Original Article

Evaluation of the relationship between fungal infection, neutrophil leukocytes and macrophages in cervicovaginal smears: Light microscopic examination

ABSTRACT

Background: Right after opportunistic fungi become pathogenic, they face immune system cells including macrophages and neutrophil leukocytes. Although the relationship between fungi and immune cells are being widely studied by using animal models and culture techniques, cervicovaginal smears have not been used to evaluate this interaction yet.

Aim: The aim of this study was to investigate the interactions between fungal infection, macrophages and neutrophil leukocytes in cervicovaginal smear.

Materials and Methods: Papanicolaou-stained cervicovaginal smears from 2307 women, aged between 18 and 73 years, were examined by light microscopy. Periodic acid–Schiff stain was also used to confirm the presence of fungal cell walls.

Results: Fungal infections were detected in 239 of 2307 patients (10.4%), and these cases were taken as the study group. Cases without any infectious agents ($n = 1800$, 78%) were considered as the control group. When the study and control groups were statistically compared in view of macrophages and neutrophil leukocytes, a significant relationship between presence of fungal infection, macrophages and neutrophil leukocytes was detected ($P < 0.05$). Furthermore, macrophages and neutrophil leukocytes were found to work against the fungal infection together ($P < 0.05$). Additionally, when the relationship between the existence of yeast or filamentous forms and these immune cells were evaluated, a significant correlation was not found ($P > 0.05$).

Conclusions: Our findings indicate that macrophages and neutrophils may play a determining role in host defense against fungal infection together, but neither yeast nor filamentous forms affect the presence of neutrophil leukocytes and macrophages. As a result of this, both yeast and filamentous forms may have pathogenic effects.

Key words: Candida; cervicovaginal smear; fungal infection; innate immune system; macrophage; neutrophil

Introduction

Fungal infections can affect various parts of the body, including the skin and respiratory and urogenital tracts.[1] In the genital mucosa, some fungi are members of the normal flora.[2,3] With a percentage of 80-90%, the most commonly isolated fungus in the genital tract is *Candida albicans*, followed by *Candida glabrata*, *Candida krusei* and *Candida tropicalis*.[4] In various conditions such as weakened immune system, diabetes mellitus and pregnancy, these opportunistic fungi become pathogenic and cause infections.[5] However, the underlying mechanism of how such a transition occurs is still unknown.

Host defense against fungal infection depends on elimination of the fungi by phagocytic cells of the innate immune system, especially neutrophils and macrophages.[6] Neutrophil leukocytes or polymorphonuclear leukocytes (PMNLs) are reported as major phagocytic immune cells that play a

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major role against fungal infection. They activate various antimicrobial mechanisms in addition to phagocytosis, including the producing of reactive oxygen species (ROS), the release of granular enzymes and antimicrobial proteins.[7] In addition, a neutrophil extracellular trap (NET) composed of a neutrophil chromatin is another significant protective mechanism against the fungal infection.[8]

In recent years, the relationship between fungal infection and macrophages has been widely studied. However, the importance of macrophages in host defense against fungal infection is still controversial.[9] While macrophages were not accepted as major effectors in defense against fungal infection in various studies, the fact that the decrease of macrophage activity causes susceptibility to systemic candidiasis was reported.[10,11]

Fungi exist as different morphological forms such as the yeast and the filamentous forms. Some previous reports indicate that the filamentous form is more pathogenic compared with the yeast form. However, a mutant fungus that cannot switch the yeast to the filamentous form causes invasive fungal infections.[12,13] These data suggest that the transition from the yeast to the filamentous form is an essential virulence factor; however, both these forms of fungi have pathogenic activities. This phenomenon is not fully understood.

Most of the studies related to fungal infection were carried out by using animal models and cell culture. There is no previous documentation based on cervicovaginal smears in enlightening the relationship between fungal infection and innate immune system cells. The purpose of the present study is to evaluate the association between existence of fungal infection, PMNLs and macrophages in cervicovaginal smears by light microscopy. To assess such an association, it is crucial to understand the interaction of fungi with the host immune system to determine the fungal infection pathogenesis. Furthermore, we have also investigated whether different morphological forms like yeast or filamentous forms affect the presence of these cells.

