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Effect of an Extract Based on the Medicinal Mushroom *Agaricus blazei* Murill on Expression of Cytokines and Calprotectin in Patients with Ulcerative Colitis and Crohn's disease

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Abstract

An immunomodulatory extract (AndoSan™) based on the medicinal mushroom *Agaricus blazei* Murill (AbM) has shown to reduce blood cytokine levels in healthy volunteers after 12 days' ingestion, pointing to an anti-inflammatory effect. The aim was to study whether AndoSan™ had similar effects on cytokines in patients with ulcerative colitis (UC) and Crohn's disease (CD). Calprotectin, a marker for inflammatory bowel disease (IBD), was also measured. Patients with CD (n = 11) and with UC (n = 10) consumed 60 ml/day of AndoSan™. Patient blood plasma was harvested before and after 6 h LPS (1 ng/ml) stimulation *ex vivo*. Plasma and faecal calprotectin levels were analysed using ELISA and 17 cytokines [IL-2, IFN-γ, IL-12 (Th1), IL-4, IL-5, IL-13 (Th2), IL-7, IL-17, IL-1β, IL-6, TNF-α, IL-8, MIP-1β, MCP-1, G-CSF, GM-CSF and IL-10] by multiplex assay. After 12 days' ingestion of AndoSan™, baseline plasma cytokine levels in UC was reduced for MCP-1 (40%) and in LPS-stimulated blood for MIP-1β (78%), IL-6 (44%), IL-1β (41%), IL-8 (30%), G-CSF (29%), MCP-1 (18%) and GM-CSF (17%). There were corresponding reductions in CD: IL-2 (100%), IL-17 (55%) and IL-8 (29%) and for IL-1β (35%), MIP-1β (30%), MCP-1 (22%), IL-8 (18%), IL-17 (17%) and G-CSF (14%), respectively. Baseline concentrations for the 17 cytokines in the UC and CD patient groups were largely similar. Faecal calprotectin was reduced in the UC group. Ingestion of an AbM-based medicinal mushroom by patients with IBD resulted in interesting anti-inflammatory effects as demonstrated by declined levels of pathogenic cytokines in blood and calprotectin in faeces.

Introduction

The *Agaricus blazei* Murill mushroom (AbM) (jap.: Himematsutake) of the *Basidiomycetes* family grows wildly in the coastal Piedade area outside of São Paulo, Brazil. People in this area have traditionally used AbM as a health-food ingredient. The frequency of serious diseases like atherosclerosis, hepatitis, hyperlipidaemia, diabetes and cancer [1] was lower in Piedade than in neighbouring regions, supposedly because of the AbM intake. In 1966, the mushroom was taken to Japan and introduced to the health-food market, and later AbM was also subjected to an increasing research effort. Other interesting medicinal *Basidiomycetes* mushrooms that have been studied are *Grifola frondosa* (Gf) (Maitake) [2] and *Hericeum erinaceum* (He) (Yamabushitake) [3].

Agaricus blazei Murill, like Gf, is rich in immunostimulatory mixture of β(1-3)-, β(1-4)- and β(1-6)-D-glucans with antitumour activity [4], probably secondary to modulation of NK-cells [5] and monocytes/macrophages of native immunity [6–8].

In vitro AbM stimulates mononuclear phagocytes to secrete nitric oxide [9] and pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α and chemokine IL-8 [9, 10]. Recently, the stimulatory effect of the AbM-based mushroom extract (AndoSan™; ACE Co. Ltd., Gifu, Japan) on cytokine production (TNF-α, IL-1β, IL-6, IL-8, G-CSF and MIP-1β) in monocyte-derived dendritic cells has also

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been demonstrated [11]. The effects are probably mediated by binding of sugars in AbM to Toll-like receptor-2 (TLR-2) [12], but also to dectin-1 [13] and the lectin-binding site of CD11b/18 [14] and possibly CD11c/18 [15]. Gene microarray expression analysis of promonocytic THP-1 tumour cells [16] supported these results because stimulation with AbM strongly upregulated genes for IL-1β and IL-8, moderately for TLR-2 and co-operative molecule MyD88, but not for TLR-4. On the other hand, daily consumption of 60 ml of the current AbM-based extract for 7 days in patients with chronic hepatitis C [17] had no effect *in vivo* on the expression of these genes in blood cells.

Recently, we reported that AbM stimulation of whole blood *ex vivo* [18] stimulated the release of all the 17 different cytokines, chemokines and leucocyte growth factors tested. The cytokines were pro-inflammatory (IL-1β, IL-6, TNF-α), anti-inflammatory (IL-10) and pleiotropic (IL-7, IL-17) and of the Th1- (IFN-γ, IL-2, IL-12) and Th2 types (IL-4, IL-5, IL-13). In addition, chemokines IL-8, MIP-1β, MCP-1 and leucocyte growth factors G-CSF and GM-CSF were also studied. On the other hand, when blood was collected from volunteers prior to and 12 days after their daily intake (60 ml) of AbM, there was *in vivo* either a significant reduction in cytokine levels for IL-1β, TNF-α, IL-6, IL-2 and IL-17 or unaltered levels of the remaining 12 factors. This pointed to a stabilizing and anti-inflammatory effect of AbM *in vivo* when given via the oral route.

