Retrovirus in salivary glands from patients with Sjögren's syndrome

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Abstract

Aims—To investigate the possibility of an immune response to retroviral antigens or of detecting retrovirus in Sjögren's syndrome.

Methods—Retroviruses were sought in labial salivary glands and peripheral blood mononuclear cells from patients with Sjögren's syndrome by immunoblotting assay, immunohistochemical assay, polymerase chain reaction (PCR), reverse transcriptase (RT) activity assay, and transmission electron microscopy.

Results—Sera from five of 15 patients with Sjögren's syndrome (33%) reacted against p24 group specific antigen (*gag*) of human immunodeficiency virus (HIV). Labial salivary gland biopsy specimens from seven of the 15 patients with Sjögren's syndrome (47%) contained an epithelial cytoplasmic protein reactive with a monoclonal antibody to p24 of HIV. PCR was performed to detect HIV and human T lymphotropic virus type I (HTLV-I) genes from salivary gland tissues and peripheral blood mononuclear cells from patients with Sjögren's syndrome. Mn²⁺ dependent, Mg²⁺ independent RT activity was detected in the salivary gland tissues in three of 10 patients. A-type-like retroviral particles were observed in epithelial cells of salivary glands by transmission electron microscopy. Target genes for HIV and HTLV-I were not found in any of the salivary gland tissues or peripheral blood mononuclear cells from Sjögren's syndrome patients.

Conclusions—The data suggest the presence of an unknown retrovirus similar to HIV in the salivary gland which might be involved in the pathogenesis of a subpopulation in Sjögren's syndrome.

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Sjögren's syndrome is an autoimmune disease characterised by inflammation of the exocrine glands and systemic production of auto-antibodies.¹⁻⁷ The characteristic sicca symptoms of dry eyes⁸ and dry mouth⁹ are due to a primary pathological process in the lacrimal and salivary glands. Examination of salivary glands is therefore an ideal means of investigating this disease.¹⁰⁻¹¹ Sjögren's syndrome is thought to arise from an interaction of genetic, hormonal, and environmental factors. Viruses have long been considered potential aetiopathological

agents,¹² and retroviruses are receiving increasing attention for their involvement in the induction of autoimmunity.¹³ Retroviruses are known to infect cells of the immune system,¹⁴ where they can cause abnormalities in immune regulation such as destruction or overstimulation of T cells, excess antibody production, or lymphomas.¹⁵

Sicca symptoms mimicking Sjögren's syndrome have been described in diseases associated with retroviruses. Sjögren's syndrome is one of several autoimmune diseases that overlap clinically with diseases induced by human immunodeficiency virus (HIV).¹⁶⁻¹⁹ The characteristic symptom of Sjögren's syndrome is dryness of the mouth and eyes, which is also sometimes observed as a manifestation of HIV infection.²⁰⁻²⁴ The dryness in both Sjögren's syndrome and HIV disease is due to loss of salivary and lacrimal gland function and is accompanied by lymphocytic infiltration of these glands. An additional link between Sjögren's syndrome and HIV disease is the observation that 30% of patients with primary Sjögren's syndrome produce serum antibodies that react with the p24 core (*gag*) protein of HIV.²⁵ A human intracisternal A-type retroviral particle that is antigenically related to HIV has also been identified in lymphoblastoid cells cocultured with homogenates of salivary glands from Sjögren's syndrome patients.²⁶ Similarity to the increased soluble CD4 molecules observed in HIV infected sera²⁷ has been noted in sera from Sjögren's syndrome patients.²⁸ An association between Sjögren's syndrome and human T lymphotropic virus type I (HTLV-I) has also been suggested.²⁹⁻³⁰ Some HTLV-I infected patients with tropical spastic paraparesis develop features of Sjögren's syndrome.³¹ Recently, using in situ hybridisation and polymerase chain reaction (PCR), the *tax* gene but not the *gag*, *pol*, or *env* genes of HTLV-I was detected in labial salivary gland sections from two of nine patients with Sjögren's syndrome but not in those from control subjects.³² These observations suggest the possibility of a retroviral aetiology in Sjögren's syndrome.

In this study we investigated the possibility of detecting retrovirus in labial salivary glands and peripheral blood mononuclear cells (PBMC) from patients with primary Sjögren's syndrome by immunohistochemical assay and PCR. In addition, we examined whether RNA dependent DNA polymerase activity (reverse transcriptase, RT) specific for retrovirus can be detected in salivary gland tissues from patients with Sjögren's syndrome. We also looked for retroviral particles in salivary gland tissues

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HIV: LTR (533 bp fragment)
 Forward: 5'-AACTACACACCAGGGCCAGG-3' (20 mers)
 Reverse: 5'-ACACTGACTAAAAGGGTCTG-3' (20 mers)
 gag (920 bp fragment)
 Forward: 5'-GTAATACCCATGTTTTTCAGC-3' (20 mers)
 Reverse: 5'-TGAGAGGGAGTTGTTGTGTC-3' (20 mers)
 HTLV-I: LTR-gag (240 bp fragment)
 Forward: 5'-CCACCCCTTTCCCTTTCAT-3' (19 mers)
 Reverse: 5'-ATGAGCGGCCAGCCCCCGG-3' (20 mers)
 tax (158 bp fragment)
 Forward: 5'-CGGATACCCAGTCTACGTGT-3' (20 mers)
 Reverse: 5'-GAGCCGATAACGCGTCCATCG-3' (21 mers)

Figure 1 Primer sequences polymerase chain reaction (PCR) for HIV and HTLV-I.

from Sjögren's syndrome patients using transmission electron microscopy.

