A Comprehensive Study of *Candida*-Specific Antibodies in the Saliva of Human Immunodeficiency Virus–Positive Individuals with Oropharyngeal Candidiasis

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Oropharyngeal candidiasis (OPC) is a common oral opportunistic infection among human immunodeficiency virus (HIV)–positive individuals. Although most cases of OPC correlate with reduced systemic levels of CD4⁺ T cells, the role of humoral immunity in protection against mucosal candidiasis, including OPC, remains questionable. In this study, a comprehensive analysis of saliva from 33 HIV-negative and 68 HIV-positive individuals, stratified by OPC status and peripheral CD4⁺ cell count, was conducted to measure levels of total and *Candida*-specific immunoglobulin A (IgA) and IgG antibodies, including subclasses and secretory IgA. Despite changes in total immunoglobulin levels, when levels of *Candida*-specific antibodies were normalized to total protein or total immunoglobulin of the corresponding isotype, no distinct differences in IgG (including subclasses), IgA (including subclasses), or secretory IgA levels were seen, regardless of HIV status, OPC status, or CD4⁺ cell count. These data suggest that when a complete repertoire of antibodies is evaluated, with appropriate normalization of data, there is no evidence of appreciable changes in levels of *Candida*-specific antibodies in saliva that would account for the prevalence of OPC among HIV-positive individuals.

In HIV-positive individuals, the incidence of OPC correlates with reduced systemic CD4⁺ T cell counts, which indicates that systemic T cells have a role in protecting against OPC [2, 5, 6]. However, some individuals with low CD4⁺ cell counts do not experience OPC, whereas others experience recurrent OPC. Some studies suggest that humoral immunity may be important in protection against infection [7–11]. In fact, for some medically important fungi, the action of antibodies is dependent on the presence of T cells and Th1/Th2 cytokines [12]. However, the role of antibodies in protection against mucosal candidiasis remains controversial and may be dependent on the relative concentrations of protective versus nonprotective/indifferent antibodies toward *C. albicans* and/or on the relative concentrations of Th1 and Th2 cytokines [13, 14]. In one study of HIV-negative persons with systemic candidiasis, the presence of a 47-kDa anti-*Candida* antibody correlated with recovery from infection, suggesting that antibodies may be important in protection against or clearance of *Candida* [15, 16]. In animals, a mannan-specific antibody has been shown to be protective against systemic and mucosal candidiasis [17–21]. On the other hand, women with recurrent vulvovaginal candidiasis (RVVC) have similar or elevated levels of *Candida*-specific mucosal and/or serum antibodies, compared with levels in women who do not have RVVC [22, 23].

In studies that examined humoral immunity in saliva during HIV infection, it was reported that, among several changes in total IgA and IgG antibody levels, levels of *Candida*-specific IgA antibodies (including IgA1 and IgA2) [7, 8] were elevated in HIV-positive persons, compared with levels in HIV-negative
persons [7–9]. In one study, in which saliva samples were examined from individuals with OPC (OPC+) and without OPC (OPC−), elevated levels of IgA antibodies specific to C. albicans–secreted asparagine proteases were found in OPC+ persons [10], and another study showed elevated levels of IgG and reduced levels of IgA in OPC+ persons [11]. However, no single study to date has evaluated a complete repertoire of antibodies in saliva, with careful attention given to CD4+ cell counts or adequate normalization of data, to fully evaluate potential changes in antibody levels that may play a role in susceptibility to OPC.

For the past several years, this laboratory (Louisiana State University Health Sciences Center [LSUHSC], New Orleans) has been studying innate and Candida-specific adaptive immunity in a cohort of HIV-negative individuals and HIV-positive individuals stratified by OPC status and CD4+ cell count. When we evaluated adaptive cell-mediated immunity, we found that Candida-specific T cell responses are not deficient, even in individuals with low CD4+ cell counts, which suggests that susceptibility to OPC is dependent on a threshold number of available CD4+ T cells or, alternatively, that deficiencies of local innate, humoral, or T cell–mediated immunity exist in these individuals.

