Oral Microbiol Immunol 2001: 16: 270–278
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Oral Microbiology and Immunology
ISSN 0902-0055

Salivary anticandidal activity and saliva composition in an HIV-infected cohort

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Lin AL, Johnson DA, Patterson TF, Wu Y, Lu DL, Shi Q, Yeh C-K. Salivary anticandidal activity and saliva composition in an HIV-infected cohort. Oral Microbiol Immunol 2001: 16: 270–278. © Munksgaard, 2001.

This study investigated salivary anticandidal activity and salivary composition in stimulated whole saliva of 18 advanced HIV-infected patients and compared these values to healthy controls. Stimulated whole saliva from HIV-infected patients showed decreased anticandidal activity. The flow rate was reduced by 40% as compared with controls. The saliva flow rate for HIV-infected patients who had recoverable yeast in their saliva was reduced as compared to HIV-infected patients without recoverable yeast. For HIV-infected patients, the saliva concentrations of lactoferrin, secretory IgA and Cl^- were increased while the secretion rate of lysozyme, total protein and K^+ were reduced. There was no difference in any parameter as a function of taking the antifungal drug fluconazole. There was no association between salivary anticandidal activity and any salivary component. This study shows reduced anticandidal activity and salivary flow rate in HIV-infected patients. These alterations may contribute to their increased incidence of oral candidal infections.

Key words: saliva composition; HIV/AIDS; anticandidal activity

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Accepted for publication February 3, 2001

Oral candidiasis occurs in more than 95% of patients with acquired immunodeficiency syndrome (AIDS) (25). Extensive lesions of this infection can be quite painful and cause impairment of food intake thus setting the stage for wasting and malnutrition, conditions commonly observed in AIDS patients. Although little information regarding the pathogenesis of oral candidiasis in HIV-infected patients is available, it has been suggested that altered salivary gland function predisposes HIV/AIDS patients to oral candidiasis.

Saliva plays an important role in maintaining the health of the oral cavity, and there are at least two mechanisms by which saliva can prevent oral infection by opportunistic pathogens such as *Candida albicans*. On the one hand, saliva serves as a cleansing agent for the oral cavity. Saliva mixes with bacteria and debris in the oral cavity. This is then cleared from the mouth by swallowing. Secondly, it contains several antifungal proteins. Indeed human saliva from healthy subjects strongly inhibits both *C. albicans* blastoconidial viability and *C. albicans* germ-tube formation *in vitro* (33). There are reports that salivary anticandidal activities are compromised in AIDS patients. A pilot study of 12 AIDS patients showed that salivary anticandidal activity was decreased as compared with healthy controls (31). This decrease in salivary anticandidal activity in AIDS patients has been attributed to a decrease in the concentration of salivary histatins and/or to dysfunction of these proteins (18). Histatins are a family of histidine-rich polypeptides and are the major antifungal proteins present in human saliva.

Other studies have concentrated on the effect of HIV infection on salivary function and composition. Several investigators report a reduction in salivary flow rates (3, 5, 22, 42); others find no alterations (27, 31). In stimulated parotid saliva and in other glandular saliva collected from HIV patients, there is an elevation of Cl^- (22, 42). Sodium is increased in stimulated parotid saliva (22). With respect to antimicrobial and antifungal proteins in saliva, some investigators report that the concentrations of histatins, lysozyme, lactoferrin and secretory IgA (sIgA) are increased in HIV-infected patients (3, 5, 22, 24, 27, 34, 38, 42), whereas others report either no change or a decrease in these proteins (18, 22, 26, 27, 31, 36). The discrepancies in findings for these studies might be due to saliva analyses taking place during different stages of the HIV disease process or to different methods employed for salivary analyses. With respect to oral *Candida* status, several studies re-

port an association between oral candidal status and salivary antifungal proteins (histatins, sIgA and lysozyme) (3, 12, 38, 41). However, the relationship between salivary gland dysfunction and the occurrence of oral candidiasis has not been well established in HIV infection.

In this report, we describe the characteristics of salivary anticandidal activities and their relationship to salivary function and sialochemistry in an HIV-infected patient cohort.

Material and methods

Subjects

Eighteen HIV/AIDS patients (17 men and 1 woman) with a mean age of 39±6.7 (±SD) years were recruited from participants in a longitudinal trial of oropharyngeal candidiasis conducted by T. F. Patterson, Department of Medicine, University of Texas Health Science Center at San Antonio (UTHSCSA). Approval to undertake this study was obtained from the Institutional Review Boards of both UTHSCSA and the Audie L. Murphy V. A. Hospital (ALMVAH) and informed consent was obtained from all subjects. All patients were taking anti-HIV medications including nucleoside analogs [lamivudine (3TC), stavudine (d4T), zudovudine (ZDV), didanosine (ddI)], non-nucleoside analogs [delavirdine, nevirapine], and/or protease inhibitors (indinavir, nelfinavir, ritonavir, saquinavir). One stimulated whole saliva sample was collected from each subject at each visit. Multiple samples of whole saliva were obtained from several patients (up to six samples) within a 4-month period. In addition, a single stimulated whole saliva sample was collected from 34 healthy volunteers (30 men and 4 women) of comparable age.

