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Characterization of the cellular immune function of patients with chronic mucocutaneous candidiasis

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SUMMARY

Chronic mucocutaneous candidiasis (CMC) is a rare syndrome characterized by persistent and refractory infections of the skin, nails and mucosal tissues by yeasts of the genus *Candida.* Defects in the cellular limb of the immune system are well documented in CMC patients, but non-specific immune defects, such as myeloperoxidase deficiency or phagocyte chemotaxis disorders, have also been described. Nonetheless, the underlying defect(s) remains poorly understood, and further studies are required. We studied eight CMC patients without endocrinopathies, who showed (i) low normal proliferative response to phytohaemagglutinin (PHA), (ii) partially defective response to pokeweed mitogen (PWM), and (iii) impaired response to *Candida and* PPD antigens. Furthermore, peripheral blood mononuclear cells (PBMC) from CMC patients produced lower levels of type-1 cytokines (IL-2 and interferon-gamma) in response to *Candida* antigens, compared with control individuals. Conversely, we did not observe an enhancement of IL-4 and IL-10 in the patients, suggesting that, even though Th1 cytokines are decreased, the Th2 response is not increased in CMC. Nevertheless, the synthesis of these cytokines was normal when induced by PHA. We also observed an increased antigen-induced apoptosis in lymphocytes from the patients compared with controls, and this applied both to *Candida* and PPD antigens. Lastly, innate immunity defects were investigated. We observed an impairment of natural killer activity against K-562 target cells in half of the studied patients. These findings corroborate the extensive clinical and laboratory variability of CMC, which requires further studies on a larger number of patients to be better understood.

Keywords chronic mucocutaneous candidiasis cellular immunity cytokines apoptosis activation-induced cell death

INTRODUCTION

Chronic mucocutaneous candidiasis (CMC) is a rare and complex disorder, characterized by persistent or recurrent infections of the skin, nails and mucosal tissues by *Candida*; *C. albicans* in the majority of the cases [1]. Patients with CMC characteristically do not develop systemic disease and/or septicaemia. The disease is often more severe when presented early in infancy and in patients with disseminated lesions [2–4].

The diversity of clinical features among patients suggests that there are several different disorders still classified under a common clinical denominator. Most reports have shown that the defects are almost exclusively in the cellular branch of the immune system, mainly the specific responses to antigens of *Candida* species. Some CMC patients present serum factors that inhibit the proliferative responses of peripheral blood mononuclear cells (PBMC) from *Candida*-sensitized normal subjects [5–9], and a wide spectrum of immune dysfunctions has been observed. The importance of type-1 cytokines in an effective cellular immunity and the requirement of type-2 cytokines in the development of an adequate type-1 response have recently been demonstrated in murine models of candidiasis [10]. Moreover, other recent studies—aimed at clarifying the pathogenic mechanisms of the disease—showed deficient production and secretion of IL-2 by PBMC in response to *Candida* antigen [11,12].

Therefore, due to the several controversial aspects of CMC, our study proposed to evaluate the proliferative responses and the cytokine synthesis of PBMC after mitogen and antigen stimulation. Furthermore, we also evaluated the lymphocyte apoptosis after non-specific and specific stimulation, and also the natural killer (NK) activity of mononuclear cells

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PATIENTS AND METHODS

We studied eight CMC patients (four male and four female) between 3 and 38 years of age, fulfilling clinical criteria for persistent and refractory candidiasis of skin, nails and mucosal tissues. There were six patients with childhood onset, who presented the most severe clinical features, and two female patients with adult onset of the disease. All patients presented oral thrush and nail lesions; five had skin involvement (nos 3, 5, 6, 7 and 8); four oesophageal lesions (nos 1, 2, 3 and 8) and three vulvovaginitis (nos 1, 2 and 3). One patient presented sinusitis by *Microsporum canis* (no. 8). All patients were submitted to a routine investigation (Table 1). The control group consisted of eight age- and sex-matched healthy individuals. The Ethical Committee of the Hospital das Clínicas da Universidade de São Paulo granted ethical approval, and informed consent of patients or parents was obtained in all cases.

Methods

Sample collection. Fasting blood samples were collected in the morning, from clinically stable patients, without associated infections and without systemic anti-mycotic therapy.

