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Effects of *Pityrosporum ovale* on proliferation, immunoglobulin (IgA, G, M) synthesis and cytokine (IL-2, IL-10, IFNγ) production of peripheral blood mononuclear cells from patients with seborrhoeic dermatitis

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Abstract The aetiology of seborrhoeic dermatitis (SD) is still unknown. An increased number of *Pityrosposurm ovale* in lesional skin of patients with SD has been suggested to play a crucial role in the pathogenesis of the disease since double-blind trials with antifungal drugs (e.g. ketoconazole) have shown that these agents result in a significantly reduced disease intensity. The frequent association of HIV infection and SD may be due to a suppressed cell-mediated immunity. In order to characterize the role of the humoral and cellular immune response in patients with SD the effects of a *P. ovale* extract on the proliferation of, and interleukin-2 (IL-2), IL-10 by an interferon-γ (IFNγ) production, and immunoglobulin (IgA, IgG, IgM) synthesis by peripheral blood mononuclear cells (PBMC) from patients with SD were studied in vitro. Healthy volunteers served as controls. PBMC from normal donors responded with a significantly increased proliferation to *P. ovale* antigen, whereas cells from patients with SD did not. Additionally, IL-2 and IFNγ production by PBMC from patients with SD was markedly depressed compared with normal cells after stimulation with *P. ovale*. However, stimulation of PBMC from SD patients with *P. ovale* antigen induced significantly increased IL-10 synthesis. IgA, IgG and IgM synthesis was significantly increased in cultures of PBMC from patients with SD whether the cells were antigen-stimulated or not. Our results support the assumption that strong skin colonization with *P. ovale* in SD is due to an altered cellular immunity, which may be induced by increased IL-10 secretion.

Key words Seborrhoeic dermatitis · Cellular immunity · *Pityrosporum ovale* · Cytokines · Immunoglobulins

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Introduction

Seborrhoeic dermatitis (SD) ia a chronic skin disease associated with seborrhoea of the scalp and the sebaceous follicle-rich areas of the face and trunk. Histologically, the sebacceous glands are often enlarged [23]. The total amount of skin surface lipids is not elevated, but an increased proportion of cholesterol, triglycerides and paraffin, and a decrease in squalene, free fatty acids and wax esters have been reported [12]. SD is often associated with neurological diseases (e.g. postencephalitic parkinsonism , facial paralysis, unilateral injury to the ganglion of Gasser, poliomyelitis, syringomyelia and quadriplegia), and treatment with levodopa and a reduction in skin oiliness improves SD [23]. Other concepts of SD favour an aetiology involving bacteria and yeasts, which can be isolated in great quantities from affected skin sites [19]. The lipophilic yeast *Pityrosporum ovale* has received particular attention. The genus *Pityrosporum* includes *P. orbiculare*, *P. ovale*, and *P. pachydermatitis*. Recently, a close antigenic relationship between *P. orbiculare* and *P. ovale* has been demonstrated, and today they are understood to be representatives of differnt stages in the cell cycle of the same species [10]. A possible role for this yeast in the pathogenesis of SD is supported by the fact that SD-like lesions have been shown to be reproducible in two animal models by inoculation of *P. ovale* [7, 9]. Additionally, ketoconazole, an antimycotic drug with pronounced pityrosporicidal activity [29], is therapeutically effective in SD when given either systemically [11], or topically [8, 26]. In healthy adults the highest numbers of *P. ovale* are found on the mid sternum area of the chest and interscapular area of the back (about $3 \times 10^2/cm^2$ each). In patients with SD, numbers up to $6.7 \times 10^5/cm^2$ have been detected [19, 25]. The prevalence of SD is high in patients with acquired immunodeficiency syndrome (83%) and in patients with AIDS-related complex (42%) [5, 18, 27]. This indicates that a defective cell-mediated response to *P. ovale* may be a component of the pathogenesis of SD. Thus, the effects of *P. ovale* antigens on peripheral blood mononu-

clear cells (PBMC) proliferation, immunoglobulin synthesis and cytokine production (IL-2, IFNγ and IL-10) were investigated in patients with SD.

