ORIGINAL ARTICLE

NOD2 3020insC mutation and the pathogenesis of Crohn's disease: impaired IL-1β production points to a loss-of-function phenotype

M.G. Netea[1,3*], G. Ferwerda[1,3], D.J. de Jong[2], S.E. Girardin[4], B.J. Kullberg[1,3], J.W.M. van der Meer[1,3]

Departments of [1]Medicine (541) and [2]Gastroenterology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, the Netherlands, tel.: +31 (0)24-361 88 19, fax: +31 (0)24-354 17 34, e-mail: M.Netea@aig.umcn.nl, [3]Nijmegen University Centre for Infectious Diseases, Nijmegen, the Netherlands, [4]Unité Pathogénie Microbienne Moléculaire, Institut Pasteur Paris, France, [*]corresponding author

ABSTRACT

Background: Mutations of the *NOD2* gene increase the susceptibility of humans to Crohn's disease. *NOD2* is a cytoplasmic receptor for the bacterial product peptidoglycan. There is considerable controversy in the literature whether the most common mutation in Crohn's disease, the 3020insC NOD2, leads to a loss of function, i.e. decreased cytokine production, or to the reverse, i.e. a gain of function. In previous papers we proposed the former, since we could show decreased cytokine production with a net proinflammatory status after exposure to muramyl dipeptide (MDP).
Methods: Because of recent data in the literature showing increased interleukin-β (IL-1β) production in mice with the corresponding *NOD2* mutation, we investigated the production of this cytokine by cells of patients with Crohn's disease, either homozygous or heterozygous for the 3020insC mutation, and compared it with that of patients with Crohn's disease bearing the wild-type allele.
Results: A strongly decreased production of IL-1β by peripheral mononuclear cells was found upon exposure to either peptidoglycan or peptidoglycan-derived MDP in homozygous patients bearing the 3020insC *NOD2* mutation.
Conclusion: This sustains the hypothesis that the 3020insC mutation in the human *NOD2* gene leads to a loss-of-function phenotype.

KEYWORDS

Crohn's disease, cytokine, IL-1, *NOD2*

In recent years, the insight into genetic susceptibility to Crohn's disease has greatly increased. A susceptibility locus for Crohn's disease was detected on chromosome 16,[1] and subsequently the candidate *NOD2* gene has been identified as the susceptibility locus IBD1.[2-4] NOD2 is a member of the NOD-leucine-rich repeat (LRR) protein family (also called the CATERPILLER family), known to be involved in recognition of microbial structures, and is expressed intracellularly in antigen-presenting cells.[5] Initially, NOD2 was believed to be an intracellular pattern recognition receptor for lipopolysaccharide (LPS),[4] similar to NOD1,[6] but further investigations have demonstrated that NOD2 is the intracellular receptor for the muramyl dipeptide (MDP) component of bacterial peptidoglycan (PGN).[7,8]

The mutated *NOD2* associated with Crohn's disease has been reported to be unable to sense MDP and this would suggest that the mutation would result in a loss-of-function phenotype. This is consistent with the finding that peripheral blood cells of patients with the *NOD2* mutation exposed to NOD2 ligands produce low amounts of the proinflammatory cytokines tumour necrosis factor alpha (TNF), interleukin-6 (IL-6) and IL-8, as well as the anti-inflammatory cytokine IL-10.[9,10] Conceptually this poses an enigma, because Crohn's disease is an inflammatory disease. In essence, two basically opposite hypotheses have been put forward: one advocating that the *NOD2* mutation leads to defective anti-inflammatory control ('loss-of function'), the other advocating that the mutation leads to activated inflammation ('gain of function'). So far, we have been more in favour of the loss-of-function hypothesis as we obtained experimental evidence for a defective response to peptidoglycan and MDP in cells with this mutation. Recently, however, Maeda *et al*. proposed a gain-of-function effect of *NOD2* mutations based on the finding of greater IL-1β release in MDP-stimulated cells of mice bearing an NOD2 mutation that corresponds to the human 3020insC mutation. Prompted by the intriguing findings of Maeda *et al*. on the increased IL-1β processing, we measured mature IL-1β released by the mononuclear cells of patients with the 3020insC mutation, after stimulation with either peptidoglycan, or a combination of MDP with the lipoprotein MALP-2, a Toll-like receptor-2 (TLR2) agonist. The latter stimulation was investigated, because we recently demonstrated synergy between the cell surface pattern recognition receptor TLR2 and the cytoplasmic NOD2.[11]