Cell separation and cell cultures. PBMC were cultured in RPMI 1640 medium (Sigma, St Louis, MO, USA) with 10% of human AB + serum (Sigma), and stimulated either by *Candida* metabolic antigen (CMA; Diagnostics Pasteur, Marnes-la-coquette, France, code 52952) (5 μg/ml) or *Mycobacterium bovis* PPD (Staten Serum Institut, Copenhagen, Denmark) (5 μg/ml) for 6 days, or by phytohaemagglutinin (PHA; 2·5 μg/ml; GIBCO BRL, Rockville, MD, USA) or pokeweed mitogen (PWM; 5 μg/ml; Sigma) for 3 and 6 days, respectively. Eighteen hours before harvesting, cells were pulsed with 1 μCi of ^{3}H-TdR, then harvested and counted in a Beta Plate scintillation counter (Wallac OY, Turku, Finland). The results are presented as stimulation index (SI). Lymphocytes from patients and controls were tested for immunomodulatory effects of patients' sera by comparing lymphocyte responses in normal AB serum and sera from four CMC patients.

Cytokine quantification. Quantification of cytokines in the supernatant was obtained by stimulation of 2×10^6 cells/ml in RPMI/10% AB serum in 24-well plates with PHA 2·5 μg/ml, *Candida* antigen 5 μg/ml, and the appropriate controls. PBMC from patients and controls were further tested in the presence of patients' serum to evaluate a possible serum-dependent modulatory effect. The supernatant samples were harvested 24 h after PHA stimulation, and 72 h after *Candida* stimulation, according to preliminary experiments in which harvesting time was determined (data not shown). The supernatants were stored in aliquots at −70°C until quantification of cytokines. Cytokine quantification was performed by a capture ELISA method using kits from Genzyme Diagnostics (Cambridge, MA, USA) (IL-2) and Endogen Inc (Woburn, MA, USA) (IL-4, IL-10 and interferon-gamma (IFN-γ)), following the manufacturer's instructions.

NK activity assay. NK activity was evaluated by a micromethod, as previously described [13], with some modifications. In brief, K-562 target cells were incubated with 100 μCi Na[^{51}Cr]O_4 (CNEN, São Paulo, SP, Brazil). The effector cells (PBMC) were maintained in RPMI/10% fetal calf serum (FCS) and adjusted to 4×10^6, 2×10^6, 1×10^6, and 5×10^5 cells/ml. PBMC from four patients and four controls were also tested with patients' serum in order to evaluate a possible serum-dependent suppressive effect on the cytotoxic activity. One hundred microlitres of each sample suspension were plated in triplicates in 96-well round-bottomed microplates; 10^4 previously cromated target cells were added to each well, in order to get 40:1, 20:1, 10:1 and 5:1 effector/target cell ratios, along with the adequate controls. After 4 h, the plates were gently spun and the supernatant collected for radioactivity counting by a gamma counter (Wallac). The lytic activity was calculated and expressed as the percentage of lysed cells for each effector/target cell ratio.

The apoptosis assay. To study the influence of cellular activation on peripheral blood lymphocyte apoptosis, 1×10^6 cells were cultured in RPMI/10% AB + serum in 24-well plates with either 5 μg/ml *Candida* antigen or 5 μg/ml PPD, or 2·5 μg/ml PHA. Four patient and control samples were also tested with patients' serum in order to evaluate a possible modulatory effect on apoptosis. PBMC from controls and patients were evaluated for spontaneous apoptosis immediately after blood collection and 72 h after antigen or mitogen stimulation. The cells were isolated and 2·5 μg/ml of anti-CD95 antibody (PharMingen, San Diego, CA, USA) were added to 2×10^6 cells/ml. As negative control cells were incubated with culture medium (without anti-CD95) for the same 4 h at room temperature. Cells were then washed twice with cold PBS and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7·4, 140 mM NaCl and 2·5 mM $CaCl_2$) at 10^6 cells/ml. An aliquot of 100 μl of cell suspension was transferred to a 5-ml cytometer tube, and 5 μl of Annexin V–FITC (PharMingen) and 10 μl of propidium iodide (PI; Sigma) at 50 μg/ml were added to each tube. Cells were then gently vortexed and incubated in the dark for 15 min at room temperature, followed by the addition of 400 μl of binding buffer for analysis in the flow cytometer. The quantification of apoptotic cells was evaluated by Annexin V–FITC binding [14]. The final results were expressed as the percentage of apoptotic cells in the lymphocyte gate (identified by Annexin V–FITC staining and exclusion of PI) per 10 000 counted cells.

Statistical analysis of the data was done by Mann–Whitney test, when the same parameter was compared for patients and controls. We considered significant values $\leq 0{\cdot}05$.