Material and methods

Patients and controls

PBMC were obtained from ten healthy volunteers and ten patients suffering from SD. The typical clinical features of SD, pink oedematous skin covered with yellow-brown scales and crusts, were seen in all patients. None of the SD patients was HIV positive. The ten healthy volunteers had no personal history of SD or other diseases associated with *P. ovale*, e.g. atopic eczema.

Isolation of PBMC

Isolation of PBMC was performed by centrifugation on a Ficoll-sodium metrizoate (Sigma, München, Germany) gradient according to the method of Böyum [6]. Briefly, heparinized venous blood (40 ml) was layered over Ficoll-sodium metrizoate (density 1075 g/ml) and centrifuged at 375 *g* for 35 min. Cells at the interface above the Ficoll-metrizoate were removed and washed three times with RPMI-1640.

Culture conditions

The basic culture medium was RPMI-1640 supplemented with 2 m*M* glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin. Medium containing 10% fetal calf serum is referred to as RPMI-1640 with 10% FCS.

Cell suspensions containing 1×10^6/ml viable cells in RPMI-1640 and 10% FCS were dispensed into each well of 24- and 96-well plates (Nunc, Roskilde, Denmark). Stimuli were added and the cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air.

Culture of *P. ovale*

An isolate of *P. ovale* purchased from the German Collection of Microorganisms (strain DSM no. 6170) was grown for 4 days at 37° C on a glucose–peptone agar containing 1.0% bacteriological peptone, 4.0% glucose, 0.01% yeast extract, 0.25% glycerol monostearate, 0.2% Tween 80, 2.0% olive oil and 1.2% agar as described by Faergemann et al. [9, 10]. The yeasts were identified microscopically and only the cream-yellow coloured colonies were harvested.

Extracts of *P. ovale*

The *P. ovale* cells were carefully harvested from the solid agar medium and a thick cell suspension was obtained. The cells were washed three times with distilled water, resuspended in 0.125 *M* NH_4HCO_3 buffer, pH 7.8, and sonicated for 5 min [17]. The cell suspension was extracted overnight at 4° C, centrifuged at 1200 *g* for 10 min. The supernatant was membrane-filtered (pore size 0.45 µm). The protein concentration of the *P. ovale* extract was estimated by the method of Lowry [16]. All extracts were lyophilized by vacuum centrifugation at 4° C for 8 h and stored at –20° C until tested.

Cell stimulation

Freshly prepared PBMC were resuspended in culture medium with different concentrations of the *P. ovale* extract: 0.01, 0.1, 1.0, 10.0 µg/l × 10^6 PBMC. When cytokines were measured, cells were stimulated only with 1.0 µg/l × 10^6 PBMC. This concentration produced optimal stimulatory effects.

[^{3}H]Thymidine incorporation

Proliferation was measured by adding 7.4 kBq/well [^{3}H]thymidine (Amersham Buchler, Braunschweig, Germany) to PBMC during the final 4 h of a 4-day overall incubation period. PBMC were then harvested and 200 µl of each cell suspension was transferred into 96-well culture plates. Triplicate cultures were harvested onto glass fibre filters, and radioactivity was counted by liquid scintillation. Data are expressed as follows: stimulation index (SI) = (counts per minute in stimulated culture)/(counts per minute in control culture).