Saliva collection and treatment

Stimulated whole saliva (WS) was collected by chewing paraffin wax for 5 min. The collected saliva was put on ice immediately. An aliquot of this saliva was stored in a −70°C freezer for sialochemical analysis. Other aliquots (2 µl and 20 µl) were plated on Sabouraud agar plates and incubated for 24 h at 37°C to determine yeast colony-forming units in whole saliva. The remaining saliva was used to assess antifungal activity. This saliva was acidified to pH 4.5 with glacial acetic acid and a protease inhibitor, phenylmethylsulfonylfluoride (PMSF), was added to 2 mM. The treated saliva was heated in a boiling water bath for 10 min. After cooling on ice for 20 min, the samples were centrifuged at 16,000×*g* for 30 min at 4°C. The supernatant was frozen and stored at −70°C. Saliva processed in this manner maintains its antifungal activity after freezing (20).

Salivary blastoconidial viability assay

The salivary blastoconidial viability microassay was developed in our laboratory (20) using methods modified after those described by Pollock et al. (31). Two fluconazole susceptible candidal isolates (#1215 and #540) were used to assess salivary inhibition of blastoconidial viability. These isolates were grown to late-log phase (optical density of 1.4–1.6 at 600 nm). After centrifugation (16,000×*g* for 5 min at 4°C) the cells were washed with sterilized water and the candidal suspension was adjusted to 3×10^6 colony-forming units (CFU)/ml in sodium acetate buffer, pH 4.5. The incubation mixture contained 95 µl of processed saliva or of acetate buffer (25 mM, control) and 5 µl of candidal suspension (final candidal concentration, 1.5×10^5 CFU/ml). The mixture was incubated for 4 h at 37°C, diluted 100-fold with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6, and incubated at 37°C for an additional 3 h. One hundred µl of the diluted mixture was plated on a Sabouraud dextrose agar plate. The colonies were counted after 24 h of incubation at 37°C. The percentage inhibition of blastoconidial viability was calculated according to the following formula: [1−(CFU of saliva treatment/CFU of acetate buffer)×100]. Duplicate determinations were performed for each sample.

Salivary germ tube inhibition assay

The microassay for salivary inhibition of germ tube formation (20) was developed based on methods described by Santarpia et al. (33). This assay used candidal isolates #566 (fluconozole resistant) and #1215 (fluconazole susceptible). A static colony from an agar plate was used to make the candidal suspension (3×10^7 CFU/ml water). The assay mixture contained 6.5 µl of filter-sterilized N-acetylglucosamine (27.2 mg/ml), 32.5 µl of fetal bovine serum (FBS), 50 µl of saliva and 10 µl of freshly prepared candidal suspension. For controls, 50 µl of 20 mM acetate buffer containing 2 mM PMSF was used in place of the saliva. After incubation for 3 h at 37°C, an aliquot of the mixture was examined at ×400 under an Olympus microscope. A total of 300 blastoconidia and germ tubes were counted and the percentage inhibition of blastoconidial germination was calculated according to the following formula: [1−(% germination of saliva treatment/% germination of acetate buffer)×100].

Sialochemical assays

Except for histatins, an unprocessed saliva sample stored at −70°C was used in the following sialochemical assays. The electrolytes [Na^+] and [K^+] were determined by atomic absorption spectrophotometry (Perkin Elmer Model 3030) and [Cl^-] was determined using a chloridometer (Labconco, Model 442–5000). Total protein of saliva was determined by absorption at 215 nm with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard (2).

Lactoferrin, lysozyme and secretory IgA were determined using ELISA. Lysozyme utilized a method (41) adapted after that of MacKay et al. (21). For this assay, the coating antibody was human lysozyme monoclonal antibody (Biomedical Technologies, Inc., #BT-577), the second antibody was sheep anti-human lysozyme, IgG fraction (Cortex Biochem, San Leandro, CA, #CR2030SP) and the conjugate antibody was peroxidase conjugated rabbit IgG to sheep IgG (Cappel Organon Teknika Corp., Durham, NC, #55814). The standard was lysozyme from human milk (Sigma Chemical Co., L6394). The lactoferrin assay was adapted after Antonsen et al. (1). The coating antibody was anti-human lactoferrin [AffiniPure $F(ab')_2$ Fragment Rabbit Anti-human Lactoferrin, Jackson ImmunoResearch Laboratories, #309–006–015] while the conjugated antibody was alkaline phosphatase conjugated AffiniPure $F(ab')_2$ fragment rabbit antihuman lactoferrin (Jackson ImmunoResearch Laboratories, #309-056-015). The standard was lactoferrin from human milk (Sigma Chemical Co., L 0520). The assay for secretory IgA was adapted after the method of Nagao et al. (28). The coating antibody was goat IgG fraction to human secretory

IgA (Cappel #55001), and the conjugate antibody was peroxidase conjugated goat IgG fraction to secretory IgA (Cappel, #55215).

