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In-Vitro Cytokine Induction and Neutrophil Respiratory Burst Activity by *Candida* Isolates from HIV Seropositive Patients with Oropharyngeal Candidiasis.

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ABSTRACT

The importance of host defense against candidiasis and role of cell mediated immunity in host defense has been the subject of many studies. Increased expression of virulence factors in candida isolates from human immunodeficiency virus (HIV) seropositive patients with oropharyngeal candidiasis (OPC) has also been reported. We conducted an experiment to study the difference in T helper type 2 (T_h2) and T helper type 1 (Th1) responses and neutrophil respiratory burst responses, of naive peripheral blood mononuclear cells (PBMC's) and neutrophils respectively, by using *Candida* isolates from study and control groups as stimulus. Thirty HIV seropositive patients with clinical evidence of OPC and thirty healthy volunteers were studied. IL-10 release was uniformly low at all concentrations of *Candida albicans* antigen isolated from healthy controls as compared to when *C. albicans* isolated from HIV seropositive patients was used as antigenic stimulus. Mean IFN-γ concentration was highest when *C.albicans,* isolated from healthy carriers was used as antigenic stimulus for PBMCs. The observations highlight a significant IL-10 dominance, which may be an indicator of the fine tuning of host immune system towards a chronic progressive disease condition, indicating the pathogenic potential of select *Candida* strains.

INTRODUCTION

Candidiasis is the commonest fungal disease of humans affecting mucosa, skin, nails and internal organs of the body. There are more than 160 species of *Candida,* about twenty are pathogenic for human beings [1-2].*Candida* species are found as commensals on mucosal surfaces of the body. However, in immunocompromised individuals [Human Immunodeficiency Virus (HIV) infection, neutropenia, intensive care patients, organ transplantation], in diabetes mellitus (DM) patients and in those receiving antibiotic treatment some form of mucosal candidiasis, most commonly oropharyngeal candidiasis (OPC), appear to manifest clinically[2].

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C.albicans has several known virulence factors contributing to its pathogenicity that includes adherence to the epithelial and endothelial cells, expression of several enzymes formation, molecular mimicry and antigenic modulation as a result of pseudohyphae formation.

In addition, cell wall associated mannan seems to have immunomodulatory effect on host cells [3,4].

HIV infection is seen to be associated with the selection of strains of *C.albicans* with an enhanced ability to adhere to buccal epithelial cells.

The selection of more adherent *C.albicans* may contribute to the predisposition of HIV-infected individuals to oral candidiasis [3].

Also increased expression of secreted aspartyl proteinases (saps) seems to be associated with *C.albicans* isolated from HIV seropositive individuals [5,6].

These evidences suggest that *Candida* species colonizing the oral cavities of HIV-infected individuals are subject to selective pressures that may lead to the emergence of strains with altered genotypic and phenotypic characteristics and enhanced expression of known and putative virulence determinants[7].

T-cell mediated immunity is important in host defense against *Candida* colonization. *Candida* infection at the mucosal surfaces can spread into tissues with a Th2 response whereas it is believed that Th1 responses are protective [8]. The response of naïve peripheral blood mononuclear cells (PBMC's) to antigen stimulation may also vary.

When *Candida* penetrates the mucous membrane the effector cells responsible for its destruction, get activated. Macrophages and PMNs have the ability to kill both *C.albicans* blastoconidia and hyphae through oxygen-dependent and -independent mechanisms. Oxygen-dependent killing by PMNs is mediated by superoxide anion and the myeloperoxidase-hydrogen peroxide-halide system, with the added participation of reactive nitrogen intermediates [9].

This in- vitro study was designed to examine the *Candida*-specific Th1/Th2 reactivity of the naïve peripheral blood mononuclear cells (PBMC's) from healthy volunteer by using *Candida* antigens from oral lesions of HIV seropositive patients and healthy carriers (controls) as stimulants, and the respiratory burst activity of neutrophils from healthy volunteers by using whole cell *Candida* isolates from HIV seropositive cases and controls.

