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Relationship of Fungal Infection with Neutrophils and Macrophages in Cervical Smears

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ABSTRACT

Introduction:
Elimination of fungal infection depends on host defense mechanism by phagocytic cells, especially neutrophils, macrophages and monocytes. A limited study has been done to establish the relationship between fungal infection and innate immune system cells in cervical smears. The present study was conducted to evaluate the relationship between fungal infection, neutrophils and macrophages in cervical smears.

Methods:
This was an observational, hospital-based study conducted in Chitwan Medical College Teaching Hospital, Nepal. Papanicolaou-stained cervical smears from 4334 women, aged 18 to 49 years were examined under light microscopy. The presence of fungal cell walls was confirmed by Periodic acid–Schiff stain.

Results:
Cases with fungal infection were taken as the study group. Fungi were detected in 1.2% patients. Cases without any type of infections (83.5 %) were taken as the control group. A significant relationship between presence of fungal infection, macrophages and neutrophils was detected ($P < 0.05$) when the study and control groups were compared. We also found that macrophages and neutrophils work together against the fungal infection ($P < 0.05$). However, there was no significance ($P > 0.05$) between the existence of yeast or filamentous forms and these immune cells.

Conclusions:
The findings in the present study suggest that macrophages and neutrophils together may play a role in host defense against fungal infection; however, the different C. albicans morphologies did not affect the presence of neutrophils and macrophages. Thus, it indicates both forms of C. albicans may have pathogenic effects.

Keywords: Candida, cervical smear, fungal infection, innate immune system cells

INTRODUCTION

Candida species are the harmless commensal organisms that colonize mucocutaneous surfaces of the oral cavity, gastrointestinal tract, and vagina of the healthy human host. Candida does not normally cause any pathology. However, when immunity is compromised or patient with long-term antibiotic treatment or when the normal microflora balance is disrupted, these fungi become opportunistic pathogens. The underlying mechanism of such a transition of a harmless to pathogenic organism is still unknown. Candida species are the most common cause of fungal diseases in premature infants, pregnancy; and diabetic, surgical and AIDS patients. The most frequently isolated fungus in the genital tract is Candida albicans (80-95%), followed non-albicans Candida species including Candida glabrata, Candida parapsilosis, Candida krusei and Candida tropicalis [1].

The host-fungal interaction can be considered as an encounter between fungal virulence and host defense mechanisms. Candida albicans (C. albicans) have various putative virulence characteristics that are responsible for general survival, fitness and persistence within the host and also specific factors for adhesion, invasion, cell damage and induction/evasion of host responses [2–4].

The immune response depends on the fungal species encountered by the host. Depending upon the organism and anatomical site of infection, the importance of specific innate and adaptive defense mechanisms varies. Different fungal morphological forms may be considered an important determinant of the host response. The smaller size of yeasts and spores make them effectively phagocytosed, whereas the larger size of hyphae or filamentous forms often prevents them from effective ingestion [5]. Thus, the hyphae or filamentous form is more pathogenic compared with the yeast form. However, the mutant yeast that cannot transform to the filamentous form can be responsible for fungal infections [6, 7].

Cell-mediated immunity is an important defense mechanism for the elimination of fungal infections but there are certain types of antibody response which are protective against pathogenic fungi [8]. Neutrophils, macrophages and monocytes are essential antifungal effector cells. Neutrophis are especially considered as the main phagocytic immune cells that play a major role to control fungal infection; macrophages are recruited in lower numbers than neutrophils. These kill invading microbes by phagocytosis. Furthermore, they can release neutrophil extracellular traps (NETs) that are considered to be part of the human innate immunity. These are fibers composed of granule proteins and chromatin to trap and kill microbes [9]. NETs kill both yeast and filamentous forms of C. albicans, and those granule components mediate fungal killing [10].