Patients with inflammatory bowel disease (IBD) like ulcerative colitis (UC) and Crohn's disease (CD) have in the colon mucosa an unselective increase in chemokine expression including that of MIP1-β, MCP-1 and IL-8 [19] as well as cytokines IL-1β [20], IL-6 and TNF-α [21]. Cytokine levels in serum, however, are less extensively studied, but increased levels of IL-6 [22] and TNF-α [23, 24] have been detected in patients with UC and CD. Recently, increased serum levels of the chemokine MIP-1β were found in patients with UC [25]. The IBD, UC and CD are autoimmune diseases of Th2 and Th1 nature, respectively.

Based on the anti-inflammatory and stabilizing effect of the AbM-based mushroom extract AndoSan™ on cytokine release in blood *in vivo* and *ex vivo* in healthy volunteers after 12 days consumption, the study aimed to investigate whether the same effect was valid in patients with IBD. In addition, calprotectin, an abundant cytosolic protein in neutrophils and a surrogate marker for degree of intestinal inflammation [26, 27], was measured in blood and faeces of these patients.

Materials and methods

Reagents. The mushroom extract (AndoSan™) used in our experiments was obtained from ACE Co. Ltd. It was stored at 4 °C in dark bottles and used under sterile conditions *ex vivo* and kept sterile until taken by volunteers for *in vivo* experiments. This mushroom extract is a commercial product and its extract contained a business secret, part of which has not been revealed until very recently. The AbM mixed powder contains per 100 g the following constituents: moisture 5.8 g, protein 2.6 g, fat 0.3 g, carbohydrates 89.4 g, of which β-glucan constitutes 2.8 g, and ash 1.9 g. The AndoSan™ extract contains 82.4% of *Basidiomycetes* mushroom derived from AbM (jap.: Himematsutake), 14.7% from *H. erinaceum* (Yamabushitake) [2] and 2.9% from Gf (Maitake) [3], and its final concentration was 340 g/l. The amount per litre of the extract was sodium 11 mg, phosphorus 254 mg, calcium 35 mg, potassium 483 mg, magnesium 99 mg and zinc 60 mg. The LPS content of AndoSan™ was found, using the *Limulus* amebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA) with detection limit 0.005 EU/ml (1 EU = 0.1 ng/ml), to be a miniscule concentration of <0.5 pg/ml. The results from tests for heavy metals were conformable with strict Japanese regulations for health foods. AndoSan™ had been heat-sterilized (124 °C for 1 h) by the producer. LPS was from *Escherichia coli* (*E. coli* 026:B6) (Sigma Co., St. Louis, MO, USA).

Experimental design. Twelve patients (nine men) with UC of median age 42 (range 33–66) years and 12 patients (five men) with CD of median age 41 (range 21–67) years volunteered to participate in the study of oral intake of low dose AndoSan™, 20 ml thrice daily for 12 days. This dose of 60 ml of the mushroom extract per day was chosen based on previous results in healthy volunteers [18] and as recommended by the manufacturer for regular use of AndoSan™ as a health-food product. The time interval between each dose should be from 6 to 10 h. Participants were asked to avoid mushroom-containing foods for 3 days prior to and during the experimental period. The diagnosis of IBD was based on histological examination of mucosal biopsies of colon, rectum and jejunum.

Two patients with UC and one with CD were excluded, because of lack of complete data. Based on clinical evaluation, the included patients with IBD had moderate disease activity and none used anti-TNF antibodies (adalimumab; Humira®, Abbott, Ludwigshafen, Germany) or azathioprine (Imurel®, GlaxoSmithKline, Solna, Sweden). In the UC patient group, all used mesalazine (Pentasa®, Ferring Legemidler AS, St. Prex, Switzerland) in daily doses of 0.8–4 g, given orally or as suppositories. One patient used antihypertensive medication (kandesartancileksetil; Atacand®, AstraZeneca, Södertälje, Sweden). In the patients with CD, one used sulphasalazine (Salazopyrin®, Pfizer, New York, NY, USA) (4 g) and one mesalazine (2 g) daily. A third patient with CD used nabumeton (Relifex®, Meda, Solna, Sweden) for

arthrosis. All the included participants with UC (n = 10) and CD (n = 11) denied regular smoking.

Prior to (day 0) and during (days 2 and 12) the intake of AndoSan™, heparinized blood collected from the included participants was, in one set of experiments, also immediately stimulated *ex vivo* with LPS (1 ng/ml) for 6 h at 37 °C in a 5% CO_2 incubator. During this incubation, the tubes were shortly manually shaken each hour. Then, plasma was harvested and samples stored at −70 °C until analysis for levels of cytokines. The included UC and CD patients had median disease duration of 15 (2–29) and 10 (2–29) years, respectively. The patients with UC had pancolitis (n = 3), left-sided colitis (n = 3), proctosigmoiditis (n = 1) and proctitis (n = 3), of whom two had been treated in hospital for acute colitis. Disease location in CD was ileal (n = 1), ileocolic (n = 6) and colic (n = 4). Three patients had had ileocolic resections.

To obtain baseline values of cytokine levels in healthy volunteers, equally treated plasma samples from unstimulated blood were also analysed for this purpose. The 15 healthy volunteers (eight men) had median age 36 (range 26–51) years and denied regular smoking and use of steady medication.