MATERIALS AND METHODS

Patients and controls

This laboratory based study was conducted in the Department of Microbiology and Antiretroviral Therapy (ART) Clinic at a tertiary care hospital, Delhi, India. The study was conducted over a period of 12 months including the enrollment, analysis and compilation of data. The sample size was a randomly selected thirty subjects that included HIV seropositive patients with clinical evidence of oral candidiasis, attending the ART clinic, constituting the study group and thirty age matched healthy volunteers as controls. Patients in the study group were from 18 to 60 years of age, diagnosed as HIV seropositive according to National AIDS Control Organization (NACO) guidelines [10] and manifesting oral candidiasis clinically.

Informed consent was obtained from the patients and ethical clearance was obtained as per institutional guidelines. Every patient was given the right to opt out from study at any stage of the study. Identity of patients in study group was concealed. Patients on corticosteroid, anti-fungals or anti-neoplastic drug therapy and persons diagnosed with malignancies, blood dyscrasias, xerostomia or chronic renal disease were excluded from the study.

Two sterile cotton-tipped moist oral swabs or scrapings with blunt spatula were used to collect material from the oral lesions in both study and control groups. 3 ml blood samples were taken from healthy volunteers in Ethylenediaminetetraacetic acid (EDTA) vials, maintaining proper aseptic precautions, for separation of PBMCs and neutrophils.

Swabs were subjected to direct microscopy and culture on Sabouraud dextrose agar (SDA) with antibiotics (Chloramphenicol 5mg/ml & Gentamicin 2mg/ml), using standard laboratory techniques. *Candida* speciation was done using conventional phenotypic methods as per standard procedure [1,2].

Candida antigen was prepared following the protocol of Rimek et al. [11]. Each fungal isolate was grown on SDA plates for one to two days at 25° C temperature. For the preparation of antigen extracts, four to five colonies of yeasts (confirmed by microscopic examination) were suspended in 2 ml of sterile phosphate-buffered saline (PBS; HiMedia, Mumbai, India; pH 7.2). The fungal suspensions were vortexed and adjusted to a density of McFarland Barium sulphate standard of 0.5. Aliquots of the standard suspensions (500 µl) in PBS were transferred into 1.5-ml tubes, kept in a boiling water bath for 5 min, and subsequently centrifuged at 10,000 x *g* for 10 min. Supernatant was distributed in 1.5 ml tubes and stored at -20 C temperature. Protein concentration of the antigen extract was estimated by absorbance at 280 nm and at 260 nm employing the formula

Concentration (mg/ml) = (1.55 × absorbance at 280 nm) – (0.76 × absorbance at 260 nm) [12, 13].

Isolation of PBMCs

PBMC isolation was done from peripheral venous blood of healthy volunteers, using HiSep (HiMedia, Mumbai, India). Blood was gently layered over HiSep along the sides of the walls of the tube to avoid mixing of the blood with solution and centrifuged at 250 x g for 30 min at room temperature. PBMCs were aspirated at junction of HiSep and plasma. The upper plasma layer was carefully aspirated with a pipette to within 0.5 cm of opaque interface, to avoid contamination with other cell types, and was discarded. The opaque interface was transferred to a sterile conical tube to which 10 ml of Hanks balanced salt solution (HBSS; HiMedia, Mumbai, India) was added and centrifuged at low speed to remove extraneous platelets. Supernatant was then discarded and cell pellet was washed twice with 5 ml of HBSS at 250 x g for ten minutes. The viability of cells was measured by Trypan blue exclusion in Neubauer's chamber. The cells were resuspended in 1 ml of Roswell Park Memorial Institute medium (RPMI) 1640 medium with 10 percent fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin B, 2 mM L- glutamine (HiMedia, Mumbai, India) at a final cell concentration of 1×10^6 cells/ml [14].

Stimulation of PBMCs with *Candida* antigens

PBMCs (1×10^6/ml) were distributed in 24 well plates in 1 ml of RPMI 1640 with antibiotics and 10 percent fetal calf serum and stimulated with 0.05 µg/ml, 0.5µg/ml and 5µg/ml of *Candida* antigens. Phorbol myristate-12 acetate (PMA; Sigma-Aldrich, India) at a concentration of 100ng/ml was used as positive control. Antigen concentrations were selected based on published literature and preliminary titration experiments at our laboratory [15]. Plates were incubated for 48 hours in CO_2 incubator at 5 percent CO_2 concentration and temperature of 37° C. Supernatants were transferred in 1.5 ml tubes stored at a temperature of – 70℃ for cytokine analysis.