ELISA for IgG, IgA and IgM

The IgG, IgA and IgM contents of the culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) on day 7. Cell viability was assessed microscopically by trypan blue exclusion analysis. Microtitre plates (96-well; Nunc; Roskilde, Denmark) were coated with rabbit antihuman IgG (100 µl, diluted 1 : 1000 in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20, incubated for 1 h at 37° C), rabbit antihuman IgA (100 µl, diluted 1 : 2000 in PBS, 0.05% Tween 20, incubated for 24 h at 4° C) or rabbit antihuman IgM (100 µl, diluted 1 : 2000 in PBS, 0.05% Tween 20, incubated for 24 h at 4° C). Anti-IgM and anti-IgA were obtained from Jackson Immuno Research Laboratories, Baltimore, Md., USA, and anti-IgG was from Immunotech S.A., Marseille, France. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS, 0.05% Tween 20 (1 h at 37° C). A 100-µl aliquot of supernatant (IgA and IgG ELISA: 1 : 10–1 : 20 dilution) was added and incubated for 1 h at 37° C. In parallel, standard curves (0.3–300 ng/ml) were prepared. (Behring, Marburg, Germany). The detection limits for IgA, IgG and IgM were 0.4 ng/ml. The specifity of the assays was confirmed by adding immunoglobulin of other isotypes to rule out the possibility of crossreactivity. Finally, *o*-phenylenediamine (OPD; Sigma, St. Louis, Mo., USA), 1 mg/ml in 33 m*M* phosphate-citrate buffer, pH 5.0, was added as substrate and the optical density was measured at 490 nm.

Cytokine production

The amounts of IL-2, IFNγ and IL-10 were measured after 4 days of culture by quantitative sandwich enzyme immunoassay kits distributed by Hermann Biermann, (Bad Nauheim, Germany). Briefly, samples and standards were incubated in microtitre wells coated with a monoclonal antibody specific for each cytokine. Afterwards, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells. After a further incubation period the excess detector complex was removed by washing and a substrate solution was added to the wells and colour developed in proportion to the amount of the cytokine. Absorbance was measured at 490 nm, 450 nm and 405 nm, respectively.

Statistical analysis

All experiments were performed with ten different donors each in the control group and the SD group. The data were calculated as means ± standard deviation. The significance was evaluated using Wilcoxon's signed ranks test for paired data and Student's *t*-test for independent means; $P < 0.05$ was considered significant.

Results

Proliferation

In healthy volunteers, *P. ovale* antigen (0.1 and 1.0 µg/ml) induced a significantly enhanced lymphocyte prolifera-

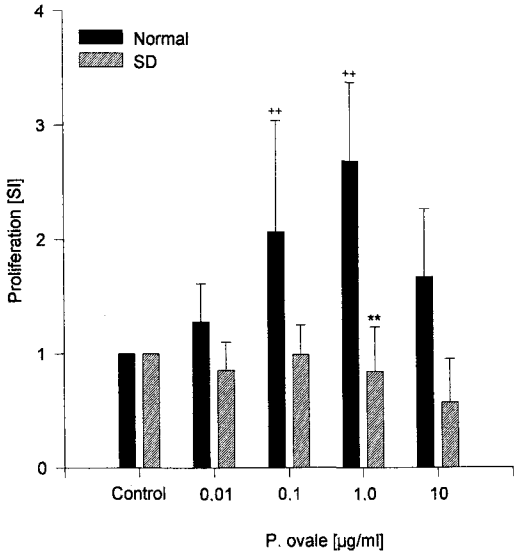

Fig. 1 Effect of *P. ovale* antigen (0.01, 0.1, 1.0 and 10 µg/10^6 cells) on [^{3}H]thymidine uptake by PBMC. Results are given as stimulation index [SI] = $cpm_{stimulated}/cpm_{control}$. ($^{++}P < 0.01$ versus spontaneous proliferation, ** $P < 0.01$ versus healthy donors)

tion compared with the unstimulated control ($P < 0.01$). In contrast, PBMC from SD patients were not stimulated by *P. ovale* antigen. After stimulation of PBMC from SD patients with 1 µg/ml *P. ovale* antigen, proliferation was significantly lower than in healthy donors ($P < 0.01$; Fig. 1).

IgA, IgG IgM synthesis

A lower spontaneous IgA, IgG and IgM synthesis was detected in normal donors than in patients with SD (Table 1). After stimulation with *P. ovale* antigen no significant modulation of IgA, IgG or IgM synthesis compared with spontaneous Ig secretion was observed either in the normal donor group or in the SD group (Table 1). However, PBMC from patients with SD produced significantly higher amounts of IgA and IgM after stimulation with 1.0 µg/ml *P. ovale* antigen ($P < 0.05$) compared with healthy volunteers.