There is a very limited study on cervicovaginal smears till date in establishing the relationship between fungal infection and innate immune system cells. The aim of the present study was to evaluate the relationship between fungal infection, neutrophils and macrophages in cervicovag inal smears by light microscopy. The present study also analyzed whether yeast or filamentous forms affect the presence of innate immune system cells.

MATERIALS AND METHODS

This was an observational, hospital-based study conducted at Chitwan Medical College, Nepal from January 2016 to December 2019. Cervical smears from 4334 women who visited Gynecology outpatient door with various gynecological complaints were evaluated. Ethical approval was taken from the Chitwan Medical College-Institutional Review Committee. Postmenopausal and pregnant women were excluded from this study. Thus, women aged 18 to 49 years were included. Samples were taken by the gynecologists using modified Ayres wooden spatula which was inserted and rotated 360° over cervix to be sampled from both ectocervix and endocervix. Smears were prepared on the slides, fixed in 95% ethyl alcohol immediately and sent to the Pathology laboratory. Smears were stained by Papanicolaou (Pap) staining method. The slides were examined under light microscopy. Cases diagnosed as fungal infection with detection of yeast and filamentous forms of fungi were studied and included as the study group. Cases without any type of infections were considered as the control group. The presence of fungal cell wall was confirmed by Periodic acid–Schiff (PAS) stain in each case. Informed consent was taken from all the selected subjects.

The statistical analysis of data was analyzed by using the Chi-square test in the Statistical Package for the Social Sciences (SPSS statistical software version 16.0; SPSS inc., Chicago IL, USA). Diff erences were considered statistically significant at $P < 0.05$.

RESULTS

On light microscopy, fungi as yeasts and filamentous forms were detected in 51 out of 4334 patients (1.2%) which were taken as the study group. Those cases without any type of infections (n = 3618, 83.5 %) were taken as the control group. It was observed that the yeast cells had attached to the epithelial cell membrane causing degradation of membrane as curve-like invagination and yeast cell had entered into the epithelial cell (Figure 1).

Neutrophils were present in both study and control groups. The percentage of macrophages was higher in the study group (66.7%) when compared with the

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control group (48.5%) (Table 1). There was also a statistically significant relationship between the presence of fungal infection and neutrophils ($P < 0.05$). We detected that the filamentous and yeast forms were surrounded by abundant neutrophils (Figure 2). The yeast forms were present in the cytoplasm of neutrophils (Figure 3).

We also compared the presence of macrophages in the study and control groups. Macrophages were more in the study group (23.5%) when compared to the control group (6%). A significant relationship between the presence of fungal infection and macrophages was noted ($P < 0.05$) (Table 1). Microscopically, macrophages have a kidney bean-shaped nucleus with foamy cytoplasm. Cytoplasm of macrophage showed ingested yeast forms. Multinucleated giant macrophages were also identified.

PAS stain was also carried out in all cases of the study group to confirm the presence of fungal cell wall. Both yeast and filamentous forms was stained pink, and their cell wall had taken darker pink color (Figure 4). Neutrophils and macrophages were also noted in the surroundings of the fungal cells. Neutrophils and macrophages worked together against the fungal infection when compared within the study group ($P < 0.05$) (Table 2).

The study group (n = 51) was divided into three groups: Yeast (+), filamentous (+); and yeast and filamentous (+) (Table 3). There were 17 out of 51 (33.3%) cases of yeast forms. The filamentous forms were seen in 10 out of 51 cases (19.6%). The morphology of the filamentous forms was as hyphae or pseudohyphae. Both yeast and filamentous forms were present in 24 of 51 cases (47.1%). Neutrophils and macrophages were detected in all three cases; however, there was no significance ($P > 0.05$) between the presence of yeast or filamentous forms and these immune cells (Table 3).