MATERIALS AND METHODS

Genotyping of NOD2 variants

Blood was collected from 74 patients with Crohn's disease and ten healthy volunteers. Polymerase chain reaction (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.[10] The 3020insC polymorphism was analysed by Genescan analysis on an ABI Prism 3100 Genetic Analyser according to the manufacturer's protocol (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands).

Four patients with Crohn's disease were found homozygous for the 3020insC mutation, and they were further investigated in the cytokine studies. As control groups, five patients with Crohn's disease heterozygous for the 3020insC *NOD2* mutation, five patients with Crohn's disease bearing the wild-type allele, and five healthy volunteers homozygous for the wild-type *NOD2* allele were included.

Isolation of mononuclear cells and stimulation of cytokine production

After obtaining informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10 ml EDTA tubes (Monoject, s-Hertogenbosch, the Netherlands). The mononuclear cell (MNC) fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, 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 5×10^6 cells/ml.

Next, 5×10^5 MNC in a 100 μl volume were added to round-bottom 96-well plates (Greiner, Alphen a/d Rijn, the Netherlands) and incubated with either 100 μl of culture medium (negative control), MDP (10 μg/ml, Sigma Chemical Co, St. Louis), purified staphylococcal peptidoglycan (1 μg/ml), or MALP2 lipopeptides (1 μg/ml, EMC Microcollections, Tübingen, Germany).

Cytokine measurements

Human IL-1β concentrations were determined by specific radioimmunoassays as previously described.[12]

Statistical analysis

The experiments were performed in triplicate with blood obtained from patients and volunteers. The differences between groups were analysed by the Mann-Whitney U test, and where appropriate by the Kruskal-Wallis ANOVA test. The level of significance between groups was set at $p<0.05$. The data are given as means ± SEM.

RESULTS

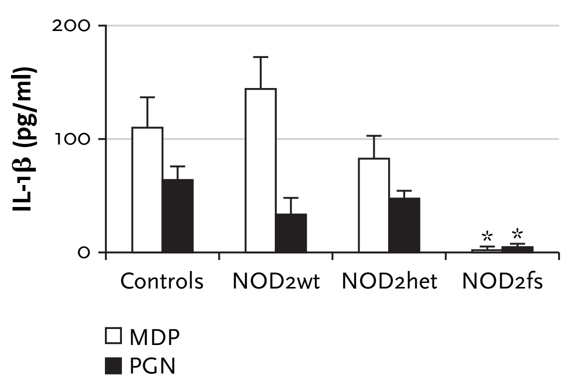
The release of IL-1β after stimulation with peptidoglycan or MDP did not differ between healthy volunteers and patients with Crohn's disease who were either heterozygous for the mutation or had no mutation (*figure 1*). In contrast, patients homozygous for the mutation exhibited a strongly decreased IL-1β synthesis in response to both peptidoglycan or MDP (*figure 1*).

To investigate whether the mutated *NOD2* leads to modified synergism between NOD2- and TLR2-mediated signalling, cells from the patients with the 3020insC mutation were stimulated with a combination of MDP and the lipoprotein MALP-2, a specific TLR2 agonist (*figure 2*). MDP and MALP-2 appeared to have synergistic effects on IL-1β release of normal cells; these effects were absent in patients with the 3020insC mutation, arguing that the 3020insC mutation induces a loss-of-function phenotype.