Histatins (HRPs) in saliva were determined by a method outlined previously (12). The acidified and boiled saliva sample was used in these analyses. Briefly gels containing both saliva and HRP standards were subjected to cationic gel electrophoresis (29). The gels were stained with Coomassie Brilliant Blue R250, destained and scanned densitometrically. The images was captured in PhotoShop (Adobe) and then analyzed using the NIH Imaging Analysis Program (version 1.47). Synthesized histatin standards (HRP1, HRP3 and HRP5) were used to determine histatin concentrations in saliva. Because of the difficulty of phosphorylating the serine in position 2 of HRP1, unphosphorylated HRP1 was used as the HRP1 standard in this study. Total HRP was defined as the sum of HRP1, HRP3 and HRP5.

Assay standardization

For all assays, an internal control was used to insure assay reproducibility. Whole saliva was collected from several donors, pooled and then stored at −70°C in small aliquots. A new aliquot was used for each day's assay. For the anticandidal assays, human whole saliva was collected from several healthy donors, pooled and treated as the subject saliva samples, i.e., acidified, treated with PMSF, heated and centrifuged. It was stored in small aliquots. Again, one aliquot was used for each anticandidal assay. The values obtained for these pools were monitored and if a value for this pool was outside a predetermined range for that assay, the saliva samples for the subjects were repeated.

Data presentation and statistical analysis

Multiple saliva samples were collected from 13 of the 18 HIV-infected patients, while a single sample was collected from the remaining five. A total of 47 samples were collected from the 18 patients. The interval between collection times varied from 1 week to 4 months. For the patients who provided saliva more than once (range from two to six times), for each assay an average was calculated for that patient and this was used in the data analysis (nonparametric Mann-Whitney *U* test). To determine an effect of fluconazole on any of the saliva variables, patients were subdivided into groups depending on whether or not they were taking fluconazole (100 to 800 mg/day) at the time the saliva sample used in the analysis was collected. Some patients ($n=7$) had saliva collected both on and off fluconazole therapy and, in some cases, there were multiple saliva samples under each condition. Again, a mean was calculated for that patient both on and off fluconazole therapy and this mean was used in the analyses (Mann-Whitney *U* test). When the same patients were seen both on and off fluconazole, a paired nonparametric analysis (Wilcoxon rank sum test) was done to determine whether fluconazole status affected the salivary variable. In some cases the patients were divided into groups based on whether or not they had recoverable yeast in their saliva. Again, many had multiple saliva samples in each group. Two patients had saliva samples that at times contained yeast and at times did not. For each variable, means for each patient were calculated for each yeast group and used in the analyses (Mann-Whitney *U* test). Spearman rank correlation analysis was used to study the relationship between factors. The data given in the tables represents the group median with the 25th to 75th percentile in parentheses. A difference was considered significant for any P value ≤ 0.05.

Results

Characteristics of the HIV-positive and control population: age, whole saliva flow rates, $CD4^+$ counts, $CD4^+$% and viral load (Table 1)

The age range is comparable for the control and HIV-positive groups. However, the stimulated whole saliva flow rates were significantly decreased for the HIV-positive group ($P<0.0001$). The median flow rate for the HIV-positive group was 40% less than that for the control group. The values for $CD4^+$ number, $CD4^+$% and viral load were obtained from patient records. The median values for $CD4^+$ number, $CD4^+$% and viral load for the HIV-positive group were 115 cells/μl, 9% and 13,800/μl, respectively. However, there was great variability within this group. The $CD4^+$ number varied from 0 to 435 cells/μl, and the percentage of $CD4^+$ lymphocytes varied from 0 to 29%. The viral load also varied greatly with a range of from less than 400/μl up to 724,000/μl. In 12 of the 18 patients, the $CD4^+$ number was less than 200 cells/μl; in the remaining six it varied from 200 to 435 cell/μl. The $CD4^+$% was less than or equal to 13% for 14 of the patients. For the remaining four the range was 15% to 23%. These data indicate that most patients were at an advanced stage of HIV infection.

