

## MINIREVIEW

# Anticandidal Immunity and Vaginitis: Novel Opportunities for Immune Intervention<sup>∇</sup>

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The majority of human *Candida* infections occur at a mucosal surface. One of these infections, vulvovaginal candidiasis (VVC), is a widespread, common disease affecting a large proportion of otherwise healthy women. Some women undergo recurrent forms of VVC (RVVC); in these cases the quality of life is devastated, the associated cost of medical visits is high, there is substantial use of unprescribed therapy, and there is a possible increase in drug resistance. However, relatively little is known about the pathogenic and immunoregulatory mechanisms underlying VVC and RVVC. While there is consensus that local rather than systemic factors play a decisive role in controlling the infection, there is no consensus about the nature of the local factors. The results obtained with animal models and human investigations have not provided an overall consistent picture; rather, they have generated divergent interpretations about the role of innate and adaptive immunity against vaginal infection. Nonetheless, recent experimental evidence has resulted in some optimism concerning the challenge of clarifying the immunological basis of susceptibility to, and protection from, vaginal candidiasis through the development of appropriate immune interventions to integrate with or even replace antifungal chemotherapy.

The aim of this review is to provide a short update of the evidence mentioned above and the resulting optimism. Here, we critically consider the linkage between the virulence traits of the fungus and the host responses to these traits, two interrelated aspects which have rarely been treated together in other reviews of the topic (36, 39–41, 48, 54, 56, 57, 65, 66, 74, 94, 102). On this basis, we suggest that despite substantial belief to the contrary, novel tools derived from adaptive immunity, in particular virulence-neutralizing antibodies (Abs), may become part of the anti-*Candida* armamentarium to fight vaginal infection.

### A PREMISE

*Candida albicans* is a human opportunistic fungus capable of stably colonizing the vaginal mucosa, thereby stimulating mucosal immunity in the absence of any sign or symptom of disease. While it is usually assumed that the transition from

asymptomatic colonization to symptomatic candidiasis occurs following a perturbation or loss of local defense mechanisms, this transition may also occur because of factors that enhance fungus virulence. Virulence factors are expressed by this fungus on the mucosal surface and have been shown to play a role in infection. Enzymes of the secreted aspartic proteinase (Sap) family and a number of protein or glycoprotein adhesins are key players in *Candida* pathogenicity (19, 39, 48, 68, 73, 74, 102, 123, 132). Virulence expression is also promoted by the capacity of this fungus to form hyphae (i.e., long, apically growing threads, which also have potential immunoescape properties) (39, 53, 87, 92, 137, 142). We hypothesized that the presence of such a “commensal” on the mucosal surface cannot be unnoticed or simply “tolerated” by the host; rather, humoral and cellular factors of both innate and adaptive immunity come into play to restrict fungus growth and neutralize its virulence (25, 28).

This hypothesis is supported by recent concurrent observations. For example, in human reconstituted vaginal epithelial tissue, some Sap family members (namely, Sap1 and Sap2) attack and derange the epithelial architecture, eliminating its physical and functional antifungal properties (121). In response to this severe offense, the epithelial keratinocytes produce a cascade of inflammatory cytokines (122), some of which (e.g., tumor necrosis factor alpha, interleukin-10 [IL-10], and gamma interferon) would typically induce a regulated and protective immune response in the normal host (114,115). A critical role may be played by Abs which neutralize the virulence traits. In fact, anti-Sap2, Fc-free domain antibodies (dAbs), which cannot rely on help from host immunoeffectors, inhibit Sap activity and adherence of fungal cells to rat vaginal tissue sections and by this mechanism provide strong pre- and post-challenge protection in the rat vaginal infection model (51). All these findings support the notion that there is a balanced interplay between fungus virulence and host immunity in the vaginal mucosal environment and suggest that commensalism results from such a balance and that disease results from perturbation of the balance.

Several epidemiological studies have documented that VVC is a widespread mucosal infection that may affect up to 75% of women of child-bearing age and that there are several predisposing factors, including antibiotic and oral contraceptive usage, hormone replacement therapy, pregnancy, and uncontrolled diabetes mellitus (7, 12, 54, 57, 99, 126). In turn, RVVC, usually defined as idiopathic with no known predis-

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posing factors, may affect up to 5% of all women who have a primary sporadic episode of VVC (57, 112, 126, 128). Antifungal therapy is highly effective for individual symptomatic attacks but does not prevent recurrences. In fact, maintenance therapy with an efficacious anti-*Candida* drug lengthens the time to recurrence but does not provide a long-term cure (127). Furthermore, there is concern that repeated treatments might induce drug resistance, shift the spectrum of causative *Candida* species, and result in an increased incidence of non-*C. albicans*, intrinsically resistant species (7, 31, 54, 81, 95, 111, 112, 140). The pathogenesis of RVVC remains unknown. Despite previous indications of a systemic immune deficit (147), there is now evidence that patients with RVVC are systemically immunocompetent. An anti-*Candida* immune dysfunction in these subjects, if there is any, must be rather subtle and restricted at the very local level and, for some forms of recurrences, could be genetically determined (2, 3, 32).

Epidemiology is of course very useful for inferring possible immunopathogenic mechanisms from prevalence and incidence data in defined population groups, provided that both sides of the coin are considered. In the case of VVC emphasis is always given to the estimated 5% of women with RVVC and the lack of demonstrated immune dysfunctions in these women. Equal, if not greater, emphasis should be placed on the 95% of women who, even after a first acute VVC attack, are not going to be affected by RVVC. These women may be colonized, but they remain largely disease-free for the rest of their lives, with only occasional, limited, and perfectly curable episodes during pregnancy or following some antibiotic therapy. This occurs despite more-than-frequent exposure to *Candida* and colonization from the gastrointestinal (GI) tract, suggesting that specific immunity is induced by the initial, even subclinical episodes; this immunity may be boosted by commensalism and effectively prevents the onset of chronic infection.

#### **CANDIDA COMMENSALISM IN THE HUMAN VAGINAL MUCOSA: A BALANCE BETWEEN AGGRESSIVE FUNGAL TRAITS AND HOST ACTIVE IMMUNE SURVEILLANCE**

The mucosa of the female reproductive tract, with its tissue architecture, cervicovaginal secretions, and fluid, has been shown to contain humoral and cellular constituents of innate immunity, together with cell populations required for initiating, recruiting, and maintaining an efficient adaptive immune response (36, 144).