Analyses. Blood was harvested from the antecubital vein into glass tubes containing 15 IU heparin per ml or 10 mmol EDTA per ml. The EDTA blood was each time (days 0, 1, 2, 5, 8, 12) analysed for haemoglobin, haematocrite, mean cellular volume, mean cellular haemoglobin, reticulocytes, immature reticulocytes, leucocytes including a differential count of neutrophils, basophils, eosinophils, lymphocytes and monocytes, thrombocytes, C-reactive protein (CRP), urea, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamine transferase, alkaline phosphatase and pancreatic amylase.

The harvested heparinized blood was immediately centrifuged (2300 ***g***, 12 min) and plasma pipetted off and immediately stored at −70 °C till analysis for micro CRP (days 0, 2, 12) and cytokines (days 0, 2, 12). The CRP was analysed by both ordinary routine laboratory technique from EDTA blood and micro-CRP from plasma by the high sensitive Tina-quant CRP particle-enhanced immunoturbidimetric method performed using a COBAS INTEGRA 400 analyser (Roche Diagnostics, Indianapolis, IN, USA) [28]. This micro-CRP method is especially sensitive in concentrations ≤20 mg/l.

Faecal calprotectin concentrations (mg/kg) (normal values <50 mg/kg) at days 0 and 12 were determined in duplicates as reported [18, 29]. Plasma calprotectin concentrations (μg/l) were analysed in duplicates by ELISA technique [30]. Briefly, the samples were diluted in assay buffer and added to microtiter plate wells coated with an IgG fraction of rabbit anti-calprotectin as previously described [31]. After washing four times in buffer, the substrate (p-phenyl-phosphate) was added. Optical density was recorded after 15–25 min. Intra-assay and interassay variation coefficients were 5% and 13%, respectively.

We used the multiplex bead-based sandwich immunoassay technology (Luminex, Austin, TX, USA) and a human cytokine 17-plex kit (Bio-Rad laboratories, Hercules, TX, USA), strictly following the manufacturers' instructions, to measure the concentrations in individual heparinized plasma samples of the following cytokines, chemokines and growth factors [lower detection limits (in pg/ml) in parentheses]; IL-1β (2.0), IL-2 (1.2), IL-4 (0.3), IL-5 (2.3), IL-6 (2.1), IL-7 (3.0), IL-8 (1.6), IL-10 (1.8), IL-12 (3.0), IL-13 (0.9), IL-17 (2.5), G-CSF (1.9), GM-CSF (0.8), IFN-γ (2.0), MCP-1 (1.7), MIP-1β (2.0) and TNF-α (5.4). As there was no reduction in cytokine levels in healthy volunteers using 60 ml daily for only 2 days, we chose to compare between cytokines levels prior to (day 0) and after 12 days of AndoSan™ consumption [18].

Statistical analysis. Data are presented as median and range values, unless otherwise specified. Prospective differences in cytokine levels in blood *in vivo* and *ex vivo* and calprotectin in faeces and plasma between prior to (day 0) and after (day 12) AndoSan™ consumption were assessed with non-parametric Wilcoxon's paired sample test, unless otherwise specified. Blood values analysed for at least three time points were evaluated by analysis of variance (ANOVA) for paired data with Dunn's multiple comparisons. Instat for Windows™ statistics software package (Graphpad Software, San Diego, CA, USA) was used. P values below 0.05 were considered statistically significant.

Ethics. The study was approved by the regional ethics committee and followed the guidelines of the Helsinki declaration. The participants were also informed in written form and signed an agreement of consent for participation in the study. The study was registered with unique protocol ID AbM2009-IBD and clinical trials gov ID NTC01106742.

Results

Effect of AbM *in vivo* on general blood parameters

We obtained sufficient data from 10 of the 12 patients with UC and 11 of the 12 patients with CD, who had ingested 60 ml of AndoSan™ daily for 12 days. Haematological-, kidney-, liver- and pancreatic-function tests were obtained prior to (day 0) and after intake of AndoSan™ (days 1, 2, 5, 8 and 12). There were no significant differences whatsoever at any time point from normal range values in the following parameters tested: haemoglobin, haematocrite, mean cellular volume, mean

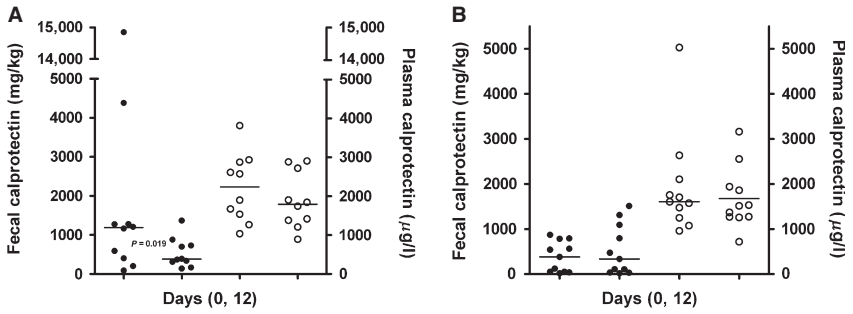

Figure 1 Scatter plots with median and individual levels of calprotectin in faeces (mg/kg) and plasma (μg/l) in 10 patients with ulcerative colitis (A) and 11 patients with Crohn's disease (B) prior to and after daily consumption of AndoSan™ for 12 days. Days 0 and 12 are represented by plots from left and right, respectively. The plots with closed circles and open circles refer to faecal and plasma calprotectin, respectively. Significant *P* value placed between the relevant plots compared with the calprotectin levels at day 0 prior to intake of AndoSan™.

cellular haemoglobin, reticulocytes, immature reticulocytes, thrombocytes, urea, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamine transferase, alkaline phosphatase and pancreatic amylase (data not shown).

