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Chronic mucocutaneous candidiasis. I. Altered antigen-stimulated IL-2, IL-4, IL-6 and interferon-gamma (IFN-γ) production

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SUMMARY

Patients with chronic mucocutaneous candidiasis (CMC) present with persistent infections with the opportunistic yeast *Candida*. Impaired cell-mediated responses to *Candida* have been documented in CMC patients, but the defect remains poorly understood. The importance of Th1 cytokines in resistance and Th2 in susceptibility to *Candida* infections has recently been demonstrated in murine models. In our studies we evaluated production of IL-2 and IFN-γ (markers of Th1 type responses) as well as IL-4 and IL-6 (Th2 type markers) following stimulation with two kinds of *Candida* antigens (CAgs), polysaccharide antigens, tetanus toxoid and pokeweed mitogen. Our results demonstrate that CMC patients have impaired cytokine production upon *in vitro* stimulation with CAgs resulting in low or absent IL-2, increased IL-6 and either absent or increased IFN-γ production. Cytokine production following stimulation by other antigens was unaltered. The overall cytokine-producing capacity assessed through mitogen stimulation was also intact. Addition of IFN-α or IFN-γ to culture in an attempt to modify cytokine production did not have significant effects. Levels of soluble IL-6 receptors were not increased and could not account for increased IL-6 production. Our studies support the hypothesis that *Candida* antigens trigger a predominantly Th2 instead of a Th1 cytokine response in patients with CMC.

Keywords chronic mucocutaneous candidiasis cytokines IL-2 IL-4 IL-6 interferon-gamma soluble IL-6 receptors

INTRODUCTION

Chronic mucocutaneous candidiasis (CMC) is a primary immunodeficiency disease presenting with debilitating, persistent and refractory infections with the opportunistic yeast *Candida albicans* [1]. Infections vary from mild to lethal, are characteristically localized to the skin, mucous membranes and/or nails, and as a rule do not progress to disseminated disease or sepsis [2]. They are often associated with thymoma, polyendocrinopathies and other phenomena thought to be autoimmune in nature. Onset is frequently in childhood but can be in adult life; the disease may be sporadic or show familial association [1]. The underlying immune defect is poorly understood [3], although it has been appreciated that mucocutaneous infections with *Candida* other than in CMC frequently accompany impairment of T cell-mediated immunity (HIV, Di George syndrome, etc.) [2]. Studies performed over a decade ago suggested that the crucial defect is selective impairment of cell-mediated immunity, resulting in the inability to clear *Candida* infection [4]. It soon became clear that immune defects in CMC differed, the most consistent finding being defective cell-mediated responses to *Candida* antigen (skin test reactivity, proliferative response, migration inhibitory factor (MIF) production) and frequently to other antigens (diphtheria toxoid, streptokinase-streptodornase, dinitrochlorbenzene (DNCB)) [1,3,4]. About a third of CMC patients were shown to have no detectable immune impairments. Other defects (serum inhibitory factors, T suppressor cells, etc.) have been sporadically described (>10% CMC patients) [3]. Some reports suggested that *Candida* itself (i.e. the mannan constituent of its cell wall) had an immunosuppressive effect leading to impaired immunity in CMC patients [5]. Several recent studies have drawn attention to the fact that patients with CMC are also susceptible to other, non-*Candida* infections, especially those caused by encapsulated bacteria where immune responses are directed toward bacterial polysaccharide (PS) cell wall components [6]. This may relate to findings demonstrating IgG2 and IgG4 subclass deficiency in some patients with CMC [7], bearing in mind that humoral responses to PS antigens are predominantly of the IgG2 isotype. These findings suggest that the immune defects seen in CMC are perhaps not as selective or subtle as previously supposed. Data obtained on animal (murine) models have shown that resistance to systemic and mucosal

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candidiasis is closely associated with a Th1 type of response characterized by increased production of IFN-γ, IL-2, and enhanced DTH, while susceptibility is accompanied by a Th2 type response with increased IL-4 and IL-6 production [8]. Administration of anti-IL-4 antibody in this model resulted in increased survival [9]. It has also been demonstrated that other cytokines, which are not produced by Th cells, can influence the balance of cytokines produced, as is the case with IFN-α, which stimulates Th1 responses—probably by enhancing IFN-γ production [10]. The earlier concept of T cell independency of B cell responses, as postulated for T-independent type two antigens (TI-2, i.e. PS antigens), is also under question, as recent data demonstrated that responses to TI-2 antigens are strongly dependent on T cell regulatory factors (cytokines) and can be significantly modified by them [11].

Taken together, these data suggest that the manifestations seen in CMC, i.e impairment of cell-mediated immunity to *Candida* and susceptibility to certain types of bacterial infections, could be the consequence of imbalanced cytokine production and regulation. The disease could, thus, result from impaired Th1 cell function and defective cytokine production by this subset with decreased DTH responses, accompanied by normal or perhaps enhanced Th2 cell function with adequate or increased cytokine production and antibody responses. Given the dependency of TI-2 antigen responses on T cell cytokines, we postulated that altered cytokine production could also underly the increased susceptibility of CMC patients to infections with encapsulated bacteria.

