu/ml Y. enterocolitica, a sizeable cytokine response was seen, the IL-6 response being the most accentuated (figure 1A). Strong stimulation of IL-10 (1444 ± 383 pg/ml) and tumour necrosis factor (714 ± 215 pg/ml) by 5 x 10^4 cfu/ml Y. enterocolitica was also measured. The response to LcrV in a dose range between 1.25 and 5 µg/ml was lower for all cytokines: low amounts of IL-10 and TNF, but a clear dose response for IL-6 (figure 1B).

PMBC responses to Y. enterocolitica and TLR/NOD2 ligands

Interestingly, when PBMC of the patient with chronic yersiniosis were exposed to whole Yersinia micro-organisms, a strongly blunted IL-6 response was observed. The IL-6 response to LcrV in the patient was in the normal range (figure 2A). The IL-10 response to LcrV was low and also did not differ between patient and controls (not shown).

Based on these findings we assessed the response to well-defined TLR ligands.

In these experiments we found virtually no cytokine response to flagellin, the ligand for TLR5, and MDP, the ligand for NOD2, and normal responses to ligands for TLR2, TLR3 and TLR4 (figure 2B). We repeated these studies four times, always with the same results.

Figure 1. Cytokine production induced by Y. enterocolitica and LcrV

A. Freshly isolated peripheral blood mononuclear cells from human volunteers were stimulated with Y. enterocolitica (5 x 10^3 and 5 x 10^4 cfu/ml) (panel A) or LcrV in various concentrations (panel B), and IL-6 was measured by ELISA 24 hours later (n=5, mean ± SD).

Figure 2. Cytokine responses to Y. enterocolitica, TLR and NOD2 ligands

A. Peripheral blood mononuclear cells of the patient with chronic yersiniosis and of five control volunteers were exposed to whole Yersinia micro-organisms (5 x 10^4 cfu/ml) or LcrV (5 µg/ml). IL-6 concentrations were measured after 24-hour incubation. B. Cytokine responses after stimulation with various TLR and NOD2 ligands: Pam3Cys (TLR2, 1 µg/ml), poly I:C (TLR3 50 µg/ml), LPS (TLR4, 1 ng/ml), flagellin (10 ng/ml) and MDP (10 µg/ml). IL-6 concentrations were measured after 24 hours of incubation.
Genetic studies
To explain the strong defects in specific stimulation with flagellin and MDP, we hypothesised that these are due to genetic defects in their receptors TLR5 and NOD2. TLR5 gene was sequenced in both the patient, his three sisters and parents. The three most common NOD2 mutations known to be associated with a decreased recognition of MDP were also determined in the family. Sequencing showed that the patient possessed TLR5 as well as NOD2 variants. The TLR5 gene of the patient contained five single nucleotide polymorphisms, three of which had already been described, namely A253G (lys-lys), T1846C (phe-leu) and A1775G (asp-ser).18 Two new single nucleotide polymorphisms were discovered: A1930T (ile-phe) and A2357G (asp-gly). Comparison of the human DNA sequence with that of other species (mouse, rat, chimpanzee) shows that the A in the 1930 position is highly conserved among different species, suggesting this is an important residue. The position 2357 is more variable (G in other species and A in human).

The mutations are part of two haplotypes which were inherited from his mother (A1930T, A2357G and T1846C) and his father (A1775G and A2523G) (figure 3), leading to compound heterozygosity for TLR5 in the patient. As both chromosomes inherited from his parents contained non-synonymous mutations, very likely leading to defects in the function of the molecule, it is highly probable that the complete incapacity of the cells of the patient to respond to flagellin was due to these mutations in the TLR5 gene. Interestingly, one sister was also compound heterozygous with severely impaired flagellin responses (figure 3), whereas the other two sisters were heterozygous for mutations inherited either from their mother or their father (figure 3). Their cytokine responses, as well as those of their parents, were low compared with control volunteers, but significantly higher than those of their compound heterozygous brother and sister (figure 3).

With regard to the NOD2 mutation, the patient was found to be homozygous for the NOD2 3020insC mutation, while his parents are heterozygous. One sister was also found to be heterozygous for the mutation, whereas the other two sisters were homozygous for the wild-type allele (figure 3). The 3020insC NOD2 mutation is a well-known mutation resulting in a total loss of MDP-recognition capacity.19,20

Pro- and anti-inflammatory cytokine responses in patient, family and patients with Crohn’s disease and defective NOD2
Next we investigated production of the pro-inflammatory cytokines TNF, IL-17 and the anti-inflammatory cytokine IL-10 by PBMC of the patient and the family, when the cells were exposed to Y. enterocolitica. As can be seen in figure 4, the production of TNF and IL-17 by cells of the patient is considerably lower than that of family and Crohn’s disease patients, whereas IL-10 production is completely normal.

DISCUSSION
In this report we present a patient with presumed chronic yersiniosis, who was found to have a virtually absent pro-inflammatory cytokine response when his cells were exposed specifically to Y. enterocolitica. This defect was due to the loss of recognition of flagellin by TLR5, and the failure to induce a response through NOD2. Genetic studies revealed that the patient was compound heterozygous for several mutations in the gene coding TLR5 (two of which not reported before) and that he was homozygous for the 3020insC NOD2 mutation.