DISCUSSION

Genital candidiasis is a common infection caused by an overgrowth of Candida species. It is estimated that 75% of women will experience an episode of genital candidiasis in their lifetimes. C. albicans is the most frequently isolated fungus in the genital tract accounting for 80–95% of genital candidiasis worldwide [1]. The prevalence of fungal infections in cervicovaginal smears varied in different studies, ranging from 0.6% and 36% [11-17]. The present study noted 1.2% of vulvovaginal candidiasis.

Reports have suggested that C. albicans has ability to bind to epithelial cells resulting in adhesion and epithelial invasion by two mechanisms such as, induced endocytosis and active penetration [18]. The present study also noted the yeast forms attached to the epithelial cell membranes that might have entered the epithelial cell cytoplasm after binding to the cell membrane. This finding was comparable to the other study; in addition to attachment of yeast forms, there was also curve-like invagination on the surface of the epithelial cells [15]. Epithelial cells have very important antifungal role. For example, epithelial cells upregulate Toll-like receptor 4 (TLR) and subsequently protect against tissue damage caused by C. albicans [18].
The proinflammatory cytokines and chemokines are secreted in response to epithelial activation that recruit neutrophils, dendritic cells and T- cells to the site of infection, causing either a return to the non-activatory colonization 'commensal' state or clearance of fungal infection [19].

The phagocytic cells are able to recognise C. albicans via multiple classes of receptors [20], including pattern recognition receptors (PRRs) such as TLR 2 and TLR 4, and C-type lectin receptors (CLRs) such as Dectin-1 and the mannose receptor [21-23]. These receptors can induce phagocytosis independently of complement; however, opsonization significantly enhances the efficiency of phagocytosis of Candida in the absence of complement [24]. Complement receptor, CR3 present on phagocytes is able to recognize complement (iC3b) deposited on the opsonised fungal cell surface and promote phagocytosis. This process is also able to occur through complement receptor immunoglobulin (CRIg) in macrophages [25]. Agents such as dexamethasone that upregulated CRIg expression, increased the rate of phagocytosis of C. albicans by monocyte-derived macrophages (MDM) [26], suggesting that CRIg rather than CR3 plays an important role in the phagocytosis of C. albicans in macrophages.

The role of macrophages in host defense against fungal infection had been reported by many molecular-based studies. Their findings strongly

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support that macrophages have a substantial role in the defense against fungal infection. Gantner et al [27] had shown a mechanism by which the shape or structure of C. albicans alone directly impacts to the method by which phagocytes recognize the fungus. Dectin-1 is a receptor that binds β-glucan and is important for macrophage to phagocytose fungi. This receptor also collaborates with TLRs for inflammatory activation of phagocytes by fungi. Their report had demonstrated that β-glucan of yeast cell wall is shielded from Dectin-1 by outer wall components. During the normal mechanisms of yeast budding growth deformities produced in the cell wall expose patches of β-glucan that are recognized by Dectin-1 to trigger antimicrobial responses in macrophages. However, no cell separation or subsequent β-glucan exposure occurs during filamentous growth, thus it fails to activate Dectin-1. The other study mentioned that the yeast form induced TLR4, and stimulated the synthesis of interleukin (IL-1β and IL-6) cytokines by binding to Dectin-1, which induced an inflammatory reaction and the activation of macrophages by increasing interferon- γ (IFN-γ) synthesis. In addition, by increasing nitric oxide (NO) synthesis, macrophages phagocytosis was ultimately induced to eliminate C. albicans. In contrast, the hyphae form was bound to TLR2 and Dectin-2, thus IFN-γ synthesis was not induced. Hence, the activation of macrophages was reduced and IL-10 synthesis was increased so that the induction of inflammatory reactions could be blocked. Hence, decrease of expression of Dectin-1 that is closely associated with phagocytosis and the reduction of NO synthesis leads the hyphae-form to resist macrophage phagocytosis and evade the host immune response [28]. Thus, the morphological switch between the yeast phase and the hyphal phase is considered to be the main virulence factor of C. albicans. However, Wellington et al [29] mentioned that glycosylation of the cell wall component of fungi is more critical and transition is not a virulence factor. In addition, reports by Ganter et al [27] and Han et al [28] suggested that morphological changes cannot affect the presence of macrophages, but they affect the receptor expression pattern as mentioned above. The present study has the findings similar to these studies that both morphological forms seem to be considerable for virulence and do not affect the presence of macrophages and neutrophils.