In this and an accompanying study [12] we present results of our investigations addressing the question of cytokine production and antibody responses to antigens found on microorganisms poorly handled by CMC patients (*Candida* and PS antigens) and those handled well (protein antigens). We also assess various factors known to be involved in regulating levels of cytokine production: effects of *in vitro* addition of IFN-α and IFN-γ on cytokine production, as both agents have been shown to exert profound effects on the types of cytokines produced, shifting the balance in favour of Th1 type cytokines [10,13]; soluble IL-6 (sIL-6R) receptors were measured, as they have been shown to up-regulate IL-6 production by associating with secreted IL-6, thus promoting binding to cell membrane receptors and stimulating further IL-6 secretion [14].

Our studies demonstrate that CMC patients do indeed have altered patterns of cytokine production, primarily in response to *Candida* antigen, which seems unable to trigger adequate production of 'appropriate' (Th1) cytokines, but instead triggers increased production of 'inappropriate' (Th2) cytokines.

PATIENTS AND METHODS

Patients

Ten patients and 20 age- and sex-matched healthy controls were studied. All patients fulfilled clinical criteria of having childhood onset, persistent, refractory candidiasis of skin, mucous membranes and/or nail, varying in severity from mild to lethal (one patient died). Most patients were not on therapy or were receiving only local anti-fungal treatment at the time of assessment, while in patients requiring systemic treatment, blood samples were taken before initiating therapy. In all but one patient, who had diabetes mellitus and was on insulin therapy, endocrinopathy was excluded as judged by absence of clinical manifestations and laboratory evidence of autoimmune endocrinopathy. One patient was receiving intravenous immunoglobulin (IVIG) substitution therapy for recurrent lower respiratory infections associated with IgG2 subclass deficiency. The patient group consisted of four adults (median age 26 years, range 19–45 years) and six children (median age 9 years, range 3–13 years). One adult and two children had familial occurrence of CMC (mother, son and daughter). The control group comprised 10 heathy adults (median age 32 years, range 20–44 years), volunteer laboratory personnel and 10 children (median age 9 years, range 2–15 years) undergoing general anaesthesia for minor surgery for non-infectious causes (e.g. ingrown toenail, epispadia, oesophageal stricture, etc.) and were otherwise healthy by clinical and routine laboratory criteria. All subjects were tested at one time point. Ethical approval was granted by the Newcastle Ethical Committee and informed consent of patients or parents was obtained in all cases. The patient group was designated CMC (all patients), CMCa (only adults with CMC), CMCc (only children with CMC), Ca (adult controls), Cc (children controls) and CaCc (all controls, both adults and children).

Cell and plasma preparation

Heparinized, peripheral blood (20–30 ml) was centrifuged, plasma collected and stored as aliquots at -40°C until use. All cultures were performed on peripheral blood mononuclear cells (PBMC) separated on a density gradient (Histopaque; Sigma, Poole, UK) and adjusted to 1×10^6/ml in tissue culture medium (TCM) (RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS; Hyclone Europe Ltd), L-glutamine (GIBCO, Paisley, UK) and penicillin/streptomycin).

Antigens and mitogen

Two types of *Candida* antigens (CAgs) were used obtained from PHLS laboratory isolates of *Candida albicans* cultured and propagated in yeast broth (kind gifts from Dr L. Morgan, Regional Immunology Department, Newcastle General Hospital): a crude preparation of sonicated, heat-inactivated, whole organisms of *C. albicans* (CS), protein content 0·24 mg/ml, and a dialysed NH_2SO_4 protein precipitate (ppt) of the sonicate (CP), protein content 0·02 mg/ml, both in dilutions of 1:10 000. The rationale for using these two types of CAgs was to compare putative differences in responses to the whole organism consisting of both protein and polysaccharide antigens (which CMC patients do not clear *in vivo*) and a ppt of the same organism, anticipating that responses to the ppt might be present or more vigorous, as CMC patients have been shown to respond well to protein antigens; pneumococcal polysaccharide antigens (PPS) from 23 pneumococcal types, in the Pneumovax II Vaccine (Merck, Rathway, NJ), protein content 1·15 mg/ml, diluted 1:5000; tetanus toxoid antigen (TT) in the Tetanus Vaccine BP in simple solution (Wellcome, Beckenham, UK) diluted 1:2000; and pokeweed mitogen (PWM; GIBCO) diluted 1:1000.

Modulation with IFN-α and IFN-γ in vitro

IFN-α (IntronA; kind gift from Schering-Plough), and IFN-γ (Immukin; Boehringer-Ingleheim), 100 U/ml, were added at the beginning and were present for the whole period of antigen- and mitogen-stimulated cultures.