On the side of innate immunity, several humoral and cellular factors have recently received particular attention. The mannose-binding lectin (MBL) is one such factor (89, 104). Reduced vaginal levels of MBL and nitric oxide and increased occurrence of polymorphism of the IL-4 (T-589) gene were found to correlate with high prevalence of RVVC in Latvian and Chinese women (3, 90). MBL has been shown to enhance complement activation (79), to be bound by *C. albicans* infecting mouse tissues, and to provide protection against experimental infection (89). Other humoral factors of innate immunity include a number of soluble proteins (e.g., lactoferrin) and cationic antimicrobial peptides, particularly the defensins (36).

However, a definite anti-*Candida* role for any specific peptide in the vaginal environment has not been established.

A critical antimicrobial defensive role is played by the epithelial cells (EC) lining the whole reproductive tract (145, 146). These cells not only provide a mechanical barrier but also function as sentinels that recognize and process antigens, and they secrete a plethora of immune mediators (chemokines, cytokines, defensive peptides) which orchestrate innate immunity and regulate adaptive immunity (38, 145). They are under the influence of sex hormones in a delicate balance which allows maintenance of immune surveillance against pathogens while providing the degree of immune tolerance needed to accept semen and a fetus (78, 142). In particular, human vaginal EC have been shown to possess intrinsic anti-*Candida* activity which was not influenced by the menstrual cycle but was lower in RVVC patients than in healthy subjects (4, 65). This activity has been shown to be estrogen dependent, suggesting that estrogens permit vaginal infection not only by allowing the well-known histological modifications of the vaginal tissue but also by decreasing the EC inhibitory potential. Nonetheless, the anticandidal activity of vaginal EC was found to be low in adolescents, who intriguingly are most resistant to a high vaginal *Candida* burden (5, 65), and does not require cellular viability (65, 66). Coupled with the observation that the human vaginal epithelium can be easily damaged by virulent *C. albicans* (42, 48, 121), the protection conferred in vivo by these cells is likely to be overcome by a virulent fungus. To be protective in clinical situations, EC probably require the cooperation of other factors of both innate or adaptive immunity, including Abs. It would be of interest to know whether Abs enhance the antifungal activity of the EC, particularly in view of the EC capacity to release complement (145). Complement fixation on opsonized fungal cells may be a very efficient mechanism whereby polymorphonuclear cells (PMN) (neutrophils) normally present in the genital tract (145) and the EC themselves may remove fungal cells from the vaginal cavity (however, see below for the role of PMN in vaginal infection).

Dendritic cells (DC) represent the link between innate and adaptive immunity and play a critical role in driving and orchestrating cell-mediated and humoral immunity. DC are scattered throughout different portions of the vagina and cervical epithelia and express surface receptors which bind *Candida* molecular patterns and transmit intracellular signals leading to cell activation, inflammation, and induction of adaptive immunity. The major and most intensely studied receptors are those of the Toll-like family (6, 8, 110, 113), but non-Toll receptors also play critical roles in fungal pathogen recognition. Some of these receptors (e.g., dectin-1) recognize molecular patterns common to all fungi ( $\beta$ -glucan) (16), while others bind specific *Candida* components. For instance,  $\beta$ -1,2-mannosides, which are mannan-type constituents present exclusively in pathogenic *Candida* spp., are recognized by the S-lectin galectin-3, which cooperates with Toll-like receptor 2 for signaling (82). Importantly, galectin-3 binding may directly cause the death of fungal cells without cellular internalization. This activity may be relevant for anti-*Candida* defense at the mucosal level since the galectin-3 receptor is present on EC as well as on macrophages and DC (86). A critical role for vaginal DC in modulating resistance to vaginal infection by *Candida* has recently emerged

TABLE 1. Similarities, differences, and uncertainties between human *Candida* vaginitis and animal models of *Candida* vaginal infection

Condition	<i>Candida</i> vaginal infection			References
	Human	Rat	Mouse	
Estrogen dependence	Yes	Yes	Yes	35, 54, 78, 83, 126, 130, 133
<i>Candida</i> colonization	Yes	No	No	54, 57, 65, 66
Protective immunization after resolution of primary infection	Unknown <sup>a</sup>	Yes	Yes	26, 48, 62, 65
CD4 <sup>+</sup> T cell/CD8 <sup>+</sup> T cell ratio in the vaginal mucosa	CD8 <sup>+</sup> T cells prevalent	CD8 <sup>+</sup> T cells prevalent	CD4 <sup>+</sup> T cells prevalent <sup>b</sup>	26, 47, 63, 67, 78, 110
Role of vaginal T cells	Probably protective <sup>c</sup>	Protective	Uncertain <sup>d</sup>	47, 50, 59, 61, 100, 129, 145–147
Role of vaginal Abs	Uncertain	Yes	Uncertain <sup>d</sup>	26, 45, 49, 70, 71
Role of vaginal DC	Probably protective <sup>c</sup>	Protective	Tolerogenic <sup>e</sup>	50, 88, 145, 146
Role of neutrophils in disease	Yes <sup>f</sup>	No	No <sup>g</sup>	10, 64
Role of Sap and adhesins in disease	Yes	Yes	Probably yes <sup>h</sup>	19, 29, 46, 49, 51, 59, 73, 101, 117, 122
Role of hyphal transition in disease	Yes	Yes	Probably yes <sup>h</sup>	24, 51, 64, 70, 117

<sup>a</sup> As hypothesized in this review, healing of the primary infection and commensalism at least partially immunize against disease in most women.

<sup>b</sup> May be dependent on the mouse strain.

<sup>c</sup> Indirectly inferred by mucosal immunology and pathology of candidiasis at other mucosal sites.

<sup>d</sup> Contrasting results are obtained with different mouse models.

<sup>e</sup> Inferred from the DC phenotype.

<sup>f</sup> Clear data were obtained from experimental infections in volunteers, but data are not consistent for routine clinical specimens.

<sup>g</sup> Neutrophils determine inflammation but do not influence the *Candida* vaginal burden.

<sup>h</sup> Based on a few ad hoc studies.

from experimental investigations with rodents and is discussed below.