Myeloperoxidase deficiency in humans may increase susceptibility to Candida infections if other predisposing conditions are also present [30]. Other receptors have been identified, for instance Fradin et al [31] recognized galectin-3 that recognizes oligomannosides. Unlike β-glucan, ligands for these receptors are exposed at the cell surface and are unlikely to be affected by changes in cell structure. These receptors help macrophages to bind to C. albicans and may additionally trigger signaling that contribute in the inflammatory response. Futhermore, the data produced by Newman et al [32] suggested that human macrophage anti-Candida activity and the inhibition of phagolysosomal fusion in macrophages are both nonoxidative mechanisms.

The role of macrophages in host defense against Candida infection have been reported by many studies in different mouse models; however its role is still less well established. Although neutrophils play the main role in the innate immune system in fungal infection, a report showed that depletion of alveolar macrophages reduced Candida clearance and neutrophil recruitment in the lung [33], suggesting the involvement of macrophages in host defenses against systemic Candida infections. It is further supported by recent researches exhibiting that deficiency in the chemokine receptor CX_3CR1, which is associated with impaired recruitment of monocyte-derived macrophages into tissues, increased fungal growth and mortality after Candida challenge. Patients with a polymorphism leading to decreased function of CX_3CR1 also showed increased susceptibility to systemic candidiasis [34]. The present study noted higher number of macrophages in the study group (23.5%) when compared with the control group (6%) which was statistically significant. The ingested yeast was also seen in the cytoplasm of macrophage, similar to the previous study [15]. The present study also demonstrated that both macrophages and neutrophils were identified together against the fungal infection in the study group which was statistically significant ($P < 0.05$). This result is compatible with the earlier study [15]. Thus, it suggests that macrophages play critical role as neutrophils against fungal infection and both act together.

The present study observed numerous neutrophils in the study group, admixed with macrophages ($P < 0.05$), comparable to the other study [15]. The reports

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have suggested that several proinflammatory cytokines, such as IL-6, IL-8, IL-12 and TNF-α are essential for the efficient control of C. albicans infection [35-37]. In addition to proinflammatory cytokines, the hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (G-MCSF) are critical for fungicidal activity of neutrophils [38, 39]. The engulfed Candida cells are killed intracellularly within the phagolysosome by using a number of oxidative and non-oxidative mechanisms including the production of toxic reactive oxygen and nitrogen species (ROS and RNS), expression of various antimicrobial peptides and the activity of hydrolytic enzymes [40].

The filamentous form was surrounded by plenty of neutrophils, similar to the other study [15] which stated that this may be because of the large size of the filamentous form; many neutrophils may be needed to get digested as single neutrophils may not be able to do so. The previous reports showed that the filamentous form was encircled by neutrophils [10, 15] and release some chemicals for extracellular digesting and NETs for killing the filamentous form. Recently, an extracellular mechanism of killing Candida species was shown to be exerted by neutrophils. Neutrophils inhibit Candida growth by releasing NETs which contain the antifungal peptide calprotectin [41]. NETs kill both yeast and hyphal forms, and that granule components mediate fungal killing [42].

Demirezen et al [15] noted multinucleated giant macrophages ingesting the filamentous form in some Pap smears. The present study also identified multinucleated giant cells. According to Lewis et al [43] the hyphal form of Candida albicans inhibits cell division in macrophages undergoing mitosis and leads to multinucleated macrophages formation. This might be the reason of a potential escape mechanism of fungi from host immune defense.