Human IL-10 and IFN-γ ELISA

Cytokine analysis was done using IL-10 and IFN-γ ELISA kit (Diaclone, Weldon Biotech, India), which are solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of IL-10 and IFN-γ, in supernatants. IL-10 and IFN-γ in each sample from antigen stimulated PBMCs was quantified in a 96 well commercially available kit using 16 wells for standards. Samples were tested in quadruplicate for both study and control groups to obtain mean peak IL-10 and IFN-γ concentrations, after stimulation with different antigen concentrations derived from *C. albicans* (from HIV seropositive patients) viz. 0.05 µg/ml, 0.5 µg/ml and 5 µg/ml. The plotted standard curve was used to accurately determine the concentration of cytokines in the samples tested.

Neutrophil separation by dextran sedimentation method [16]

Two ml of 5 percent dextran (Sigma-Aldrich, India) solution was taken in a wide bottom glass test tube. Two ml of whole blood was added and diluted with two ml of sterile normal saline. The whole suspension was mixed and made to stand at room temperature for thirty minutes. Supernatant plasma separated and layered over two ml of Hisep (HiMedia, Mumbai, India) and centrifuged at 1800 revolutions / min for 30 min. Supernatant removed and discarded, cell pellet washed with 1 ml HBSS (HiMedia,

Mumbai, India). Residual erythrocytes were lysed by adding 0.5 ml of 0.2 percent saline at 37°C for one minute. Tonicity was restored by adding 0.5 ml of 1.6 percent saline.

Resulting suspension was again centrifuged at 1400 revolution/min for five minutes.

Supernatant was discarded and sediment was resuspended in one ml of HBSS(HiMedia, Mumbai, India). Cells were counted in Neubauer's chamber and adjusted to a concentration of 2×10^6 /ml in HBSS(HiMedia, Mumbai, India).

Preparation of *Candida* isolates suspension [17]

Four to five colonies of each *Candida* isolates were picked up from SDA plate and cultured in five ml of nutrient broth with 1 percent glucose, at 30°C temperature for seven to eight days. Suspension of *Candida* isolates were centrifuged at 1500×g for ten minutes.

Supernatant were discarded, and resulting pellet was washed twice with PBS (HiMedia, Mumbai, India; pH 7.2). Pellet was resuspended in gelatin-HBSS (HiMedia, Mumbai, India) at concentration of 1-2 × 10^7 cells / ml by counting in Neubauer's chamber.

Nitroblue tetrazolium (NBT) dye reduction for assay of phagocytosis [17]

Three horizontal wells of ELISA plate were marked as test, positive control and negative control.100 µl of suspended neutrophils (2 × 10^6/ml), 100µl of NBT, 100 µl of *Candida* cells and 50 µl of HBSS (HiMedia, Mumbai, India) was added in test well. 50 µl of PMA (Sigma-Aldrich, India) and 50 µl of HBSS (HiMedia, Mumbai, India) were used as positive control and negative control, respectively. The ELISA plate was covered and incubated at 37°C for 30 min. The amount of formazan produced was measured at 550 nm in an ELISA reader. The results were expressed as absorbance at 550 nm per 2 × 10^6 cells.

Statistical Analysis

Standard error (SE) and 95 percent Confidence Intervals (CI) were calculated. Quantitative difference in cytokine production by PBMCs to mitogenic and/or antigenic stimuli and quantitative difference in formazan production by NBT dye reduction test, for study and control groups, was identified by the Mann-Whitney *U* post hoc test. Quantitative variables between study and control groups were tested by independent t-test. In all cases, significance was defined as $p<0.05$.

RESULTS

On examination of oral cavity of HIV seropositive patients, white patches on tongue or mucosa were found to be the predominant lesions, in both males (65 percent) and females (60 percent). Mixed lesions were seen in some of the patients as well (Table 1).