RESULTS

The proliferative lymphocyte responses were analysed after stimulation with PHA, PWM, *Candida* and PPD antigens (Fig. 1). The majority of the patients (5/8; 62·5%) showed a low proliferative response to *Candida* antigens (Fig. 1c), but the proliferative response to PPD was also below the lower normal limit (SI $< 4{\cdot}17$) in four of seven patients tested (Fig. 1d). These proportions were significantly different in relation to the control group: *Candida* ($P = 0{\cdot}0207$) and PPD ($P = 0{\cdot}0401$). The response to PWM was more heterogeneous, as three of them presented low SIs ($< 8{\cdot}42$) (Fig. 1b). Nevertheless, all patients presented normal proliferative responses to PHA ($> 18{\cdot}28$), despite a trend to lower median SI values ($39{\cdot}78 \times 99{\cdot}94$ for patients and controls, respectively) (Fig. 1a).

The patients' PBMC showed lower NK activity than those of the controls (Fig. 2). Since NK activity was low, as were T cell responses, it was decided to investigate whether cytokines, known to activate both subsets, were involved in these immunological disturbances.

The analysis of IL-2 production by PBMC of the patients (Fig. 3a) revealed significantly lower levels than those found for the control group; i.e. 4/8 patients and only 1/8 control secreted

Table 1. Clinical and general laboratory features of the patients

Patient no., age/sex	First symptoms	Age at diagnosis	PPD DTH	Tp DTH	Ca DTH	SK/SD DTH	IgA	IgE	IgG	IgM	AutoAb	C′3	C′4	Leucoc	Eos	Lymph	$CD4^+$	$CD8^+$
1 38/F	33 years	36 years	Neg	10	8	25	185	27	1200	159	None	95	21	5800	87	1856	945	393
2 35/F	31 years	33 years	8	Neg	Neg	15	257	34	1635	196	None	96	16	8300	232	2822	1343	807
3 25/F	2 years	19 years	Neg	Neg	Neg	Neg	251	48	2985	162	dsDNA 1/1280	97	19	12 900	258	1574	334	283
4 3/F	18 months	20 months	NP	NP	NP	NP	125	15	1659	205	None	NP	NP	14 900	241	4879	1678	732
5 6/M	7 months	1 year	Neg	Neg	14	NP	289	25	1870	172	None	NP	NP	NA	NA	2500	975	700
6 38/M	2 years	23 years	Neg	10	Neg	Neg	402	31	1978	87	None	100	40	8900	9	2027	446	487
7 7/M	2 months	18 months	Neg	Neg	Neg	Neg	184	17	1912	115	TPO-92.9 TG-834	90	30	10 900	218	2986	1075	478
8 17/M	2 years	15 years	Neg	Neg	Neg	Neg	400	27	3048	165	None	NP	NP	7500	0	2810	1121	556

NP, Not performed; NA, not available.
Tp, Trichophytin; Ca, candidin; SK/SD, varidase.

low levels of IL-2, when stimulated by *Candida* antigen ($P = 0{\cdot}0281$). There was no statistical difference upon PHA stimulus. IFN-γ levels (Fig. 3b) also showed a highly significant difference among patients and controls when stimulated by *Candida* antigen; i.e. 5/8 patients and none of the controls produced low levels of IFN-γ ($P = 0{\cdot}0047$). Conversely, data from IL-4 and IL-10 failed to show any statistical difference among the groups, under any conditions (Fig. 3c,d).

The analysis of patient and control lymphocyte apoptosis (Fig. 4) under different stimulation conditions showed that the behaviour of peripheral blood lymphocytes was similar in both situations: in basal conditions, stimulated by suboptimal doses of anti-CD95 antibody (Fig. 4a) and when stimulated by PHA, enhanced or not by anti-CD95 antibody (Fig. 4b). However, when cells were stimulated by *Candida* antigen, there was a trend to higher levels of apoptosis in the patient group (5·75 *versus* 9·10), that achieved a statistically significant difference when the stimulation was enhanced by anti-CD95 antibody (8·85 *versus* 19·4) ($P = 0{\cdot}007$) (Fig. 4c). The same trend of activation-induced cell death was observed upon PPD stimulation (3·10 *versus* 7·60), which was also significant when enhanced by anti-CD95 (5·0 *versus* 16·0) ($P = 0{\cdot}0205$) (Fig. 4d).