The present study also analyzed whether yeast or filamentous forms affect the presence of innate immune system cells. Hence, the study group was classified as three groups, such as the yeast form, the filamentous form and both forms. Neutrophils and macrophages were seen in all three groups similar to the other previous study [15]. There was no statistical significance between different morphological forms and the presence of immune cells ($P > 0.05$).

Postmenopausal women were excluded from this study. A report had identified histiocytes from 108 postmenopausal women on otherwise-normal cervical smears, except for endometrial pathology in 13 of cases [44]. When other clinical or cytologic findings are absent the presence of histiocytes alone in cervicovaginal smears from peri- or post-menopausal is non-specific [45].

CONCLUSIONS

The findings in the present study suggest that macrophages and neutrophils together may play a role in host defense against fungal infection; however the different C. albicans morphologies did not affect the presence of neutrophils and macrophages. Thus, it indicates both yeast and filamentous forms may have pathogenic effects.

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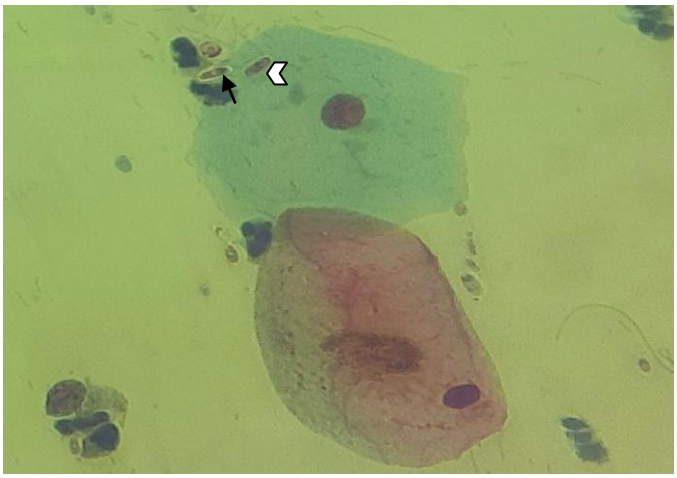

Figure 1: Yeast forms attached to the cell membrane with degrading membrane as curve-like invagination (arrow) and entering the epithelial cell (arrowhead) (Pap, ×400)

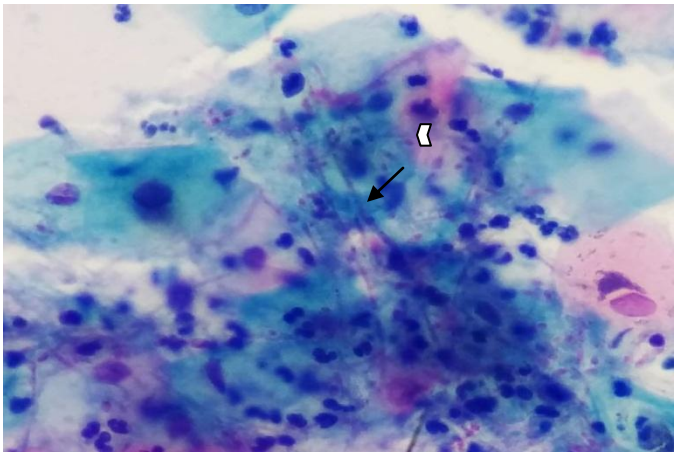

Figure 2: The filamentous (arrow) and yeast forms (arrowhead) were surrounded by abundant neutrophils (Pap, ×400)

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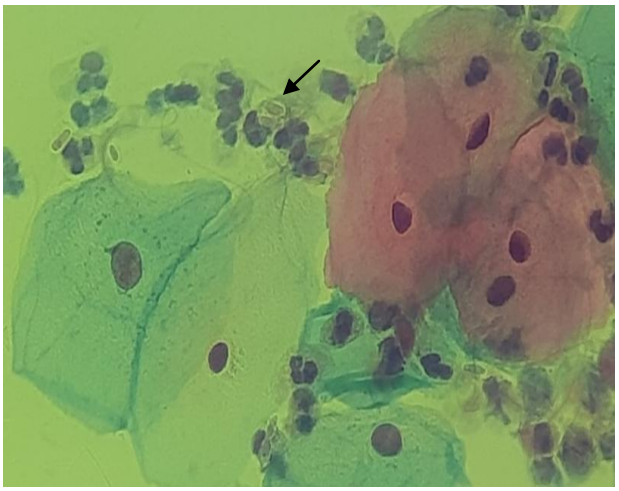