The purpose of this study was to conduct a comprehensive analysis of total and Candida-specific IgA and IgG antibodies, including subclasses, in the saliva of the cohort of HIV-negative persons and HIV-positive persons with and without OPC to come to a consensus on the role of local humoral immunity in resistance or susceptibility to OPC during HIV disease.

Subjects, Materials, and Methods

Subjects. We enrolled 101 (68 HIV-positive and 33 HIV-negative) individuals from the Medical Center of Louisiana and the HIV Outpatient Program, LSUHSC. HIV status was verified in serum by ELISA and Western blot analyses. Peripheral blood CD4+ cell counts were measured by flow cytometry in the Clinical Immunology Laboratory at Charity Hospital, LSUHSC. HIV-negative persons were identified as being at high risk (n = 18) or low risk (n = 15) for exposure to HIV, on the basis of intravenous drug use, sexual contact with individuals who used intravenous drugs, or high-risk sexual behavior. HIV-positive persons were stratified by CD4+ cell count and OPC status. Stratification of HIV-positive individuals by OPC status and CD4+ cell count was as follows: HIV-positive, OPC+ individuals (n = 31) with CD4+ cell counts < 200 cells/mm³ (n = 16) or ≥ 200 cells/mm³ (n = 15) and HIV-positive, OPC− individuals (n = 37) with CD4+ cell counts < 200 cells/mm³ (n = 17) or ≥ 200 cells/mm³ (n = 20). The mean CD4+ cell count for all HIV-positive individuals was 264 cells/mm³; for individuals with CD4+ cell counts < 200 cells/mm³, the mean was 64 cells/mm³; and for those with CD4+ cell counts ≥ 200 cells/mm³, the mean was 453 cells/mm³. The mean CD4+ cell counts for OPC+ and OPC− individuals were 270 and 259 cells/mm³, respectively. Among the HIV-positive subjects, 52 were taking antiretroviral medications; 29 of these patients were receiving highly active antiretroviral therapy (HAART), which was defined as a regimen of ≥ 3 antiretroviral medications, whereas monotherapy or dual therapy that did not include a protease inhibitor was defined as a non-HAART regimen. The ages of the subjects ranged from 18 to 55 years; ~50% were male, and 63% were defined as minorities (predominantly black).

Diagnosis of oropharyngeal candidiasis and detection of yeast colonization. Diagnosis of pseudomembranous or erythematous OPC was made on the basis of a clinical appearance of removable white plaques or atrophic erythematous areas of the oral mucosa, confirmed by a positive potassium hydroxide stain of an oral smear. OPC lesions were swabbed, and smears were streaked onto Sabouraud dextrose (SAB) agar (Becton Dickinson) and Chromagar plates (CHROMagar Microbiology). After incubation at 34°C (SAB) or 37°C (Chromagar) for 48 h, colony-forming units were counted. Additionally, colonies that appeared light-green on Chromagar were tested for germ-tube formation (incubation in fetal bovine serum [FBS] for 2 h at 37°C), and colonies in which germ tubes were found were identified as C. albicans. Colonies of all other colors were categorized as non-albicans.

None of the 33 HIV-negative patients showed any clinical signs or symptoms of OPC or had a history of OPC. An oral swab was obtained from each patient and was streaked onto SAB and Chromagar for positive identification of yeast. Identification was made by colony morphology and wet-mount slide preparation.

Of the HIV-negative patients, 45% were asymptptomatically colonized with Candida in the oral cavity, and C. albicans was identified in 86% of yeast-positive cultures by the criteria described above. Among the HIV-positive, OPC− patients, 76% were asymptptomatically colonized with Candida in the oral cavity, and C. albicans was identified in 66% of yeast-positive cultures.

