Outpatient biopsies of the palatine tonsil: Access to lymphoid tissue for assessment of human immunodeficiency virus RNA titers

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OBJECTIVES: **Our objective was to assess the feasibility of using tonsillar lymphoid biopsy specimens obtained on an outpatient basis to quantitate a patient's lymphoid human immunodeficiency virus (HIV) RNA titers.**
DESIGN: **A pilot cohort study was performed.**
PATIENTS: **We evaluated ten HIV-seropositive patients who ranged in age from 26 to 48 years and had CD4+ cell counts ranging from 110 to 833 at enrollment.**
MAIN OUTCOME MEASURES: **The main outcome measures were tolerance and safety of outpatient tonsil biopsies and quantitation of HIV RNA titers in tonsillar lymphoid biopsy specimens, plasma, and peripheral blood mononuclear cells determined by a new method of HIV RNA signal amplification with branched DNA probes.**
RESULTS: **Outpatient tonsil biopsies were well tolerated and were performed without complications. Nine of 10 tonsil biopsies from the HIV-seropositive patients examined were positive for significant concentrations of HIV RNA, ranging from 10^6 to 10^9 HIV RNA equivalents per gram of tissue. All of the HIV RNA-positive tonsillar lymphoid specimens had HIV RNA titers that were 10^2 to 10^4 times greater than those recovered from plasma (per milliliter) of the same patient obtained at the time of biopsy.**
CONCLUSIONS: **Sufficient tonsillar tissue can be obtained in an outpatient clinic setting to quantitate lymphoid HIV titers by the new branched-DNA signal amplification method with relative ease and without complication. The biopsy method described here affords ready access to the lymphoreticular system, which may help to advance our understanding of the pathogenesis of myriad immune diseases without the need for excisional node biopsies.**
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The CD4+ cell count has been the key criterion for following the progression of immunodestruction from the human immunodeficiency virus (HIV). Monitoring of therapy and disease progression are based on following this cell count and clinical events that herald further immunosuppression. However, more than 90% of CD4+ lymphocytes are sequestered in the lymphoid tissues. It is estimated that by the time "early therapy" is initiated (at CD4+ cells ≤ 500), between 80% and 90% of the body's CD4+ cells have already been destroyed. Determination of the peripheral CD4+ lymphocyte count is therefore an incomplete assessment of the immune system with regard to HIV progression. Similarly, assays for circulating HIV p24 antigen and culturable HIV in peripheral blood falsely suggest minimal or absent viral replication during the clinically silent period of immune destruction.

It has recently been shown that lymphoid tissue

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including the palatine tonsil represents a covert reservoir of HIV during the latent seropositive yet clinically negative period of HIV infection.[1] Determinations of lymphoid HIV titers in living patients are limited by the relative inaccessibility of the lymphoid tissue compartment. Such studies in fresh tissues have required excisional lymph node biopsies, with inherent risks to both the patient and the surgical team. As a result many studies to date have been performed on postmortem specimens.

In a postmortem study of lymphoid tissues from deceased intravenous drug users who were HIV-seropositive, tonsils were positive for HIV more often than any other lymphoid tissue examined, with 78% of the cases tonsil-positive for HIV RNA.[2] Tonsil biopsies are routinely performed during otolaryngologic procedures to rule out carcinoma of the tonsil. We therefore reasoned that similar biopsies performed on an outpatient basis might provide a sufficient lymphoid specimen on which to determine lymphoid HIV RNA titers in living HIV-positive patients.

Current methods available for quantitation of HIV viral RNA in tissues are not suitable for routine use in a clinical setting. Quantitative culture techniques are labor-intensive and are limited by lack of reproducibility. Although polymerase chain reaction techniques can provide the necessary level of sensitivity, they are similarly labor-intensive and remain merely semiquantitative.[3] Competitive quantitative polymerase chain reaction provides greater accuracy but is technically demanding and is therefore difficult to apply in the clinical setting.[3-5]

In this study we addressed the feasibility of determining HIV RNA titers in tonsillar lymphoid tissue obtained in an outpatient setting from seropositive patients with no symptoms. We used a new technique for the quantitation of HIV viral RNA in tonsil lymphoid tissue based on solid-phase RNA capture with oligonucleotide probes and branched DNA (bDNA) signal amplification technology in a 96-well microplate format well suited to automation. The viral RNA is detected directly at its physiological level without replication of the target sequence. The combination of outpatient tonsil biopsy with this new method of HIV RNA quantitation has the potential to provide a window into the covert lymphoid HIV reservoir of living subjects.