Materials and Methods

In this study, cervicovaginal smears obtained from 2307 patients with varied gynecological complaints were analyzed at the Gynecology and Obstetrics Clinic in Hacettepe University, Ankara, Turkey. The age group of these patients varied from 18 to 73 years. Pregnant women were not included in the study. Cervicovaginal samples were taken with a cytobrush from each patient before a pelvic examination and fixed with 96% ethanol for 30 min without drying in air. After the routine Papanicolaou (PAP) staining method, slides were examined by light microscopy in detail. In the cytological examination, the diagnosis of fungal infection was established by detecting yeast and filamentous forms of fungi. In order to observe the cell wall of the yeast and filamentous forms, Periodic acid–Schiff (PAS) stain was also performed in one case where fungal infection was detected by the PAP method. All findings were analyzed by using the Chi-square test in the Statistical Package for the Social Sciences (SPSS) 11.5. *P*-values <0.05 were accepted as significant.

Results

As a result of light microscopic examination, yeast and filamentous forms that showed fungal infection were detected in 239 of 2307 cases (10.4%), and these cases were included in the study group [Figure 1a]. Patients without any infection ($n = 1800$, 78%) were accepted as the control group. We especially noted that yeast cells had attached to the epithelial cell membrane and had formed curve-like invagination on the surface of the epithelial cell [Figure 1b]. Furthermore, Figure 1c demonstrated that yeasts were almost engulfed by the epithelial cell.

In the cytological examination, it was detected that macrophages have a kidney bean-shaped nucleus and foamy cytoplasm. As seen in Table 1, the presence of macrophages was observed in both study and control groups; however, the percentage of macrophages was higher in the study group (9.2%) compared with that of the control group (4.7%). In statistical analyses, a significant relationship between the existence of fungal infection and macrophages was also

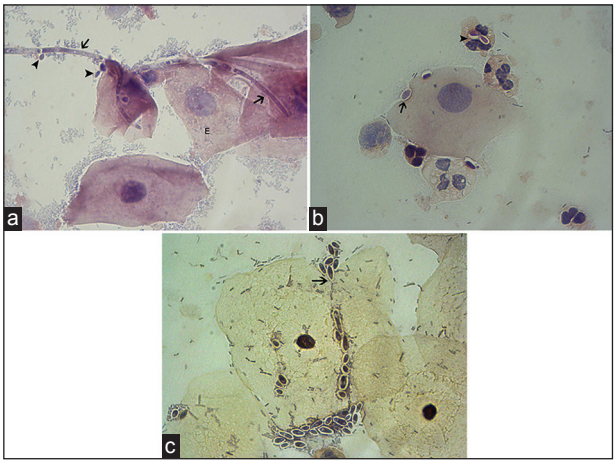

Figure 1: (a) Yeast (arrowhead) and filamentous forms (arrow) were seen near the epithelial cell (E) (Pap, ×1000). (b) Yeasts degraded membranes as curve-like invagination (arrow) and yeast forms in the cytoplasm of polymorphonuclear leukocytes (arrowhead) (Pap, ×1000). (c) Yeast forms attached to the cell membrane and almost entering the epithelial cell (arrow) (Pap, ×1000)

detected ($P < 0.05$). Furthermore, we observed cytoplasmic inclusions in the cytoplasm of macrophages containing digested materials such as the yeast form [Figure 2a]. Multinucleated giant macrophages were also examined [Figure 2b].

In our study, a comparison of PMNLs was carried out in the control (147/239, 61.5%) and study groups (953/1800, 52.9%). Statistical analysis of the results indicated that there was a significant relationship between the presence of fungal infection and PMNLs ($P < 0.05$) [Table 1]. An interesting finding was the observation of large numbers of intact and budding yeast forms in the cytoplasm of PMNLs [Figure 3a]. As shown in Figure 3b, some PMNLs were lysed. Furthermore, it was noted that large filamentous forms were surrounded by PMNLs [Figure 3c]. As shown in Table 2, when the presence of macrophages and PMNLs were statistically compared within the study group, it was figured out that these cells act together against fungal infection ($P < 0.05$).