Figure 3: Yeast forms in the cytoplasm of neutrophil (arrow) (Pap, ×400)

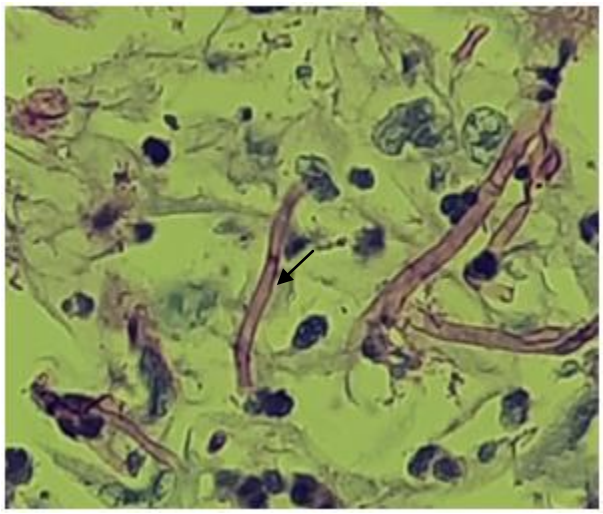

Figure 4: PAS stain showed the fungi with pink color and their cell wall (arrow) stained with a stronger and darker pink color (PAS, ×1000)

Table 1: Comparison of the study and control groups with the presence of neutrophils and macrophages

Type of immune cells	Study group n = 51 (%)	Control group n = 3618 (%)	*P*-value
Neutrophils	34 (66.7)	1756 (48.5)	**P* = 0.010
Macrophages	12 (23.5)	214 (6)	**P* < 0.000

* $P < 0.05$

Table 2: Relationship between the presence of macrophages and neutrophils

Macrophages (+/-)	Neutrophils (+) n (%)	Neutrophils (-) n (%)	*P*-value
Macrophages (+), n= 12	11 (91.7)	1 (8.3)	**P* = 0.035
Macrophages (-), n= 39	23 (59)	16 (41)	

* $P < 0.05$

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Table 3: Relationship between the presence of yeast or filamentous forms, neutrophils and macrophages

Type of immune cells	Yeast (+) n = 17 (%)	Filamentous (+) n = 10 (%)	Yeast and filamentous (+) n = 24 (%)	*P*-value
Neutrophils (n = 34)	10 (29.4)	6 (17.6)	18 (53)	**P* = 0.491
Macrophages (n = 12)	5 (41.7)	3 (25)	4 (33.3)	**P* = 0.552

**P* > 0.05

ABBREVIATIONS

AIDS Acquired immunodeficiency syndrome

C. albicans Candida albicans

CLRs C-type lectin receptors

CR Complement receptor

CRIg Complement receptor immunoglobulin

CX_3CR1Chemokine receptor

G-CSF Granulocyte colony-stimulating factor

G-MCSF Granulocyte-macrophage colony-stimulating factor

IFN-γ Interferon *gamma*

IL Interleukin

MDM Monocyte-derived macrophages

NETs Neutrophil extracellular traps

NO Nitric oxide

PAP Papanicolaou

PAS Periodic acid–Schiff

PRRs Pattern recognition receptors

RNS Reactive nitrogen species

ROS Reactive oxygen species

SPSS Statistical Package for the Social Sciences

TLR Toll-like receptor

TNF-α Tumor necrosis factor alpha

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