

Oral Fluids as an Alternative to Serum for Measurement of Markers of Immune Activation

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Oral fluids are convenient alternatives to blood sampling for evaluating significant metabolic components. Two forms of oral fluids, oral mucosal transudates (OMT) and saliva, were collected and compared for content of soluble products of immune activation. The data confirm that OMT and saliva represent distinct body fluids. The concentrations, outputs, and analyte/protein ratios of β -2-microglobulin (β 2M), soluble tumor necrosis factor alpha receptor II (sTNF α RII), and neopterin were measured. Both the OMT and the saliva of most of the individuals in the control healthy populations had measurable levels of all three activation markers. When the immune system is activated, as in human immunodeficiency virus (HIV) infection, the levels of β 2M and sTNF α RII are increased in both OMT and saliva compared to those in a healthy control population. OMT levels correlated better with levels in serum than did saliva and appear to reflect systemic immune activation in HIV infection. Because acquisition of oral fluids is noninvasive and easily repeatable, measurement of β 2M and/or sTNF α RII content in OMT could be useful in the assessment of disease activity in patients with HIV infection or chronic inflammatory diseases.

Oral fluids as test specimens have several advantages over blood and are increasingly being used in diagnosis and assessment of diseases (12, 13). They are easily obtainable and can be collected repeatedly without individuals having to come to medical clinics or offices except to deliver the samples. Two types of oral fluids can be collected: oral mucosal transudates (OMT) and saliva. The former resembles a filtrate of plasma, and the latter contains enzymes and other contributions from the parotid and salivary glands. The method of collection determines the predominance of OMT or saliva. Both types of oral fluids were collected from healthy individuals and also evaluated for use in assessing immune activation markers in human immunodeficiency virus (HIV) infection.

Immune activation is recognized as a major feature of HIV pathogenesis. It has been shown that the level of immune activation is closely related to the course of HIV disease and is a strong prognostic marker (8). The level of immune cell activation in HIV infection is usually assessed by measurement of β 2-microglobulin (β 2M) and/or neopterin (NPT) (10, 14, 15, 19, 32) or soluble tumor necrosis factor alpha receptor II (sTNF α RII) (3, 5) in serum.

NPT is released by macrophages activated by gamma interferon which has been secreted by stimulated T cells (16). NPT has been detected in human saliva (18). In one study, increased concentration of NPT has been reported in stimulated saliva of HIV-infected subjects (26). However, recent results of Evans and Wansbrough-Jones revealed no significant increase (7). Müller et al. found a lower parotid NPT output and no difference in the NPT concentrations in saliva samples from HIV-infected persons and controls (23). β 2M is a product of a variety of activated lymphoid cells. β 2M has also been detected in human saliva, and significantly higher levels were found in

saliva from patients with juvenile periodontitis (1), adult primary glomerulonephritis (28), and primary Sjögren's syndrome (20). There are no reports on β 2M measurements in the saliva of HIV-infected individuals. Use of oral fluid as a diagnostic medium for several other analytes, including steroid hormones (9, 25), therapeutic drugs (22, 27), drugs of abuse (30), etc., has been discussed as well. There are no reports of measurements of sTNF α RII in oral fluid.

The aims of the present study were (i) to investigate the feasibility of measuring the concentration of immune activation markers such as NPT, β 2M, and sTNF α RII in oral fluids, (ii) to compare the analyte output of those markers in OMT and saliva, (iii) to relate the findings on these two oral fluids to those on serum, (iv) to compare the changes in these three markers in the oral fluids of HIV-infected persons who exhibit substantial immune activation versus controls, and (v) to determine the interrelationship of the three different markers in the oral fluids.