Vaginal T cells have recently been characterized and quantified in the lamina propria and the epithelia of the human vagina and cervix. There are about 240 T lymphocytes per mm<sup>2</sup> of vaginal epithelial tissue (78). Despite rather marked inter-subject variations, in all cases CD8<sup>+</sup> T cells appear to be more numerous than CD4<sup>+</sup> T cells, and the ratio approaches 1.25. CD4<sup>+</sup> T cells appear to be more abundant in the transitional zone between the exocervix and the endocervix. Little more than 100 CD8<sup>+</sup> T cells and fewer than 100 CD4<sup>+</sup> T cells per mm<sup>2</sup> of vaginal tissue were found in normal women (110). The large majority of vaginal T cells have a memory CD45RO<sup>+</sup> phenotype, and very few of them express the E-cadherin ligand CD103, which is an epithelium homing factor for lymphocytes. All this information suggests that most vaginal T cells migrate to the vaginal epithelium in response to a local antigenic stimulus and/or inflammatory chemokines. The same appears to be true for B cells and immunoglobulin (Ig)-secreting plasma cells which are normally absent in the healthy vagina but are recruited into the vaginal tissue following appropriate immunization (57, 78, 110). Notably, the CD4<sup>+</sup>/CD8<sup>+</sup> vaginal T-cell ratio in healthy women is more similar to that in the rat vagina (47) than to that in the mouse vagina (63) (Table 1). Finally, Abs to *C. albicans* constituents have been detected in the vaginal fluid of women and all other animal species tested at levels similar to those found in cervical secretions (11, 96, 141), and these levels are representative of the serum Ab composition, without discounting possible local Ab production. Although the biological significance of the proportions of the different subsets of T lymphocytes and their relationship with B cells is not known, the numbers given above support the overall contention that the reproductive tracts of women and other mammals are well equipped to fight infection with immunological weapons, although not in such an organized way as in

the lymph nodes or Peyer's patches of the GI tract. It is therefore not surprising that colonization of the vaginal mucosa by *C. albicans* induces both humoral and cell-mediated immunity which is expressed both locally and at the systemic level (58, 59, 146, 147). This highly focused immunity may not "eradicate" the fungus because of the possible interplay of fungal immunoevasion mechanisms and regulatory, immunosuppressive T cells in a particularly tolerogenic tissue, such as that of the reproductive tract. The presence of inflammatory cells (macrophages and neutrophils) and the constitutive expression of proinflammatory cytokines by epithelial and stromal vaginal cells (55, 110, 144, 145) make it conceivable that, as in the GI tract, a low-grade, sustainable "inflammation" is the normal vaginal status, with a balance between antimicrobial immunity and containment by humoral and cellular regulatory mechanisms. Hormonal factors and the resident flora itself could contribute to immunoregulation by stimulating anti-inflammatory responses (41, 56, 131). We speculate that what is probably required for avoiding disease is only control of the virulence traits of the fungus (see below), which may allow the transition from commensal status to pathogenic status, control that is likely to be better achieved by concurrent mobilization of innate and adaptive immune responses.

#### HUMORAL IMMUNITY IN *CANDIDA* VAGINITIS: A VEXATA QUÆSTIO

Vaginal colonization by *C. albicans* induces humoral responses, but these responses are not usually considered important for avoiding infection. Two clinical findings always cited in support of this conclusion are the finding that *Candida* vaginitis is not more frequent in subjects with Ig disorders and, conversely, the finding that subjects with candidiasis often have high titers of anti-*Candida* Abs. Additionally, there are reports showing that the presence of Abs does not preclude develop-

ment of mucosal and disseminated candidiasis (1, 84, 96, 114). Thus, the majority of authors tend to be dismissive of the role of Abs, and this dismissive view has been authoritatively expressed (65, 66).

The observations described above do not rule out the possibility of an anti-*Candida* protective role for Abs or, even less so, the possibility of generating immunotherapeutic Abs. First of all, vaginal Abs have rarely been investigated to determine their fine specificity, affinity, titer, and isotype; all of these qualities are essential to determine whether Abs are protective or nonprotective in a given host-microbe setting. The presence or absence of “anti-*Candida* Abs” whose qualities have not been determined is close to meaningless per se. On the other hand, the use of monoclonal Abs (MAbs) has demonstrated that only a limited number of specific epitopes can be protective, as observed with other pathogens (22, 72, 77). As recently discussed by Burton and Parren (17), pathogens that have evolved as commensals (and, probably still more, pathogens causing chronic persistent infections) usually elicit poor neutralizing and potentially eradicating Ab responses, and this is obviously relevant to their (privileged?) status in the host. Only rapidly transmissible bacteria and viruses (for example, *Streptococcus pneumoniae* and influenza virus) causing acute infections elicit strongly neutralizing Abs as their evolutionary survival is ensured not by their persistence in a given host but rather by their rapid transmission and spread in the community. Commensal microbes have something in common with microbes causing persistent infections: they can induce the generation of a plethora of irrelevant Abs to dilute and render ineffective a potentially eradicating host response. This is combined with their capacity to thrive at particularly tolerogenic sites, such as mucosa or skin.

As elegantly described for *Cryptococcus neoformans* by Casadevall and his collaborators (20–22) and also highlighted by Cutler et al. for *Candida* (40), polyclonal anti-*Candida* sera contain a plethora of irrelevant Abs targeting dominant cell-surface-located fungal antigens which not only are nonprotective but also can inhibit the activity of protective Abs. For instance, the killer-toxin-mimicking anti-idiotypic and anti- $\beta$ -1,3-glucan Abs—which express a strong fungus-eradicating potential for fungicidal action—compete strongly in vivo with Abs against other cell wall components (14, 15, 94, 107, 141). In a more subtle fashion, anti- $\beta$ -mannoside Abs are protective, whereas anti- $\alpha$ -mannoside Abs are not protective (69, 71). Configuration-dependent, epitope-selective binding with the same molecular pattern may have opposite or competing effects. The substantial presence of nonprotective epitopes on abundant cell surface molecules, such as the mannans, is probably advantageous for the fungus and may allow it to avoid eradication by candidacidal Abs and thus persist as a mucosal commensal (15, 26, 69, 94, 98, 138). In summary, all these data strongly suggest that specific Abs (and other immunological tools) can be generated in an attempt to overcome fungus persistence and the onset of vaginal infection. A strong case is represented by the clinical efficacy of an anti-heat shock protein of *C. albicans* MAb (Mycograb) against invasive candidiasis (97) in a setting where there has been a consensus, derived from experimental studies, that cell-mediated immunity is the protective mechanism (115). All this indicates that it is necessary to identify and sharply dissect *Candida* virulence traits and

protective antigens and immune responses and also to modify these factors to achieve protection. This has in part been done by use of animal models of vaginal infection and is discussed below.