Sample collection and processing. After a health questionnaire had been completed, 10 mL of unstimulated saliva was collected from each patient in a sterile centrifuge tube, and 10 mL of blood was collected in an EDTA-containing collection tube. Processing of saliva involved centrifugation of the sample at 800 g and removal of the clarified supernatant. The soluble fraction was sterile-filtered through a 0.45-μm membrane, aliquoted, and stored at −70°C until use. Blood samples were sent directly to the Clinical Immunology Laboratory.

Detection of total antibodies. IgA and IgG (including subclasses of each) and Candida-specific antibodies were quantified by ELISA, using commercial capture and detection antibodies and the respective human purified antibody isotype as the standard. For each assay, optimal coating concentrations, detection dilutions, and standard concentrations were determined. The coating antibody concentrations were as follows: IgA, 20 μg/mL; IgA1, 740 μg/mL; IgA2, 24 μg/mL; IgG, 5 μg/mL; IgG1, 10 μg/mL; IgG2, 50 μg/mL; IgG3, 10 μg/mL; IgG4, 5 μg/mL. The detection antibody dilutions were as follows: IgA, 1:30,000; IgA1, 1:20,000; IgA2, 1:20,000; secretory IgA (slgA), 1:1000; IgG1, 1:30,000; IgG1, 1:1000; IgG2, 1:1000; IgG3, 1:1000; IgG4, 1:1000. All antibodies were obtained from Sigma, except for IgA1 and IgA2 coating and detection antibodies (Biodesign), and slgA detection antibody (Accurate). Standards (Sigma, Accurate) consisted of purified antibodies of each type, with concentrations determined by a linear relationship in the assay.
For ELISAs, microtiter plates were coated with the appropriate concentration of coating antibody diluted in coating buffer (0.1 M NaHCO₃, pH 8.2) at 50 µL/well and incubated overnight at 4°C. After a wash with PBS-Tween buffer (1X PBS and 0.05% Tween 20 [Sigma]), nonspecific protein-binding sites were blocked with 10% FBS in PBS for 1 h at room temperature. After blocking, the plates were washed, and the samples (diluted 1:20) and standards were added at 50 µL/well. Standards were prepared using purified antibodies. Each standard curve was prepared, beginning at a nonsaturating concentration, with serial 1:2 dilutions performed in PBS. After a 2-h incubation at room temperature, the plates were washed; 50 µL/well of the secondary antibody, conjugated to horseradish peroxidase (HRP) and diluted in blocking buffer, or biotinylated secondary antibody with avidin-HRP diluted in blocking buffer (for IgA1 and all IgG subclasses), was added; and the solution was incubated for 1 h at room temperature. After a final washing step, o-phenylenediamine dihydrochloride (Sigma) substrate was added at 50 µL/well. Plates were incubated in the dark for 15–30 min and read at 450 nm in an automated plate-reader (Thermo Labsystems Multiskan Ascent). Concentrations were determined by extrapolation from the standard curve.

**Detection of Candida-specific antibodies.** The procedure for Candida-specific ELISAs was similar to that for ELISA for total antibodies, except that the plates were coated with a Candida antigen, soluble cytoplasmic substance (SCS; kindly provided by Judith Domer, Appalachian State University, Boone, NC) [24, 25]. A preliminary study tested a panel of different Candida antigens for ELISA reactivity, including Candida culture filtrate antigen (LSUHSC) [26], SCS [24, 25], purified mannan [27], and heat-killed blastospores. SCS was the most reactive of all antigens tested, presumably because of the presence of cytoplasmic antigens as well as antigens destined to be membrane associated, all exposed for B cell activation from dying yeast cells. Therefore, SCS was used for the complete analysis. Plates were coated with 50 µg/mL SCS, and undiluted samples were used. Standards consisted of pooled positive saliva samples, defined as those that reacted with an optical density (OD) > 2 SD higher than the mean OD of PBS. The highest concentration was arbitrarily given a value of 1000 U and serially diluted 1:2 in PBS. Concentrations in units were determined by extrapolation from the standard curve.

