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Candida-specific interferon-γ deficiency and Toll-like receptor polymorphisms in patients with chronic mucocutaneous candidiasis

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ABSTRACT

Chronic mucocutaneous candidiasis (CMC) is a group of disorders, characterised by persistent mucocutaneous infections with *Candida* species. The underlying defect of CMC has not been elucidated, but a defective cytokine response may be involved. Therefore, we investigated whether an imbalance between IFNγ and IL-10 may play a role in this disorder.

We assessed the cytokine production in whole-blood cultures from CMC patients using *Candida albicans*, lipopolysaccharide and phytohaemagglutinin as stimuli. As the Toll-like receptors are important pattern recognition receptors for *Candida* species, we also investigated Toll-like receptor polymorphisms in these patients.

Patients with CMC had a significantly decreased IFNγ production when whole blood was stimulated with *C. albicans* (232 ± 120 *vs* 2279 ± 609 pg/ml, p<0.02). When stimulated with phytohaemagglutinin, the differences were not significant (3549 ± 1320 *vs* 7631 ± 1790 pg/ml). The *Candida*-stimulated production of IL-10 tended to be higher in CMC patients, whereas TNF and IL-1β production were similar in patients and controls. Stimulation with LPS showed no differences in cytokine production between patients and controls. Two out of seven patients had the TLR4 Asp299Gly polymorphism and none had the TLR2 Arg677Trp polymorphism.

These data support the hypothesis that deficient IFNγ production is involved in the pathogenesis of CMC, whereas a role for genetic polymorphisms of Toll-like receptor 2 and 4 is not obvious in these patients.

INTRODUCTION

Chronic mucocutaneous candidiasis (CMC) is a group of disorders characterised by persistent mucocutaneous infections with *Candida* species. Several clinical variants of CMC have been described,[1] some of which are associated with endocrinopathies or autoimmune diseases, such as hypothyroidism and hypoparathyroidism.[1,2] Patients with CMC rarely develop disseminated or invasive candidiasis, suggesting a defect in the host defence limited to superficial candidal infections.[3,4]

It is generally accepted that such defence mechanisms encompass macrophages, cytotoxic lymphocytes and natural killer (NK) cells.[5] For activation of these cells, proinflammatory cytokines such as interferon (IFN)γ and tumour necrosis factor (TNF) are major mediators, whereas anti-inflammatory cytokines, such as interleukin (IL)-4 and IL-10, antagonise the cellular anticandidal defence.[5] Production of these cytokines is initiated by recognition of the micro-organism by pattern recognition receptors, especially Toll-like receptors (TLR), on the cellular surface.[6] TLR2 is the main receptor involved in induction of proinflammatory cytokines after stimulation with *Candida albicans*, while TLR4 mediates chemokine production.[6] The balance between T helper (Th)1 and Th2 cytokines is important in the initiation of the type of immune response. A Th1 cytokine response is associated with resistance to candidiasis, whereas a Th2 response results in susceptibility to infection.[6]

It has been hypothesised that a defective Th1 response may be at least partially responsible for the persistence of

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Candida infection in CMC patients.[7] To further test this hypothesis, we assessed the pro- and anti-inflammatory cytokine response in a whole-blood culture model after stimulation with *C. albicans*, lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) in patients with chronic mucocutaneous candidiasis. In addition, we investigated whether known polymorphisms in TLR2 or TLR4 genes, which are associated with impaired cytokine production, could be involved in the pathogenesis of CMC.

PATIENTS AND METHODS

Seven patients with CMC (three males and four females, aged from 8 to 55 years) were studied. For each patient, two healthy age- and sex-matched controls were included. During the study, the CMC patients did not suffer from other concurrent disorders or acute infections. After obtaining informed consent, blood samples were obtained from both patients and controls at the same time, using 2 ml glass tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ).