### EXPERIMENTAL CANDIDAL VAGINITIS

Animal models have been useful for studying various aspects of *Candida* pathogenicity and the anti-*Candida* immune response under conditions possibly mimicking human infection. Experimental *Candida* vaginitis has been reproduced especially in mice and rats under pseudoestrus conditions, but a primate model has also been explored (130). With estrogen administration, the vaginal epithelium is thicker and fully keratinized (83), which offers a substrate to the fungus for attachment, growth, and biofilm formation (33). Hypha formation, adherence, and Sap production are also enhanced by the production of the keratin layer. In addition, the estrogens down-modulate cell-mediated immunity and reduce leukocyte infiltration (78, 83), which helps fungal growth and establishment of infection (see below for additional discussion of the role of leukocytes in candidiasis). Direct estrogen binding to a protein receptor of *C. albicans* has also been suggested to be a determinant for vaginal infection in rats (133). In both mouse and rat models, *Candida* infection is acquired only with estrogen induction, and animal susceptibility to infection strongly correlates with estrogen sensitivity (35), which are facts that are important for extrapolation to the human clinical situation. In fact, human vaginal infections occur almost exclusively during the fertile years and are extremely rare in premenarchal and postmenopausal women, and the prevalence and severity increase in the premenstrual week (54, 126), conditions that are all clearly influenced by estrogens. There is therefore consensus that the models described above are somewhat representative of human disease and that knowledge of the expression of virulence by the fungus and the related immune responses in the animal models may help us understand mechanisms of *Candida* infection and immunity in women. Two main caveats must, however, be considered when parallels between humans and animals are discussed. First, *C. albicans* is a commensal in the vaginas of most women, whereas it is not a commensal in either the mouse or rat vagina. Thus, women are immunologically “primed” against the fungus, while the animals are not. Second, experimental vaginal infection in rodents is achieved by direct, intravaginal inoculation of a rather large fungus inoculum (usually between  $10^5$  and  $10^6$  cells) that is unlikely to be the typical mechanism of human infection, which probably occurs by “exacerbation” of the resident *Candida* or influx of fungal cells from the intestinal reservoir. In addition, little or no effort has been made to establish experimental models reflecting different presentations of vaginal candidiasis, including allergic or atopic forms and candidiasis of immunosuppressed subjects. Some data on vaginal infection in neutropenic mice have been reported (10). Finally, there are no experimental models of RVVC, the syndrome that is most clinically relevant. In fact, in the two best-studied experimental models (mouse and rat) the experimental infection is either self-healing or persistent with a low fungus burden, and in both cases it effectively immunizes the animals against any secondary challenge (26, 61, 62). In other words, experimental vaginal

infection in rodents elicits memory responses that protect against reinfection.

Efforts have also been made to reproduce the vaginal infection in *in vitro* studies by using reconstituted human vaginal epithelial tissue (121–123) or vaginal tissue sections (51). Useful information has been obtained, particularly with the former model, but again, caution is necessary when direct comparisons between the *in vivo* data and the data obtained with this model, which is based on a keratinocyte tumor cell line, does not require estrogenic conditioning, and has no afferent immune control, are made.

Special mention should be made of the experimental vaginal infection of women performed by Fidel and collaborators (64). These authors used live fungal challenge with volunteers with different susceptibilities to vaginal infection by *C. albicans*. The clinical signs of the vaginal infection caused in predisposed subjects were all attributed to PMN infiltration into the vaginal tissue, the intensity of which correlated with the intravaginal fungus growth. Importantly, no signs of inflammation were detected in the subjects without predisposing factors, most of whom resisted the challenge. These interesting data were interpreted in terms of varied susceptibility or resistance of vaginal EC to *Candida* and the related chemical signals allowing massive recruitment of PMN in the vagina (i.e., inflammation) (64, 66). A direct correlation between *Candida* burden and vaginal disease was also established (64), somewhat strengthening the relationship between clinical and experimental vaginitis, which is actually measured by the burden of fungal cells in the animal vagina. However, no data were provided for the virulence traits or other *C. albicans* factors interacting with EC and stimulating their proinflammatory signaling.

Despite all the caveats, the experimental models of vaginal candidiasis have provided unique, invaluable information about *Candida* pathogenicity and the immune response, which constitutes the logical basis for immune intervention against vaginal candidiasis in women, as outlined below.

***Candida* attack.** The virulence factors of *Candida* that have been studied most by using experimental vaginal candidiasis include various adhesins, the yeast-to-hypha transition, phenotype switching, antigen variation, and the production of enzymes, especially Saps. However, formal demonstration of a role in infection has been obtained for only some of these factors by use of knockout mutants and reinsertion of relevant genes (46, 87, 117). Adhesins may play both a direct role in pathogenesis (by fostering adherence to vaginal tissue) and an indirect role (by exerting a cell wall-remodeling activity needed for hypha transition). Cases in point are some glycosylphosphatidylinositol-anchored proteins (73, 129, 132) or other cell wall  $\beta$ -glucanase and/or transglucosidase enzymes (52). For example, the MP65 mannoprotein has the typical sequence and structural motifs of a  $\beta$ -glucanase or transglucosidase enzyme and has recently been shown to be involved in the adherence of *C. albicans* to vaginal epithelial cells and, at the same time, to be critical for hypha formation (51, 117). Interestingly, MP65 has long been recognized as a major target of the human cell-mediated immune response against *Candida* (28). Conversely, the pathogenicity potential attributed to the hyphal cells may rightly impinge on multiple and redundant adhesion expression (132).