Yeast colony-forming units in stimulated whole saliva and relationship to flow rate (Table 2)

Candidal colonies were present in only 5 of the 25 healthy subjects (20%). On at least one occasion, the whole saliva of 10 of the 18 patients (56%) contained yeast. Two of the patients had saliva samples which were collected when yeast were present and when yeast were absent in their saliva. For both the control group and the patient group, the median flow rates were lower for the patients with recoverable yeast in their saliva, although this was significant ($P=0.034$) only for the HIV-positive group. A comparison of the median flow rates between the control and HIV-positive groups for the yeast-positive and yeast-negative groups also showed differences. The flow rate for the yeast-negative control group was 51% greater than that of the yeast-negative HIV-positive group. This difference was significant ($P=0.002$). Although the flow rate for the yeast-positive control group was

Table 1. Age, salivary flow rate and HIV status of the control and patient populations

Group	*n*	Age (years)	Flow rate (ml/min)	$CD4^+$ (number/μl)	$CD4^+$%	Viral load (number/μl)
Control	34	35 (23–53)	2.00 (1.50–2.40)	ND[a]	ND[a]	
HIV	18	39 (30–53)	1.14[b] (0.79–1.37)	115 (59–298)	9 (5–13)	13,800 (400–75,400)

[a] Not determined.
[b] Significantly different from the control group ($P<0.0001$).

Table 2. Whole saliva flow rates and yeast counts

Group	*n*	CFU POS (*n*)	CFU NEG (*n*)	Flow rate (ml/min) CFU POS	Flow rate (ml/min) CFU NEG	*P* CFU POS/NEG[a]	CFU/ml (CFU POS)
Control	25	5	20	1.52 (1.1–2.14)	2.00 (1.70–2.40)	0.183	200 (140–290)
HIV[b]	18	10	10	0.96 (0.56–1.16)	1.32 (1.20–1.60)	0.034	8,400 (2,000–55,600)
P Con/HIV[c]				0.066	0.002		0.002

[a] Comparison of CFU POS and CFU NEG groups.
[b] Two patients had saliva collected at times when they had recoverable yeast in their saliva and at times when they did not and these two patients are included in both groups.
[c] Comparison of control and HIV-infected groups.

Table 3. Anticandidal activity in stimulated whole saliva collected from healthy controls and HIV-infected patients

	% inhibition of blastoconidial viability[a]			% inhibition of germ tube formation[a]		
Group	*n*	Isolate #1215	Isolate #540	*n*	Isolate #1215	Isolate #566
Control	34	100 (98.4–100)	95.6 (75.4–100)	25	100 (100–100)	100 (100–100)
HIV	18	86.1 (77.9–93.6)[b]	68.8 (52.7–92.5)[c]	18	77.3 (41.1–96.1)[b]	76.8 (48.9–100)[b]

[a] Isolates #1215 and #540 are fluconazole-susceptible; isolate #566 is fluconazole-resistant.
[b] Significantly different from control group ($P < 0.0001$).
[c] Significantly different from control group ($P < 0.01$).

also more than 50% greater than that of the yeast-positive HIV-positive group, this difference was not significant ($P = 0.066$). This may be at least in part attributable to the fact that there were so few yeast-positive subjects in the control group. A comparison of the median yeast counts for the yeast-positive saliva samples showed that, when present, the yeast counts were significantly higher ($P = 0.002$) in the HIV-positive group. For the yeast-positive patients, the median CFU/ml of whole saliva was more than 40 times greater in the yeast-positive HIV-positive group as compared to the yeast-positive control group.

Salivary anticandidal activity: control versus HIV-positive

Saliva from healthy controls consistently showed strong inhibition of blastoconidial viability to both isolates (#1215 and #540; Table 3). However, salivary inhibition of blastoconidial viability in HIV-positive patients showed a large variation. Even samples from the same patient collected at different visits had large variations in blastoconidial killing activity. Several saliva samples from HIV-positive patients completely lost their blastoconidial killing activity. Overall, the median salivary inhibition of blastoconidial viability in HIV-positive patients was significantly lower (14% for isolate #1215, $P < 0.0001$, and 28% for isolate #540, $P = 0.008$) than that for the controls (Table 3).

Similar to the salivary blastoconidial killing assays, saliva from healthy subjects always exhibited complete inhibition of candidal germ tube formation to both isolates (#1215 and #566), whereas great variation was seen in saliva from HIV-positive patients (Table 3). The median salivary inhibition of germ tube formation for the HIV-positive patients was 23% lower ($P < 0.0001$) than that for the controls for isolates #1215 and #566.