Ex vivo cytokine production

The whole blood was diluted 1:5 with RPMI 1640 Dutch Modification (ICN Biomedicals, Aurora, OH) in 24-well plates (Costar Corning, New York, NY).
Phytohaemagglutinin (PHA; 10 μg/ml; Sigma Chemical Co., St Louis, MO), *E. coli* lipopolysaccharide (LPS; 1 ng/ml; Sigma) or heat-killed *C. albicans* (1 x 10± cfu/ml or 1 x 10^7cfu/ml, heat-killed for 30 minutes at 100°C) were added. Each well contained a final volume of 1 ml. The samples were incubated for 24 or 48 hours at 37°C in 5% CO_2 atmosphere. Supernatants were collected after centrifugation and stored at -20°C until tested.

Circulating cytokine concentrations

For the analysis of circulating cytokine levels, the blood samples were centrifuged and the plasma was collected. The samples were stored at -20°C until analysis. The concentrations of TNF, IL-1β and IL-1Ra were measured by specific radioimmunoassay. Concentrations of IFNγ and IL-10 were measured by ELISA according to the guidelines of the manufacturer (CLB, Amsterdam, the Netherlands). Detection limits of the assay were IFNγ 2.5 to 200 pg/ml; IL-1β 0.04 to 1.25 ng/ml; IL-10 1.25 to 200 pg/ml; IL-1Ra 0.08 to 0.8 ng/ml; and TNF 0.02 to 1.0 ng/ml.

TLR2 and TLR4 polymorphisms

TLR2 and TLR4 polymorphisms were assessed in the CMC patients and in 200 healthy Dutch controls, participating in a health survey for recurrent venous thrombosis. Genomic DNA was isolated from blood by using the Puregene DNA isolation kit (310001, Gentra systems, BIOzym, the Netherlands). The DNA was stored at 4°C until the analysis. To determine the TLR4 genotype, the DNA was amplified with primers (forward primer: 5' ATACTTAGACTACTACCT**C**ATG 3', reverse primer 3' AAACTCAAGGCTTGGTAGATC 5'; the bold **C** in the forward primer indicates a mutation, creating an NCO-I site). The polymerase chain reaction (PCR) conditions were as follows: five minute initial denaturation at 94°C, followed by 37 cycles (94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds). The PCR products were digested with the restriction enzyme NCO-1 (New England BioLabs, MA) and separated on a 2.5% agarose gel stained with ethidium bromide.
For the determination of the TLR2 polymorphism, the DNA was amplified with primers (forward primer: 5' GATGCATTTGTTTCTTACAGTG 3' and reverse primer: 3' TGCACCACTCACTCTTCACA 5'). The PCR was as follows: five minute initial denaturation at 94°C, followed by 37 cycles (94°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds). The PCR products are digested with ACI-1 (New England BioLabs, MA) and separated on a 2.5% agarose gel, stained with ethidium bromide.

Statistical analysis

Statistical evaluation was performed by using the Mann-Whitney test. Values were considered significant at $p<0.05$.

RESULTS

Ex vivo cytokine production

IFNγ and IL-10 production

In earlier experiments, we studied the kinetics of cytokine production, after stimulation with *Candida* or LPS, and we found that the proinflammatory cytokine production is maximal at 24 hours and that of IFNγ and IL-10 at 48 hours (data not shown). After 48 hours of stimulation with 10^7 cfu/ml heat-killed *C. albicans*, the IFNγ production in patients was significantly lower than that in controls (*figure 1A*; $p<0.02$). In contrast, the IL-10 production in patients with CMC tended to be greater than that in controls (*figure 1A*; $p>0.05$). When the cells were stimulated with a lower amount of *C. albicans* (10^6 cfu/ml), the results were similar, showing a lower production of IFNγ in patients compared with controls (data not shown).
When diluted whole blood was incubated with either *E. coli* LPS (1 ng/ml) or PHA (10 μg/ml) for 48 hours, there was a tendency toward lower production of IFNγ upon stimulation with either LPS or PHA in the patient group in comparison with the control group (*figure 2*, $p>0.05$). A similar trend was observed after 24 hours of incubation with either stimulus (data not shown).