Cytokine production

In vitro production of IL-2, IL-6, IL-4, IFN-γ and soluble IL-6 receptor (sIL-6R) was assessed. Cultures of PBMC were set up as

described in plastic tubes (Falcon 2054; Becton Dickinson, Oxford, UK) and incubated with or without stimulating antigen/mitogen for 24 h in a volume of 1 ml in a humidified, 5% CO_2 atmosphere. Supernatants were collected and stored at –40°C until tested. Optimal concentrations for all antigens, mitogen, IFN-α, IFN-γ, incubation time for cytokine production and reproducibility of results were determined in preliminary titration experiments (data not shown).

Cytokine assays

Levels of IL-2, IL-6, IL-4, IFN-γ and sIL-6R in culture supernatants as well as plasma levels of IL-6 and sIL-6R were measured. IL-2 and IL-6 supernatant levels were determined in ^{3}H-thymidine (Amersham, Aylesbury, UK) uptake bioassays using CTLL2 and B9 cell line, respectively. IL-4 and IFN-γ supernatant levels, plasma IL-6 levels and confirmation of IL-6 bioassay levels in selected supernatants as well as sIL-6R levels in supernatants and plasma were determined by ELISA (AMS Biotechnology, Pharmingen and R&D, respectively), and performed as specified by the manufacturers. Levels of cytokines (pg/ml) were calculated from standard curves produced by recombinant IL-2 (Biogen, Geneva, Switzerland), IL-6 (ICI, Middlesbrough, UK), and IFN-γ (Boehringer-Ingleheim), calibrated to reference NIBSC regents (rhIL-2 86/564, rhIL-6 89/548, rhIFN-γ 87/586 and rhIL-4 88/656). Specificity of bioassays was confirmed by neutralization studies with anti-IL-2 MoAb (DMS-1 (Genzyme, Cambridge, MA), gift from J. H. Robinson (Department of Immunology, University of Newcastle) and polyclonal anti-IL-6 antibody (G150/BM 89/586; NIBSC, South Mimms, UK)).

Lymphocyte proliferation assays

PBMC, with or without stimulating antigens/mitogen, were set up in triplicate 0·2 ml TCM for 5 days in 96-well plates in a humidified 5%CO_2 atmosphere. Lymphocyte proliferation was estimated by ^{3}H-thymidine incorporation. Results are given as stimulation indices (ratio between stimulated and unstimulated cultures), an index >2 being counted as positive.

Autoanibody screen

Patient samples were screened in the Regional Immunology Department, Newcastle General Hospital, for rheumatoid factor, antinuclear, thyroid microsomal, mitochondrial, reticulin, smooth muscle, gastric parietal cell, adrenal and parathyroid antibody.

Lymphocyte markers

In all patients peripheral blood lymphocyte markers CD3, CD4 and CD8 were previously evaluated as part of a routine clinical work-up.

Statistical analysis

Results are presented as individual or median group values with s.e.m. Statistical significance for differences between two population medians was calculated with the non-parametric two-sample rank sum test (Mann–Whitney). Tests were performed on the statistical software program Minitab 9/w.

RESULTS

IL-2 production

Figure 1a demonstrates significantly decreased IL-2 production in CMC patients. Production of IL-2 in response to CP was absent or borderline (less that × 2 background) in 9/10 patients and fell below 2 s.e.m. from the median control group value, while one patient had normal IL-2 production (Table 1). However, several controls (4/20, all children) also failed to produce IL-2. Median values were highly significantly different between patients (5 ± 1 pg/ml) and controls (20 ± 3 pg/ml, cut-off level 5 pg/ml) at $P < 0{\cdot}005$ (Table 2). Interestingly, responses to the other CAg, CS, did not follow this pattern, in that most patients did produce IL-2 (24 ± 27 pg/ml, Table 1), although less than controls (45 ± 10 pg/m) (Table 2); however, all controls responded to CS, while some patients did not or produced only borderline levels (4/10) (Table 1). The level of IL-2 produced to CS was always much higher than to CP or other antigens in all groups; this was also seen in proliferation assays, although levels of proliferation did not always correlate with