MATERIALS AND METHODS

Study population. Serum, saliva, and/or OMT samples were collected after obtaining informed consent from 39 persons with HIV infection who participate in the Los Angeles cohort of the Multicenter AIDS Cohort Study (17). All patients had serum antibodies to HIV type 1 (HIV-1) as determined by enzyme-linked immunosorbent assay (Genetic Systems, Seattle, Wash.) and confirmed by Western blot analyses (Bio-Rad Laboratories, Hercules, Calif.) (24). Of the HIV-1-seropositive participants, 29 were asymptomatic and are the basis for this report. Three with clinically diagnosed AIDS and oral thrush at about the time of sample collection were compared later with the asymptomatic group. Two groups were selected as controls: (i) 10 healthy heterosexual volunteers and (ii) 16 homosexual HIV-1-seronegative participants from the Multicenter AIDS Cohort Study cohort.

Samples. Blood was collected by venipuncture, and serum was separated and stored frozen at -80°C until use. Oral fluid samples were collected by laboratory personnel between 9 and 11 a.m. without provocation with any stimulant (i.e., acids or mastication). Two commercially available collection devices were used by following the manufacturers' instructions. Samples were collected by placing the OraSure collection device (Epitope, Beaverton, Oreg.) between the lower cheek and gum for 2 min. These samples contained mainly OMT (21, 31). Samples were also collected by placing the Omni-Sal device (Saliva Diagnostic Systems, Vancouver, Wash.) under the tongue for 2 min. These samples we call

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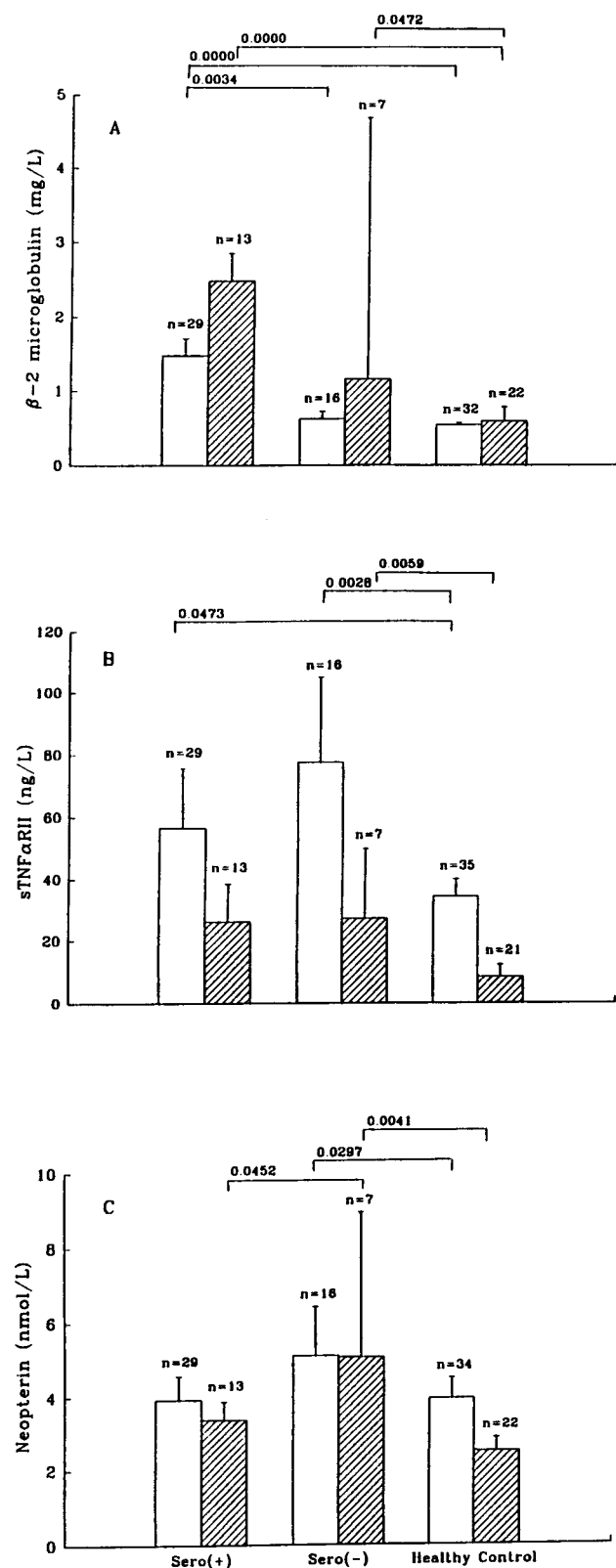