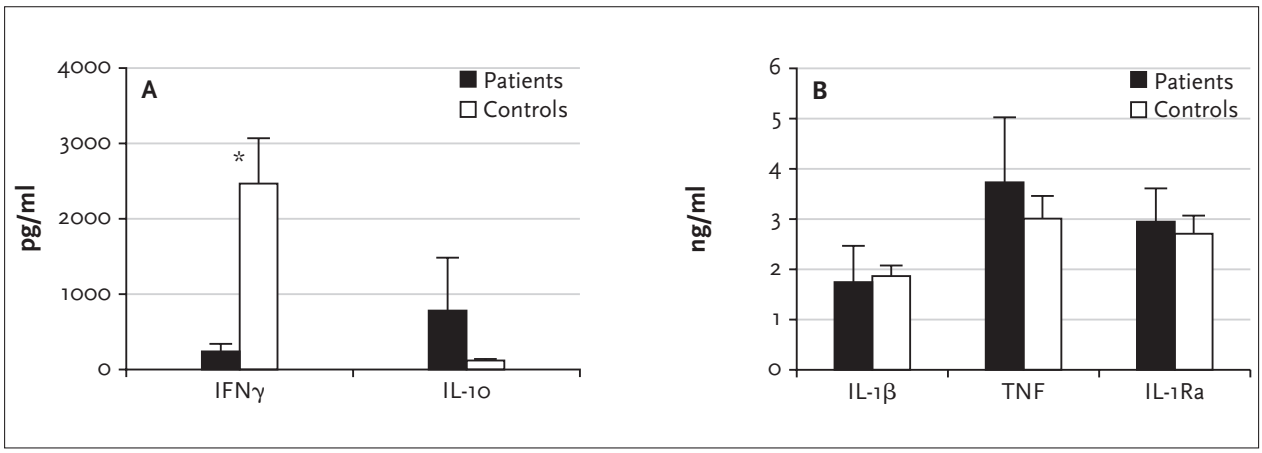

Figure 1

Diluted whole blood of patients with CMC (n=7) and healthy controls (n=14) was stimulated with heat-killed C. albicans (10^7 cfu/ml) and production of IFNγ, IL-10 (A) and IL-1β, TNF, IL-1Ra (B) was assessed after 48 hours