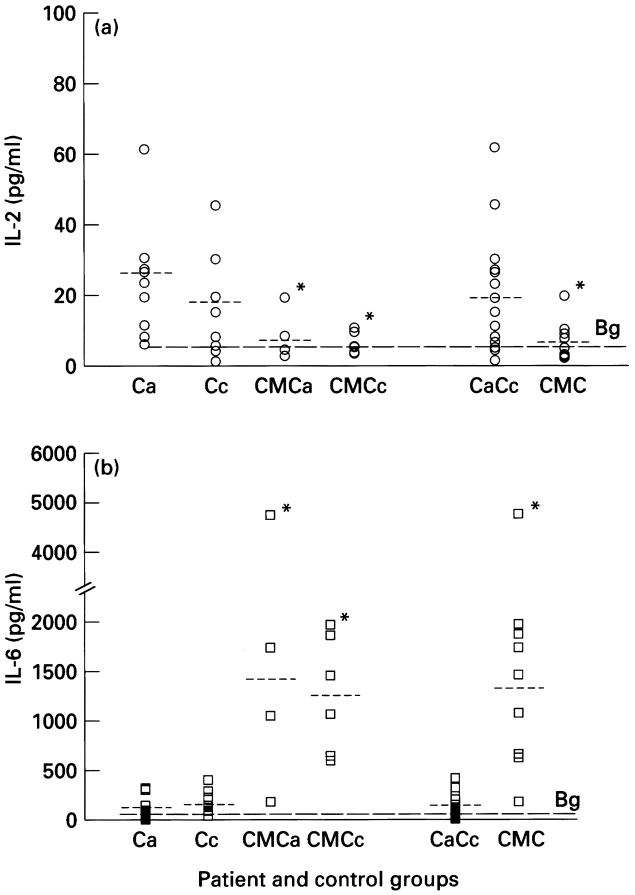

Fig. 1. Production of IL-2 and IL-6 in response to stimulation with *Candida* protein antigen (CP). Peripheral blood mononuclear cells (PBMC) from patients with chronic mucocutaneous candidiasis (CMC) and healthy controls (C) (adults (a) and children (c)) were stimulated in culture with CP and supernatants assessed for IL-2 (a) and IL-6 (b) levels. Results are presented as levels obtained for each individual and median group values. (a) Demonstrates that production of IL-2 in response to CP was absent or borderline in 9/10 patients. (b) Shows that CP stimulation resulted in very high levels of IL-6 production in 9/10 patients and in none of the 20 controls. One CMC patient demonstrated normal IL-2, while another demonstrated normal IL-6 production. (a) *CMCa:Ca $P = 0{\cdot}02$, CMCc:Cc $P = 0{\cdot}04$, CMC:CaCc $P = 0{\cdot}005$. (b) *CMCa:Ca $P = 0{\cdot}01$, CMCc:Cc $P = 0{\cdot}001$, CMC:CaCc $P > 0{\cdot}001$; Bg, Background, unstimulated cytokine production.

Table 1. Comparative levels of cytokine production and lymphocyte proliferation to *Candida* antigens in individual patients with chronic mucocutaneous candidiasis

	IL-2 (pg/ml) (Bg = 5)		IL-6 (pg/ml) (Bg = 71)		IFN-γ (pg/ml) (Bg = 10)		IL-4 (pg/ml) (Bg = 4)		Ly P (SI) (Bg = 2)	
Patient	CS	CP	CS	CP	CS	CP	CS	CP	CS	CP
1(a)	197	19	72	1066	10	10	5	7	14·2	1·1
2(a)	8	2	6000	4760	10	10	15	4	1·7	1·8
3(a)	23	8	1544	1749	65	80	3	4	2·6	1·0
4(a)	8	4	733	187	210	160	5	5	14·5	2·2
5(c)	29	9	1166	1086	10	14	1	1	5·5	1·0
6(c)	68	5	45	2000	45	45	4	4	7·0	3·3
7(c)	53	4	400	616	750	105	3	4	13·0	3·0
8(c)	4	4	548	666	10	30	10	5	4·0	2·0
9(c)	26	9	900	1883	370	120	1	1	10·0	2·0
10(c)	9	4	916	1466	380	100	1	1	1·6	0·5

CS, *Candida* sonicated antigen; CP, *Candida* protein precipitate antigen; Ly P, lymphocyte proliferation; SI, stimulation index; a, adult; c, child; Bg, background.

IL-2 production (Table 1). No major differences were seen between adults and children with CMC in either IL-2 production or lymphocyte proliferation. TT-stimulated IL-2 production was comparable in all groups tested, and levels produced were similar to those produced by controls in response to CP (Table 2). As expected, PPS did not stimulate IL-2 production (data not shown). PWM stimulated high levels of IL-2 in all patients as well as healthy controls (Table 2).

IL-6 production

Production of IL-6 differed markedly between CMC patients and controls. CS and CP stimulated production of very high levels of IL-6 of at least 2 s.e.m. above median control group values in 8/10 and in 9/10 patients, respectively; this high production was not seen in any of the 20 controls (Fig. 1b and Table 1). Table 2 gives median values of IL-6 production induced by different antigens: CS-stimulated IL-6 production in patients resulted in levels of 733 ± 209 pg/ml compared with 110 ± 22 pg/ml for controls ($P < 0{\cdot}007$), while values detected after CP stimulation were 1266 ± 403 pg/ml and 97 ± 25 pg/ml, respectively ($P > 0{\cdot}001$). Both children and adults with CMC produced high IL-6 levels. High levels measured in the B9 bioassay were confirmed in ELISA. Unstimulated background production of IL-6 in patients was low and did not differ significantly from control levels (71 ± 25 pg/ml and 34 ± 7 pg/ml, respectively). PPS stimulated moderate and comparable levels of IL-6 both in patients and controls, as did TT (Table 2). PWM-stimulated IL-6 production was high, but there were no significant differences between patient and control groups (Table 2). All groups were tested for plasma IL-6 concentration in ELISA and not one individual was found to have levels above background.