We performed another series of experiments comparing serum from four patients with AB serum; only patient no. 6 serum modulated several functions of the immune response, such as the proliferation of patient and control PBMC under all stimuli

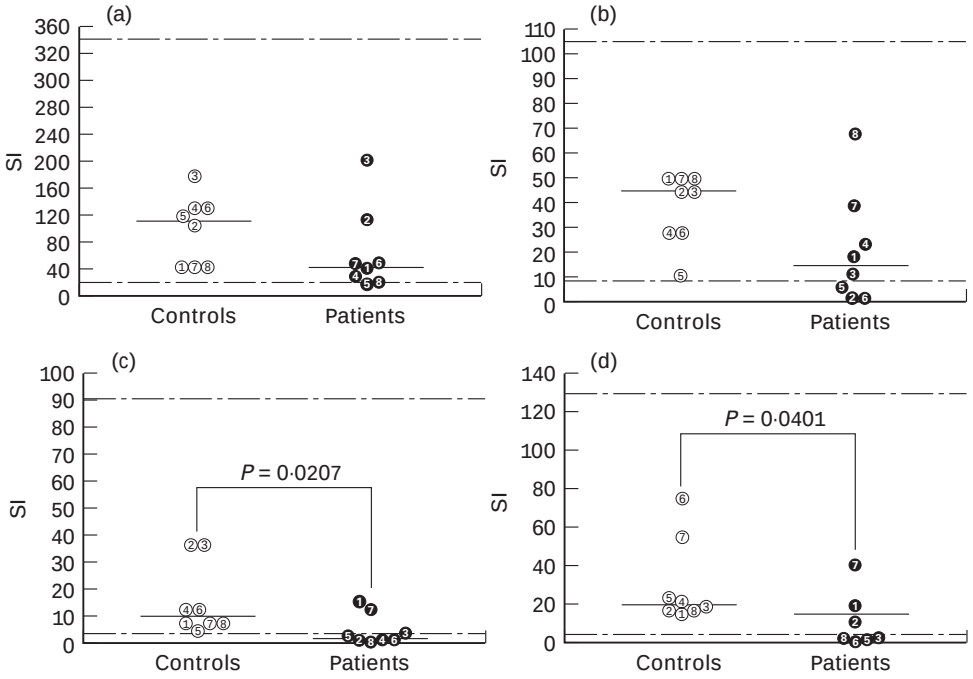

Fig. 1. Proliferative response of mononuclear cells from patients and controls under different stimuli. (a) Phytohaemagglutinin (PHA). (b) Pokeweed mitogen (PWM). (c) *Candida* antigen (CMA). (d) *Mycobacterium tuberculosis* PPD. Data expressed as levels obtained for each individual and median of stimulation index (SI). The respective normal value limits (percentile 5 and 95) established by the analysis of a normal population studied at LIM/56 are: PHA = 18·28–343·00; PWM = 8·42–107·40; CMA = 3·35–91·40; PPD = 4·17–129·20.

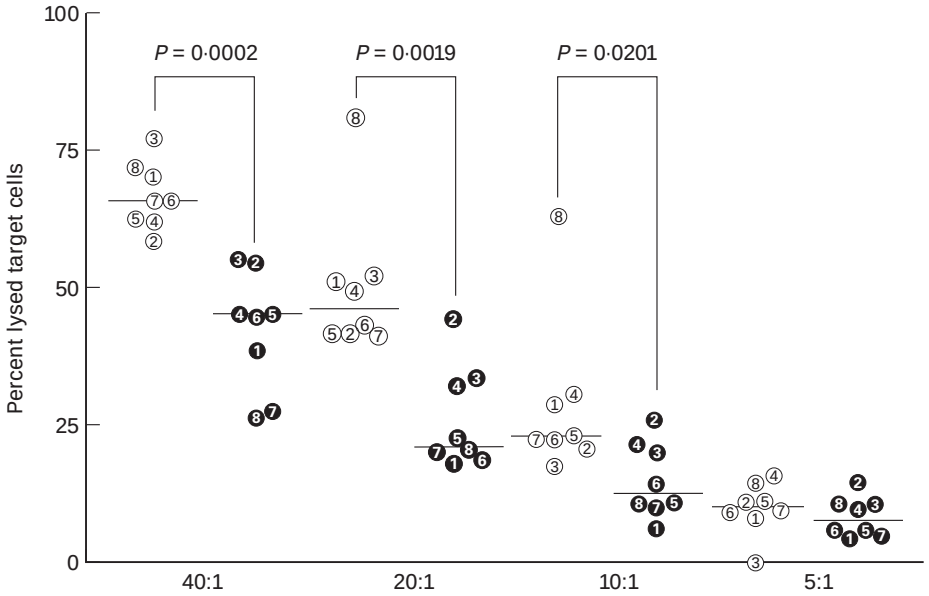

Fig. 2. Natural killer activity of peripheral blood mononuclear cells from chronic mucocutaneous candidiasis (CMC) patients (filled symbols) and healthy controls (open symbols). Results presented as the percentage of lysed target cells at different effector:target (E:T) cell ratios for individual patients and median group values.