METHODS AND PATIENTS

Patients

Patients at the Saint Paul Ramsey HIV Program Clinic were surveyed for volunteers to participate in this study; approval by the Institutional Review Board and informed consent were obtained. Inclusion criteria consisted of (1) documentation of HIV antibody seropositivity by criteria of the Centers for Disease Control on enzyme-linked immunosorbent assay and Western blot analysis, (2) accessible tonsillar tissue, and (3) written informed consent. Exclusion criteria were limited to any medical condition precluding tonsillar biopsy or any opportunistic infection at time of study. Table 1 summarizes the demographic and clinical data from these 10 patients; all the patients were men.

Specimens

All patients were screened for accessibility of tonsillar tissue at a clinic visit before the time of biopsy; a protime and partial thromboplastin time were also obtained at that time. At the time of biopsy, 30 cc of peripheral blood was collected for determination of CD4+ cell counts and HIV RNA titers in plasma and peripheral blood mononuclear cells (PBMC). The otolaryngologist was protected in an impermeable surgical gown and a surgical mask with a face shield and was double-gloved for the procedure. The patient was placed in a seated, upright position in a standard examination chair in the outpatient surgery department of the Otolaryngology Outpatient Clinic at St. Paul Ramsey Medical Center, and the headrest was adjusted for comfort and security. Topical anesthetic (10% Xylocaine) was sprayed over the right posterolateral oropharynx including the right tonsil, tonsillar pillars, and soft palate. This procedure was followed by infiltration of 0.5 cc 2% Xylocaine containing epinephrine (1:100,000 dilution) into the superior pole of the right tonsillar capsule with a 30 gauge needle; an attempt was made to instill this local anesthetic into the potential peritonsillar space between the tonsillar capsule and the tonsillar fossa, as for a tonsillectomy with the patient under local anesthesia. After a period of 3 minutes anesthesia and suppression of gag response were tested by manipulation of the right tonsil with a tongue blade. Rarely a second injection of 0.5 cc of local infiltrate was required to achieve complete local anesthesia. The tonsil biopsy specimen was then obtained from the superior portion of the tonsil with a triangular adenoid punch biopsy forceps. The tonsil biopsy specimen was immediately sharply divided into approximately two equal portions, and the specimens were placed into media for assays (see following text). Hemostasis at the biopsy site was achieved with a chemical cautery application ($AgNO_3$), followed by cold water oral

Table 1. Age and HIV status of patients undergoing tonsillar biopsy

Patient no.	Age (yr)	HIV+*	CDC Class
1	48	11/86	I
2	40	4/86	I
3	31	6/86	I
4	45	12/92	I
5	32	3/86	I
6	32	5/94	I
7	31	1/86	I
8	31	8/86	I
9	32	9/93	I
10	26	4/92	II

CDC, Centers for Disease Control and Prevention.
*HIV+ column indicates documentation date of seropositivity.

rinses. The patients were examined again at 15 minutes before they were discharged from the clinic. Patients were discharged from the clinic with acetaminophen, acetaminophen-codeine analgesics, and Cetocaine topical spray. Follow-up of patients was carried out by telephone contact at 5 to 7 days and a clinic follow-up at 4 weeks.

HIV RNA Signal Amplification With bDNA

Determination of HIV RNA was performed by quantitative bDNA signal amplification assay as described,[6] as modified and applied to lymphoid tissue.[7] Quantitation of HIV viral RNA levels in PBMC and plasma with bDNA signal amplifcation was performed essentially as described.[8]