Out of a total of 30 samples from HIV seropositive patients, *C. albicans* was isolated from 70 percent of the oral cavity lesions and both *C. albicans* and *C. krusie* were found coexisting in one sample. Among healthy controls, all (23.33 percent) showed the growth of *C. albicans.* The mean IL-10 concentrations of 68.13 pg/ml, 77.50 pg/ml and 93.93 pg/ml, respectively were higher than the mean IFN-γ concentrations of 12.33 pg/ml, 16 pg/ml, 22.5 pg/ml respectively at similar antigen concentrations. The IL-10 concentrations of 93.93 pg/ml and IFN-γ concentrations of 22.5pg/ml were considered optimal for further analysis.

IL-10 release from antigen stimulated PBMCs were uniformly low at any concentration when the antigen was derived from *C. albicans* isolated from healthy volunteers. The levels for IL-10 were 19.50pg/ml, 30.95pg/ml and 49.90pg/ml for antigen concentrations 0.05 µg/ml, 0.5µg/ml and 5µg/ml respectively (Figure 1). IFN-γ levels were 40.45pg/ml, 51.50pg/ml and 61.75pg/ml respectively at similar antigen concentrations, which was higher for all antigen concentrations (Table 3). Mitogen (PMA at 100ng/ml) stimulated PBMCs, secreted IL-10 at a concentration of 351.38 pg/ml (Table 2) and IFN-γ at a concentration of 366.3 pg/ml (Table 3).

Neutrophil response on stimulation with *C.albicans* obtained from HIV seropositive patients showed mean OD of 0.8497 which did not vary significantly with neutrophil response on stimulation with

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C.albicans obtained from healthy carriers (OD of 0.8235). PMA taken as positive control, showed mean OD value of 0.8233 indicating, the viable state of neutrophils.

Figure 1: Interleukin (IL)-10 response of peripheral blood mononuclear cells (PBMCs) from healthy volunteers to stimulation by *Candida* antigens from Human Immunodeficiency Virus (HIV) seropositive patients and healthy controls.

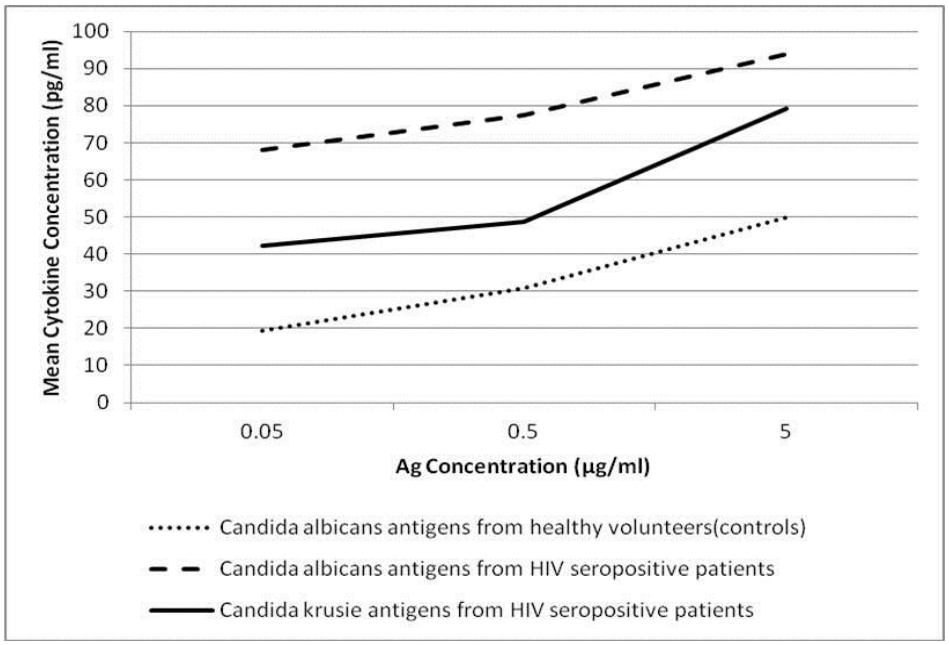

Figure 2: Interferon (IFN)- γ response of peripheral blood mononuclear cells (PBMCs) from healthy volunteers to stimulation by *Candida* antigens from Human Immunodeficiency Virus (HIV) seropositive patients and healthy controls.