FIG. 1. Comparison of concentrations of β 2M (A), sTNF α R II (B), and NPT (C) in oral fluids of seropositive and seronegative homosexual men and a reference population. For OMT (open bars) and saliva (crossed bars) specimens, the number of samples (n) in each group is shown. Data are medians and standard errors. The *P* values of differences that are statistically significant are shown at the top.

saliva. For study participants who donated both blood and oral fluid, the samples were collected during the same visit. Both OMT and saliva were collected simultaneously from some subjects by placing one OraSure device between the lower cheek and gum and one Omni-Sal device under the tongue for 2 min. After collection, the oral fluid was transferred into a tube containing preservative buffer and thus diluted. The data for each sample were normalized by using a dilution factor (*F*) that was calculated by using the formula $F_{\text{OMT}} = (V_E + 0.1)/(V_E - 0.63)$ (for the OraSure device) or $F_{\text{SALIVA}} = (V_E + 0.45)/(V_E - 0.55)$ (for the Omni-Sal device), where V_E is the measured volume of liquid extracted from the absorbent pad of the collection device and 0.63 ml is the difference between the volume of preservative buffer (0.73 ml for the specific lot of OraSure devices used) and the volume of liquid (0.1 ml) that remained unextracted from the absorbent pad after centrifugation. The difference between the volume of preservative buffer (1.0 ml for the specific lot of Omni-Sal devices used) and the volume of liquid (0.45 ml) that remained unextracted from the absorbent pad when a serum separator was used for extraction was 0.55 ml. The 0.1- and 0.45-ml volumes were determined in preliminary test tube experiments by using a known volume of oral fluid. The ranges of collected volumes were 0.4 to 1.65 (average, 1.13 ± 0.24) ml of OMT and 0.35 to 2.2 (average, 1.21 ± 0.36) ml of saliva. Oral fluid samples were tested within 3 days of collection or stored at -80°C until use. Preliminary experiments comparing fresh samples and those frozen for 3 months indicated that freezing did not harm the analytes in these samples.

NPT quantification. NPT in serum was measured by radioimmunoassay (Hening Test Neopterin; B.R.A.H.M.S. Diagnostics GmbH, Berlin, Germany), purchased from Polymedco (New York, N.Y.), by following the manufacturer's instructions. As oral fluids have lower concentrations of NPT than serum does, a sample volume of 100 μl was used for OMT and saliva instead of the 20 μl used for serum testing. In preliminary experiments, this modification demonstrated that NPT could be measured in oral fluids. In this modified assay, the standards supplied with the kits were used as recommended at 20 μl /well and OMT and saliva samples were used at 100 μl /well. A factor of 5.9 was determined by testing five-times-diluted standards at 100 μl /well and used to transform experimental results to real NPT concentrations.

β 2M assay. β 2M in serum and oral fluids was measured by using the IMx automated microparticle enzyme immunoassay system and following the manufacturer's instructions for IMx β 2M (Abbott, Abbott Park, Ill.).

Quantitation of sTNF α R II. sTNF α R II was measured by using a huTNF α R II enzyme-linked immunosorbent assay kit manufactured by HyCult Biotechnology (Uden, The Netherlands) and purchased from CALTAG Laboratories (San Francisco, Calif.) and following the manufacturer's instructions. In preliminary recovery studies, no interference of oral fluids with the NPT, β 2M, and sTNF α R II assays was found.