The Saps constitute a family of 10 aspartyl proteinase en-

zymes whose sequences, biochemical properties, and *in vitro* and *in vivo* expression differ so markedly that they constitute at least three different lineages (74, 102, 123, 143). Sap1 to Sap3 share around 70% homology, have an optimal pH for activity in the acidic range (pH 3 to 5), and are thought to be expressed mostly in cutaneous and mucosal infections, both experimental and clinical (101). Sap4 to Sap6 share around 85% homology, have an optimal pH for activity in the near-neutral range, and exhibit preferential expression in systemic infections (102). However, in a model of vaginal infection of BALB/c mice, Sap4 and Sap5 were also expressed, and this was attributed to the higher pH of mouse vaginal fluid than of the vaginal fluids of rats and women (135). Moreover, Sap5 has recently been demonstrated to degrade E-cadherin, a major protein of epithelial cell junctions, thus facilitating invasion of oral epithelial cells in an *in vitro* culture with a human tumor origin (141). All this suggests that substrate specificity and local tissue factors probably add to pH restraints for the expression and activity of the various Saps. There is relatively little information on the other four Sap family members, but they have varied properties and exhibit low homology with the two groups described above and some of them are nonsecretory but glycosylphosphatidylinositol-anchored proteins (74). Overall, they may be less relevant for mucosal infection by *C. albicans*.

Old correlative phenotypic studies suggested that Saps could play a role in vaginal candidiasis. In fact, candidal vaginitis in women was strongly associated with high levels of *in vitro* Sap expression by vaginal isolates of *C. albicans* and elevated Sap levels in the vaginal fluid (24, 42). Moreover, Sap2 gene expression correlated with vaginal infection in rats (44). These findings were later corroborated by the observations that (i) inhibitors of Sap activity, such as pepstatin A and human immunodeficiency virus protease inhibitors (human immunodeficiency virus protease is also an aspartyl proteinase), are therapeutic in experimental models of candidal vaginitis (29, 37, 74) and (ii) Sap-deficient (116) or Sap1 to Sap3 knockout mutant strains of *C. albicans* (46) do not cause vaginal infection in rats and are not capable of damaging the reconstituted human vaginal epithelium (both pathogenic properties were regained by strains in which the Sap1 to Sap3 genes were reinserted) (46, 121). This did not occur with Sap4, Sap5, and Sap6 individual or triple-knockout mutants (46). More recently, the expression of some Saps has been clearly correlated with active vaginal infection by *Candida* in women. Naglik et al. (101) have shown that Sap2 is the enzyme that is constantly and permanently expressed, both during commensalism and during active vaginal infection.

The more recent studies have shown how complex the *in vivo* profile of Sap expression can be and how the different Saps could cooperate and sequentially affect virulence expression at different times during *C. albicans* infection (102). Nonetheless, based on consideration of all published data, Sap2 (together with the highly homologous enzymes Sap1 and Sap3) has emerged as a protein that plays a particularly consistent role in the relationship between *C. albicans* and vaginal tissue, probably more than any other single virulence trait. Sap2 is the member of Sap family with the broadest substrate specificity. It is capable of hydrolyzing structural host proteins, and the keratin of the skin and the keratin lining the surface of the vaginal epithelium are quite good substrates. Furthermore, practically

all humoral factors of innate and adaptive immunity, including complement and Ig, are highly susceptible to Sap2 activity (74). By this combined action, Sap2 can degrade the whole epithelial tissue, as clearly seen with the reconstituted human vaginal epithelium (121). The optimum pH is between 2.5 and 5, and the vaginal environment, which is rich in proteins and peptides of host and microbial origin, is very well suited to the mechanism of Sap2 induction and expression (74, 102). Overall, in three different experimental and clinical settings (i.e., reconstituted human vaginal epithelial tissue, rat vagina, and human vagina), similar decisive roles of Sap2 in colonization and infection have been reported. Not surprisingly, therefore, active immunization with Sap2 protein is preventive, and anti-Sap2 Abs are therapeutic in the rat vaginitis model (26, 45–47, 51).

What is really not known is the precise mechanism whereby Sap2 confers high colonizing and vaginopathic potential to the fungus. Whether this has to do with (i) destruction by proteolytic cleavage of humoral and cellular factors of the immune response, (ii) adherence and degradation of the EC keratin component, (iii) direct damage to the EC themselves, or (iv) simultaneous or sequential combinations of these three or other factors is not known, and further investigations are necessary.

There is also clear evidence that the capacity to develop hyphae is required for vaginal infection, similar to *C. albicans* infection of other tissues (87, 92, 117, 120). Tissue sections of animal vaginas show that hyphae strongly adhere to the keratinized surface of the vaginal epithelium, with some hyphal tips slightly infiltrating the subepithelial layer (43, 51). Some adhesin-encoding genes are critically expressed during hypha formation (87, 117, 132). Moreover, the dimorphic switch has been associated with hyphal elongation and expression of a specific Sap family member (74). As reviewed by Kunamoto and Vences (87), genes controlling hyphal morphogenesis either upstream (encoding transcriptional factors) or downstream (encoding cell wall proteins) are all coregulated with other genes encoding virulence traits, such as some Saps and some adhesins, in such a manner that hyphal transition is directly bound to pathogenicity. This is clearly demonstrated by the fact that each deletion of relevant genes affecting hyphal transition directly translates into a decrease in or abolition of experimental pathogenicity (87). Although most of the evidence for this relationship has been derived from systemic rather than mucosal infection models, it is logical to expect that deletions of genes affecting hyphal transition will have a similar impact on vaginal infection. Recent examples are the Als (73, 132) and MP65  $\beta$ -glucanase (117) adhesins, whose deletion impacts hyphal morphogenesis and virulence in vaginal infections, as well as in systemic infections.

Overall, Sap expression, adherence, and hypha formation appear to be intimately interconnected in the vaginal environment, although it is not clear which one is the key orchestrating factor.