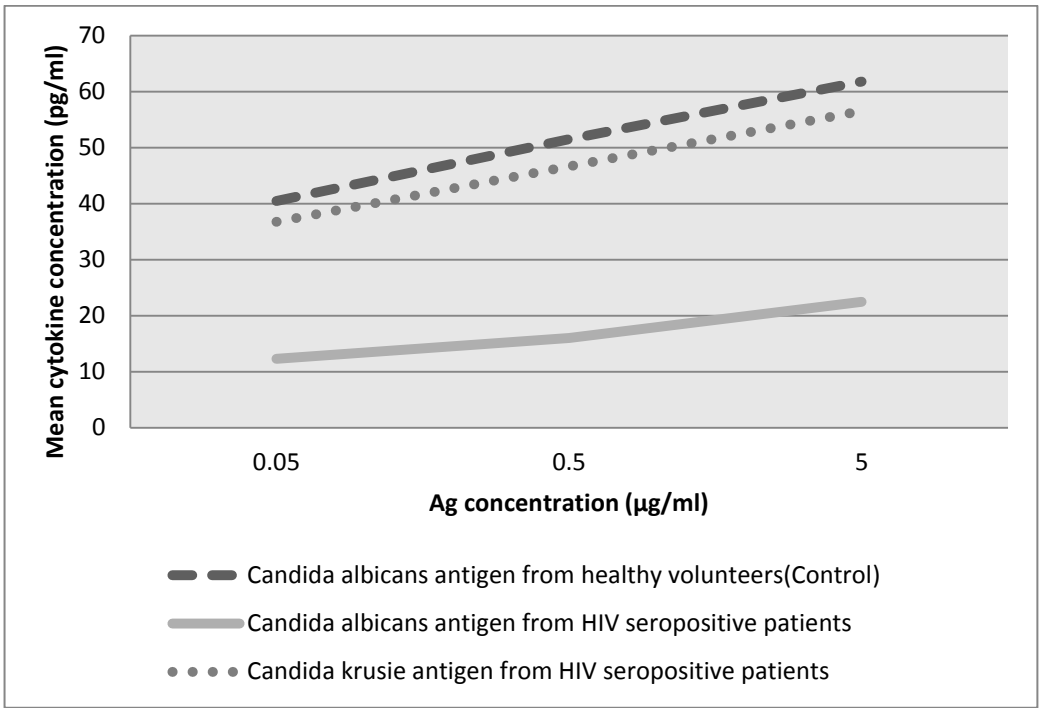

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Table 1: Distribution of oral cavity lesions among HIV seropositive patients.

Oral cavity lesions	HIV seropositive patients	
	Male (n=20) n (%)	Female (n=10) n (%)
White patches on tongue or mucosa	13(65)	6(60)
Erythematous lesion on tongue	3(15)	1(10)
Angular cheilosis	2(10)	1(10)
Ulcer on mucosa or tongue	1(5)	1(10)
Erythematous lesion and angular cheilosis	1(5)	0
White patches and angular cheilosis	0	1(10)
White patches and ulcer on mucosa	0	0
Angular cheilosis and ulcer on mucosa	0	0

Table 2: IL-10 response after stimulation of PBMCs with *Candida* antigens obtained from study groups

Study groups & Control	Antigen conc. (µg/ml)	Mean IL-10 cytokine levels (pg/ml)	SE[b]	CV[c]	95% CI[d]	
Candida albicans isolated from healthy volunteers	0.05	19.5[e,h]	2.13	4.17	15.33	23.67
	0.5	30.95[f,i]	1.73	3.39	27.56	34.33
	5	49.9[g,j]	6.53	12.80	37.09	62.70
Candida albicans isolated from HIV seropositive patients	0.05	68.13[e]	12.77	25.03	43.09	93.16
	0.5	77.5[f]	11.92	35.66	41.84	113.16
	5	93.93[g]	11.83	23.18	70.74	117.11
Candida krusie isolated from HIV seropositive patients	0.05	42.5[h]	3.76	7.37	34.83	49.57
	0.5	48.8[i]	5.95	11.66	37.14	60.46
	5	79.25[j]	6.65	12.83	66.42	92.08
Positive control PMA[a]	100 (ng/ml)	351.38	34.06	66.76	284.61	434.06

[a] PMA: Phorbol myristate-12 acetate; [b]SE: Standard Error; [c]CV: Confidence Value;[d] CI: Confidence Interval
e,f,g,h,i,j:P value-0.0095, P value-0.0083, P value- .0173 , P value- 0.0019 , P value- 0.028, P value- 0.0192 respectively.