IFN-γ production

Levels of IFN-γ produced in response to CS and CP were similar in patients and controls, when median values for the two groups were compared (Table 3). However, Table 1 demonstrates that when stimulated with CS, 4/10 patients did not produce IFN-γ above background levels ('non-responders') compared with 20/20

Table 2. Culture supernatant levels of antigen- and mitogen-stimulated cytokine production in patients with chronic mucocutaneous candidiasis (CMC) and controls (C)

	IL-2 (pg/ml)					IL-6 (pg/ml)					
	CS	CP	TT	PWM	Bg	CS	CP	PPS	TT	PWM	Bg
Ca	74 ± 14	26 ± 5	24 ± 6	1522 ± 299	6 ± 2	69 ± 35	74 ± 36	36 ± 16	20 ± 14	3916 ± 674	17 ± 5
CMCa	15 ± 46	6 ± 4*	15 ± 5	1223 ± 216	6 ± 3	1139 ± 839*	1407 ± 993*	195 ± 168	87 ± 56	5055 ± 1804	81 ± 8
Cc	26 ± 12	19 ± 6	10 ± 2	371 ± 202	4 ± 2	165 ± 26	135 ± 37	99 ± 25	80 ± 15	1680 ± 550**	52 ± 1
CMCc	29 ± 31	5 ± 2*	15 ± 3	379 ± 67	4 ± 2	724 ± 167*	1266 ± 244*	187 ± 55	125 ± 38	3277 ± 1341	51 ± 1
CaCc	45 ± 10	20 ± 3	21 ± 4	1007 ± 206	5 ± 1	110 ± 22	97 ± 25	53 ± 16	47 ± 11	2333 ± 450	34 ± 7
CMC	24 ± 27	5 ± 1*	15 ± 2	590 ± 155	4 ± 2	733 ± 209*	1266 ± 403*	187 ± 66	106 ± 49	3916 ± 1028	71 ± 2

a, Adults; c, children. *, $P < 0{\cdot}05 - 0{\cdot}001$ compared with controls; **, $P < 0{\cdot}05$ compared with healthy adults. CS, *Candida* sonicated antigen; CP, *Candida* protein precipitate antigen; TT, tetanus toxoid antigen; PPS, pneumoccocal polysaccharide antigen; PWM, pokeweed mitogen; Bg, background.
Values are given as medians $\pm$ s.e.m.

Table 3. Culture supernatant levels of antigen- and mitogen-stimulated cytokine production in patients with chronic mucocutaneous candidiasis (CMC) and controls (C)

	IFN-γ (pg/mg)						IL-4 (pg/mg)	
	CS		CP					
	R + non-R	R	R + non-R	R	PWM	Bg	PWM	BG
C	40 ± 22	40 ± 22	45 ± 16	40 ± 29	3800 ± 649	15 ± 2	16 ± 4	3 ± 1
CMC	45 ± 72	290 ± 36*	34 ± 20	100 ± 17*	3800 ± 967	12 ± 1	21 ± 4	2 ± 2

* C:CMC $P < 0{\cdot}006$. CS, *Candida* sonicated antigen; PWM, pokeweed mitogen; Bg, background; R, responders above Bg. Values are given as medians $\pm$ s.e.m.

controls. Of the patients that did produce IFN-γ (6/10; 'responders'), most produced much higher levels resulting in significantly higher median values compared with controls (290 ± 107 pg/ml and 40 ± 22 pg/ml, respectively; $P > 0{\cdot}006$) (Table 3). A similar pattern was also seen with CP (100 $\pm$ 17 pg/ml and 40 ± 29 pg/ml, respectively), although in this case a substantial number of individuals in both groups did not respond (3/10 patients and 6/20 controls) (Table 1). PPS and TT induced low and similar levels of IFN-γ in all groups tested (not shown). Not all individuals (from both control and patient groups) produced IFN-γ when stimulated with PPS or TT. PWM induced very high levels of IFN-γ in all groups and in all individuals tested (both responders and non-responders to CS; Table 3). In CMC patients, no correlation could be seen between IFN-γ and IL-2 or IL-6 levels produced (Table 1).

IL-4 production

Production of this cytokine in response to stimulation with either CS or CP was absent or low in half of the adults in both groups and in all children (both patients and controls). Two CMC adults did, however, produce somewhat higher levels of IL-4 to CS stimulation (Table 1). PPS and TT stimulated only background levels of IL-4 production. However, PWM did stimulate IL-4 production in all individuals and showed similar median levels in CMC patients and controls (Table 3).