To confirm the presence of fungal cell wall, PAS stain was also performed. Yeast and filamentous forms stained pink, while their cell wall stained with a stronger and darker pink color. Furthermore, yeast and filamentous forms juxtaposed to the epithelial cell membrane and some points of the membrane were found to be fused to the filamentous form. Macrophage and PMNLs were also observed in the surroundings of the fungal cells [Figure 2c].

As seen in Table 3, the study group ($n = 239$) was classified into three groups: Yeast (+), filamentous (+) and yeast + filamentous (+). This classification was based on light microscopic observations. In 83 of 239 cases (34.7%), these cases are accepted as the yeast (+) group by the fact that there were only the yeast forms. The filamentous (+) group had only hyphae or pseudohyphae forms and were found in 92 of 239 cases (38.5%). Both yeast and filamentous forms were present in the remaining 64 of 239 cases (26.8%), and were classified as the yeast + filamentous (+) group. Macrophages and PMNLs were found in all three cases, and the association

Table 1: Comparison of the study and control group in view of macrophages and PMNLs

Type of cells	Study group ($n = 239\%$)	Control group ($n = 1800\%$)	*P*-value
Macrophages	22 (9.2)	85 (4.7)	$^*P = 0.005$
PMNLs	147 (61.5)	953 (52.9)	$^*P = 0.007$

$^*P < 0.05$

Table 2: Relationship between presence of macrophages and PMNLs

Presence of PMNLs	Macrophages (+) ($n = 22\%$)	Macrophages (−) ($n = 217\%$)	*P*-value
PMNLs (+)	21 (95.4)	126 (58.1)	$^*P = 0.000$
PMNLs (−)	1 (5.6)	91 (41.9)	

$^*P < 0.05$

Table 3: Evaluation of the relationship between the presence of yeast or filamentous forms, macrophages and PMNLs

Type of cells	Yeast (+) ($n = 83\%$)	Filamentous (+) ($n = 64\%$)	Yeast and filamentous (+) ($n = 92\%$)	*P*-value
Macrophages ($n = 22$)	12 (54.5)	4 (18.2)	6 (27.3)	$P = 0.069$
PMNLs ($n = 147$)	56 (38.1)	52 (35.4)	39 (26.5)	$P = 0.329$

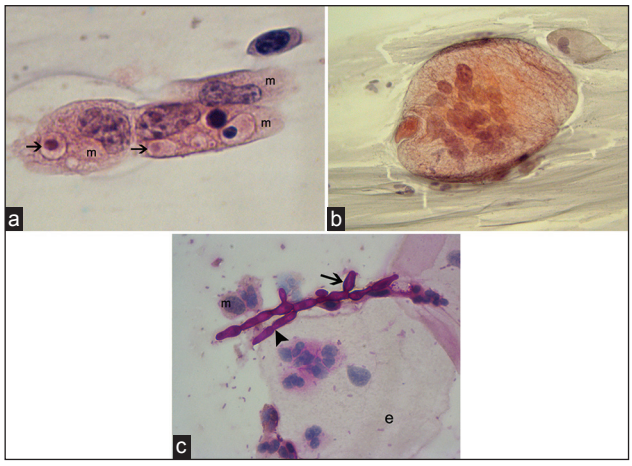

Figure 2: (a) Cytoplasmic inclusions in the cytoplasm of macrophages (m) containing digested materials (arrow) such as the yeast form were seen (Pap, ×1000). (b) A giant multinucleated macrophage (Pap, ×1000). (c) The yeast and filamentous forms were pink and their cell wall (arrow) stained with a stronger and darker pink color by using the Periodic acid — Schiff (PAS) method. Some points of the epithelial cell (e) membrane (arrowhead) were fused to the filamentous form (PAS, ×1000)