* **Significant difference between patients and controls, p<0.02.**

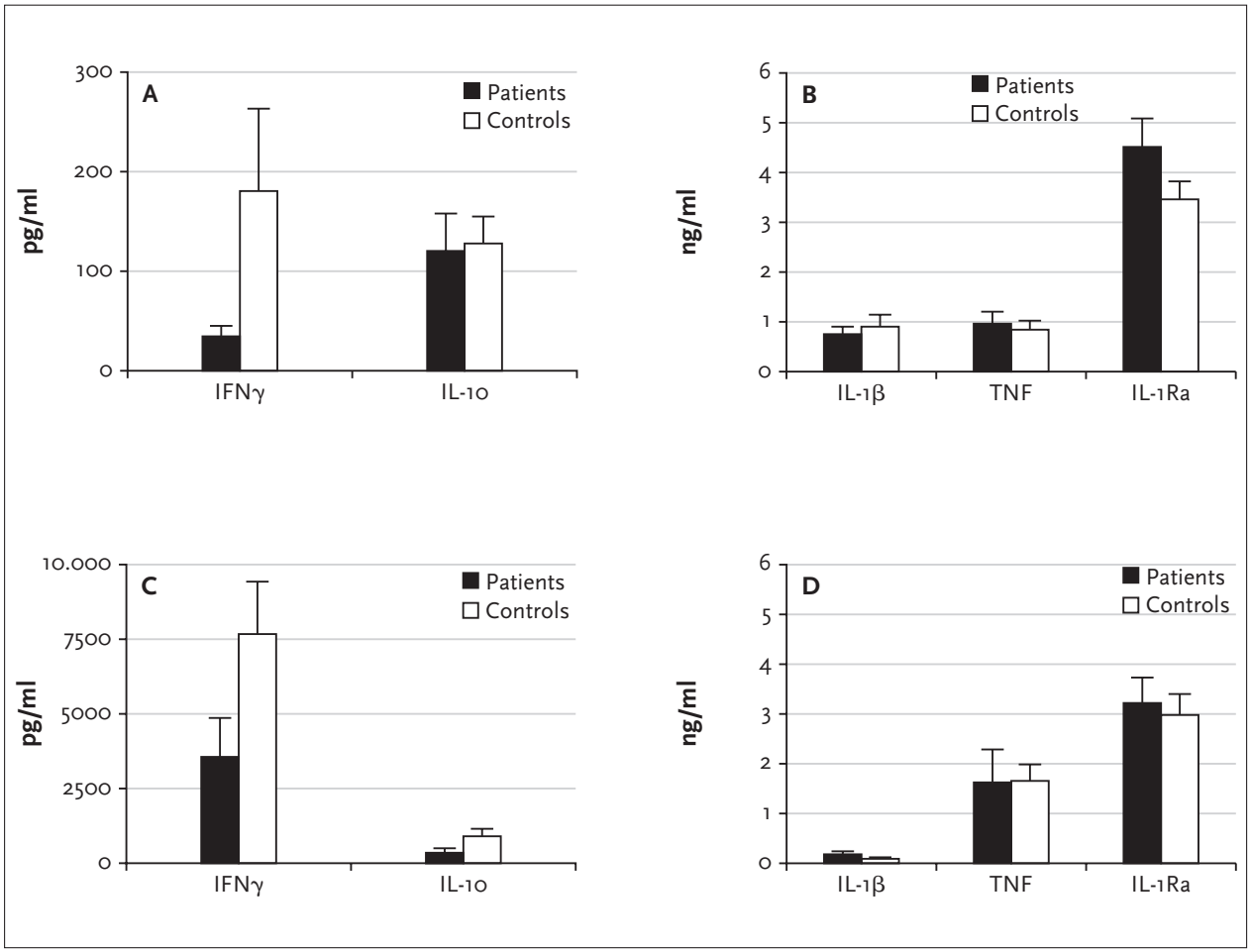

Figure 2

Diluted whole blood of patients with CMC (n=7) and healthy controls (n=14) was stimulated with LPS (A and B) or PHA (C and D) and production of IFNγ, IL-10 and IL-1β, TNF, IL-1Ra was assessed after 48 hours

No significant differences between patients and controls were found.

TNF, IL-1β and IL-1Ra production

Diluted whole blood of patients with CMC and healthy volunteers was incubated with heat-killed *C. albicans*, and the cytokine response was analysed after 24 and 48 hours (*figure 1B*). After 24 hours of incubation, there were no significant differences in the production of IL-1β, IL-1Ra and TNF between patients and controls (*table 1*). Similarly, there was no significant difference in production of TNF, IL-1β or IL-1Ra between patients with CMC and healthy controls when stimulated with either LPS or PHA for 24 hours (*table 1*) or 48 hours (*figure 2*). In all experiments, the cytokine production in the absence of specific stimuli was very low, and no significant differences were observed between patients and controls.

Toll-like receptor 2 and 4 polymorphisms

The TLR4 Asp299Gly polymorphism and the TLR2 Arg677Trp polymorphism were assessed in blood. In the healthy control population (200 subjects), TLR4 polymorphism was present in 21 cases (11%), whereas none of them had a TLR2 polymorphism. Of the seven CMC patients, two (father and son) were heterozygous for the TLR4 Asp299Gly mutation. None of the CMC patients were positive for the TLR2 Arg677Trp mutation. After *C. albicans* stimulation, lowest IFNγ production was seen in the two patients with the TLR4 mutation. One of these patients had a IFNγ concentration below the detection limit, in the other heterozygous patient, IFNγ was 28 pg/ml, versus 340 ± 155 pg/ml in the other CMC patients and 2279 ± 609 pg/ml in healthy controls.