Table 3: IFN- γ response after stimulation of PBMCs with *Candida* antigens obtained from study groups

	Ag (µg/ml)	Average	SE[b]	CV[c]	95% CI[d]	
Candida albicans isolated from healthy volunteers	0.05	40.45[e,h]	1.64	0.55	39.90	41
	0.5	51.50[f,i]	2.04	0.69	50.81	52.19
	5	61.75[g,j]	3.62	1.22	60.53	62.97
Candida albicans isolated from HIV seropositive patients	0.05	12.33[e]	3.03	5.15	7.19	17.48
	0.5	16[f]	4.46	8.74	7.27	24.74
	5	22.5[g]	10	13.86	8.64	36.36
Candida krusie isolated from HIV seropositive patients	0.05	36.75[h]	1.32	0.45	36.30	37.19
	0.5	46.66[i]	1.13	0.38	46.28	47.04
	5	56.54[j]	1.27	0.43	56.11	56.97
Positive control PMA[a]	100 ng/ml	366.3	22.98	7.75	358.55	374.05

[a] PMA: Phorbol myristate-12 acetate; [b]SE: Standard Error; [c]CV: Confidence Value;[d] CI: Confidence Interval
e,f,g,h,i,j:P value-0.0013, P value-0.0068, P value-0 .0044 , P value- 0.0013 , P value- 0.0206, P value- 0.0953 respectively

DISCUSSION

Candida species are found as commensals on mucosal surface of the body. In immunocompromised individuals, diabetics and in persons receiving antibiotic treatment, some form of mucosal candidiasis often manifest, most commonly as OPC. *C. albicans* is the most common species found amongst these OPC patients as was observed in our study. Mechanism of development of OPC involves the interplay of increased expression of virulence attributes of *C.albicans* and defects in immune defence mechanism in infected groups of patients [3,5,6]. OPC is the most frequent opportunistic fungal

infection among HIV infected patients, and it has been estimated that more than 90 percent of HIV-infected patients develop this debilitating infection, at some time during progression of their disease [18]. CMI has the predominant role among immune defences both at local and systemic level in these groups of patients. In acute phase response, PMNs play a major role and a defect in their response, as seen in HIV infected patients can become detrimental to the host [19,20]. Adaptive immune responses also play major role in containing oral candidiasis. The cytokines produced by monocytes and Th1/Th2 subsets of T helper cells determine the progression of an early OPC to a non-healing chronic mucocutaneous candidiasis (CMC), or may even be responsible for its resolution [21]. The role of *Candida* strain selection under these underlying stressful conditions may lead to an increased expression of virulence attributes resulting in a more chronic and fulminant course of oral candidiasis, as established by other workers [22-23].

This study was done to determine the prevalence of different species of *Candida*, isolated from HIV seropositive patients with oral candidiasis. This study also evaluated difference in the acute and adaptive (chronic) host immune responses to different strains of *Candida* species isolated from HIV seropositive patients and healthy carriers (controls).

In our study Pseudomembranous variant (white patch) was the commonest clinical presentation of OPC as described in previous studies [24]. Out of thirty HIV seropositive patients 65 percent of males and 60 percent of females presented with white patches over tongue or mucosa of oral cavity. *C. albicans* was present in 70 percent of patients presenting with white patches on tongue and mucosa in HIV seropositive patients. In one patient both *C. albicans* and *C. krusie* were isolated from the same lesion, suggestive of a mixed infection; symptoms were severe in this patient and difficult to treat.

C. albicans carriage among healthy controls was 23.33 percent. Earlier studies have reported a carriage rate ranging from 31percent to 55 percent without any symptoms in healthy adults[25].*Candida* carriage as low as 20 percent has also been reported in general population, which matches the results of our study.

Histopathological findings have shown, only few *Candida* hyphae associated with the atrophic epithelium in erythematous candidiasis, whereas numerous organisms were found invading the prickle cell layer of oral epithelium in pseudomembranous candidiasis indicating a higher pathogenic potential of these isolates in high risk conditions.