Neither differential leucocyte counts nor CRP levels were significantly altered throughout the experiment. More specifically, median (range) leucocyte counts (10^9/l) at days 0, 1, 2, 5, 8 and 12 were for UC 6.8 (4.7–14.7), 6.7 (4.5–11.0), 6.2 (4.7–11.2), 7.3 (5.7–12.1), 6.8 (4.8–19.4) (n = 9) and 5.9 (4.4–14.5) and for CD 7.3 (3.6–12.6), 6.3 (4.5–13.5), 7.3 (3.9–11.8), 7.0 (4.5–10.4), 6.3 (4.7–12.0) (n = 10) and 7.3 (4.7–10.2). Corresponding values using the routine technique for CRP (mg/l) were for UC 3.5 (0.8–11.6), 3.1 (0.7–13), 2.9 (0.5–14.9), 4.9 (0.6–19.3), 4.5 (0.6–20.6) (n = 9) and 4.1 (0.5–26.2) and for CD 3.1 (0.6–32.3), 3.4 (0.5–52.2), 3.9 (0.06–49.6), 5.2 (1.4–46.7) (n = 10), 4.1 (0.5–30.6) and 3.2 (0.6–18.2). Using the micro-CRP technique, corresponding levels for days 0, 2 and 12 were comparable with 3.5 (0.8–11.6), 2.9 (0.5–14.9) and 4.1 (0.5–26.2) for UC and 3.1 (0.6–32.3), 3.9 (0.06–49.6) and 3.2 (0.6–18.2) for CD.

Calprotectin in faeces and blood

There was a significant reduction (Fig. 1A) in faecal calprotectin only in patients with UC from prior to and 12 days after AndoSan™ consumption. In some patients with UC (n = 6) and CD (n = 6) who were tested 1 week after the termination of AndoSan™ consumption (day 19), the faecal calprotectin levels were still unaltered. Respective median (range) values (mg/kg) comparing days 12 and 19 were 379 (139–1678) versus 476 (128–1683) for UC and 383 (16–1272) versus 237 (16–884) for CD. In contrast to patients with IBD, three middle-aged healthy volunteers had normal initial values of 16, 16 and 19 mg/kg of faecal calprotectin that did not alter over 12 days (data not shown) when consuming same dose of AndoSan™.

There were no alterations in plasma calprotectin levels of patients with IBD. Levels of plasma calprotectin (μg/l) in the three AndoSan™-consuming volunteers were also unaffected (data not shown), also with lower initial plasma values (1603, 1531 and 869 at day 0) than patients with IBD. Interestingly, the median ratio of calprotectin in plasma and faeces in patients with UC (1.8 (2229/1186)) was increased more than twofold [4.2 (1606/382)] in patients with CD and 50-fold [90 (1531/17)] in the three healthy volunteers.

Cytokine release in whole blood

In blood collected from the 10 patients with UC, there was a significant reduction (40%) in MCP-1 from before (day 0) and after 12 days intake of AndoSan™ (Fig. 2D), whilst the concentration of the remaining 16 cytokines was not significantly reduced. When the collected blood from these AndoSan™-consuming patients also was stimulated *ex vivo* for 6 h with a low concentration of LPS (1 ng/ml) to increase cytokine release, there was a significant reduction in seven of the 17 cytokines studied (Fig. 2A–G). These cytokines (percentage reduction given in parentheses) were MIP-1β (78%), IL-6 (44%), IL-1β (41%), IL-8 (30%), G-CSF (29%), MCP-1 (18%) and GM-CSF (17%).

In the 11 patients with CD (Fig. 3C,F,H), there was a significant reduction (day 0 versus day 12) in IL-2 (100%), IL-17 (55%) and IL-8 (29%) as a consequence of AndoSan™ consumption. LPS stimulation *ex vivo* of such blood resulted in significant reduction in seven cytokines (Fig. 3A–H), of which five (MIP-1β, IL-1β, IL-8, MCP-1, G-CSF) were identical to those for the patients with UC. The reduction in five cytokines was IL-1β 35%, MCP-1 22%, IL-8 18%, IL-17 17% and G-CSF 14%. Despite no reduction in median levels of IL-2, there was a significant reduction (P = 0.01) from day 0 to day 12 (Fig. 3H). Because all MIP-1β values measured after LPS stimulation at day 0 were out of range (upper limit:

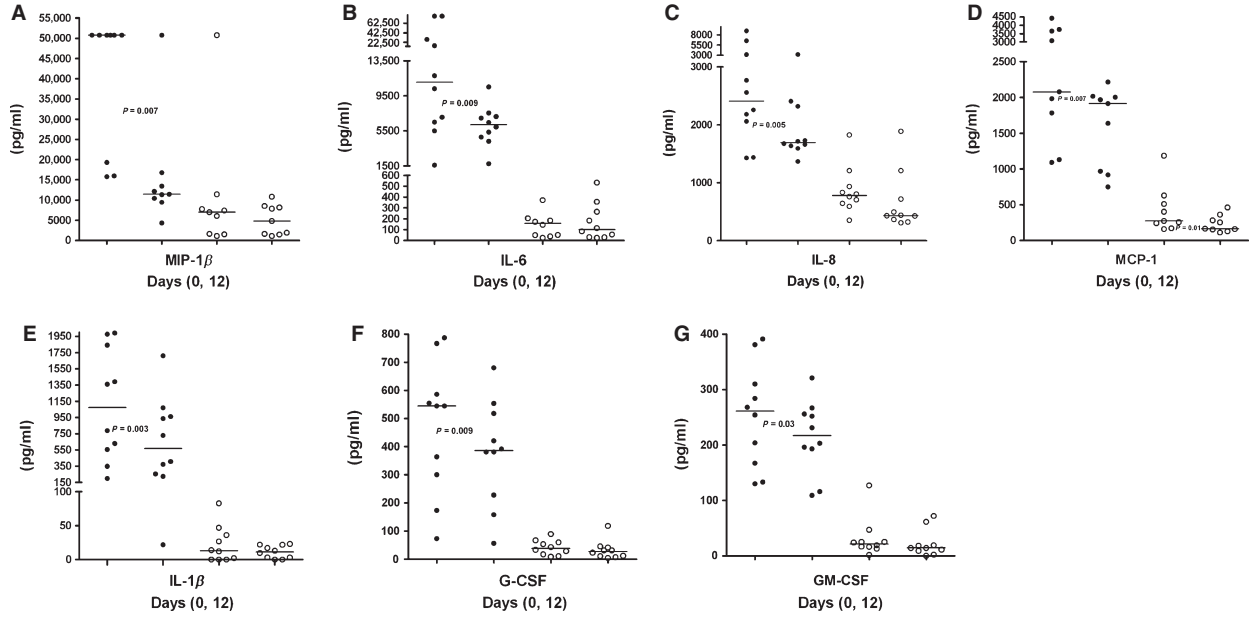

Figure 2 (A–G) Scatter plots with median and individual levels (pg/ml) of cytokines [MIP-1β (A), IL-6 (B), IL-8 (C), MCP-1 (D), IL-1β (E), G-CSF (F), GM-CSF (G)] in unstimulated or stimulated (LPS 1 ng/ml) whole blood *ex vivo* from 10 patients (unless otherwise stated) with ulcerative colitis prior to (day 0) and after AndoSan™ consumption for 12 days. Days 0 and 12 after stimulation are depicted by the two-first plots with closed circles from the left, respectively. Corresponding measurements from unstimulated blood are presented by plots by open circles. The *P* values placed between the relevant plots compared with the cytokine levels at day 0 prior to intake of AndoSan™. For MIP-1β and MCP-1, measurements for nine out of ten patients were available.