DISCUSSION

In our study of patients with CMC, we investigated cytokine production in whole blood stimulated with specific microbial stimuli such as *C. albicans*, or LPS, and PHA, a direct stimulus of T cells. After stimulation with *C. albicans*, IFNγ production was 70 to 90% lower in CMC patients as compared with healthy controls. In contrast, the production of the anti-inflammatory cytokine IL-10 tended to be higher in CMC patients, whereas no difference in the release of TNF, IL-1 and IL-1Ra was seen between CMC patients and healthy volunteers.

The defective IFNγ release appeared to be rather specific for candidal stimulation. Microbial components stimulate IFNγ production through intermediary release of monocyte products such as IL-12 and IL-18,[10] while PHA directly stimulates T lymphocytes. Thus, the difference between *Candida* and PHA stimulation suggests that the defect in CMC patients may be localised at the level of monocyte.

In contrast to the release of IFNγ, production of IL-10 upon stimulation of whole blood with *Candida* tended to be higher in CMC patients compared with controls. As IL-10 is a potent anti-inflammatory cytokine which counteracts the actions of IFNγ, the IFNγ/IL-10 ratio is considered to be important in defence against *C. albicans*.[11] Therefore, the greater release of IL-10 in CMC patients further contributes to a reduced IFN/IL-10 ratio and is likely to also be involved in the defective activation of anticandidal mechanisms.

Our data are in accordance with those of Gravenor *et al.* demonstrating higher IL-10 levels and deficient IL-12 production in CMC patients after *C. albicans* stimulation,[12] whereas the expression of the IL-12 receptor appears to be normal in CMC patients.[13] Since there was a tendency for lower IFNγ production after stimulation with PHA, an additional defect at the level of the T lymphocyte cannot be ruled out. Not all studies in CMC patients have observed decreased Candida-specific IFNγ release.[7,14,15] The difference between these studies and ours probably lies in the experimental conditions: we used a whole-blood stimulation, whereas the other studies used cultures of isolated PBMC, in which IFNγ production may be suboptimal.[7]

Additional studies have also reported increased release of other anti-inflammatory cytokines such as IL-4[15] and IL-6.[7] All these data suggest a strong Th2 bias in patients with CMC. Several experimental studies have demonstrated the deleterious effects of Th2-like cytokines for the anti-candida defence, in contrast to the beneficial effects of Th1 cytokines.[11,16]

Two out of seven CMC patients were heterozygous for the TLR4 Asp299Gly polymorphism, whereas the TLR2 Arg677Trp polymorphism was detected in none of the CMC patients. In the general population, the incidence of

Table 1

Cytokine production after 24h stimulation

CYTOKINE	RPMI		*C. ALBICANS*		LPS		PHA	
	CONTROLS	CMC	CONTROLS	CMC	CONTROLS	CMC	CONTROLS	CMC
TNF (ng/ml)	0.2 ± 0.0	0.2 ± 0.1	3.6 ± 0.5	4.7 ± 1.8	1.3 ± 0.3	1.1 ± 0.3	1.2 ± 0.3	1.7 ± 1.0
IL-1β (ng/ml)	0.0 ± 0.0	0.1 ± 0.0	2.2 ± 0.9	1.8 ± 0.9	1.1 ± 0.2	0.9 ± 0.3	0.1 ± 0.04	0.1 ± 0.03
IL-1Ra (ng/ml)	0.8 ± 0.2	1.2 ± 0.6	2.8 ± 0.5	2.6 ± 0.7	3.2 ± 0.3	4.0 ± 0.5	2.6 ± 0.4	3.1 ± 0.8

Whole blood of CMC patients (n=7) and healthy controls (n=14) was stimulated with different stimuli. Values are given as means ± SEM.

the TLR4 mutation varies between 6 and 11%,[8] whereas the TLR2 mutation is very rare, although only limited data are available.[9] The present study is limited due to the small number of patients. Therefore, no epidemiological conclusions can be drawn from the observation on TLR polymorphisms. The only conclusion to be made is that the TLR polymorphisms that have been identified so far are not the major cause of the immunological abnormalities in CMC patients, since not all of the CMC patients had the polymorphism. Interestingly, the two patients with the TLR4 polymorphism had the lowest IFNγ production on *Candida* stimulation among all tested individuals. This suggests that TLR4 plays a role in Candida-specific IFNγ production.