Not surprisingly, all the virulence traits described above have been associated with the immunoescape potential of the fungus. The transition from the commensal yeast cells to the pathogenic hyphae is accompanied by genetic reprogramming ultimately leading to remarkable antigenic and structural variations in the cell wall, avoidance of phagocyte internalization and killing, and perturbation in DC function in *in vitro* and *ex*

*vivo* experiments (53, 136, 137, 143). Germ tubes (i.e., the precursors of hyphal forms) are found on the vaginal epithelium of the rat vagina a few hours after intravaginal challenge and continue to overlay the epithelia without any sign of leukocyte infiltration from distant tissue. Production of PMN-attracting chemokines, such as MIP-1 $\alpha$  and -1 $\beta$ , monocyte chemoattractant protein 1, and IL-8, by human monocytes *in vitro* is induced well by yeast forms of *C. albicans* and is induced much less by hyphal forms (34, 136). Notably, the observation that activated leukocytes may kill hyphae extracellularly (139) suggests that hypha formation may be instrumental in keeping these candidacidal cells away from a very sensitive target. An anti-Sap immunoevasion mechanism is also suggested by the observation that some anti-Sap Abs do not inhibit enzyme activity (103). However, human engineered dAbs exclusively made up of Ab variable regions inhibit Sap2 enzyme activity, as well as adherence to rat vaginal tissue sections, and are protective *in vivo* (51). A caveat here is that there is little *in vivo* evidence of any of these immunoescape mechanisms. Moreover, the redundancy of innate and adaptive anti-*Candida* immunity suggests that these potential immunoescape mechanisms would probably be irrelevant in a normal host. However, they could enhance fungus spread in an immunocompromised host.

**Vaginal immune response.** Mucosal tolerance and innate immunity are believed by some authors to be the only relevant defensive factors against vaginal infection by *Candida*. In particular, extensive work by Fidel and his collaborators with a murine model of candidal vaginitis has given no apparent indication that local factors of adaptive anti-*Candida* immune responses have an impact on the outcome of infection (58–61; for reviews, see references 65 and 66). However, other animal models have provided valid data showing that adaptive humoral and cellular immunity may add to the innate defense and play a relevant protective role.

In the rat model of vaginal infection, primary and secondary challenges with the fungus induce adaptive immunity and protection clearly related to each other, as demonstrated by passive transfer of vaginal Abs and adoptive transfer of vaginal T cells (26, 45, 47, 119). In addition, animals could be actively and passively vaccinated against infection by both intravaginal and intranasal routes by use of protective *Candida* antigens and Abs, respectively (45, 47, 49). There are doubtless differences between our rat model and the mouse model studied by Fidel and collaborators which could, in part, explain the different results. The two models, for instance, differ in the ratio of vaginal CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In particular, CD8<sup>+</sup> T cells, which are more numerous than CD4<sup>+</sup> T cells both in the rat and in the human vaginal mucosa, have been reported to be present in very low numbers compared to CD4<sup>+</sup> T cells in the CBA/J mouse (H-2<sup>k</sup> haplotype) (63). Intriguingly, in other mice (BALB/c mice; H-2<sup>d</sup> haplotype) CD8<sup>+</sup> T cells have been found to be more numerous than CD4<sup>+</sup> T cells, and it has been suggested that they play a protective role (67). A relative deficiency of CD8<sup>+</sup> T cells may be of particular significance given that the number of these cells increases during vaginal infection in BALB/c mice (67) and rats (47). In an unrelated model, recent evidence showed that CD8<sup>+</sup> T cells, together with CD1 expression, provide help for Ab production against polysaccharide antigens (85). CD3<sup>-</sup> CD5<sup>+</sup> B cells have been identified in

rat vaginas and have been shown to participate in the protection obtained by adoptive lymphocyte transfer (47, 50). In another mouse model (100), protection from vaginal candidiasis was achieved by adoptive transfer of splenic T lymphocyte from *C. albicans*-immunized mice. Depletion of CD3<sup>+</sup> or CD4<sup>+</sup> T cells before transfer completely abrogated protection.

Together with the induction of protective memory responses following resolution of the primary infection in both mouse and rat models (see above), classical tools of adaptive immunity, such as vaccines and Abs, have been shown to efficiently control vaginal infection in animal models. Han et al. (70) obtained strong evidence that the protection induced by a mannan-liposome (L-mann) vaccine against vaginal infection in mice was mediated by Abs. In this model, two cell wall mannan-directed IgM MAbs, MAbs B6 and B6-1, which agglutinated *C. albicans* yeast cells were generated. However, MAb B6 recognized an epitope located in the acid-stable part of phosphomannan, while MAb B6-1 recognized a  $\beta$ -1,2-mannotriose which is the acid-labile component of phosphomannan. Intravaginal administration of MAb 6-1, but not intravaginal administration of MAb B6, resulted in a highly effective reduction in vaginal colonization by *C. albicans*. These results are further proof that cell surface-located epitopes of *C. albicans* may induce either protective or nonprotective Abs. It is rather intriguing that the  $\beta$ -1,2-oligomannosides constitute a molecular pattern which is specifically recognized by galectin-3 (82), a lectin which has recently been shown to induce *C. albicans* cell death (86).

A particularly important role in the anti-*Candida* protection at the vaginal level could be played by the antigen-presenting cells which transduce differentiation and activation signals from innate to adaptive immunity. The most important of these cells are the DC, which, despite their functional plasticity, have been divided into different subsets with distinctive biological properties (91).

Data on identification and the function of vaginal DC were reported almost simultaneously for the mouse (88) and rat (50) models. A remarkable difference that may help explain the different immunological outcomes and relative interpretations has emerged. In the murine model, the predominant DC subset during infection was plasmacytoid DC lacking maturation markers, consistent with tolerance induction, although no data on functional tolerance in terms of absence of priming for T-cell proliferation and production of cytokines (for instance, IL-10 [80]) were obtained. In contrast, myeloid and lymphoid OX62<sup>+</sup> DC subsets were isolated from the rat vagina, and the two subsets acquired maturation markers during infection and had different cytokine production patterns. Notably, no plasmacytoid DC (lacking the OX62 DC-specific marker [75]) were isolated. Rat vaginal DC expressed CD134L, a marker of the tumor necrosis factor-tumor necrosis factor receptor superfamily, which is required for optimal B-cell responses. Moreover, relatively low numbers of vaginal DC from immunized rats that were injected into the vaginal cavities of naive rats 1 day before challenge with *C. albicans* conferred a substantial degree of protection against the vaginal infection. Protection was associated with the ability of DC to rapidly migrate to the vaginal mucosa and lymph nodes and prime T-cell proliferation (50).