Effect of fluconazole on salivary flow rates and anticandidal activities

Since many of the patients were taking the antifungal drug, fluconazole, several analyses were undertaken to determine whether fluconazole affected salivary flow as well as the assays for salivary inhibition of blastoconidial viability and/or for germ tube formation. The HIV-positive patients were divided into groups based on whether they were (FluY) or were not (FluN) taking the drug at the time the saliva sample used in the analyses was taken. For many patients ($n = 7$), saliva samples were obtained both when they were taking the drug and when they were not. When multiple samples were analyzed for the same fluconazole status, a mean value was determined for each drug condition and this mean was used in the analysis. There was no difference in stimulated whole saliva flow rate as a function of taking the drug fluconazole (Table 4). Further, there was no difference in $CD4^+$ counts, $CD4^+$% or viral load. In spite of the fact that fluconazole is an antifungal drug, there was also no difference between FluN and FluY for either anticandidal assay. In another approach, a paired nonparametric analy-

Table 4. Effect of fluconazole on salivary flow rate, disease status and anticandidal activity[a]

	FLU N ($n = 12$)	FLU Y ($n = 13$)
Flow rate (ml/min)	1.00 (0.88–1.32)	1.21 (0.64–1.35)
$CD4^+$ (cells/μl)	153 (59–340)	96 (18–204)
$CD4^+$%	12.5 (5–18)	6.5 (5–10.5)
Viral load (number/μl)	13,800 (7,100–96,800)	13,800 (500–78,300)
Yeast colony-forming units (CFU)/ml	1,200 (0–6,900)	100 (0–2,700)
% inhibition, blastoconidial viability (isolate #1215)	88.8 (70.4–95.2)	87.9 (77.6–98.9)
% inhibition, germ tube formation (isolate #1215)	80.3 (11.1–87.7)	74.9 (51.8–100)

[a] Since multiple saliva samples were collected from each patient, seven patients had saliva samples obtained while they were on (FluY) and off (FluN) fluconazole.

sis was undertaken that used the values for the anticandidal assays for the seven patients while they were on and off the drug fluconazole. There was again no significant difference in anticandidal activity as a function of taking fluconazole. This is in spite of the fact that two of the isolates are fluconazole-susceptible (#1215 and #540) while one is fluconazole-resistant (#566).

Sialochemistry: electrolytes

The median concentration of salivary Cl^- in the HIV-positive group was significantly higher than for the control ($P=0.005$, Table 5). There were no significant differences in median concentrations of K^+ and Na^+ between these two groups. When adjusted for flow rates (secretion/min), the outputs of Na^+, K^+, and Cl^- were lower in HIV-positive patients, with statistical significance only for K^+ ($P=0.0005$). When the HIV-positive group was subdivided into FluN and FluY, the concentration of K^+ was significantly higher ($P=0.027$) for the FluY group (21.2 meq/l) as compared with the FluN group (18.3 meq/l). Although the concentration of Na^+ was approximately 60% higher in the saliva of FluN (25.4 meq/l) as compared with FluY (16.1 meq/l), the difference was not significant ($P>0.05$).

Sialochemistry: total protein and antimicrobial proteins

There are at least four salivary proteins that show anticandidal activity *in vitro*: histatins, lactoferrin, sIgA and lysozyme. In order to elucidate the hypothesis that increased oral candidiasis in HIV-positive patients may be due to fluctuations of these antifungal proteins, the concentrations of salivary total protein, lysozyme, lactoferrin, secretory IgA and histatins were determined (Table 5). Of these five components, only lactoferrin and secretory IgA of the HIV-positive group showed significant alterations as compared with the control. The concentration of salivary lactoferrin for the HIV-positive group was 6.3 μg/ml, which was almost 40% higher than the 4.6 μg/ml for the healthy controls ($P=0.005$). Further analysis showed that the median concentration of lactoferrin was 80% higher ($P=0.025$) for those taking fluconazole (FluY, 9.5 μg/ml) as compared with those that were not (FluN, 5.3 μg/ml). The concentration of secretory IgA was increased by 32% in the saliva of the HIV-positive patients ($P=0.05$). The concentration of histatins was almost 50% greater in the treated saliva samples of the HIV-positive patients, but this was not significant ($P=0.06$). The concentration of total histatin is the sum of HRP1, HRP3 and HRP5. Analysis indicated no significant differences in the individual components used to derive total histatin concentration. Except for lactoferrin, when the HIV-positive patients were subdivided into FluY and FluN, there were no significant differences between the FluN and FluY subgroups for any of the variables (data not shown).

With respect to secretion or output, that of total protein and lysozyme were significantly reduced for HIV-positive patients. The secretion of total protein was reduced by 40% ($P=0.0155$), whereas that of lysozyme was reduced by 33% ($P=0.0069$). While the median values for secretion of the other antimicrobial/antifungal proteins were somewhat less for the HIV-positive group, they were not significantly reduced. Again, when the patients were divided in FluN and FluY groups, there was not a significant difference in output of total protein or in output of any individual protein.

Relationship between salivary parameters and anticandidal activities

The correlation of salivary flow rates and sialochemistry with salivary anticandidal activities was tested. There was no specific association between anticandidal activity and stimulated whole saliva flow rate, and concentration or secretion of electrolytes and antimicrobial proteins (data not shown).