Modulation with IFN-α and IFN-γ

Although addition of IFN-α or IFN-γ altered cytokine production to a certain degree, sometimes even markedly, this was never statistically significant, nor were the effects consistent between individuals in the various groups. Depending on the stimulating antigen and the group of subjects tested, we observed both increases and decreases of the four cytokines assessed. Thus, both IFN-α and IFN-γ increased CP-stimulated IL-2 production in adult CMC patients, while only IFN-γ had this effect on CMC and healthy children (Fig. 2a); in all groups both IFN-α and IFN-γ increased PWM-stimulated IL-2 production, while TT-stimulated IL-2 production was either increased or unaltered (not shown). As opposed to these effects, IFN-α markedly decreased the very high CP-stimulated IL-6 production in CMC adults, but again had no effect on the other groups tested, while IFN-γ markedly decreased IL-6 production in both adults and children with CMC (Fig. 2b). IL-6 production following stimulation with other antigens and PWM was not significantly altered by addition of either interferon. This was also the case with IL-4 and IFN-γ production, which were

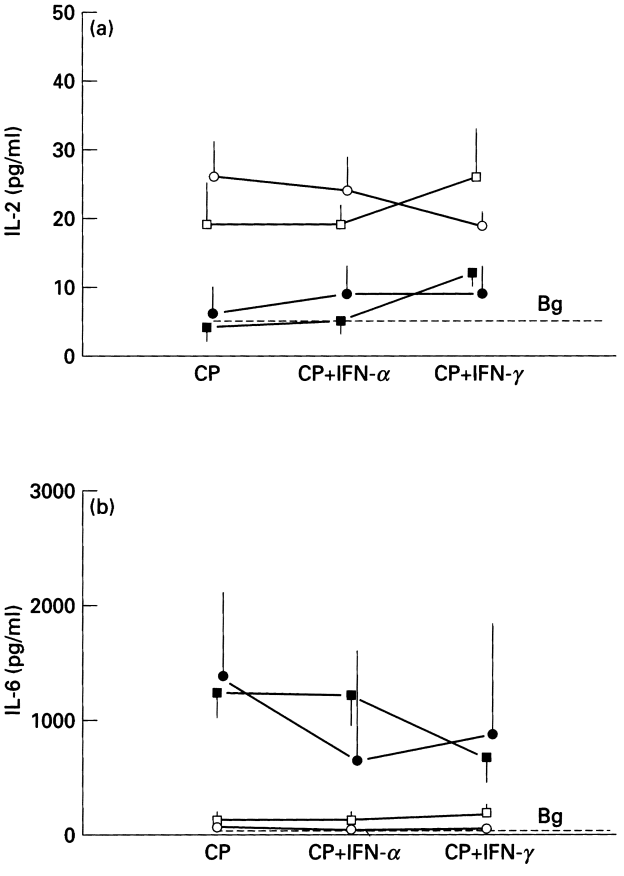

Fig. 2. Effects of IFN-α or IFN-γ on Candida protein antigen (CP)-induced IL-2 and IL-6 production. Peripheral blood mononuclear cells (PBMC) from patients with chronic mucocutaneous candidiasis (CMC) and healthy controls (C) (adults (a) and children (c)) were stimulated in culture with different antigens in the presence or absence of IFN-α or IFN-γ. Both IFN-α and IFN-γ increased CP-stimulated IL-2 production in adult CMC patients, while only IFN-γ had this effect on children, both CMC and healthy (a). As opposed to these effects, IFN-α markedly decreased the very high CP-stimulated IL-6 production in CMC adults, but again had no effect on the other groups tested, while IFN-γ markedly decreased IL-6 production in both adults and children with CMC (b). ○, Ca; □, Cc; ●, CMCa; ■, CMCc.

not significantly modified by addition of either IFN-α or IFN-γ. Taken together, these data suggest that addition of IFN-α or IFN-γ did modify cytokine production, albeit not to a significant degree, with a tendency to increase IL-2 and decrease IL-6 production, but had no effect on IL-4 or IFN-γ production.

sIL-6R levels

In vivo, all studied groups had substantial sIL-6R levels in plasma (Fig. 3). Although the highest values were seen in patients (both adults and children), there was no statistically significant difference between any of the groups. *In vitro*, levels of sIL-6R produced were low but detectable with all of the antigens or mitogen tested, and did not show any correlation with the high levels of IL-6 produced by CMC patients. Stimulation with CS induced highest levels of sIL-6R, although not significantly higher than levels induced by other antigens and mitogen (for CMC 83 $\pm$ 14 ng/ml, for CaCc 101 $\pm$ 12 ng/ml) with relatively high spontaneous production in all groups (background levels for CMC and CaCc were 46 $\pm$ 9 and 57 $\pm$ 6 ng/ml, respectively). No significant differences were observed between groups, although some children, mostly patients, produced higher levels than adults (data not shown).

Autoantibodies

All patients were negative for all antibodies tested, apart from one adult patient who was found to have a low titre (1:1600) of thyroid microsomal antibodies.

Lymphocyte markers

In all patients previous routine evaluation of lymphocyte markers demonstrated normal numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cells in peripheral blood (data not shown).

DISCUSSION

Our results demonstrated significant alterations in the patterns of cytokine production seen in patients with CMC, specifically in response to stimulation with *Candida* antigens, presenting as an impaired ability to produce IL-2, a vigorous production of IL-6, and an absent or increased production of IFN-γ. Cytokine production to other stimuli was not affected. The alterations were not correctable by either IFN-α or IFN-γ. Our data suggest that responses may in part depend on the type of CAg used to stimulate responses.