Protein assay. Protein in oral fluids was quantified by the Bradford method (4) using the Bio-Rad protein assay kit with bovine plasma albumin as the standard. To normalize the data for an analyte in every sample tested, the ratio of the experimental value for the analyte to the protein concentration in the same test sample was used.

Oral fluid flow rate. Oral fluid was collected for 2 min with a collection device. The specimen was extracted from the absorbent pad of the device with a serum separator in the case of the Omni-Sal collection device or by centrifugation at $1,000 \times g$ for 15 min in the case of the OraSure collection device. The volume of the eluate (V_E) was measured. The volume of oral fluid (V_{OMT}) or V_{SALIVA} in the eluate was calculated by using the formula $V_{\text{OMT}} = V_E - 0.63$ (for the OraSure device) or $V_{\text{SALIVA}} = V_E - 0.55$ (for the Omni-Sal device). The output of a marker in the oral fluid was determined as its concentration was multiplied by the flow rate.

Measurement of CD4 $^+$ T-cell numbers. Whole blood samples were stained with anti-Leu3-phycoerythrin (CD4) and anti-Leu4-fluorescein (CD3) conjugated monoclonal antibodies (Becton Dickinson, Mountain View, Calif.). The percentage of CD4 $^+$ T cells was determined by using an EPICS C flow cytometer (Coulter Electronics, Hialeah, Fla.). The CD4 $^+$ T-cell numbers were determined by obtaining total and differential leukocyte counts as previously described (11).

Statistical analysis. The SAS program (29) (SAS Institute, Cary, N.C.) was used for statistical analysis. Data are presented as medians and 25th to 75th percentiles. Comparisons between groups were performed by using the nonparametric *t* test, the matched paired *t* test, and/or the Wilcoxon rank sum test. The Pearson rank test was used to assess the correlation between two variables. *P* values of less than 0.05 were accepted as significant.

RESULTS

Activation marker levels in normal OMT and saliva. β 2M, sTNF α R II, and NPT are detectable in normal OMT and saliva (Fig. 1). Levels of sTNF α R II were similar or higher in OMT. β 2M was similar or higher in saliva. Seronegative homosexual men generally had higher levels in both OMT and saliva than the general reference population.

Levels of immune activation markers in OMT and saliva of HIV-infected persons. In HIV-positive men without AIDS, the

TABLE 1. Correlation of analyte levels in oral fluids from HIV-seropositive persons

Sample and parameter	Correlation coefficient (<i>P</i> value)			
	NPT	β 2M	sTNF α RII	Protein
OMT^a				
β 2M	0.6104 (0.0004)			
sTNF α RII	0.5522 (0.0019)	0.8214 (0.0001)		
Protein	0.9234 (0.0001)	0.5825 (0.0009)	0.4949 (0.0063)	
Flow rate	-0.8193 (0.0001)	-0.4482 (0.0148)	-0.4309 (0.0196)	-0.7193 (0.0001)
CD4 ⁺ count	-0.1380 (0.4837)	-0.3666 (0.0550)	-0.2481 (0.2031)	-0.0502 (0.7997)
Saliva^b				
β 2M	0.4900 (0.0891)			
sTNF α RII	0.5497 (0.0516)	0.6581 (0.0145)		
Protein	0.8560 (0.0002)	0.6215 (0.0234)	0.8136 (0.0007)	
Flow rate	-0.9241 (0.0001)	-0.3665 (0.2180)	-0.4081 (0.1663)	-0.7858 (0.0015)
CD4 ⁺ count	0.2162 (0.4781)	-0.0234 (0.9396)	-0.0356 (0.9080)	0.0388 (0.8998)

^a *n* = 29.^b *n* = 13.

levels of β 2M and sTNF α RII were significantly elevated (Fig. 1) in both OMT and saliva. In OMT, the increase in the β 2M level was significant compared to the level in both the seronegative and healthy control groups. Correlational analyses showed that the levels of all three analytes were highly correlated (Table 1).