**Detection of total and Candida-specific sIgA.** For measurement of total sIgA, the plates were coated with anti-human IgA (Sigma); purified sIgA (IgA with secretory component attached; Accurate) was used as the standard, and anti-human sIgA conjugated to HRP (Accurate) was used as the detection antibody. For measurement of Candida-specific sIgA, the plates were coated with SCS, and anti-human sIgA conjugated to HRP was used as the detection antibody.

**Protein assay.** The protein content in each saliva sample was quantified by the BCA protein assay kit (Pierce) according to the manufacturer’s instructions, using bovine serum albumin as the standard. The protein was quantified on an automated plate-reader (Multiskan Ascent) at 595 nm and expressed in milligrams per milliliter.

**Data expression.** Concentrations of all antibodies were normalized to total protein of that sample and expressed in nanograms or units per milligram of protein. Additionally, concentrations of Candida-specific antibodies were normalized to total immunoglobulin of that sample and expressed in units per nanogram of total immunoglobulin. Candida-specific sIgA was expressed as a ratio of ODs of Candida-specific IgA to total sIgA of each sample, run in parallel.

**Albumin content.** The albumin content of each saliva sample was quantified by ELISA, using the same procedures that were used to measure antibody levels. For this, 2 µg/mL capture antibody and a 1:20,000 dilution of detection antibody were used (ICN Pharmaceuticals). The standard included purified human albumin serially diluted from 8000 ng/mL.

**Hemoglobin content.** The hemoglobin content of saliva samples was evaluated by placement of 10 µL on the reagent end of a Hemastix strip (Bayer). Sixty seconds later, each strip was read, according to the color chart provided, and the results were numerically rated from 0 to +3.

**Statistical analysis.** The Mann-Whitney U test was used to analyze all data. P < .05, using a 2-tailed test, was considered to be significant. All statistics were evaluated using GraphPad Prism software.

**Results**

To adequately evaluate changes in levels of Candida-specific antibodies in the saliva of HIV-positive, OPC † individuals, saliva from specific groups of HIV-negative persons (stratified by risk for HIV exposure) and HIV-positive persons (stratified by OPC status and CD4+ cell count) was tested for a full complement of total and Candida-specific IgG and IgA antibodies, including subclasses. Figure 1 shows individual and median concentrations of total IgA, normalized to total protein (figure 1A), and of Candida-specific IgA, normalized to total protein (figure 1B) and to total IgA (figure 1C), in the saliva samples. Evaluation of total IgA in saliva showed higher levels of IgA among HIV-negative persons with high-risk behavior than among those with low-risk behavior (P = .0014) or among all HIV-positive persons (P < .0002) (figure 1A). No differences were observed for Candida-specific IgA when those data were normalized to total protein (figure 1B). In contrast, concentrations of Candida-specific IgA, normalized to total IgA, in saliva from HIV-positive, OPC † persons with CD4+ cell counts ≥ 200 cells/mm³ were higher than those in saliva from low-risk, HIV-negative persons (figure 1C). No other statistically significant differences were detected. Evaluation of Candida-specific IgA subclasses showed that both Candida-specific IgA1 and IgA2 were detectable in saliva, but no significant differences were found between any groups (figure 2). Finally, total sIgA was higher among HIV-positive persons, regardless of OPC status or CD4+ cell counts, than among HIV-negative persons (P < .03) (figure 3A). However, similar to the results for Candida-specific IgA, no differences were detected for Candida-specific sIgA in saliva between any groups (figure 3B).

Evaluation of total IgG showed higher levels among HIV-positive, OPC † persons than among high- or low-risk HIV-negative persons (P < .005) (figure 4A). When Candida-specific IgG in saliva was normalized to total protein (figure 4B) or total IgG (figure 4C), no significant differences were observed between...
any groups, including OPC+ individuals with CD4+ cell counts < 200 or ≥ 200 cells/mm³. Of the IgG subclasses (IgG1–IgG4), only Candida-specific IgG4 was detectable in saliva. As was true for Candida-specific IgG, no significant differences were observed between concentrations of Candida-specific IgG4 in the saliva of HIV-positive and HIV-negative persons when normalized to total protein (figure 4D). No statistically significant differences were seen between groups when Candida-specific IgG4 was normalized to total IgG4 (data not shown).