Table 1 IgA, IgG and IgM synthesis by P.ovale-stimulated PBMC (*Normal* PBMC from healthy volunteers, *SD* PBMC from seborrhoeic dermatitis patients)

	P. ovale antigen				
	Control	0.01 µg/ml	0.1 µg/ml	1.0 µg/ml	10.0 µg/ml
IgA (ng/ml)					
Normal	195.1 ± 63.2	190.6 ± 58.3	158.4 ± 18.9	151.2 ± 20.5	103.3 ± 60.1
SD	403.4 ± 192.9	378.8 ± 198.3	364.9 ± 175.2	368.6 ± 155.7*	254.3 ± 99.2
IgG (ng/ml)					
Normal	209.5 ± 166.3	170.2 ± 40.8	144.4 ± 61.3	126.5 ± 109.5	84.9 ± 53.5
SD	552.4 ± 348.8	340.0 ± 187.4	351.4 ± 229.6	210.4 ± 138.6	250.9 ± 119.1
IgM (ng/ml)					
Normal	20.2 ± 1.8	24.0 ± 4.9	21.6 ± 0.9	20.5 ± 1.0	15.3 ± 8.9
SD	32.0 ± 3.9*	45.2 ± 22.7	32.7 ± 12.3	34.0 ± 10.6*	42.1 ± 18.5

*$P < 0.05$ versus normal PBMC

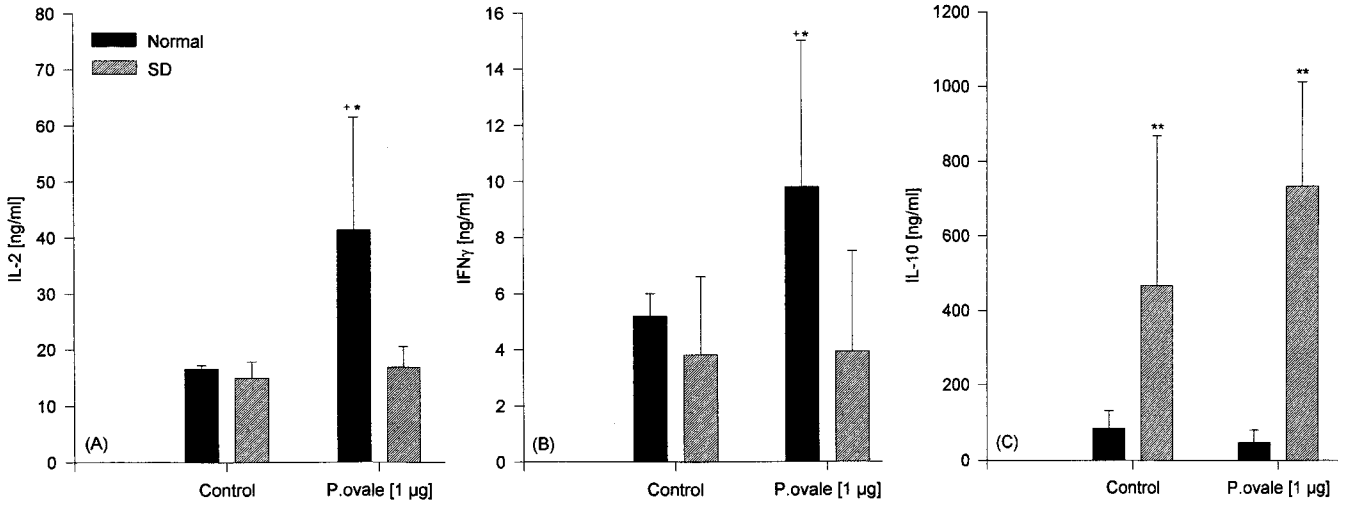

Fig. 2 A–C IL-2 **(A)**, IFNγ **(B)** and IL-10 **(C)** production measured in the culture supernatants of PBMC from patients with SD and from normal donors. *$P < 0.05$, **$P < 0.01$ versus cytokine secretion by normal PBMC; $^+P < 0.05$ versus spontaneous cytokine secretion