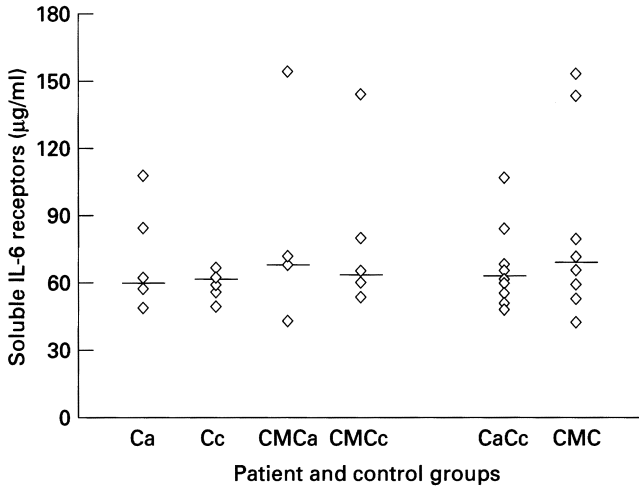

Fig. 3. Soluble IL-6 receptor plasma levels. Plasma was obtained from patients with chronic mucocutaneous candidiasis (CMC) and healthy controls (C) (adults (a) and children (c)) and evaluated for sIL-6R levels in ELISA. Results are presented as levels obtained for each individual and median group values. All groups had substantial and comparable sIL-6R plasma levels.

Decreased or absent IL-2 production in CMC patients was specifically seen in response to CAg, while IL-2 production to other antigens and mitogen was normal. A similar lack of responsiveness was noted in proliferation assays. However, there was often poor correlation between IL-2 production and proliferation, which could reflect the influence of other factors on proliferation (such as IL-4) or different incubation times before assay. CS, however, did stimulate IL-2 responses in some patients. Interestingly, the levels of IL-2 produced to CS in both patients and controls were in all cases much higher than those produced to other antigens, as were proliferation rates. The CAg preparations used in this study were crude preparations of either sonicated whole organisms or of *Candida* proteins with multiple antigenic components present. It would be interesting to determine whether the abnormalities of cytokine production observed in CMC patients reflect aberrant response to some, but not other (protein? polysaccharide?) antigens present [15] or to an immunodominant component of the yeast. Low or absent IL-2 production in response to CAg could largely explain impaired cell-mediated immunity previously reported in these patients [1,3]. It is unlikely that low IL-2 levels are the result of decreased $CD4^+$ cell numbers, as all patients had been previously shown to have normal lymphocyte and lymphocyte subset counts (CD3, CD4, CD8); normal levels of IL-2 produced to other antigens and mitogen in CMC patients would also argue against this possibility. High levels of IL-2 produced to CAgs and TT in controls in spite of a relatively short incubation period might be due to increased numbers of antigen-specific T cells present *in vivo* as a result of either constant stimulation with *Candida* organisms colonizing skin and mucous membranes of most healthy people, or as a result of previous immunizations to TT. Alternatively, high IL-2 production might be due to a 'bystander' effect, or may be secondary to macrophage stimulation by components of *Candida* organisms (lipopolysaccharide, mannan?) leading to release of lymphocyte stimulating factors. Our previous time-course studies of IL-2 production (data not shown) demonstrated highest levels after 24 h and 48 h incubation, followed by gradually declining levels, probably due to 'mopping-up' of the secreted IL-2 by activated cells expressing IL-2 receptors.

The most impressive finding was the very high level of IL-6 produced by nearly all CMC patients in response to either of the CAgs used. The finding was again specific for CAg stimulation. It was not the result of high spontaneous production, which might have been triggered by existing infection *in vivo*. The absence of a systemic *in vivo* reaction was also confirmed by negative IL-6 plasma levels in all patients. At the time of study patients were not receiving therapy that might have influenced our findings. The mechanism of this over-expressed IL-6 response remains elusive. Levels of sIL-6R were not increased in either plasma or culture supernatants and can not account for increased IL-6 production [14]. Further studies will also have to address the question of the cell type producing IL-6: although produced by Th2 lymphocytes, monocytes and other non-lymphoid cells are also potent producers

of this cytokine [16]. Consequently, IL-6 is not an exclusive marker of a Th2 response, although in our studies the specificity of the increased IL-6 response only to CAg would suggest T cell involvement. The fact that these alterations are seen specifically in response to *Candida* antigens might suggest defects in the processing and/or presentation of these antigens in CMC patients [17], which could also result in preferential stimulation of Th2 type lymphocytes to produce IL-6. Alternatively, increased IL-6 production by macrophages activated through binding of *Candida* to mannan receptors present on their surface [17] cannot be excluded. Increased IL-6 production stimulated by CAg in patients that failed to produce IL-2 suggests that unresponsiveness to CAg in CMC is not as complete as previously suggested [18]. Taken together, the absence of a systemic *in vivo* reaction confirmed by negative IL-6 plasma levels in all patients, the absence of systemic therapy at the time of study, as well as a vigorous response limited to only *Candida* antigens strongly suggest that the markedly increased IL-6 production seen in patients with CMC is not a non-specific effect, but rather a finding suggesting imbalances in cytokine production inherent to disease pathogenesis itself.