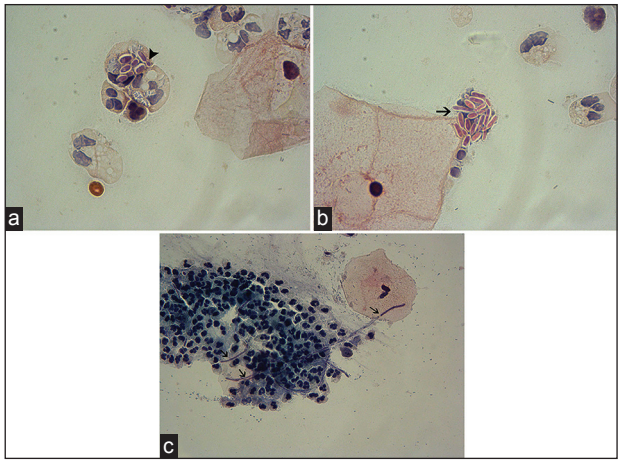

Figure 3: (a) Yeasts and a budding yeast (arrowhead) were seen in the cytoplasm of polymorphonuclear leukocytes (PMNLs) (Pap, ×1000). (b) Disintegrated, fully filled PMNL cytoplasm (arrow) and budding yeast were also seen (Pap, ×1000). (c) The filamentous form (arrows) was surrounded by abundant PMNLs (Pap, ×400)

between different morphological forms and the presence of these cells was not statistically significant ($P > 0.05$).

Discussion

In this study, fungal cells were examined by using both PAP and PAS methods. In both these methods, yeast and filamentous forms were observed to be attached and closely interacting with epithelial cells, macrophages and PMNLs. In addition to the PAP stain used for the purpose of detecting yeast and filamentous forms, PAS, which gives off a characteristic pink color different than that of PAP, was also used in order to confirm the presence of fungal cell wall [Figures 1a and 2c].

Genital fungal infections, especially Candidiasis, are the most common genital disease in women.[14] There are many reports assessing the prevalence of fungal infections in cervicovaginal smears, and percentages vary between 2% and 36%.[14-16] Our findings of 10.4% show resemblance to previous studies.

Some reports indicate that fungal infection develops with adhesion of the yeast form to the epithelial cells, and this is accepted as a critical virulence factor.[17,18] Consistent with these reports, we observed that yeast forms had juxtaposed to the epithelial cell membranes and had formed curve-like invaginations on the surface of the epithelial cell [Figure 1b]. After such an attachment, it was observed that the yeast forms had penetrated to the cytoplasm of the epithelial cells. Two mechanisms are involved for such an entry. The first one induced by epithelial cells is called "induced endocytosis" and the second one that is mediated by fungi is described as "active penetration."[17] We also observed that the yeast form may enter the epithelial cell cytoplasm after binding to the cell membrane [Figure 1c]. Our light microscopic observation was parallel to that of other reports.

Several studies on the role of macrophages in host defense against fungal infection have been previously reported. In one study, macrophages were eliminated in mouse and enhanced susceptibility to fungal infection was shown.[10] However, in another study, macrophages were not accepted as major effectors against *Candida albicans*.[19] Thus, the significance of macrophages in the host defense against fungal infection is still controversial.[9] In our study, the presence of macrophages was observed to be statistically higher in the study group (9.2%) compared with that of the control group (4.7%). Furthermore, we observed that the yeast form was present in cytoplasm of macrophages [Figure 2a]. As a result of our findings, macrophages might have important roles against fungal infection.

In recent years, according to molecular-based studies, the recognition of fungal structure by macrophages via cell membrane receptors is termed as pattern-recognition receptors (PPRs). This recognition triggers the activation of macrophages. Findings from preceding studies suggest that Toll-like receptor-2 (TLR2) and TLR4 bind to mannose residues of the fungal cell wall while dectin-1 and dectin-2 receptors recognize β-glucan.[20,21] In addition to these cell membrane receptors, TLR-7 and TLR-9, which can recognize fungal nucleic acids, were found in the endosome membrane.[22] The recognition of fungi by macrophages causes phagocytosis and activation of proinflammatory processes. All these reports support that macrophages have a substantial role in the defense against fungal infection.