The bDNA method is based on the hybridization of HIV-1 viral RNA in the sample to 39 oligonucleotide probes (target probes). Each target probe comprises 33 bases complementary to the most conserved regions of the HIV-1 *pol* gene.[6] Each target probe also contains a common base sequence that remains as a single-stranded overhang after hybridization of the target probe to the HIV RNA. Assay hybridizations are carried out in wells of a 96-well microwell plate. Capture of the viral RNA to the surface of the microwell, which is coated with an oligonucleotide capture probe (complementary to the 20-base overhang sequence), is mediated through this overhang. A subset of different target probes, which contain a second common 20-base overhang sequence, binds bDNA amplifier molecules to the RNA-target-probe complex. Alkaline phosphatase-conjugated probes then bind to the bDNA side chains. The RNA-probe complex is finally detected with the chemiluminescent dioxetane substrate for alkaline phosphatase (Lumiphos 530, Lumigen, Detroit, Mich.), and light emission is quantitated with a standard curve that is run on every assay plate. With 39 target probes per molecule of HIV RNA, each capable of binding a bDNA probe with 15 branches able to bind three alkaline phosphatase probes per branch, the potential amplification factor per HIV RNA is 1755. The results are expressed as HIV-RNA equivalent per gram of tissue for tonsillar lymphoid tissue, equivalent per 10^6 cells for PBMC, or equivalent per milliliter for plasma.

Tissue Processing and Assay

Tonsil tissue intended for HIV RNA quantitation was placed immediately on collection into a medium of 150 mmol/L NaCl, 50 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid pH 7.5, 0.5% sarcosyl, 8 mmol/L ethylenediaminetetraacetic acid, 0.04% Na Azide, transferred to the laboratory on ice, and frozen at $-70°$ C until the time assay was performed. For assay, samples were thawed and homogenized in 8 mol/L guanidine human cultured lymphoblasts and 0.5% sarcosyl, and RNA was selectively precipitated with ethanol. The precipitated RNA underwent resolubilization in the bDNA assay sample buffer containing proteinase K and 0.5% sarcosyl and was added directly to a 96-well microplate for quantitation of HIV RNA (Quantiplex HIV-RNA, Chiron Corporation, Emeryville, Calif.) as described.[6] Most of the specimens required dilution (400 mmol/L LiCl, 100 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid pH 7.5, 8 mmol/L ethylenediaminetetraacetic acid, 0.5% sarcosyl, 12 μg/ml sonicated salmon sperm DNA, 0.04% Na Azide, 0.04% Proclin 300 [Supelco Inc., Bellefonte, Pa.]) to bring them within the range of detection.

RESULTS

Survey of the Saint Paul Ramsey HIV Program Clinic for volunteers for this study yielded a 60% positive response rate; 80% of enrolled patients returned for a second follow-up biopsy at 4 weeks. All patients were well healed when examined at that time; many had no evidence of their previous biopsy. All patients tolerated the biopsy procedure well. No significant postoperative complications occurred. Discomfort at the biopsy site resolved within 48 hours after biopsy in 8 of 10 patients, with 2 patients reporting persistent sore throats for 5 and 10 days, respectively; this problem fully resolved without residual discomfort and without further intervention. One of 10 patients reported otalgia ipsilateral to the tonsil that had undergone biopsy; this condition had

Table 2. Simultaneous CD4+ cell counts and HIV RNA levels in the plasma, PBMC, and tonsil compartments

Patient no.	CD4+ count/mm^3	Plasma HIV*	PBMC HIV	Tonsil HIV
1	782	26,380	<5,000	2,000,000
2	821	<5,000	10,700	Not detected
3	587	7,400	<5,000	49,000,000
4	551	10,110	<5,000	1,100,000,000
5	833	75,870	13,000	580,000,000
6	294	<5,000	7,070	1,100,000
7	204	<5,000	<5,000	280,000,000
8	347	21,430	15,100	550,000,000
9	342	5,810	7,880	82,000,000
10	110	433,700	72,090	400,000,000

*Plasma HIV RNA concentrations expressed as HIV equivalents per milliliter.
PBMC HIV RNA concentrations expressed as HIV equivalents per 10^6 cells.
Tonsil HIV RNA concentrations expressed as HIV equivalents per gram of tissue.
The limit of sensitivity of the bDNA signal amplification method was 5000 HIV equivalents per unit of tissue.

lasted for 2 days after the biopsy had been performed. One patient had an aphthous ulcer at or near the biopsy site, which resolved spontaneously over a 2-week period without further intervention.