Comparison of levels of activation markers in serum and oral fluid of HIV-seropositive persons. β 2M levels were similar in saliva and serum (Fig. 2) but different in OMT, indicating a difference between these fluids. However, sTNF α RII and NPT concentrations were much higher in serum than in OMT or saliva. Correlation analyses (Table 2) revealed that β 2M and sTNF α RII levels in OMT correlated well with the levels in serum. The marker content of saliva, however, did not correlate significantly with the levels in serum. This is another difference between saliva and OMT.

Activation marker content of oral fluids considered as output and as ratio to protein content. The quantity of each marker was related to the flow rate (output) of oral fluid secretion and to the protein content (Table 3). Seropositive men, compared to healthy controls, demonstrated significantly increased β 2M and sTNF α RII outputs and ratios in both OMT and saliva. NPT output and analyte/protein ratio were not increased significantly in either OMT or saliva. Healthy controls, HIV-seronegative, and HIV-seropositive persons all had lower β 2M and higher sTNF α RII outputs and ratios in OMT than in saliva. These findings are similar to those revealed by direct analyses of the concentrations in OMT and saliva, indi-

cating that the extra measurements (e.g., of flow rate and protein content) may not be necessary.

NPT levels in OMT or saliva, however, were not substantially increased in HIV-infected persons, whether measured by concentration (Fig. 1) or in relation to oral fluid secretion flow rate or protein content (Table 3).

Relationship between marker levels in oral fluids and oral infection or stage of HIV disease. Eight HIV-seropositive persons who had oral thrush at the time of sample collection and three patients who had diagnosed AIDS were compared with the 29 seropositive, thrush- and AIDS-free men reported here. In the HIV-infected participants with AIDS, only NPT output in OMT was significantly different (3.08 ± 1.56 pmol/min, *n* = 3) from that of the asymptomatic, HIV-seropositive group (2.17 ± 0.56 pmol/min, *n* = 36) (*P* = 0.0284). Neither significant differences nor significant correlations between any other marker in OMT versus saliva (measured as a concentration, output, or ratio) and the diagnosis of AIDS were noted. The eight HIV-infected persons with oral thrush showed no significant differences from the thrush-free group. Apparently, oral thrush in HIV infection is not the cause of an increased level of oral fluids. Also, no significant correlations were found between CD4⁺ T-cell numbers (as a marker for the stage of HIV disease) and the concentration, output, or ratio of NPT, β 2M, or sTNF α RII in OMT or saliva (Table 1). This is in accord with previous reports that activation marker levels in serum provide information that differs from that provided by

TABLE 2. Correlation of analyte levels in oral fluids with levels in serum during immune activation in HIV-seropositive persons

Sample and parameter	Correlation coefficient (<i>P</i> value), no. of observations			
	NPT in serum	β 2M in serum	sTNF α RII in serum	CD4 ⁺ counts
OMT				
NPT	0.2996 (0.0676), 38	0.4365 (0.0061), 38	0.5509 (0.0016), 30	-0.1857 (0.2643), 38
β 2M	0.5327 (0.0006), 38	0.6474 (0.0001), 38	0.6190 (0.0003), 30	-0.3046 (0.0630), 38
sTNF α RII	0.2597 (0.1154), 38	0.5924 (0.0001), 38	0.4072 (0.0255), 30	-0.2607 (0.1139), 38
CD4 ⁺ count	-0.4103 (0.0117), 37	-0.5393 (0.0006), 37	-0.5194 (0.0039), 29	
Saliva				
NPT	0.0570 (0.8222), 18	0.1382 (0.5845), 18	0.3400 (0.1675), 18	-0.2703 (0.2781), 18
β 2M	0.1142 (0.6518), 18	0.3195 (0.1963), 18	0.4438 (0.0651), 18	-0.2940 (0.2363), 18
sTNF α RII	0.0016 (0.9949), 18	0.4961 (0.0363), 18	0.3578 (0.1449), 18	-0.1995 (0.4274), 18
CD4 ⁺ count	-0.5206 (0.0268), 18	-0.6360 (0.0046), 18	-0.5236 (0.0258), 18	