The conclusions of the experimental studies summarized

above indicate that in the mouse and rat models used so far there are intrinsic differences in the immune responses following *Candida* infection and immunization, which can also depend on the mouse strain and different methodological approaches used to identify and characterize the vaginal immunoeffectors. Nonetheless, the findings derived from the rat model are positive and suggest that it may be possible to induce and expand adaptive immunity that may prevent and control vaginal infection by *Candida*. The observations made with the murine models are somewhat contrasting but do not rule out the possibility of intervention by the specific immunological tools of adaptive immunity. Table 1 summarizes the main differences observed between the rat and mouse models and also shows the relationship to human infection.

#### RECENT EVIDENCE FOR PROTECTION BY Abs AND Ab-MEDIATED VACCINATION

From the experimental data reported above, a remarkable piece of evidence suggests that Abs directed against virulence traits of *C. albicans* can confer protection against vaginal infection in a preclinical setting. A number of protective Abs with different formats (polyclonal, monoclonal, single chain/variable fragment, Ab domains, murine or human) have been generated (26) and have been shown to work both as opsonins (18) and as adhesin inhibitors (118) or by exerting direct candidacidal or candidastatic effects (23, 27, 93, 94, 98). Some Abs have been generated by various types of anti-*Candida* vaccines (30, 98, 107, 108, 138). The Abs recognizing fungal  $\beta$ -glucan have recently been shown to inhibit hyphal growth and provide protection against vaginal (and systemic) infections (30, 138).

Recently, the mechanisms by which anti-MP65 adhesin and anti-Sap2 Abs protect against vaginal candidiasis have been investigated, particularly by use of human dAbs (51, 117). These dAbs are genetically engineered human Ab fragments comprising individual V<sub>H</sub> or V<sub>K</sub> variable domains and lacking the Fc region. Anti-MP65 and anti-Sap2 dAbs which strongly inhibited the adherence of *C. albicans* cells to rat vaginal tissue were generated (51). These dAbs were highly protective against rat vaginal infection with both prechallenge and potentially therapeutic, postchallenge administration schedules. In particular, the administration of heterodimeric bispecific dAbs targeting both MP65 and Sap2 cleared the infection with an efficacy equivalent to that of treatment with fluconazole for a drug-susceptible strain, and it was clearly superior when the challenge organism was a fluconazole-resistant strain (51). This result strengthens previous observations made when single-chain, variable-fragment Abs or peptides from Ab CDR regions were used (9, 109) and demonstrates that protective Abs directed against some virulence traits of the fungus may not require the participation of inflammation and natural immunoeffectors for protection.

#### A POSSIBLE UNIFYING HYPOTHESIS

As in all models of host-parasite interactions, protective cell-mediated and humoral responses need to be finely tuned and regulated to avoid overwhelming stimulation which may turn into local inflammation and host damage. Together with

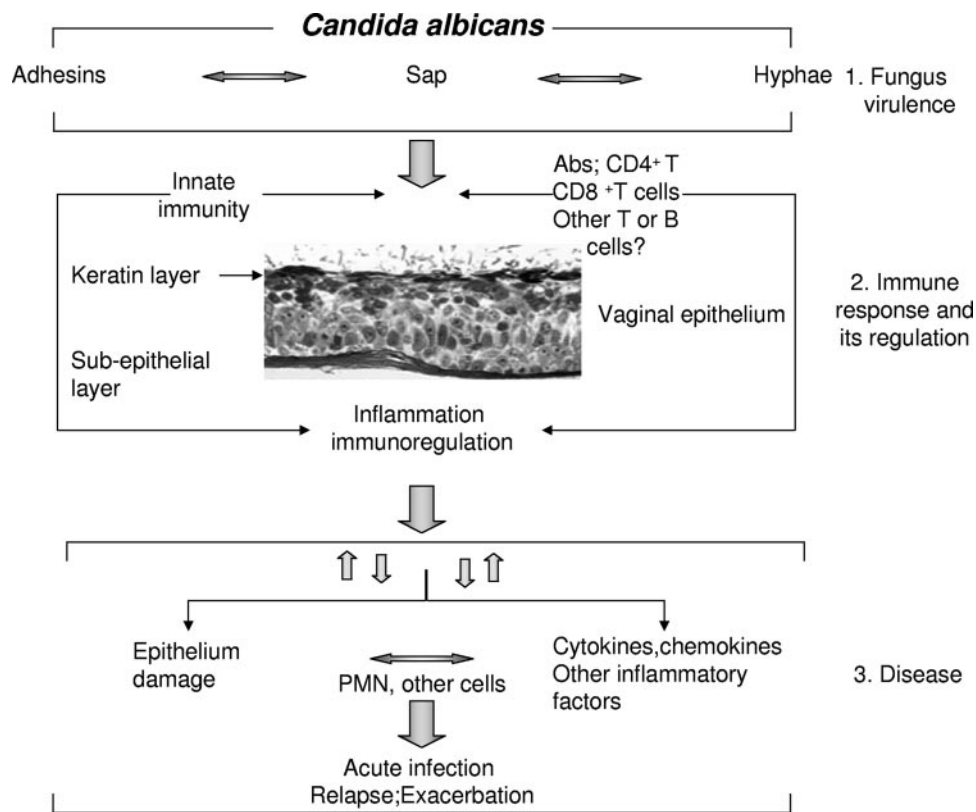


FIG. 1. Schematic, mostly theoretical representation of the *Candida*-host relationship in candidal vaginitis, as inferred from experimental models and clinical considerations, as discussed in the text. The three stages are obviously closely linked (as indicated by bidirectional arrows) and are represented as sharply separate stages only to illustrate mechanisms and consequences. The basic assumption (stages 1 and 2) is that the fungus may reside commensally in the vagina because of the equilibrium reached between the expression of virulence traits (stage 1) and the immune response, both innate and adaptive, of which an epithelial functional barrier, fungus immunoescape, immunoregulatory mechanisms, and local tolerance are important components (stage 2). Disease (stage 3) occurs when excess virulence, which may also be due simply to an overburden of fungal cells (attack numerator), causes damage to epithelial cells and overwhelms immune responses and their regulation (defense denominator). In some women, the denominator may be particularly low for unknown (possibly genetic?) reasons, and these women would be prone to repeated episodes and exacerbation of vaginitis. Two noteworthy consequences of this model are that *C. albicans* adherence and damage to the epithelial cells constitute the most critical pathogenicity event and that factors inhibiting adherence and damage are likely to be protective. Abs (from active or passive vaccination) are direct therapeutic options; less direct options are those affecting inflammation and immunoregulation (cytokines, probiotics, etc.). The model does not consider the seemingly small proportion of women in whom vaginitis may be due to allergy to *Candida* products (atopic subjects).