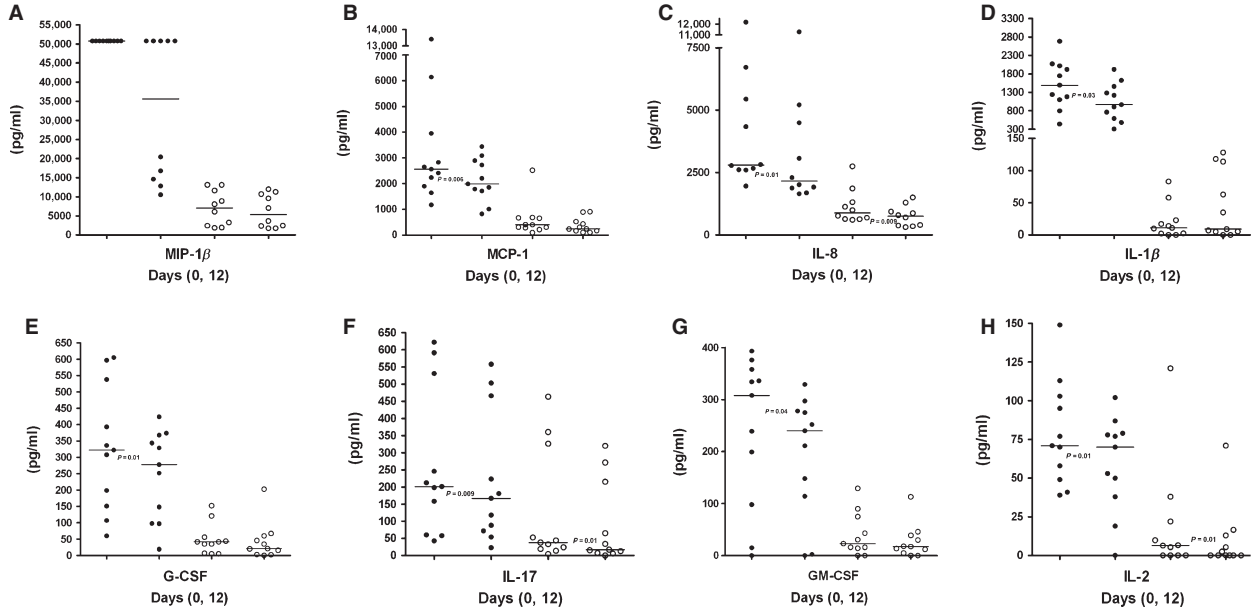

Figure 3 (A–H) Scatter plots with median and individual levels (pg/ml) of cytokines [MIP-1β (A), MCP-1 (B), IL-8 (C), IL-1β (D), G-CSF (E), IL-17 (F), GM-CSF (G), IL-2 (H)] in unstimulated or stimulated (LPS 1 ng/ml) whole blood *ex vivo* from 11 patients (unless otherwise stated) with Crohn's disease prior to (day 0) and after AndoSan™ consumption for 12 days. Days 0 and 12 after stimulation are depicted by the first two-first plots with closed circles from the left, respectively. Corresponding measurements from unstimulated blood are presented by plots with open circles. Despite remeasurement of MIP-1β after dilution of plasma 1/10, high out of range values still occurred. Accordingly, the true concentrations of MIP-1β were even higher. The *P* values between the relevant plots compared with the cytokine levels at day 0 prior to intake of AndoSan™. For MIP-1β and IL-8, measurements in ten out of eleven patients were available.

Table 1 Insignificant alterations in levels (pg/ml) of cytokines [median (range)] in unstimulated or stimulated (LPS 1 ng/ml) whole blood *ex vivo* from 10 patients with UC prior to (day 0) and after AndoSan™ consumption for 12 days.

	Patients with UC			
	Unstimulated		LPS-Stimulated	
Analyte	Day 0	Day 12	Day 0	Day 12
TNF-α	114 (14–307)	65 (11–707)	4203 (1458–8920)	2960 (1241–8412)
IFN-γ	120 (17–199)	65 (9.7–605)	1319 (360–1951)	1059 (266–2308)
IL-17	39 (0–289)	8.5 (0–374)	186 (53–484)	150 (46–489)
IL-12	34 (7–110)	35 (1.6–150)	66 (10–140)	60 (7–139)
IL-2	5 (0–80)	0 (0–73)	70 (19–141)	52 (6–98)
IL-10	4.8 (0–11)	2.8 (0–28)	61 (16–427)	55 (11–298)
IL-13	2.3 (0.7–7.7)	2.0 (0.6–5.9)	4.6 (2–8.5)	4 (1.6–7.4)
IL-5	1.7 (0–7.9)	1.5 (0–4.9)	2.1 (0–7.6)	2.1 (0–6.8)
IL-4	0.9 (0–5.1)	0.2 (0–8.4)	12 (4.6–23)	9 (3–23)
IL-7	0.03 (0–7.1)	0.06 (0–9)	4.5 (1.3–7.1)	2.9 (0.6–7.3)

UC, ulcerative colitis.

Table 2 Insignificant alterations in levels (pg/ml) of cytokines [median (range)] in unstimulated or stimulated (LPS 1 ng/ml) whole blood *ex vivo* from 11 patients with CD prior to (day 0) and after AndoSan™ consumption for 12 days.

	Patients with CD			
	Unstimulated		LPS-Stimulated	
Analyte	Day 0	Day 12	Day 0	Day 12
IL-6	140 (43–12,914)	140 (39–3820)	14,777 (4025–77,494)	9162 (3772–21,947)
TNF-α	110 (19–1526)	119 (11–532)	5838 (3172–28,143)	5417 (1499–18,275)
IFN-γ	89 (0–1080)	78 (0–520)	1448 (293–3157)	1229 (153–2488)
IL-12	35 (1.1–139)	31 (0.1–137)	67 (4.7–158)	66 (3–154)
IL-10	3.8 (0–306)	2.1 (0–281)	116 (8–471)	125 (2.4–400)
IL-13	1.8 (0.3–11)	1.0 (0.6–7.0)	4.6 (2.6–13)	3.7 (0.6–13)
IL-4	0.6 (0.1–13)	0.4 (0–5.3)	13 (4–34)	9.7 (3–27)
IL-7	1.2 (0–8.9)	0.5 (0–7.3)	5.2 (1.5–15)	4.5 (0.3–7.3)
IL-5	1.2 (0.1–3.5)	0.9 (0–15)	1.8 (1.4–15)	1.6 (0.5–11)

CD, Crohn's disease.

Table 3 Comparison of 17 baseline cytokine levels [median and (range)] in patients with UC (n = 10) and CD (n = 11) versus in healthy volunteers (n = 15) before oral intake of AndoSan™.

Analyte	Ratio UC/ normal	Ratio CD/ normal	Normal baseline level (pg/ml)
TNF-α	5.7	5.5	20 (0–273)
IFN-γ	6.3	4.6	19 (3–206)
IL-1β	1.4	1.2	9 (4–575)
IL-2	2.0	2.5	2.5 (0.5–3)
IL-4	ND	ND	0 (0–2.4)
IL-5	1.0	0.7	1.7 (1.4–5)
IL-6	4.6	4.1	34 (13–931)
IL-7	0.006	0.2	4.9 (1.4–32)
IL-8	2.1	2.7	359 (78–1433)
IL-10	1.5	1.1	3.2 (2.1–13)
IL-12	5.6	5.8	6 (1.9–25)
IL-13	1.2	0.9	1.9 (1–7.3)
IL-17	2.4	2.3	16 (10–41)
MCP-1	2.5	3.8	105 (62–884)
G-CSF	0.6	0.9	43 (10–157)
GM-CSF	2.3	2.5	9 (0.1–54)
MIP-1β	6.8	6.8	1026 (337–12,300)

CD, Crohn's disease; ND, not detectable; UC, ulcerative colitis.