Table 5. Comparison of stimulated whole saliva composition for healthy control and HIV-infected patients

	Concentration		Secretion	
Component	Control ($n=34$)	HIV ($n=18$)	Control ($n=34$)	HIV ($n=18$)
Na^+ [a]	15.3 (11.0–24.8)	16.9 (13.2–27.2)	33.5 (16.1–59.1)	18.7 (11.0–33.5)
K^+ [a]	19.6 (18.2–21.6)	19.4 (18.3–23.0)	36.2 (30.6–43.0)	22.2*** (20.7–26.0)
Cl^- [a]	18.7 (15.6–26.5)	29.7** (22.6–38.8)	39.5 (23.4–60.2)	30.8 (21.7–46.9)
Total protein[b]	3.5 (3.1–4.1)	4.3 (3.6–5.2)	7.4 (5.0–9.1)	4.5* (3.3–6.9)
Lysozyme[c]	14.1 (10.9–18.0)	15.0 (12.6–17.8)	27.3 (21.1–32.0)	18.3** (15.2–20.6)
Lactoferrin[c]	4.6 (3.4–7.1)	6.3** (5.1–14.0)	7.7 (5.6–12.0)	7.6 (5.7–13.3)
Secretory IgA[c]	196 (143–234)	259* (182–339)	352 (271–447)	296 (194–359)
Histatins[c]	7.7 (5.7–11.9)	11.4 (7.9–14.1)	13.6 (9.2–26.7)	12.1 (7.1–17.5)

[a] Concentration is meq/l while secretion is meq/min.
[b] Concentration is mg/ml while secretion is mg/min.
[c] Concentration is μg/ml while secretion is μg/min.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared with control.

Discussion

Our data show that stimulated whole saliva from HIV-infected patients having advanced disease has decreased salivary anticandidal activity, specifically decreased inhibition of blastoconidial viability and decreased ability to inhibit germ tube formation. This observation is consistent with the report of Pollock et al. (31), which demonstrated that stimulated whole, parotid or submandibular/sublingual saliva from 12 AIDS patients had decreased anticandidal activity. In our studies, we obtained 47 stimulated whole saliva samples from 18 advanced HIV-infected patients. Although the numbers of HIV-infected patients in both studies are small, both obtained similar results.

Oral candidiasis is a hallmark infection of HIV-infected patients. Many people have the *Candida* organism in their mouths without overt infection.

The infection occurs when the yeast colonize the oral mucosa. The attachment of yeast to the oral mucosa is thought to be mediated by the hyphae form of the organism. Thus, mechanisms that inhibit the ability of the *Candida* to replicate (i.e., inhibition of blastoconidial viability) and/or the ability of *Candida* to form hyphae (inhibition of germ tube formation) may contribute to controlling oral candidiasis (6). Other mechanisms that may aid in the control of oral candidal infections are the cleansing mechanisms of saliva as well as specific protein components. The debris and foreign substances (i.e., microorganisms) within the mouth are mixed with saliva and this mixture is then swallowed. Saliva also has specific components such as mucins that coat the oral surfaces and keep them moist. Mucins also contain aggregates of other proteins such as secretory IgA, histatins, etc., and these proteins are therefore concentrated at the oral mucosal surfaces. This in turn may exert an antibacterial/antifungal protective effect. Many of the specific proteins secreted by the salivary glands have antifungal activity. Among these are histatins, secretory IgA, lactoferrin and lysozyme.

This study has shown that an important component of oral host defense, that of salivary flow, is reduced by 40% in HIV-infected patients. This differs from the findings of Pollock et al. (31), who found no change in salivary flow rate in their HIV-infected cohort. It is possible that the reduction in flow rate found in our study relates to the powerful anti-HIV medications that the patients in this study were taking. It is quite likely that these medicines were unavailable for the patients in the earlier studies. The 40% reduction in flow rate was not a function of taking fluconazole, as there was no significant difference between the FluN and FluY groups. Although the median flow rate, for the FluN group was 20% higher than that of the FluY group, the 25th to 75th percentile ranges for each group were comparable. When the groups were divided on the basis of the presence or absence of *Candida* in saliva, although not statistically significant, the median flow rate for those in the control group who were *Candida*-positive was lower than for the *Candida*-negative group. For the HIV-infected patients, there was a significant reduction in the stimulated whole saliva flow rate for the *Candida*-positive group. For both the *Candida*-positive and *Candida*-negative groups, the median flow rates for the control group were more than 50% greater than for the HIV-infected group. This difference was significant only for the *Candida*-negative control and HIV groups ($P=0.0018$), although the difference did approach significance for the control and HIV *Candida*-positive groups ($P=0.066$). Past studies from our laboratory have shown reduced flow rates for individuals with recoverable yeast in their saliva (12, 41). Since saliva is important for cleansing the mouth and for delivery of the salivary host-defense factors into the oral cavity, a reduction in flow rate could lead to compromises in these important components of oral host defense.