IL-2, IL-10 and IFNγ synthesis

The effect of *P. ovale* antigen stimulation on IL-2, IL-10 and IFNγ synthesis by PBMC from healthy volunteers and SD patients are shown in Fig. 2. Stimulation with 1.0 μg/ml *P. ovale* antigen enhanced IL-2 ($P < 0.05$) and IFNγ ($P < 0.05$) synthesis by PBMC from normal donors but not by PBMC from patients with SD. In contrast, IL-10 synthesis by PBMC from patients with SD significantly increased after stimulation with *P. ovale* antigen up to 733.3 ± 279.7 ng/ml. In normal PBMC no difference compared with spontaneous IL-10 secretion was observed when the PBMC were stimulated with *P. ovale* antigen. Spontaneous and stimulated IL-10 secretion was markedly higher in PBMC from patients with SD than in those from healthy volunteers.

Discussion

Our results clearly demonstrate that PBMC from patients with SD show a diminished cellular immune response to *P. ovale* antigens. An increased lymphocyte proliferation response to *P. ovale* antigen was observed in the control group, but not in patients with SD. These results correspond to those reported by Bergbrant [2] who observed a low response of lymphocytes from patients with SD to mitogenic stimulation with ConA and PHA. The mitogenic capacity of *P. ovale* antigens is lower than that of other microbial antigens. For example, *Candida albicans* extracts are 66.6 times more active in inducing lymphocyte proliferation then *P. ovale* extracts [22]. This might explain the low stimulation indices in the proliferation experiments with *P. ovale* extracts.

The assumption that T cells from patients with SD show a diminshed response to *P. ovale* antigens is supported by low IL-2 and IFNγ production compared with those from healthy donors after stimulation. However, IL-10 secretion was significantly increased after stimulation with *P. ovale* extract. IL-10 is known to inhibit monocyte/macrophage-dependent T-cell proliferation and cytokine synthesis [21]. Therefore, increased IL-10 secretion in SD may explain low proliferation as well as reduced IL-2 and IFNγ secretion. The mechanism by which IL-10 inhibits monocyte/macrophage-dependent T-cell functions is not yet completely understood [21]. It has been proposed that downregulation of monocyte/macrophage class II MHC expression induced by IL-10 may explain the inhibition of T-cell functions [21]. In SD such mechanisms may explain the disturbed cellular immunity to *P. ovale*.

Alterations in humoral immunity in patients with SD are a matter of controversy. Increased titres of total serum antibodies [2] as well as specific serum antibodies to *P. ovale* antigens have been reported [8]. On the other hand, significantly lower serum antibody titres against a *P. ovale* cell-wall protein have been observed [2]. Other studies have indicated no differences in specific [20] or nonspecific [3, 4] antibody synthesis between patients with SD and healthy controls in vivo.

Our results show an enhanced IgA, IgM and IgG synthesis by unstimulated and *P. ovale*-stimulated PBMC from patients with SD but not by those from normal donors. IL-10 augments both the proliferation of B cells and their differentiation into antibody-secreting cells [13]. Thus, increased immunoglobulin synthesis in SD may be due to high IL-10 secretion. Whether the enhanced immunoglobulin synthesis is due to polyclonal activation or to the production of specific antibodies is still unclear.

The mechanisms by which *P. ovale* induces skin inflammation are currently unknown. The lipophilic yeast shows lipase activity [8, 24, 30] and is able to activate the alternative pathway of complement [1, 28]. These processes may induce nonspecific skin inflammation. Furthermore, superficial infections may stimulate cellular immune reactions against *P. ovale*, e.g. T-cell proliferation and cytokine secretion [15]. However, lack of proliferation as well as reduced IL-2 and IFNγ secretion in SD patients supports the assumption that increased skin colonization with *P. ovale* is due to a diminished cellular immunity against this microorganism in SD. Furthermore, increased IL-10 production might be responsible for disturbed T-cell function in SD.

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