DISCUSSION

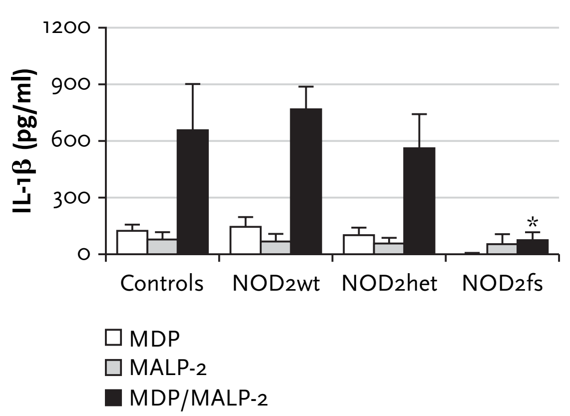
In this paper, we demonstrate that peripheral blood mononuclear cells of patients suffering from Crohn's disease with the 3020insC *NOD2* mutation are defective in terms of IL-1β production when stimulated with the NOD2 ligands MDP and peptidoglycan. These results argue for a lack-of-function character of the mutation and are fundamentally different from those obtained in mice with the equivalent mutation.[13]

Figure 1 *PGN and MDP stimulation of cytokines: requirement of NOD2*



MNC isolated from four patients with Crohn's disease homozygous for the 3020insC *NOD2* mutation (NOD2fs), five patients heterozygous for *NOD2* mutations (NOD2het), five patients with the wild-type *NOD2* allele (NOD2wt) and five healthy volunteers with wild-type *NOD2* (controls), were stimulated with either 5 μg/ml MDP (solid bars) or 10 μg/ml PGN (hatched bars), for 24h at 37°C. IL-1β concentrations were measured by ELISA. Data are presented as means ± SD, and compared by Mann-Whitney U-test (*p<0.05).

Figure 2 *TLR2 and NOD2 pathways have synergistic effects on IL-1β production*



MNC isolated from four patients with Crohn's disease homozygous for the 3020insC *NOD2* mutation (NOD2fs), five patients heterozygous for *NOD2* mutations (NOD2het), five patients with the wild-type *NOD2* allele (NOD2wt) and five healthy volunteers with wild-type *NOD2* (controls), were stimulated with either 5 μg/ml MDP (open bars), 5 μg/ml MALP-2 (hatched bars), or a combination of both (solid bars) for 24h at 37°C. IL-1β concentrations were measured by ELISA. Data are presented as means ± SD, and compared by Mann-Whitney U-test (*p<0.05).

The low IL-1β production in humans with the mutation is in agreement with previous studies from both our laboratory and others demonstrating decreased production of other proinflammatory cytokines in these patients.[9-11,14] Likewise, Li *et al*. have shown reduced IL-1β release in mononuclear cells from two patients with homozygous 3020insC mutation when stimulated with a combination of MDP and TNF.[15] These human studies are, however, at odds with the increased IL-1μ production in mice genetically engineered to have the same *NOD2* mutation as the human 3020insC mutation,[13] which suggested a gain-of-function phenotype of this mutation. Proponents of the gain-of-function hypothesis have argued that the cells from patients with the 3020inC mutation suffer from active inflammatory disease and therefore may have down-regulated cytokine production,[16] as is commonly found in other inflammatory conditions.[17] This is, however, very unlikely for a variety of reasons. First of all, patients with Crohn's disease bearing the 3020insC mutation had lower cytokine production only after stimulation with the NOD2 ligands peptidoglycan and MDP, but not after the TLR2 agonist MALP-2 in this study (*figure 2*), or other TLR ligands as shown in previous studies.[11,14] If an inhibition of cytokine production due to inflammation had been present, a general downregulation of both NOD2- and TLR-induced cytokines should have been found. Secondly, an inflammation-driven downregulated pro-inflammatory cytokine production tends to be associated with an upregulated anti-inflammatory cytokine response.[17] This is not the case here: we have previously demonstrated that the anti-inflammatory response as exemplified by IL-10 production is strongly inhibited.[10]
An explanation for the increased inflammation in mice bearing the variant *NOD2* could have been the lack of inhibitory signals on TLR2-induced cytokine release, leading to increased cytokine production, as recently proposed by Watanabe *et al.*[18] Unfortunately, Maeda *et al.* inappropriately tested this hypothesis by using PGN as a putative TLR2 ligand, and stimulating cells with a combination of MDP and PGN.[13] PGN is in fact the bacterial product containing the MDP motif, and thus an NOD2 ligand. The TLR2-dependent activity of PGN has been convincingly shown to be due to contamination with lipoteichoic acid.[19] To settle the argument whether the lack of functional NOD2 would lead to enhanced TLR2-mediated signals, we stimulated cells from the patients bearing the 3020insC mutation with a combination of MDP and the lipoprotein MALP-2, a specific TLR2 agonist (*figure 2*). We have shown here that MDP and MALP-2 have synergistic effects on IL-1β release, and these effects were absent in patients with the 3020insC mutation, arguing that an *NOD2*-mediated suppression of TLR2 signals does not play an important role in patients with Crohn's disease.