In our study group ($n = 239$), there were abundant PMNLs in addition to macrophages ($P < 0.05$). The reason for this observation is the fact that PMNLs are major players of the innate immune system against fungi as the cells are the first ones arriving at the site of infection.

Macrophages and PMNLs were detected together against the fungal infection in the study group ($n = 239$, $P < 0.05$). Although PMNLs are the main cells in the innate immune system in attacking fungi, a previous study suggests that the depletion of macrophages enhance susceptibility against fungal infection.[10] Thus, our results show that macrophages are as important as PMNLs, and they collaborate against fungal infection.

The interaction between innate immune cells and fungi is not fully understood and studies are still being conducted. In our cytological examination, the cytoplasm of some PMNLs was fully filled with yeast forms and some PMNLs were degraded. Interestingly, budding yeasts were observed in the cytoplasm of PMNLs [Figure 3a and b]. These findings may suggest that the yeast form has the ability to survive from PMNLs attack. While innate immune cells play a significant role against fungal infection, fungi attempt to survive from phagocytes by inhibiting the production of ROS, by preventing phagolysosome fusion or increasing the pH of phagolysosome. In addition, fungi can transform itself from yeast into the filamentous form in the cytoplasm of immune cells, inhibit mitosis and cause apoptosis.[23-25]

In cytological examination, we also observed giant macrophages in some cases, which include the filamentous form [Figure 2b]. We thought that one macrophage may not have enough digestive activity for the filamentous form. However, Lewis *et al*. stated that the hyphal form of *Candida albicans* induces to fail cell division of macrophages and leads

to formation of multinucleated macrophages.[24] This may also be a potential escape mechanism of fungi from host immune defense.

As seen in Figure 3c, the filamentous form was surrounded by numerous PMNLs. Thus, this may be because of the fact that the filamentous form is too large to be digested by a single PMNL. Consistent with our finding, previous reports showed that PMNLs encircle the filamentous form and release some chemicals for extracellular digesting and NETs for killing the filamentous form.[8] Furthermore, migration of PMNLs to the infectious area was induced by the existence of filamentous forms.[26]

In our study, we investigated whether the existence of yeast or filamentous forms effect the presence of macrophages and PMNLs. For this purpose, the study group ($n = 239$) was specified as three groups including only the yeast form, only the filamentous form and both forms. In microscopic examination, macrophages and PMNLs were found in all three groups (yeast [+], filamentous [+] and yeast + filamentous [+]), and the association between different morphological forms and the presence of immune cells was not statistically significant ($P > 0.05$).

In the literature, switching from yeast to the filamentous form was reported as a virulence factor for dimorphic fungi.[1] In recent studies, Wellington *et al*. reported that transition is not a virulence factor; glycosylation of the cell wall component of fungi is more critical.[27] Additionally, Han *et al*. declared that morphological changes cannot affect the presence of macrophages, but they affect the receptor expression pattern. When macrophages are induced with yeast, it was shown that the TLR4 and dectin-1 expression had increased. However, induction with filamentous forms had increased TLR2 and dectin-2.[28] Compatible with these studies, our data show that both morphologies may seem to be considerable for virulence and does not affect the presence of macrophages and PMNLs.

Conclusion

In conclusion, PMNLs and macrophages have essential roles against fungal infection. The yeast form might have the ability to escape from neutrophil attracts, but further studies are needed to define the exact mechanism. In addition, neither the yeast nor the filamentous form affects the presence of neutrophil leukocytes and macrophages. This may suggest that both forms have pathogenic activity. A better understanding of host defense against fungal infection and escape strategies of fungi should help clarify the pathogenesis and could possibly lead to a new approach for therapies.

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