The HIV RNA titers in tonsil lymphoid tissue of 10 patients who are HIV-seropositive and in HIV titers in plasma and PBMC collected at the time of tonsil biopsy are summarized in Table 2. Nine (90%) of 10 tonsil lymphoid specimens from the HIV-positive patients examined had significant HIV RNA titers; HIV RNA was undetectable in tonsillar lymphoid tissue of one patient.

Figure 1 depicts a histologic preparation of a portion (20% to 25%) of a tonsillar biopsy specimen typically obtained (100 mg) with the triangular adenoid biopsy forceps. The mass of tonsil biopsy specimens obtained in this manner ranged from 20 to 200 mg. As apparent in Fig. 1, this small portion of a specimen yields approximately five to eight germinal follicles. Biopsy specimens examined in this study therefore contained a total of 4 to 40 lymphoid follicles each. All biopsy specimens in this study contained lymphoid tissue.

HIV RNA was readily detectable in portions of tonsillar lymphoid tissue as small as an estimated 2 mg by the bDNA signal amplification method. HIV RNA titers of nearly all tonsillar lymphoid specimens were extremely high; most specimens required dilution to bring them into the working range of the assay. As evident from Table 2, HIV RNA titers in HIV RNA-positive tonsil lymphoid tissues were 10^2 to 10^4 times greater than plasma and PBMC titers on a per milliliter or per 10^6 cells basis, respectively.

DISCUSSION

This study demonstrates the ease and safety with which tonsillar lymphoid tissue may be accessed for investigation, thus avoiding an open surgical procedure. The method was without significant complications and was well tolerated by all patients, with 80% of those enrolled returning for follow-up biopsies at 4 weeks. One patient had an aphthous ulcer at or near the biopsy site; it healed spontaneously over the next 2 weeks. In light of the relatively common occurrence of these oral lesions in patients with HIV and the enigmatic nature of their cause regardless of HIV status, it is unclear whether the ulcer in this patient was coincidental or the result of biopsy-related inflammation. Regardless, this occurrence would not constitute a contraindication for further biopsies.

The single patient in this study without detectable HIV RNA in tonsillar lymphoid tissue also had extremely low HIV RNA titers in PBMC and plasma HIV RNA titers below the sensitivity cutoff of the bDNA assay. It may be significant that this same patient had been on antiviral medications since 1988. The effect of antiviral therapeutics on the lymphoid HIV viral load clearly merits further investigation. Encouraged by these preliminary results, we are planning to apply these methods to a prospective longitudinal analysis of the efficacy of antiviral agents on tonsillar lymphoid HIV titers in these patients and to statistically address the reproducibility of this HIV RNA assay as applied to tonsillar lymphoid tissue.

This study represents the first clinical application

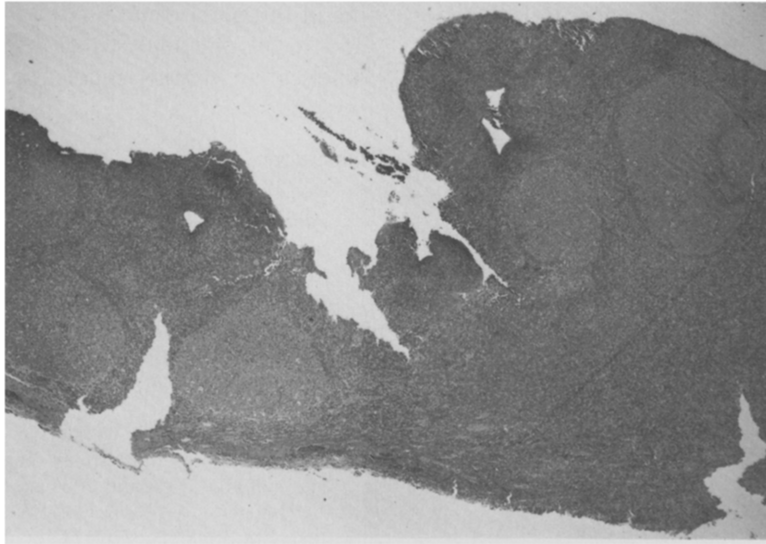

Fig. 1. Hematoxylin-eosin histologic preparation of portion of outpatient tonsil biopsy revealing tonsillar lymphoid architecture. Figure depicts only portion (20% to 25%) of 100 mg specimen. (Magnification approximately ×40.)