In this study we determined the salivary concentration and secretion of antifungal proteins as well as electrolytes. A comparison of the healthy controls with the HIV-positive patients showed higher concentrations of lactoferrin (37%) and secretory IgA (32%) in the stimulated whole saliva of the HIV group. The concentration of chloride was also increased (59%). This latter may be of importance, since the biologic activity of some proteins is dependent on ionic strength (19, 29). While the concentration (i.e., unit/ml) of a component is one means of monitoring saliva composition, overall secretion or output (units/time) is another important aspect. When flow rates are altered, the amount of a particular component in the mouth may be affected. This is clearly seen in this study. The salivary concentrations of total protein, potassium and lysozyme are similar for both the control and HIV groups. However, when flow rate is taken into account, the output of all three components is reduced in the saliva of the HIV patients (39%, 38% and 33%, respectively). On the other hand, in the case of lactoferrin and chloride, for which the concentrations are higher in the HIV patients, because of the reduced flow rate, the overall secretion is the same as that for the control group. Therefore, it is important to consider both concentration and secretion in the evaluation of salivary changes.

In this study we divided the HIV-positive patients into two groups based on whether or not they were taking the drug fluconazole at the time of saliva collection and also into two groups based on whether or not the saliva sample contained yeast. We examined both the concentration and secretion of all components for the subgroups as well as anticandidal activity (data not shown). With respect to the yeast-positive and -negative HIV groups, only the concentration of potassium in saliva was significantly different ($P=0.027$). The median concentration for the yeast positive group was 20.8 meq/l (25th to 75th percentile: 19.3–31.1 meq/l) and 17.2 meq/l (25th to 75th percentile: 15.3–19.4 meq/l) for the yeast-negative group. There was no difference in anticandidal activity between these two groups. In the case of the division using fluconazole as a factor, there was a significant difference in the concentration ($P=0.03$) and secretion ($P=0.039$) of lactoferrin. Those taking fluconazole had a higher concentration (2.94 μg/ml; 25th–75th percentile: 1.70–4.31 μg/ml) and secretion (13.2 μg/min; 25th–75th percentile: 8.9–22.9 μg/ml) of lactoferrin as compared to those not taking this drug (5.52 μg/ml; 25th–75th percentile: 4.95–6.20 μg/ml and 6.2 μg/min; 25th–75th percentile: 5.2–7.9 μg/min). There was also no significant difference in anticandidal activity as a function of taking fluconazole.

In addition to a reduction in salivary flow rate, the saliva of HIV-infected patients showed deficient antifungal activity. The difference between the control and HIV-infected group was highly significant. The reduction in anticandidal activity is in agreement with a previous study (31). Other investigations have suggested that these antifungal activities are a function of salivary histatins (19), a group of small basic proteins that have powerful anticandidal activity. Several studies have examined the concentration of histatins in the saliva of HIV-infected patients with conflicting results. For stimulated parotid saliva, histatins may be increased (3), decreased (18) or show no change (22). In stimulated submandibular/sublingual saliva, one investigation showed an increase in concentration (3) while another showed a decrease (18). In the present study, we determined salivary histatin concentrations in the acidified stimulated whole saliva sample. Although the median concentration was almost 50% higher for the HIV-infected patients, this increase was not significant. It should be noted that the concentrations of histatins in treated whole saliva are several fold lower than the values reported in our previous studies

for stimulated parotid and stimulated submandibular/sublingual saliva (12, 14). Others have reported lower histatin concentrations in whole saliva as compared to glandular saliva (4, 13, 30). The reason is unknown, but histatins may be rapidly degraded, absorbed or aggregated with other components in whole saliva or the oral cavity (11, 30 40).

There are other salivary proteins that are known to have anticandidal activity, i.e., lysozyme, lactoferrin and secretory IgA. Lysozyme shows blastoconidial killing activity (15, 23, 32) which is sensitive to the ionic concentration of the solution (19, 39). Both lactoferrin and secretory IgA have also been shown to exhibit anticandidal activity *in vitro* (7, 16, 35, 37). However, for reasons outlined below, we believe that the anticandidal activity shown in our assay system may not be related to any of these proteins.