What could the explanation be for the apparent discrepancies between 3020insC-positive Crohn's patients and *NOD2* variant mice? Most likely, there are crucial differences between the murine and human *NOD2* systems. This may also explain why humans with *NOD2* mutations develop Crohn's disease, whereas mice deficient for *NOD2* do not show any signs of inflammation.[20] The mechanisms through which the mutations in the *NOD2* gene result in chronic intestinal inflammation in humans are likely mediated by two pathways: firstly through decreased defence against intestinal pathogens that trigger the initial inflammatory reaction,[21] and secondly through loss-of-control of the intestinal inflammation due to the defective release of anti-inflammatory cytokines such as IL-10 and TGFβ.[10]

ACKNOWLEDGEMENTS

M.G. Netea was supported by a VIDI grant from the Netherlands Organisation for Scientific Research (NWO-ZonMW).

REFERENCES

1. Hugot J-P, Laurent-Puig P, Gower-Rousseau C, et al. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 1996;379:821-3.
2. Hugot J-P, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.
3. Hampe J, Grebe J, Nikolaus S, et al. Association of NOD2 (CARD15) genotype with clinical course of Crohn's disease: a cohort study. Lancet 2002;359:1661-5.
4. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603-6.
5. Gutierrez O, Pipaon C, Inohara N, et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kB activation. J Biol Chem 2002;277:41701-5.
6. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. J Biol Chem 2001;276:2552-4.
7. Girardin SE, Boneca IG, Viala J, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-72.
8. Inohara N, Ogura Y, Fontalba A, et al. Host recognition of bacterial muramyl dipeptide mediated through Nod2. J Biol Chem 2003;278:5509-12.
9. Bonen DK, Ogura Y, Nicolae DL, et al. Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 2003;124:140-6.
10. Netea MG, Kullberg BJ, de Jong D, et al. NOD2 mediates induction of the antiinflammatory signals induced by TLR2-ligands: implications for Crohn's disease. Eur J Immunol 2004;34:2052-9.
11. Netea MG, Ferwerda G, De Jong DJ, et al. NOD2 modulates specific Toll-like receptor pathways for the induction of cytokine release. J Immunol 2005;174:6518-23.
12. Drenth JPH, van Deuren M, van der Ven-Jongekrijg J, Schalkwijk CG, van der Meer JWM. Cytokine activation during attacks of the hyper-immunoglobulinemia D and periodic fever syndrome. Blood 1995;85:3586-93.
13. Maeda S, Hsu L-C, Liu H, et al. NOD2 mutation in Crohn's disease potentiates NF-kB activity and IL-1beta processing. Science 2005;307:734-8.
14. Van Heel DA, Ghosh S, Hunt K, et al. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. Lancet 2005;365:1794-6.
15. Li J, Moran T, Swansioson E, et al. Regulation of IL-8 and IL-1beta expression in Crohn's disease associated NOD2/CARD15 mutations. Hum Mol Genetics 2004;13:1715-25.
16. Eckmann L, Karin M. NOD2 and Crohn's disease: loss or gain of function? Immunity 2005;22:661-7.
17. Van Deuren M, Dofferhoff ASM, van der Meer JWM. Cytokines and the response to infections. J Pathol 1992;168:349-56.
18. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 2004;advanced online publication.
19. Travassos LH, Girardin SE, Philpott DJ, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Reports 2004;5:1000-6.
20. Pauleau AL, Murray PJ. Role of NOD2 in the response of macrophages to toll-like receptor agonists. Mol Cell Biol 2003;23:7531-9.
21. Kobayashi KS, Chamaillard M, Ogura Y, et al. NOD2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005;307:731-4.