50,806 pg/ml), non-parametric statistics could not be applied. Using parametric statistics (paired t-test), there was a significant reduction (30%, P = 0.01) in MIP-1β (Fig. 3A) with mean values 50,806 (day 0) and 35,544 (day 12).

When initial unstimulated baseline values for the 17 cytokines were compared in the UC and CD patient groups, there were largely similar concentrations (Tables 1 and 2, Figs. 2 and 3). Table 3 shows the comparison of baseline cytokine levels in the patients IBD versus those of healthy volunteers before oral intake of AndoSan™.

Discussion

The present study demonstrates reduction in several cytokines in the serum of patients with UC and CD after 12 days' intake of a *Basidiomycetes* mushroom extract (AndoSan™) mainly based on AbM. For the patients with UC, there also was a concomitant reduction in levels of faecal calprotectin. Similar results showing such decline in cytokine levels have been demonstrated [18] in healthy volunteers consuming AndoSan™ in a similar

experimental set-up. Collectively, the findings support the notion of a general anti-inflammatory and stabilizing effect of AndoSan™ on cytokine release in individuals with good health or IBD.

Blood samples collected from patients with IBD had to be stimulated *ex vivo* with LPS, a well-known stimulator of innate immune cells, to reveal significant reduction in the levels of cytokines in addition to MCP-1 in UC (Fig. 2D) and IL-8, IL-17 and IL-2 in CD (Fig. 3C,F,G). The LPS stimulation effectuated the depletion of residual cytokine production and storage capacity of the harvested peripheral blood leucocytes and thus enabled our detection of the mushroom's total potential to decrease cytokine levels in blood. For comparison, in healthy volunteers likewise consuming AndoSan™ [18], there was a significant reduction in as many as five cytokines in unstimulated blood and in four other cytokines in LPS-stimulated blood *ex vivo*. From this, it can be inferred that the mushroom extract altogether reduced levels of comparable number of cytokines in healthy volunteers (n = 9) and patients with UC (n = 7) and CD (n = 7), but to a larger extent in unstimulated blood in the former healthy group. This is not unexpected because a majority of the cytokines (n = 10) in the patients with IBD were from two- to sevenfold higher (Table 3), rendering the anti-inflammatory effect of AndoSan™ less detectable in unmanipulated IBD blood samples. In addition to GM-CSF and MIP-1β (not measured in healthy volunteers after low doses AndoSan™ consumption), in patients with IBD, IL-1β, IL-2, IL-6, IL-17 and G-CSF were detected in reduced concentrations after mushroom intake both among healthy volunteers and patients. Thus, both pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (IL-8, MIP-1β, MCP-1, GM-CSF, G-CSF) were downregulated by AndoSan™ in these patients with IBD.

The three cytokines with the most marked reduction in LPS-stimulated blood from these patients were MIP-1β, IL-1β and IL-6. Chemokine MIP-1β belongs to the family of macrophage inflammatory 1 proteins, which orchestrate acute and chronic inflammatory responses at sites of injury or infection mainly by recruiting pro-inflammatory cells [32]. Recently, an unselective increase in chemokine expression in mucosa has been demonstrated by immunohistochemistry among patients with UC and CD. Such studied chemokines include MIP1-β, MCP-1 and IL-8 [19], which were reduced in collected blood from patients with UC (MIP1-β, MCP-1) and CD (MIP1-β, IL-8). IL-6 in the intestinal mucosa is synthesized by mononuclear cells [21, 24], and it is elevated in serum in both UC [25] and CD [24]. We observed a considerable decline in this cytokine (Fig. 2B) in patients with UC after consumption of the mushroom extract. Similar to our study (Tables 1–3), increased serum levels of IL-1β are seldom detected [24], but IL-1β levels are elevated [20, 33, 34] in intestinal lesions in both UC and CD. Interestingly, levels of IL-1β in LPS-stimulated blood declined in both diseases, again pointing to a net anti-inflammatory effect of AndoSan™.

The hitherto unreported reduction in pleiotropic IL-17 (Fig. 3F) in patients with CD is intriguing [35]. Because IL-17 will both convey a host defensive mechanism to various extracellular bacterial infections and pathogenic involvement in autoimmune disease, a reduced concentration of this cytokine may dampen these inflammatory reactions. The general tendency in patients with UC and CD was that cytokine levels were either significantly or insignificantly reduced after 12 days of mushroom consumption. Thus, the lack of significant reduction in concentrations especially for cytokines TNF-α, IFN-γ and IL-6 (CD) could be because of the limited number of patients included in each IBD group (type II error). For cytokines IL-4, IL-5, IL-7 and IL-13 in patients with IBD, there were no striking alterations in their concentrations throughout the experimental period.

None of the Th2 cytokines (IL-4, -5, -13) potentially relevant for UC seemed to be initially elevated or modulated by AndoSan™, whilst IL-2 was the only Th1 cytokine that was reduced after AndoSan™ ingestion in patients with CD. According to the Th2/Th1 dichotomy [36], one could also have anticipated an inverse increase in Th2 and Th1 cytokines in UC and CD, respectively.