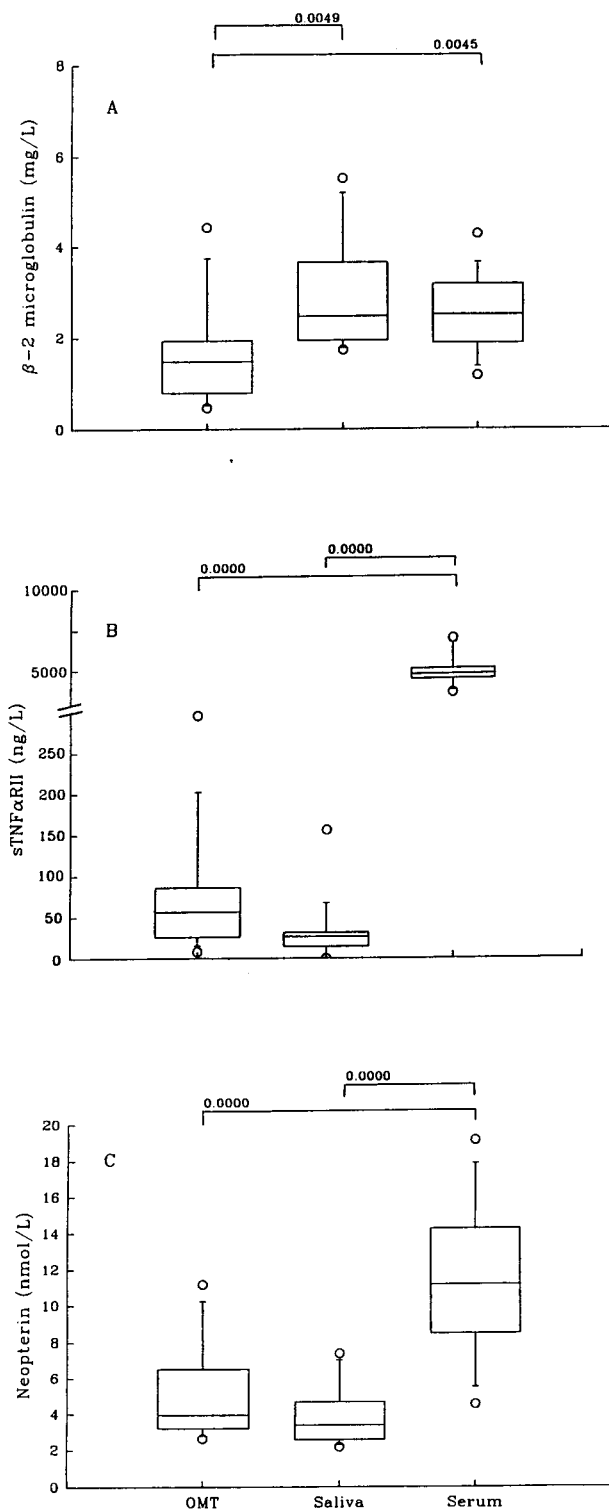


FIG. 2. Comparison of levels of immune activation markers in oral fluids and sera of HIV-1-infected persons. (A) Box plots of β 2M concentrations in OMT ($n = 39$), saliva ($n = 18$), and serum ($n = 38$). (B) Box plots of sTNF α RII concentrations in OMT ($n = 39$), saliva ($n = 18$), and serum ($n = 30$). (C) Box plots of NPT concentrations in OMT ($n = 39$), saliva ($n = 18$), and serum ($n = 38$). The line inside each box represent the median; the lower and upper limits of each box indicate the 25th and 75th percentiles, the vertical lines are the 10th and 90th percentile points, and the lower and upper circles indicate the 5th and 95th percentile points. The P values of statistically significant differences are shown at the top.