fungus attributes allowing commensalism, the immunoregulatory mechanisms may play a central role in the vaginal environment (115, 134). Some immunoregulatory disorders of bacterium-harboring peripheral tissues involve excessive stimulation of Th1-type responses with the associated production of proinflammatory cytokines and chemokines and excess recruitment of inflammatory cells to the mucosal site. Many more Th1-type T-cell clones secreting proinflammatory cytokines can be generated from the vaginas of RVVC subjects than from the vaginas of non-RVVC subjects (106), and there is some evidence that IL-23, a cytokine whose overproduction is critically associated with some types of inflammatory intestinal disorders (76), is secreted during vaginal infection (148). IL-23 induces Th17 cells and IL-17, which potently recalls inflammatory cells at the mucosal site. The two defensive but proinflammatory axes IL-12—gamma interferon and IL-23—IL-17 can both contribute to pathology when there is a great deal of stimulation or in the absence of dampening immunomodulatory mechanisms. As an attractive, although at present

merely hypothetical, view, it could be suggested that the predominant forms of vaginal candidiasis are inflammatory immune disorders to which overwhelming Th1-Th17 stimulation is coupled with inflammatory responses by the vaginal EC (and possibly stromal cells and resident phagocytes) stimulated or damaged by *Candida* virulence traits. This would be in accord with (i) the documented leukocyte-driven inflammation in experimental *Candida* challenge in women; (ii) the potential regulatory role of Th2 responses, plasmacytoid IL-10-secreting DC, and transforming growth factor  $\beta$ ; and (iii) the observation that Abs blocking virulence traits of the fungus and inhibiting adherence to EC are protective. These Abs may well inhibit the hyperstimulatory fungal burden in the vagina. In fact, Abs, through their Fc binding to inhibitory Fc-gamma receptors, may constitute a rather potent means to dampen excessive inflammation (22). Our hypothesis is shown in Fig. 1. This hypothesis links *C. albicans* virulence to immune responses, both innate and adaptive, and may be worthy of further investigation.



### IS THERE A PERSPECTIVE FOR IMMUNE INTERVENTION AGAINST CANDIDAL VAGINITIS?

Vaginal candidiasis is a disease that is easily controlled by efficacious chemotherapy when it sporadically occurs in the presence of predisposing factors. Conversely, it is a malignant infection that may destroy the quality of life for some women who are predisposed to recurrences. It seems possible that these unlucky women could be protected by local or even systemic infusion of suitable humoral and/or cellular factors of innate and adaptive host immunity. According to the hypothesis shown in Fig. 1, there are a number of attack sites for both preventive and therapeutic intervention, including the use of immunomodulatory compounds or probiotics that keep the level of vaginal immunoreactivity in balance with the resident flora and *Candida* as a commensal.

Nonetheless, we believe that most of the preclinical evidence indicates that it may be possible to induce by active immunization Abs which inhibit fungus attachment to and attack of the vaginal EC, kill the fungus or inhibit fungus growth, or even reduce the inflammatory humoral and cellular exudate associated with vaginal inflammation. Passive vaccination with these Abs is probably still more feasible. Particularly by inhibiting fungus adherence to the EC, the Abs could effectively oppose the main mechanisms of *C. albicans* pathogenicity and vaginal disease which have collectively emerged in recent studies. Anti-Sap and anti-adhesin Abs are well suited to this task as Sap and adhesin expression allows *Candida* cells both to attach to and damage a critical host defensive line such as the epithelium. Other Abs (for instance, anti- $\beta$ -glucan or toxin-mimicking anti-idiotypic Abs) may inhibit growth or even kill the fungus, working literally as antimycotics (27, 94, 125). None of these Ab functions require the participation of host inflammatory cells. We have shown in a preclinical model with some resemblance to the human situation that this protection is indeed achievable even with Fc-free single-chain variable-fragment Abs and dAbs (9, 51). Far from being regarded as a limiting factor, the lack of involvement of phagocytic cells with their intrinsic inflammatory potential may be an advantage in view of the report by Fidel et al. that excessive neutrophil recruitment in the vaginal canal may be the hallmark of disease in some women (64).

Other possible immune interventions are intravaginal infusion of *Candida* antigen-primed DC or Ab-producing B cells or T cells providing help for production of the right Ab or stimulating phagocytic killing of the fungus (13, 105). All these adoptive interventions are, however, more cumbersome and generally less predictable in terms of efficacy and side effects and thus are usually regarded with a lesser degree of acceptance by regulatory authorities (30). Moreover, a subunit vaccine is ideal under conditions where the subjects are already primed with the pathogen, as is the case here, also considering that the intravaginal route appears to be ideal for consistent IgA and IgG production in the cervicovaginal secretion (142). In this case, the vaccine formulation must take into account the required balance between the induction of antifungal immunity and tolerance at the mucosal level, which is always required for mucosal vaccines (41, 124). However, infusion of immune cells may be more appealing for those subjects in need of immune reconstitution (e.g., AIDS patients or leukemic

subjects undergoing bone marrow transplant) (105). Clearly, for local intervention with any type of vaccine investigators must carefully consider the rather strong control exerted by reproductive hormones on regulation of immune responses at the vaginal level.

*Candida* is an extracellular organism, and thus Abs against critical virulence traits of the fungus are indeed expected to play a role in protection. There is now clear experimental evidence that some Abs may be therapeutic and that protective Abs may be obscured by the plethora of irrelevant, if not disease-enhancing, Abs. Also, vaccines have been shown to be capable of preventing infection through Abs, and there are various mechanisms, including direct growth-inhibiting or even candidacidal activity (30, 40). Clinical investigations have not addressed these issues, and great caution is always necessary in extrapolating data from experimental infections to human disease. Nonetheless, the available clinical evidence does not rule out the possibility of a therapeutic Ab or an Ab-inducing vaccine against vaginal candidiasis. We remain optimistic about the possibility of reaching the goal of an efficacious immune intervention to fight vaginal candidiasis.

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