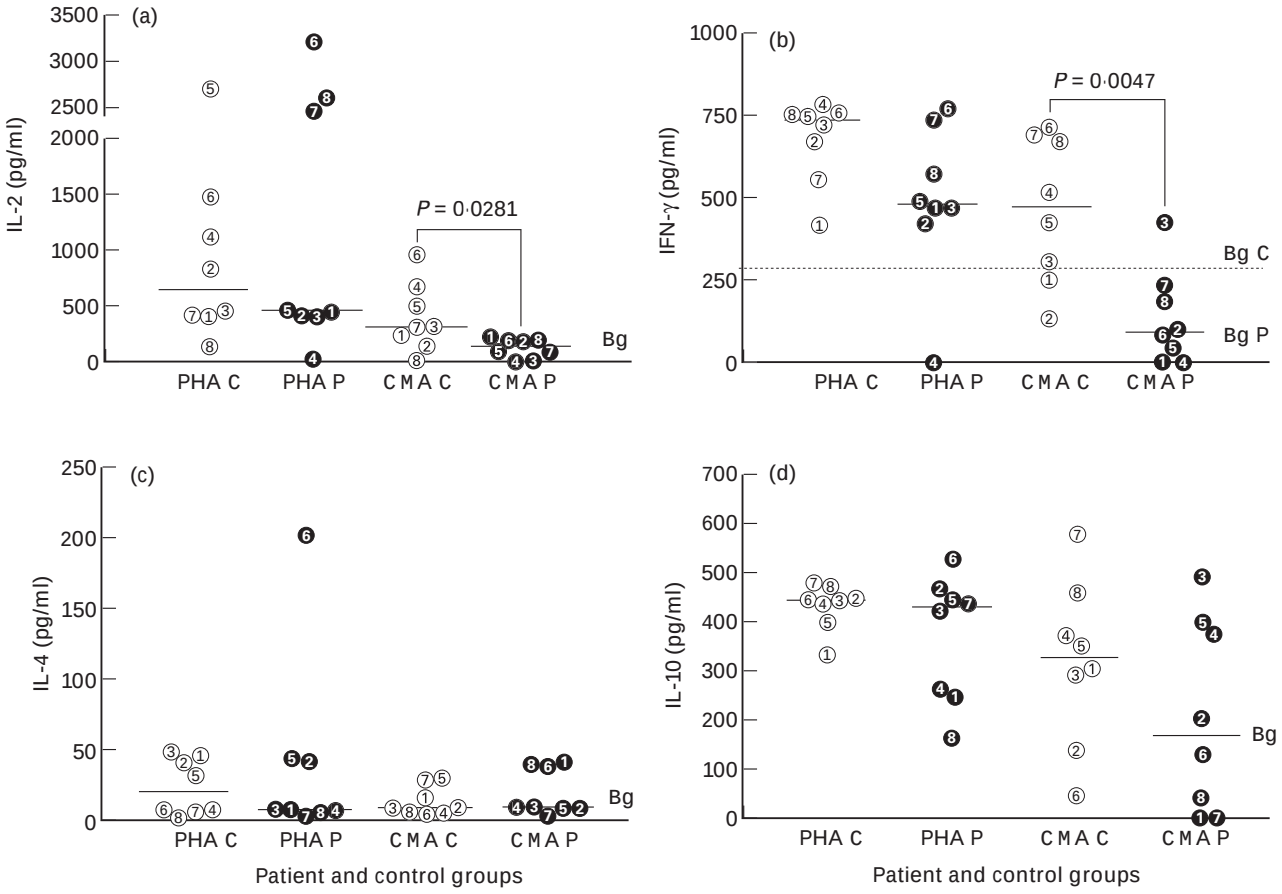

Fig. 3. Production of IL-2 and IFN-γ in response to stimulation with phytohaemagglutinin (PHA) and *Candida* antigen (CMA). Peripheral blood mononuclear cells from patients with chronic mucocutaneous candidiasis (P) and controls (C) were stimulated in culture and supernatants assessed for IL-2 (a), IFN-γ (b), IL-4 (c), and IL-10 (d). Results are presented as levels obtained for each individual and median group values. Bg, Background (unstimulated cytokine production).