of two novel methods: an outpatient application of a standard operative biopsy method and a new RNA quantitation method with bDNA probes used as a signal amplifier. Our findings corroborate earlier *qualitative* reports of HIV RNA in tonsil lymhoid tissue[1,2] but constitute the first *quantitative* analysis of tonsillar HIV titers and the first detection of HIV RNA in tonsillar lymphoid tissue obtained on an outpatient basis. Tonsil biopsies from 90% of those HIV-seropositive patients examined in this study revealed significant HIV load. This finding is in contrast to the lower percentage of detection of HIV RNA in postmortem tonsils of patients seropositive for HIV.[2] This difference may reflect technical differences between the studies that perhaps resulted from the exquisite sensitivity of the bDNA signal amplification system and the fresh nature of the biopsy specimens used in this study or from possible population differences. Regardless, it is clear that patients clinically negative for HIV may harbor extremely high HIV load in palatine tonsil lymphoid tissue.

The HIV RNA tites detected in the plasma of patients in this study with the bDNA signal amplification method are in agreement with those reported elsewhere based on quantitative polymerase chain reaction methods.[4] With the bDNA method we found that the tonsil lymphoid tissues examined bear HIV viral RNA levels that are much higher than HIV RNA titers recovered from PBMC or plasma of the same patient. Similarly, these tonsillar lymphoid viral titers are much greater than other tissues examined to date including other lymphoid tissues and tissues analyzed with this bDNA signal amplification method.[6-8] Why the palatine tonsil should harbor such high HIV RNA levels can only be a matter of speculation at this time, but it may be related to the pharyngeal and nasopharyngeal lymphoid hyperplasia commonly associated with HIV infection.[9,10]

The bDNA signal amplification assay used in this study has proved simple, reliable, rapid, and extremely sensitive. This method is readily applicable to a variety of tissues. In this study we have quantitated HIV RNA in tonsil lymphoid, PBMC, and plasma with this method. Achieving sufficient sensitivity in RNA detection techniques has typically required radioactive probes; that this method is able to do so with biochemical probes is clearly advantageous. For the purposes of tonsil lymphoid HIV RNA quantitation, as little as 2 mg of tissue contained detectable levels of RNA, quantifiable by the bDNA signal amplifcation method (data not shown). This method therefore introduces the possibility of performing similar molecular quantitations on fine-needle aspirates, which typically yield approximately 5 mg of tissue. Furthermore the bDNA amplication RNA assay format is readily applicable to large numbers of clinical samples.

Whether the HIV RNA determinations described

here are representative of HIV concentrations elsewhere in the same or opposite tonsil or of lymphoid tissue elsewhere in the same patient is not addressed by our data. However, in preliminary work that described the quantitation of HIV RNA with the same method used here, Dailey et al.[6] obtained assays of several portions of the same lymphoid specimens. They found that the difference between multiple samples from the same lymph node or spleen was less than one half log; separate samples of left and right tonsil were virtually identical in viral load in the single patient examined. Whether the reproducibility of this assay as applied to tonsil lymphoid tissue is validated and whether tonsil lymphoid HIV RNA levels can be generalized to other lymphoid tissues necessitates further examination. Another issue to be addressed is the nature of the HIV RNA being measured by the bDNA assay and how it relates to infectious viral titers.

This simple biopsy method may prove to be valuable for directly monitoring the covert HIV lymphoid reservoir, for helping to elucidate the pathogenesis of HIV immune destruction, and for monitoring both the disease progression and success of therapeutic intervention in patients with HIV. One recent report used cultured human tonsillar tissue as a model for HIV pathogenesis, confirming the values of the tonsil as a means of assessing the immune system.[11] The described method has several advantages over open-node biopsies for assessing the lymphoid system including lower patient morbidity, lower risk involved for the surgical team, and significantly reduced costs for the procedure and follow-up. Of greater value is the ability to perform repeated evaluations in the same patient, which would be difficult to justify in the case of open lymph node biopsies. Ultimately, the most important contribution made by this biopsy method may be the window that it opens into the lymphoreticular system. This view may help to advance our understanding of the pathogenesis of myriad immune diseases and to permit analyses of tissue penetration and kinetics of various medications directed at this system.

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