IL-10 and TNF-β are anti-inflammatory cytokines that inhibit the secretion of proinflammatory cytokines and impair anti-fungal effector functions by phagocytes. These cytokines potentially play a protective role later in the course of infection when resolution of the inflammatory process is necessary. Thus a careful balance needs to be maintained between the cytokines that affect the orchestration of cellular functions.

In our study *Candida* antigens isolated from study and control groups were used to stimulate the PBMCs derived from healthy volunteers. When PMA (100ng/ml) was used as stimulant for PBMCs, mean IL-10 concentration was 351.38 pg/ml and mean IFN- γ concentration was 366.3 pg/ml. Such high concentration of cytokines indicates the healthy state of the PBMCs.

Mean peak IL-10 (93.93 pg/ml) concentration was highest when *C.albicans* isolated from HIV positive patients were used as antigenic stimulus and lowest (19.5pg/ml) with *C.albicans* isolated from healthy carriers as the antigenic stimulus. The increase in IL-10 concentration in study groups as compared to the controls probably indicates a possibility of an enhanced expression of virulence factors leading to strain selection in the HIV seropositive patients, and the ability of these strains to overcome the protective Th1 responses allows them survival under stress [22,23]. Subsequently this may predispose to a more fulminant course of oral candidiasis leading to the development of chronic mucocutaneous candidiasis as a result of an imbalance in the immune system [21]. Another unusual observation was a high mean IL-10 levels at all antigen concentrations with *C.krusie* isolated from HIV seropositive patients used as stimulus, as compared to controls. In this regard, further experiments are needed to study the pathogenic potential of *C.krusie*.

Mean IFN-γ (61.75 pg/ml) concentration was highest when *C.albicans,* isolated from healthy carriers was used as antigenic stimulus for PBMCs, at a concentration not greater than 5μg/ml. This would corroborate the classic Th1-Th2 paradigm in which these two cytokines would be inversely related. In healthy carriers the overgrowth of *Candida,* is kept in check by various innate and adaptive immune response strategies, where Th1 cytokines (IFN- γ) have a predominant role to play, and maintain an

immunological balance[1]. Commensal strains, when they confront a microenvironment with suboptimal immune vigil, may express the virulence attributes, in an otherwise immunocompetent individual.

The behaviour of the *Candida* strains isolated from HIV seropositive patients on the cytokine response highlights the fact that there is a significant IL 10 dominance, which is an adaptation of the host immune system towards a chronic progressive disease condition indicating the pathogenic potential of these *Candida* strains in transit from a restrictive ambience of a competent host to a barrier free compromised host. The minimal dose response of IFN- γ only indicates a suppression of Th1 differentiation and its negligible anti candidicidal role when encountering these pathogens [25].

Neutrophil respiratory burst response was measured in terms of mean OD obtained after reduction of NBT dye to formazan (dark blue colour compound) by superoxide anion. In this study we attempted to study the host- pathogen interaction, by using *Candida* strains of patients from the study groups and stimulating neutrophils derived from healthy donors. *Candida* strains isolated from both study and controls groups stimulated the neutrophils and generated a similar acute response. In earlier in-vitro studies, dysfunctional acute phase response generated against yeast cells had been attributed to defective chemotaxis, phagocytosis and intracellular killing capacity of neutrophils and monocytes of the HIV seropositive patients [19-20]. Our study is also in agreement with previous studies, as no significant difference in acute phase response was observed, by using different strains of *Candida* as stimulant for neutrophils of healthy volunteers. It also emphasizes that neutrophils are not the only cells responsible but cell mediated immunity has a critical role to play as well.

CONCLUSION

The host pathogen interaction is a spectrum of dynamic events encompassing total silence to utter noise bordering on chaos. In this sequence, the decision to eliminate the microbe or retain it as commensal requires fine tuning of the immune response hinging on the threshold limits.

Crossing that rubicon would tilt the balance; hence elevated IFN-gamma would indicate the immunological decision of the host to eliminate the agent as undesirable. On the contrary, elevated IL-10 would indicate the decision of the host to retain the agent as the pathogen. In between these polar ends, the vacillating host before arriving at a decision is but helpless to try out its capabilities which operationally results in increased IFN- γ as well as IL-10; this is a transitory immunological state pending final decision. The pathogen factors, depending on whether it is relentless or insidious can tilt the balance either way.

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