It is most likely that the genetic changes in TLR5 led to the poor cytokine response to flagellin and strongly contributed to the defective response to whole Yersinia. Support for this hypothesis is provided by the studies that reported an increased susceptibility to another facultative intracellular pathogen, Legionella pneumophila, in individuals bearing TLR5 mutations that led to defective responses to flagellin.16 The contribution of the NOD2 3020insC mutation to the lack of response to whole Yersinia is unclear. Although we have not found an abnormal cytokine response after Y. enterocolitica stimulation of cells isolated from Crohn’s disease patients homozygous for the 3020insC NOD2 gene mutation (figure 4), one could assume synergistic effects between NOD2 and TLR5 deficiencies. The synergy between TLR5 and NOD2, however, is an area of some controversy.

Figure 3. Genetic studies in the family of the patient

TLR5 gene was sequenced in both the patient, his three sisters and parents. The three most common NOD2 mutations known to be associated with a decreased recognition of muramyl dipeptide were also determined in the family. The mutation analysis is presented for both TLR5 and NOD2.

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in the literature. In previous studies, we were unable to demonstrate significant synergy between these two receptor pathways,\(^\text{19}\) while Van Heel et al. found evidence of synergy, albeit to a much lower degree than between TLR4 and NOD2.\(^\text{20}\) Therefore, the data presented here point to a more important role of TLR5 for recognition of \textit{Y. enterocolitica} and the disease process.

The finding that our patient was homozygous for the 3020insC NOD2 mutation, while not having Crohn’s disease, is remarkable, since the relative risk for Crohn’s disease in individuals bearing this NOD2 genotype is up to 42.\(^\text{21}\) Thus, the first question to be addressed is whether the pathology observed is an infiltrate of Crohn’s disease. However, the histological picture of the biopsy taken after colonoscopy does not support this diagnosis. Moreover, the patient had normal stools, did not display any other symptoms related to Crohn’s disease, and none of the additional investigations have revealed intestinal abnormalities. A second possibility would be that the patient does not have Crohn’s disease yet, but will still develop the disease. Although this cannot be ruled out, it is of interest to note that the mean age of diagnosis in ‘genetic’ Crohn’s disease is lower than that of common Crohn’s disease.\(^\text{22}\) Yet another more interesting possibility could be that the patient is protected from Crohn’s disease because of the concomitant TLR5 defect. Indeed, it has been reported that TLR5 mutations are less frequent in Crohn’s disease,\(^\text{23}\) in agreement with the observation that flagellin is a dominant antigen in this disease.\(^\text{24}\) The defective production of pro-inflammatory cytokines in our patient, albeit most prominent with \textit{Yersinia} as a stimulant, would fit with this observation. Elsewhere, we have defended the thesis that a net pro-inflammatory cytokine status (with reduced anti-inflammatory response) contributes to the development of Crohn’s disease in individuals with defective NOD2.\(^\text{17}\) The recent finding that IL-23 receptor polymorphisms protect against Crohn’s disease is in line with this concept.\(^\text{25}\) In addition, recent studies point to IL-17 as a key mediator in Crohn’s disease\(^\text{26,27}\) and in this respect our finding of a strongly diminished IL-17 response in the patient is of great interest.

In many preclinical studies on chronic yersiniosis, a major role for IL-10 has been found, probably due to the inhibitory effects of IL-10 on an adequate antibacterial effector mechanism mediated through the proinflammatory cytokines TNF\(\alpha\), IL-6 and interferon-\(\gamma\).\(^\text{3}\) The cytokine production patterns observed in our patient closely resemble this situation: \textit{Yersinia}-induced production of proinflammatory cytokines was low, while the IL-10 production was normal. The production of the latter cytokine apparently is not TLR5 and NOD2 dependent. In the literature, the virulence factor LcrV acting through TLR2 is considered a major stimulant for IL-10 production in mice,\(^\text{14}\) although this is a somewhat controversial issue.\(^\text{28}\) In the stimulation assay used in our study LcrV did not induce significant amounts of IL-10, neither in our patient nor in the controls. Possibly other \textit{Yersinia} components, such as YopJ and Yop/J/YopP, could also induce IL-10 induction in human monocytes.\(^\text{28}\) A potential limitation is that the yersiniosis has merely been diagnosed by serology, and therefore the diagnosis

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\textbf{Figure 4. Pro- and anti-inflammatory cytokine responses in patient, family and patients with Crohn’s disease and defective NOD2}

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\caption{Pro- and anti-inflammatory cytokine responses in patient, family and patients with Crohn’s disease and defective NOD2.}
\end{figure}
is still presumptive. However, recovering *Y. enterocolitica* from chronic extraintestinal sites has proven extremely difficult. It is not unusual in such cases to rely on serology, although even the Western blot using the various *Yersinia* outer membrane proteins meets with some cross-reactivity and false-positives. The onset of the disease, the development of the abdominal mass histologically compatible with a granulomatous infection and the gradual response to antibiotics, are strong arguments in favour of chronic yersiniosis.

**CONCLUSION**

In conclusion, the patient reported here suffered from a chronic inflammatory mass due to chronic yersiniosis. The finding of mutated genes for TLR5 leading to an almost complete loss of response to flagellin puts this patient in the category of selective immunodeficiencies. This TLR5 defect also contributes to the lack of proinflammatory response to whole *Yersinia*, while the contributing role of the loss of NOD2-induced signals is unclear. In addition, it is attractive to speculate that the loss-of-function of TLR5 is protective against the development of Crohn's disease in this patient homozygous for NOD2 mutations.

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**REFERENCES**