In the present study, the HIV-infected patients and the healthy controls have similar concentrations of lysozyme in stimulated whole saliva. This suggests that lysozyme is not a component of the decreased antifungal activity shown for HIV-infected stimulated whole saliva. With respect to the association between HIV and lysozyme concentrations in isolated glandular secretions, the findings are controversial. In stimulated parotid saliva, some investigators find an increase in concentration (3, 27, 34), one shows a decrease (22) and others find no change (18). For stimulated submandibular/sublingual saliva, both reports suggest there is no change (3, 18). While lysozyme has antifungal activity, it is unlikely that lysozyme contributes to the antifungal activity reported in this article for the following reason. Others have noted that lysozyme at a concentration of up to 50 μg/ml does not inhibit germ tube formation under these anticandidal assay conditions (19). We have found that lysozyme has no anticandidal activity in our assay system or in other buffer solutions at concentrations of up to 200 μg/ml, which is more than 10 fold higher than the 15 μg/ml concentrations found in whole saliva (Table 5). While lysozyme shows no anticandidal activity in a buffer system, we find that when water is used as the medium, lysozyme at concentrations of 50 μg/ml strongly inhibits candidal blastoconidial viability and germ tube formation. Thus, the ionic environment is critical to the functional ability of lysozyme. However, in our assay system, lysozyme is not active and therefore cannot contribute to the anticandidal activity of saliva in our system.

Both lactoferrin and secretory IgA show *in vitro* anticandidal activity (7, 16, 35, 37), and we find that both proteins are increased in stimulated whole saliva collected from HIV-infected patients. Again, in saliva collected from the salivary glands, the findings with respect to both of these proteins are variable. Lactoferrin is not increased in stimulated parotid saliva (3, 22), but it is increased in stimulated submandibular/sublingual saliva (3). As for secretory IgA, Mandel et al. (22) and Schiodt et al. (34) find no change, while Atkinson et al. (3) finds a 10-fold increase in stimulated parotid saliva and a 50% increase in stimulated submandibular/sublingual saliva. Again, in our assay system, we feel that the contributions of salivary secretory IgA and lactoferrin are probably minimal, if any. Firstly because the concentration of each of these is higher in the HIV-infected patients and these patients have decreased antifungal activity. Secondly, we have assayed acidified and boiled whole saliva samples used for the anticandidal assays and have found that most of both secretory IgA and lactoferrin are removed by this treatment.

This study found a higher concentration of Cl^- in the saliva of HIV-infected patients, consistent with the observations of other studies (22, 42). There was no difference in Na^+ and K^+. It has been shown that the blastoconidial killing activity of histatins and of lysozyme is dependent on the ionic strength of the solution (39). Most of this dependence appears to be related to divalent ions. Whether this increase in chloride concentration is related to the reduction in salivary anticandidal activity is not known. We found no relationship between the concentrations of K^+, Cl^-, or Na^+ and blastoconidial killing and inhibition of germ tube formation.

Fluconozole is a relatively inert fungistatic drug (9, 10) that is widely used to treat oral candidiasis in HIV patients, and many of the patients in this study were taking this drug. The mean concentration of fluconazole in saliva is approximately 2 to 12 μg/ml after ingesting 100 mg/day (8, 17). In this study, HIV patients who were taking the drug were taking 100 to 800 mg of fluconazole/day. Although we did not measure the concentration of fluconazole in the saliva of these patients, we believe that the patients in this study would have high salivary concentrations, which might contribute to a greater ability of the patient's saliva to inhibit blastoconidial viability and germ tube formation. However, from our studies, fluconazole appears to have no effect on these antifungal assays. This is demonstrated in the following ways.

Patients were subdivided into groups based on whether they were (FluY) or were not (FluN) taking fluconazole at the time of saliva collection. There was no difference in candidal inhibitory activities between the patients in the two groups. Since multiple saliva samples were obtained over a several month interval, some patients ($n=7$) had saliva collected while they were taking fluconazole and also while they were not. The mean inhibitory activity for each patient while they were on and off the drug was not significantly different. In the case of the germ-tube inhibition assay, both a fluconazole-susceptible and a fluconazole-resistant strain were used. The patient saliva samples for which both strains were used in the analysis were divided into FluY ($n=18$) and FluN ($n=16$). Within each fluconazole group, there was no difference in inhibitory activity against the two strains. Taken together, these findings suggest that the candidal inhibitory activities measured in saliva are independent of the antifungal drug fluconazole.

This study has confirmed the observations of Pollock et al. (31) that HIV-infected patients have impaired anticandidal defense mechanisms of saliva, i.e., a reduced ability to kill yeast blastoconidia and a reduced ability to inhibit the formation of yeast germ tubes. This study also shows that the stimulated flow rate of whole saliva for HIV-infected patients is reduced by over 40% as compared with healthy control patients. A further analysis of this issue shows that the flow rate is reduced to a greater extent for those HIV-infected patients having yeast in their saliva. These data show that two components of oral host defense (salivary anticandidal activity and salivary flow rates) are reduced in HIV-infected patients. These reductions may be factors that contribute to the development of oral candidiasis.

Acknowledgments

This work was supported by Public Health Service grants DE-12188 (to C.-

K.Y.) and DE-11381 (to T.F.P.) from the National Institute of Dental and Craniofacial Research. We also acknowledge the contributions of Jose L. Lopez-Ribot, Marta Caceres and William R. Kirkpatrick.

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