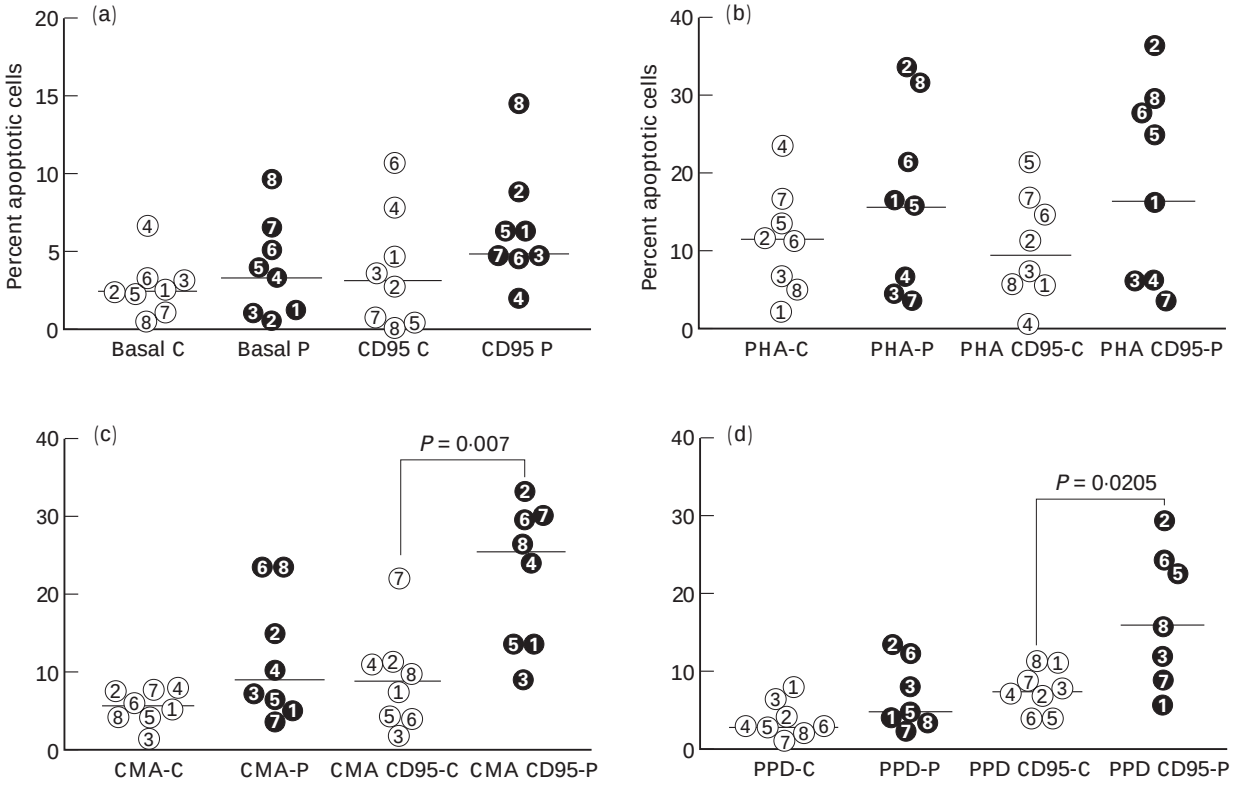

Fig. 4. Quantification of lymphocyte apoptosis from patients and controls in response to different stimulation conditions: (a) basal; (b) phytohaemagglutinin (PHA); (c) *Candida* antigen (CMA); (d) PPD. Results are presented as apoptosis levels obtained for each individual and group median of percent values.

(Fig. 5a,b). Patient no. 6 serum suppressed both his NK activity and the control subject's (Fig. 5c). The analysis of cytokine synthesis modulation showed (i) a suppression of IL-2 secretion under PHA and *Candida* stimuli for control and patient (Fig. 5d), (ii) an enhancement of IL-4 (Fig. 5f) under *Candida* stimuli for control and patient, and (iii) a suppression of IFN-γ (Fig. 5e) under PHA for control of patient no. 6. There was no influence of patients' sera over IL-10 secretion (Fig. 5g). Modulation of apoptosis occurred only for patient no. 6 under CD95 (Fig. 5h), CMA and CMA-CD95 stimuli (Fig. 5i), in which the patient's serum enhanced the activation-induced cell death.

DISCUSSION

The inconclusive reports of cellular immune response of CMC patients reflect the clinical and immunological variability of these patients. Some reports describe the heterogeneity of the immunological disturbances, suggesting the existence of several variants (or several defects) giving rise to the various clinical manifestations [1].

Due to the dominant role of the cellular compartment of the immune system in the defence against fungi, several parameters of the immune response of patients with CMC were evaluated. These were mainly the T cell response to *Candida* antigens, as well as to mitogens, and the NK activity that showed variable disturbances. Furthermore, cytokine secretion and apoptotic activation-induced cell death were also investigated.

Despite the depressed proliferative response from PBMC cultured in medium with AB serum to metabolic products of the yeast present in skin and mucous membranes, some patients presented a wider disturbance of the specific immune response, failing to recognize PPD antigen and even PWM, as observed previously [1,7,15]. However, it is important to stress that the serum of one out of four patients clearly modulated immune reactivity, suppressing the proliferation of his own PBMC and of the control subject.