In conclusion, our results show an imbalance in the cytokine network in CMC patients using *Candida* stimulation. The defective IFNγ production is likely to be involved in the chronic infections with *Candida* species. The molecular defect responsible for this syndrome still needs to be localised. However, the known Toll-like receptor 2 and 4 polymorphisms do not play a crucial role in pathogenesis of this disease.

REFERENCES

1. Kirkpatrick CH. Chronic mucocutaneous candidiasis. J Am Acad Dermatol 1994;31:S14-7.
2. Coleman R, Hay RJ. Chronic mucocutaneous candidosis associated with hypothyroidism: a distinct syndrome? Br J Dermatol 1997;136:24-9.
3. Germain M, Gourdeau M, Hebert J. Case report: familial chronic mucocutaneous candidiasis complicated by deep candida infection. Am J Med Sci 1994;307:282-3.
4. Kauffman CA, Shea MJ, Frame PT. Invasive fungal infections in patients with chronic mucocutaneous candidiasis. Arch Intern Med 1981;141:1076-9.
5. Mathews H, Witek-Janusek L. Host defenses against oral, esophageal and gastrointestinal candidiasis. In: Calderone RA (ed). Candida and candidiasis. Washington DC: ASM Press, 2002;179-92.
6. Netea MG, Graaf CA van der, Vonk AG, Verschueren I, Meer JW van der, Kullberg BJ. The role of Toll-like Receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. J Infect Dis 2002;185:1483-9.
7. Lilic D, Cant AJ, Abinun M, Calvert JE, Spickett GP. Chronic mucocutaneous candidiasis. I. Altered antigen-stimulated IL-2, IL-4, IL-6 and interferon-gamma (IFN-gamma) production. Clin Exp Immunol 1996;105:205-12.
8. Kiechl S, Lorenz E, Reindl M, et al. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med 2002;347:185-92.
9. Kang T, Chae GT. Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. FEMS Imm Med Microbiol 2001;31:53-8.
10. Ahn HJ, Maruo S, Tomura M, Mu J, et al. A mechanism underlying synergy between IL-12 and IFN-gamma-inducing factor in enhanced production of IFN-gamma. J Immunol 1997;159:2125-31.
11. Romani L. Immunity to Candida albicans: Th1, Th2 cells and beyond. Curr Opin Microbiol 1999;2:363-7.
12. Gravenor I, Robson N, Abinun M, Cant AJ, Lilic D. Pattern of altered cytokine production in chronic mucocutaneous candidiasis suggests macrophage defect. 10th Meeting of the European Society for Immunodeficiencies 2002, Weimar, Germany.
13. Lilic D, Gravenor I. Immunology of chronic mucocutaneous candidiasis. J Clin Pathol 2001;54:81-3.
14. De Moraes-Vasconcelos D, Orii NM, Romano CC, Iqueoka RY, Duarte AJ. Characterization of the cellular immune function of patients with chronic mucocutaneous candidiasis. Clin Exp Immunol 2001;123:247-53.
15. Kobrynski LJ, Tanimune L, Kilpatrick L, Campbell DE, Douglas SD. Production of T-helper cell subsets and cytokines by lymphocytes from patients with chronic mucocutaneous candidiasis. Clin Diagn Lab Immunol 1996;3:740-5.
16. Romani L. Innate and adaptive immunity in Candida albicans infections and saprophytism. J Leukoc Biol 2000;68:175-9.s