To determine whether any of the differences observed in IgG antibody concentrations were the result of blood contamination, albumin and hemoglobin levels were measured in each sample. For hemoglobin, no significant differences were seen between any groups, whereas albumin concentrations, although they were low in saliva compared with serum (~10–20 vs. 4000 µg/mL), were high in HIV-positive, OPC+ persons with CD4+ counts < 200 cells/mm³ (P = .0252) and HIV-positive, OPC+ persons with CD4+ cell counts ≥ 200 cells/mm³ (P = .0029), compared with levels in HIV-negative persons (data not shown).

Discussion

OPC continues to be a significant problem in HIV-positive persons, and there is a paucity of information about the immune parameters that are important for protection against infection. Accordingly, the role of antibodies in protection against candidiasis, including OPC, remains questionable. Although protection against vaginal and systemic candidiasis by Candida-specific antibodies has been demonstrated in animal models [16–21], there is no clinical evidence to date that a pool of naturally occurring antibodies protects against candidiasis in humans or that any deficiency in Candida-specific antibody is evident during any infectious condition. This may be explained by the fact that the animal studies used monoclonal “protective” antibodies isolat-
ed from a pool of antibodies or a model that favored antibody responses [16–21]. On the other hand, clinical studies are simply surveys of a pool of Candida-specific antibodies in biological fluids from stratified patient groups. The most formidable studies have included women with symptomatic vaginal candidiasis in whom there was no evidence for a deficiency in Candida-specific antibodies [22, 23]. In fact, antibodies were often elevated in those individuals with infection, which, in some cases, has led to the interpretation that the presence of Candida-specific antibodies may play a role in susceptibility to infection.

Some attention has been given to Candida-specific antibodies in the saliva of HIV-positive persons in an attempt to examine the potential role of Candida-specific antibodies in protection against and susceptibility to OPC [7–11]. Most studies, like the studies involving vaginal candidiasis, showed evidence of increases in Candida-specific IgA or IgG in HIV-positive persons, although this was not always true for patients with symptomatic OPC [10, 11]. However, a full complement of total and Candida-specific antibodies, including subclasses and sIgA, had not been evaluated in any one study. In addition, the lack of any attempt to normalize data to protein content or to total immunoglobulin of that isotype in saliva may have affected comparisons between groups. Finally, the groups of individuals evaluated may not have been stratified strictly enough (i.e., by risk behavior or CD4+ cell count) to draw adequate conclusions.