The NK activity against some fungal infections is important [16–20] and therefore it was evaluated. These reports demonstrated that direct activity, as well as NK-derived IFN-γ, are important in the natural immunity against fungi. The indirect effect—modulated by cytokines—is mainly secondary to nitric oxide (NO) production by macrophages. Indeed, half of the patients showed depressed NK activity, suggesting that some of them could present multiple immunological defects. Moreover, serum from patient no. 6 suppressed the immune activity of NK cells. However, none of the patients has so far presented clinical manifestations that are frequently associated with decreased NK activity, such as neoplasia, herpetic or Cryptococcal infections.

In addition, disturbances in the amplification and effector mechanisms of the immune response, or an inadequate Th2 responsiveness pattern could lead to suppression of cell-mediated processes, maintaining predominantly humoral responses. Therefore, the absence of an efficient cellular immune response in patients with persistent infectious processes by yeasts of the genus *Candida* might be due to defects in the generation or maintenance of cellular memory response, and this defect could be linked to disturbances in the production of cytokines or antigen-induced apoptotic mechanisms. Cytokine production

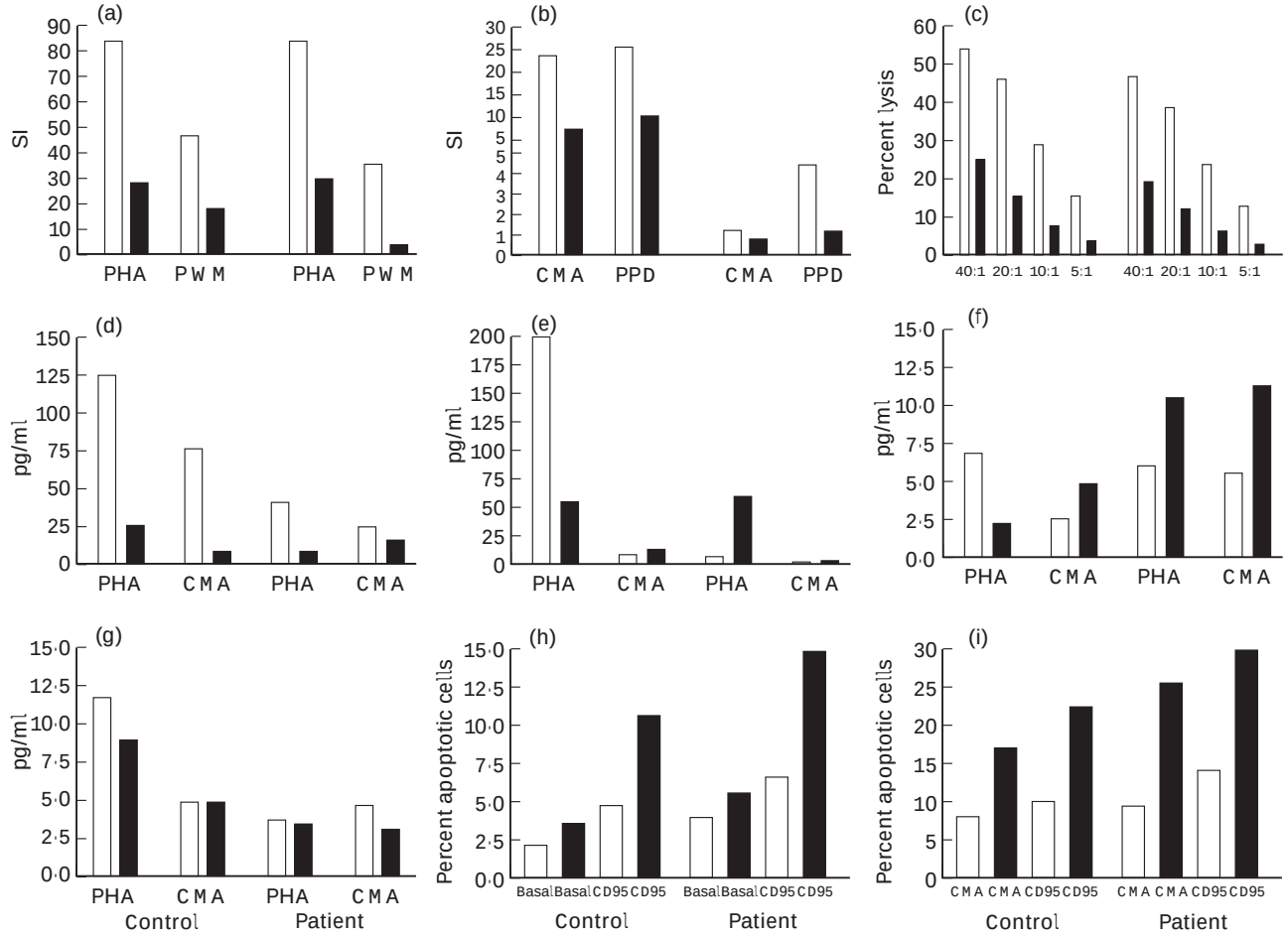

Fig. 5. Modulation of control and patient immune functions by chronic mucocutaneous candidiasis (CMC) patients' sera. (a) Phytohaemagglutinin (PHA) and pokeweed mitogen (PWM)-induced lymphoproliferation. (b) *Candida* antigen (CMA) and PPD-induced proliferation. (c) Natural killer (NK) cytotoxic activity at different effector:target ratios. (d) IL-2 secretion induced by PHA and CMA. (e) IFN-γ secretion. (f) IL-4 secretion. (g) IL-10 secretion. (h) Basal and CD95-enhanced lymphocyte apoptosis. (i) CMA and CMA-CD95-induced lymphocyte apoptosis. SI, Stimulation index. □, AB serum (fetal calf serum for NK activity assay); ■, serum from patient no. 6.

induced by non-specific stimuli, such as PHA, was preserved as previously reported [15,21].

Conversely, we observed a significant decrease in the production of Th1 cytokines, without concomitant Th2 enhancement, especially after stimulation with *C. albicans* antigen. Contrary to other reports [11,12], none of our patients presented a typical Th2 pattern with high levels of IgE and eosinophilia. It should be noted that serum from patient no. 6 suppressed both mitogen- and antigen-induced lymphocyte proliferation and the production of Th1-type but not Th2-type cytokines. This serum also enhanced stimulation-induced apoptosis and decreased NK function. It is important to stress that the cytokine disturbance did not relate to the proliferative response, nor to the clinical features of the patients. The fact that some patients secreted adequate concentrations of the Th1 cytokines can be a consequence of the heterogeneity of the disease or, on the other hand, of the clinical status at the time of evaluation. Considering that these defects are not consistently found in CMC patients, they are unlikely to be the only or the main underlying cause of susceptibility to persistent *Candida* infections.