CD4⁺ T-cell levels, thus indicating independence of these markers of HIV disease.

DISCUSSION

Oral fluids have several advantages over blood and are increasingly being used in the diagnosis and assessment of diseases. The advantages include easy collection, ability to obtain large numbers of specimens within a short time period, safety due to the lack of need for needles and the reported low viral load in oral fluids, safer disposal of waste materials, and possible low overall cost (21, 31). Oral fluid testing has been proposed as the procedure of choice for testing for antibodies to viruses, including HIV (31). Our present findings indicate that oral fluids can also be an alternative to serum or plasma for measurements of β 2M and sTNF α RII as markers of immune activation.

Specific features of oral fluid are important to consider when quantitative measurements are sought. These include (i) selection of OMT or saliva as the fluid to measure, (ii) collection of spontaneous versus stimulated fluids, and (iii) ratio of analyte to reference parameters such as oral fluid flow rate and protein content.

Oral fluids are a mixture, with saliva and OMT being the main components. Saliva is a complex mixture of secretions of several salivary glands. OMT (also called gingival crevicular fluid) (21, 31) is the fluid derived from the transport of serum components through the oral mucosa into the mouth. In our present study, efforts were made to collect and analyze OMT separately from saliva. Our analyses emphasize that OMT and saliva are distinct body fluids.

In many saliva studies, samples were collected after stimulation of oral fluid secretion by different techniques (21). It has been shown that variations in stimulation and sample collection methods caused differences in immunological responses (2) and salivary gland function (6). We assume that unstimulated oral fluid better represents the physiological state. Furthermore, as the data indicate, the levels of all of the analytes studied are inversely correlated to the flow rate. In this study, we collected unstimulated samples by using standardized procedures and collection devices, recently developed and commercially available, that include preservative buffers to prevent proteolytic degradations.

Changes in the flow rate influence the concentrations of analytes in oral fluids. Flow rate was inversely correlated with the concentrations of all analytes (Table 1), thus indicating that fluctuations in flow rate will cause differences in marker values. However, when concentrations were normalized with regard to flow rate and analyte measurements were presented as output, variations in output within a short time period were evident. This indicates that changes in analyte concentrations may reflect additional factors. Analyte concentrations in oral secretions, combined with the easy repeatability of oral tests, provide an opportunity for identification of rapid changes and follow-up of short-term changes caused by therapeutic or other interventions.

To normalize analyte concentrations, we used the protein concentration in samples as a reference and present the data as an analyte concentration-to-protein concentration ratio. When total protein is selected as a reference, two facts may be important. The total proteins are a complex mixture. Furthermore, there are large differences among the molecular weights of NPT, β 2M, and sTNF α RII. As the results indicate (Table 3), the use of this ratio provides data that are similar to the output data based on flow rates and apply to both β 2M and sTNF α RII. The differences between the levels of analytes in

TABLE 3. Analyte levels in oral fluids presented in relation to output and protein content^a