In the present study, individuals were stratified in groups by several factors, including HIV status, OPC status, and CD4+ cell count. HIV-negative individuals were further stratified by whether they were at a high or low risk for exposure to HIV, which allowed...
the inclusion of a matched control group of HIV-negative individuals with risk behaviors similar to those of HIV-positive persons. In many of the patients who had been evaluated for systemic *Candida*-specific cell-mediated immunity (peripheral blood lymphocyte responses to *Candida* antigen), no deficiencies in immunoreactivity were identified that would account for the correlation between reduced CD4$^+$ cell counts and the presence of OPC. It was postulated that local immunodeficiencies, in combination with a low threshold number of peripheral blood CD4$^+$ cells, might account for susceptibility to OPC. *Candida*-specific antibodies represented one such local immune parameter. Increases in the level of total immunoglobulins (sIgA and IgG) in the saliva of HIV-positive, OPC$^+$ and/or HIV-positive, OPC$^+$ individuals initially suggested that such differences might be attributed to *Candida*-specific antibodies. However, this was not the case; no differences in concentrations of *Candida*-specific IgG (including subclasses) or IgA (including subclasses), normalized to either total protein or total immunoglobulin, or in concentrations of *Candida*-specific sIgA, normalized to total sIgA, were detected. These comparisons included HIV-positive versus HIV-negative individuals, OPC$^+$ versus OPC$^-$ individuals, and individuals with CD4$^+$ cell counts $<200$ cells/mm$^3$ or $\geq 200$ cells/mm$^3$. The lone exception was the higher levels of *Candida*-specific IgA, normalized to total IgA, that were found in the saliva of HIV-positive, OPC$^+$ persons with CD4$^+$ counts $\geq 200$ cells/mm$^3$, compared with levels in low-risk HIV-negative individuals. However, this single difference with a single mode of normalization would not appear to provide enough evidence to suggest that increased *Candida*-specific IgA is associated with susceptibility to OPC. Moreover, no increase in total IgA was seen in this group, which would have given further support to the finding of a higher level of *Candida*-specific IgA. It should be noted that, unlike most comparisons, in which high-risk individuals are the primary control group, the low-risk group was used as the control for IgA analyses, because of an unexplained increase in total IgA in saliva seen in high-risk HIV-negative individuals but not in HIV-positive persons with similar risk behaviors. Thus,
for analysis of Candida-specific IgA, the most appropriate control group was low-risk HIV-negative persons. Why high-risk behavior was associated with higher levels of IgA in saliva and whether HIV played a role in reducing levels of IgA are unclear.

Another important issue in these analyses was the fact that the antibodies measured seem, on the basis of the low hemoglobin levels and low albumin content of saliva (compared with levels found in serum) present, to be primarily locally derived. The elevated albumin concentrations seen in OPC+ persons with CD4+ cell counts < 200 cells/mm³ and OPC+ persons with CD4+ cell counts ≥ 200 cells/mm³ did not correlate with increases observed for total or Candida-specific antibodies. Therefore, differences in antibody concentrations do not appear to be attributable to blood contamination or serum transudate. Equally important was the lack of significant differences in hemoglobin or albumin concentrations between groups with and without OPC or with high or low levels of CD4+ cells, which indicated that the source of antibody in each group was similar and allowed more-effective comparisons between groups.

When all representative controls and a complete repertoire of antibodies are included in the analysis, there is no evidence that any appreciable changes in Candida-specific antibodies occurred that would account for the high incidence of OPC in HIV-positive persons. We recognize, however, that the Candida antigen used in the assay may be a limiting factor in these interpretations because of the cytoplasmic nature of the preparation. However, because Candida SCS represented the most reactive of several soluble and particulate Candida antigens tested in a pilot study and is also one of the strongest stimulators of peripheral blood lymphocyte responses [28], SCS appears to include a broad base of antigenic determinants (both cytoplasmic and membrane associated) that are useful in surveying a wide array of Candida-specific antibodies in biological fluids. This was supported further by similar results attained when a limited number of saliva samples from each group were reevaluated for Candida-specific IgA and IgG in parallel, using heat-killed Candida blastospores and SCS (data not shown).

Although the present study is yet another survey of antibodies in a biological fluid, clearly this comprehensive analysis is definitive in suggesting that SCS-specific antibodies have little, if any, role in susceptibility to OPC. The possibility remains, however, that levels of protective antibodies directed against a specific antigenic determinant in or on Candida are low or absent in OPC+ individuals and that such antibodies are replaced in the antibody pool by nonprotective or indifferent antibodies that react with the antigens represented in SCS or by antibodies that cannot be detected through the use of SCS. If so, this could be caused by HIV, reduced CD4+ cell counts, or both. Specific antigenic mapping and additional antigen preparations will be required to evaluate this possibility. In any event, efforts to use well-characterized and functionally protective Candida-specific antibodies [20, 21] as an immunotherapeutic treatment should in no way be diminished as a result of our findings. On the contrary, regardless of whether any clear indication is found that antibody deficiency has a role in susceptibility to OPC during HIV infection, “protective” antibodies may be quite useful in treating or preventing OPC in cases of immunosuppression that may eliminate natural protective mechanisms.

References