In a wider context, production of cytokines in CMC may be compared with the fungal disease paracoccidioidomycosis. Our own data on this mycotic disease suggest several points of convergence. In the chronic form of the latter, there are typical mucosal lesions, mainly in the upper respiratory tract. Therefore, the fungal antigens chronically stimulate these patients; yet a decrease in the proliferative response to some *Paracoccidioides brasiliensis* products—but not to mitogens or to other non-related antigens, e.g. *C. albicans* antigens—was observed [22,23]. A decreased production of Th1 cytokines (IL-2 and IFN-γ) and preservation of IL-10 synthesis was also observed (G. Benard, personal communication). On the other hand, patients presenting recurrent vulvovaginal candidiasis also showed a decrease in the proliferative response and in IFN-γ synthesis when stimulated by the yeast, and this was more pronounced in the follicular phase [24]. We can conclude that in these chronic mycoses the predominance of a non-protective response might occur, with high titres of antibodies.

It is also possible that apoptosis could favour the selective depletion of the T lymphocytes responsible for the immunological memory to *Candida* sp., explaining the defective immune response among CMC patients. Our findings support this possibility, as patients' antigen-specific stimulated lymphocytes expressed activation-induced cell death at significantly higher levels than the controls, when enhanced by anti-CD95 MoAb in suboptimal doses. It is important to stress that CD95 expression is

higher in memory cells ($CD45RO^+$) [25], influencing memory cell depletion [26]. Our observation of higher levels of apoptotic cell death among patients suggests that some disturbance of cell signalling could be leading to a dissociation of the pathways for the expression of CD95 and its ligand [27,28] and cytokine synthesis [29,30], favouring apoptosis instead of proliferation. In contrast, it is also possible that activation-induced cell death could be due to repeated stimulation by widely disseminated antigens, presented by antigen-presenting cells [31]. A horizontal analysis of the data showed that antigen-specific activation-induced cell death inversely correlated with antigen-specific proliferation and IFN-γ secretion. This finding corroborates the idea that apoptosis is an important phenomenon in the pathogenesis of CMC, at least in our patients. Moreover, serum from patient no. 6 enhanced apoptosis by his own and control lymphocytes. The enhanced antigen-induced apoptosis described here opens a new approach to the understanding of more specific immunodeficiency diseases and chronic relapsing infections.

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