Sample and group (no. of men)	β 2M		sTNF α R		NPT		Protein
	Output (μ g/min)	Ratio (μ g/mg)	Output (pg/min)	Ratio (pg/mg)	Output (pmol/min)	Ratio (pmol/mg)	Output (μ g/min)
OMT							
Healthy controls (35)	0.30 (0.15)	0.39 (0.16)	20.16 (16.29)	26.57 (18.02)	1.93 (0.62)	2.69 (0.86)	744.7 (199.1)
HIV seronegative (16)	0.24 (0.18)	0.27 (0.13)	37.12 (38.76)	40.17 (31.04)	1.74 (0.41)	2.41 (0.72)	804.0 (393.1)
HIV seropositive (29)	0.77 (0.47) ^b	0.81 (0.46) ^b	32.96 (29.21) ^b	35.50 (30.30)	2.07 (0.53)	2.36 (0.67)	964.4 (439.4) ^b
Saliva							
Healthy controls (22)	0.57 (0.28)	0.68 (0.33)	6.69 (8.61)	7.51 (8.07)	2.03 (0.87)	2.36 (0.98)	920.6 (275.4)
HIV seronegative (7)	1.06 (1.32)	1.07 (0.60)	17.68 (26.46)	25.36 (28.74)	2.04 (0.65)	4.35 (4.25)	788.1 (558.4)
HIV seropositive (13)	1.77 (0.88) ^b	1.86 (0.71) ^b	16.55 (12.41) ^b	17.04 (12.84) ^b	2.09 (0.39)	2.36 (0.54)	932.9 (271.5)

^a Values are means and standard deviations (in parentheses).

^b $P < 0.05$ versus controls.

HIV-infected persons and normal controls are readily apparent.

Immune activation is an essential feature of HIV pathogenesis. Increased production of cytokines is reflected by elevated levels of the products of cytokine activation, such as β 2M, in serum and plasma. The level of β 2M (measured as a concentration, output, or ratio) in both OMT and saliva is higher in HIV-infected persons than in healthy controls. In HIV-infected persons, compared to HIV-seronegative controls, the increase in the β 2M level is significant for OMT but not for saliva. This difference between OMT and saliva and, more importantly, the lack of correlation between saliva and OMT for the activation parameter indicate that OMT and saliva are distinct body fluids.

In HIV-infected persons, the level of β 2M was significantly higher in saliva than in OMT (Fig. 2). Such an elevation could be caused by (i) a selectively higher rate of β 2M transport in salivary glands than in OMT or (ii) an increased rate of local synthesis of β 2M that reflects higher activation of oral mucosal immune cells. The level of β 2M in OMT correlated with the levels of all immune activation markers in serum (Table 2). In contrast, the β 2M level in saliva did not correlate with that of any marker in serum. Thus, it seems more likely that β 2M in OMT reflects mainly marker levels in serum and generalized immune activation, while β 2M in saliva may represent local synthesis. Together, these data strongly indicate that OMT, but not saliva, is the preferred oral fluid alternative for β 2M measurement in serum as an indicator of systemic immune activation. sTNF α RII levels in OMT correlated fairly well ($P < 0.026$) with levels in serum, but saliva did not. Thus, OMT could be an alternative to serum for measurement of sTNF α RII.

We found that NPT levels in the saliva and OMT of the HIV-seropositive group were not significantly increased compared to those in controls. Our NPT level finding in nonstimulated saliva differs from the results of Reibnegger et al., who found significantly higher NPT levels in the stimulated saliva of HIV-infected patients than in that of controls (26). However, our saliva findings agree with that of Müller et al., who have shown that NPT concentration and output in stimulated parotid saliva of HIV-infected persons were neither significantly increased over those of controls nor correlated with NPT levels in serum (23). Similar results for NPT levels in stimulated whole saliva have been reported by Evans and Wansbrough-Jones (7). An important difference between those studies and ours is that we used unstimulated saliva.

Serial assessments of NPT or β 2M levels in serum or urine have proved useful in assessing the course of diseases such as

multiple sclerosis (11a) and inflammatory bowel disease (24a). The value of measuring markers of immune activation in HIV infection is well known. In the present study, we have documented the capacity to detect generalized immune activation by measurement of the products of immune activation in OMT and saliva. Acquisition of OMT compared to serum or plasma is noninvasive and easily repeatable, and measurements of β 2M and/or sTNF α RII could be useful for identification of rapid responses or for follow-up of short-term changes caused by complications or therapeutic interventions in patients with HIV infection or autoimmune disorders or other chronic inflammatory disorders.

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