Patients with CMC differed in their ability to produce IFN-γ in response to stimulation with CAg, segregating into those that cannot respond and those who produce high IFN-γ levels. No obvious clinical correlation with this finding could be recognized. The beneficial effect of IFN-γ in CMC patients has been reported and attributed to enhanced macrophage/granulocyte fungicidal activity [19]. However, clinical effects are very variable and often absent (Lilic *et al.*, in preparation), suggesting that the role of IFN-γ in CMC may be more complex. Recent experimental findings in murine experiments demonstrate that, contrary to previous reports, IFN-γ levels do not always correlate with the outcome of *Candida* infection [20].

Detectable levels of antigen-stimulated IL-4 production were present in only a few adults in both patient and control groups, while none of the children responded. However, mitogen-stimulated responses, although low, were detectable and similar in patients and controls. It has been shown that IL-4-producing cells in the periphery are scarce [21]; this may explain the low IL-4 levels detected and suggests that further studies on IL-4 production in CMC patients are necessary.

Attempts to modify patterns of cytokines produced upon addition of IFN-α and IFN-γ did not give consistent and significant findings, although marked effects were sometimes noted. The most frequently observed effect was increased IL-2 and decreased IL-6 production, with little or no effect on IL-4 or IFN-γ production. Both interferons had been previously shown to favour production of Th1 cytokines such as IL-2 and IFN-γ [10,13], and this tendency was also observed in our studies, as expected. However, the absence of consistent findings compromises any clear-cut conclusions and suggests that other *in vitro* conditions may be necessary and/or crucial (e.g. longer incubation or different timing of interferon addition) for a clear phenomenon to emerge.

To the best of our knowledge there have been no other published studies addressing cytokine production in CMC patients. However, reported findings of over a decade ago of absent MIF production following CAg stimulation in CMC patients could be interpreted as findings of abnormal/absent cytokine production, since MIF was a monokine found in supernatants of antigen-stimulated PBMC [3], and as such would be consistent with our findings.

The finding that some patients produced normal quantities of IL-2, IL-6 or IFN-γ focuses the frequently raised question of disease heterogeneity [1,3] or alternatively, clinical status at the moment of assessment. Clinically, all our patients had childhood onset CMC, did not have clinically manifest autoimmune disease and were negative when screened for autoantibodies. The severity of infection, however, varied considerably and did not show correlation with cytokine production: the one patient that produced IL-2 had mild disease, as did at least three other patients who did not; low IL-6 levels were found in a patient with moderate infection, while very high levels were seen in patients with both the mildest and most severe infections; although four of the patients with the highest IFN-γ levels had very mild disease, this was not the case in the two other patients. Cytokine production did not correlate with presence of autoantibodies. In spite of this it should be stressed that most of the patients studied did show similar alterations of cytokine production in response to CAg, suggesting that at least some pathogenic mechanisms (if not necessarily causes) leading to CMC may be the same.

Taken together, our data suggest that altered cytokine production may be an important mechanism underlying increased susceptibility to *Candida* and other infections seen in patients with CMC, and could explain many of the reported findings of impaired cell-mediated and intact humoral immunity in these patients. Our findings of an impaired ability to produce IL-2, a vigorous production of IL-6 and an absent or increased production of IFN-γ suggest that in patients with CMC, *Candida* antigens seem unable to trigger adequate production of 'appropriate' (Th1) cytokines, but instead trigger increased production of 'inappropriate' (Th2) cytokines. This pattern has already been demonstrated in other animal and human infections such as listeriosis and leprosy [22]. The consequences of this Th2 type bias in response to CAgs would be preferential development of vigorous humoral responses, i.e. very high levels of anti-*Candida* antibodies (which we indeed demonstrate in the accompanying study [12]) together with low or absent cell-mediated responses (which has consistently been demonstrated both *in vivo* and *in vitro* in CMC patients [1,3,4]). As antibodies do not confer protection against fungi, the result would be an inability to clear *Candida* infection, as is indeed the case in CMC patients. More subtle alterations of cytokine production might well also underlie the recently recognized susceptibility of these patients to encapsulated microorganisms as well [6], a possibility that merits further study. However, even though our findings support this scenario, further studies addressing production of other cytokines characteristic of a Th1 or Th2 type response are needed to confirm this bias.

Very recent data suggest that MIF, whose production was previously shown to be impaired in most CMC patients, has important regulatory effects on cytokine production [23]. These data would indirectly suggest that cytokine regulation and/or production might be impaired in CMC patients and could contribute to the pathogenesis of disease. If this proves to be the case, patients with CMC might become candidates in which a very recent, exiting new therapeutic approach involving stimulation of Th1 cytokine production could be contemplated [24]. It is as yet impossible to say whether altered cytokine production is the cause or consequence of immune defects underlying CMC, and the question of how this defect is triggered by *Candida* antigens remains unanswered.

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