Even though LPS- and cytokine-stimulated polymorphonuclear neutrophils have been shown to synthesize and secrete cytokines like IL-1β, IL-8, TNF-α, MIP-1β, IL-12 [34] and possibly GM-CSF [37, 38], we assume that these cytokines mainly are synthesized by and released from monocytes. These cells have a far greater capacity for cytokine biosynthesis [37] as well as a longer half-life in blood (approximately 3 days) [39] than neutrophils (approximately 6.5 h) [40]. In addition, other abundant cytokines such as G-CSF, MCP-1, IL-6 and IFNγ are absent in neutrophils and were probably mainly derived from monocytes. On the other hand, IL-17 [35], IFN-γ and IL-2 [41] were exclusively derived from lymphocytes, Th17 and Th1 cells, respectively.

One explanation for the AndoSan™-promoted reduction in LPS-induced inflammatory response in blood *ex vivo* as well as in patients with IBD may be the following: AndoSan™ may actually inhibit LPS-induced TLR4 signalling because (1) AndoSan™ stimulates TLR2 [12], which has a common intracellular downstream pathway with the LPS receptor TLR4 for the activation of transcription factor NF-κB, and (2) the inflammation in patients with IBD may in fact partly be because of gram-negative bacterial (LPS)-induced inflammatory response.

The second major finding in this study was that the patients with UC had a significant reduction in faecal

calprotectin on day 12, whilst calprotectin in plasma was unaltered during the experiment. Calprotectin, an abundant cytosolic protein in neutrophils [26] can, when released to faeces, be used as a marker for disease activity in IBD [27, 29]. Also in patients with CD, reduction in faecal calprotectin has been detected in parallel with reduced degree of inflammation, but then the reported initial calprotectin values were much higher (approximately 15-fold) [27] than here and probably from more seriously affected patients than in the current study. Together with the limited time-span of AndoSan™ ingestion, this difference may contribute to explain the lack of effect on faecal calprotectin levels in our patients with CD. Interestingly, there was no reduction in plasma calprotectin by mushroom consumption, which indicates that the effect of AndoSan™ on that parameter was local in the colonic mucosa. During active inflammation, neutrophils infiltrate the lamina propria, crypt epithelium and form crypt abscesses. These histological changes return to normal levels in periods of remission [34]. Although not systematically registered, patients with both UC and CD spontaneously reported a reduction in stool frequency after a few days of AndoSan™ intake, which at least partly may be ascribed to the reduction in faecal calprotectin.

Similar to experiments with healthy volunteers consuming the AbM-based mushroom extract [18], there were no pathological effects whatsoever on haematological parameters, including CRP values and leucocyte counts, and negative clinical side effects were not registered.

The AndoSan™ mushroom extract mainly containing *A. blazei* Murill (AbM) (~83%) but also *H. erinaceum* (approximately 15%) and *G. frondosa* (approximately 3%) is rich in immunostimulatory substances like proteoglucan [42, 43] and β-glucans [4]. Accordingly, major effects of AndoSan™ are probably mediated by binding of sugars to TLR-2 [12, 44], but also to the dectin-1 receptor [13, 45] and the lectin-binding site of CD11b/18 [14, 46] and possibly CD11c/18 [15]. In line with our results on the reduction in faecal calprotectin in patients with UC, anti-inflammatory effects of the β-glucan pleuran, isolated from the fruiting bodies of *Pleurotus ostreatus*, has been reported when given orally or intraperitoneally in rats with experimentally induced colitis [47]. Thus, β-glucans seemed to have an anti-inflammatory effect on the colonic mucosa both when administered directly to the gastrointestinal tract or indirectly to the peritoneum. Accordingly, two paths of direct and indirect anti-inflammatory effects may be operating concerning both reduction in faecal calprotectin (UC) and blood levels of cytokines in these patients with IBD. In another study in rats [48] given AbM extracts orally for 1–2 weeks, several anti-inflammatory effects were observed. Examples were reductions in rat paw oedema induced by nystatin, neutrophil migration to the peritoneal cavity and arthritis induced by Freund's adjuvant.

As to the explanation why AndoSan™ in our study both had a local effect in patients with UC by reducing faecal calprotectin and probably a systemic one in patients with UC and CD by reducing foremost levels of pro-inflammatory cytokines is intriguing and has recently been discussed [18]. Briefly, it is commonly believed that carbohydrates larger than monosaccharides are not absorbed from the human gut. However, in murine models [49, 50], uptake of β-1,3-glucans across the gut wall, probably by microfold cells (M cells) and gastrointestinal macrophages in Peyer's patches for transport to the reticuloendothelial system and blood [51, 52], has been demonstrated. Presumably, a similar mechanism was operating in humans for intestinal absorption of small and immunomodulatory bioactive β-glucan fragments into the lymphoid system and blood. In addition, AbM contains absorbable low molecular weight antioxidant substances [53], which downregulate the levels of reactive oxygen species (ROS). This may be of relevance in patients with IBD concerning reduction in IL-1β because inhibitors of ROS reduce synthesis of this cytokine in macrophages [54].

In conclusion, consumption of an AbM-based medicinal mushroom extract for 12 days by patients with IBD resulted in no side effects and a decline of especially pro-inflammatory and chemotactic cytokines as well as a reduction in faecal calprotectin in patients with UC. These promising results warrant further studies on additional biological parameters and potential improvement of clinical outcome in these patients.

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