Methods

STUDY SUBJECTS

Fifteen female patients (age 39 to 85 years) who fulfilled the diagnostic criteria for primary Sjögren's syndrome were studied. The criteria used to diagnose primary Sjögren's syndrome were those proposed by Fox *et al.*³³ The patients did not have associated rheumatoid arthritis, nor were they taking corticosteroids or immunosuppressive drugs. None was in a high risk group for acquired immunodeficiency syndrome (AIDS). Ten healthy women (age 35 to 81 years) served as normal control subjects.

Salivary gland tissues were obtained by performing lip biopsies on 15 patients with primary Sjögren's syndrome and 10 healthy persons at necropsy, and were stored at -80°C until use in PCR analysis, RT activity assay, and morphological analysis.

Blood samples were obtained by venepuncture, and sera and PBMC were stored at -80°C until use in immunoblotting studies and PCR analysis.

ENZYME LINKED IMMUNOELECTROTRANSFER BLOT (WESTERN BLOT) ASSAY

Western blots were performed using commercial kits (HIV: Bio-Rad Labs, Hercules, CA,

USA; HTLV-I: Fuji-Rebio, Tokyo, Japan) to identify antibodies to HIV or HTLV-I related antigens in sera from patients with primary Sjögren's syndrome.³⁴⁻³⁹

IMMUNOHISTOCHEMICAL ASSAY

Indirect immunohistochemical analyses of labial salivary gland biopsy specimens from patients with primary Sjögren's syndrome were performed, using a monoclonal antibody to HIV specific p24 antigen (mAb 880-A, Chemicon International, Temecula, CA, USA).⁴⁰ Sections for immunohistochemistry were cut 4 μm thick and fixed in acetone at -20°C . After being washed three times in phosphate buffered saline (PBS), they were incubated with the monoclonal antibody. After testing in serial dilutions from 1:10 to 1:160, the monoclonal antibody was found to give optimal results with salivary gland at a concentration of 1:50. After 30 minutes of incubation the sections were washed three times in PBS and reacted with biotin labelled rabbit antimouse IgG for 10 minutes. The specimens were then washed in PBS and reacted with peroxidase labelled streptavidin for five minutes, washed again in PBS, and reacted with 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, St Louis, MO, USA) for 10 minutes. HIV was propagated in cultures of CEM cells, which were used as a positive control for the HIV antigens. A murine myeloma protein was used as an additional negative control.

DETECTION OF HIV AND HTLV-I GENOMES BY PCR ASSAYS

Genomic DNA was prepared from salivary gland tissues and PBMC of patients with primary Sjögren's syndrome and from normal controls.⁴¹⁻⁴² DNA was prepared from these tissues by freeze drying for 16 hours, digested with RNase (10 mg/ml) for two hours, and proteinase K (1 mg/ml) for 16 hours at 37°C , followed by phenol extraction and ethanol precipitation.⁴³ The dried DNA pellet was redissolved in diethyl pyrocarbonate treated water (DEPC-w), and the extracted DNA was read spectrophotometrically at 260 nm (DU-5; Beckman, Follerton, CA, USA). One microgram of each sample was used for PCR. This method of DNA preparation avoids the use of tissue homogenisers, a possible source of cross contamination of tissue sample DNA.

PCR was carried out with primer pairs (fig 1) which were synthesised in our laboratory on the basis of published DNA sequences.⁴⁴⁻⁴⁶ Human β globin primers (GH20/KM38, Takara-Shuzo, Kyoto, Japan) and human mitochondrial displacement loop region primers⁴⁷ were used as internal controls.

Reaction aliquots were run on a 4% NuSieve agarose gel (FMC Bio-Products, Rockland, ME, USA) in the presence of ethidium bromide, and compared with amplified DNA fragments from HIV infected CEM cells and from MT-4 cells, a chronically HTLV-I infected T cell line.

Figure 2 Detection of serially diluted plasmid DNA. Amplification (35 cycles) was done by mixing with 1 μg DNA from an uninfected T cell line (RPMI 8402) and different dilutions of pNL432 (HIV, panel A) and pATK03 (HTLV-I, panel B). Lanes: (1) 1 ng of plasmid DNA; (2) 100 pg; (3) 10 pg; (4) 1 pg; (5) 100 fg; (6) 10 fg; (7) 1 fg; (8) 0.1 fg. One tenth volume of the amplified product was examined by Southern blot analysis with an internal probe.

SENSITIVITY OF THE PCR TECHNIQUE BY AMPLIFICATION OF RECOMBINANT HIV AND HTLV-I DNAs

The sensitivity of the PCR for detecting HIV and HTLV-I was assessed by mixing serial dilutions of 100 pg cloned DNA (pNL432 for HIV^{48,49}; pATK03 for HTLV-I⁴⁶) with 1 µg of DNA from an uninfected T cell line (RPMI 8402), under conditions where the total amount of DNA remained constant. Each band represents a decreased amount of cloned

DNA (fig 2). As one tenth of the volume of amplified product was used for analysis, the band was derived from the PCR product of 1 fg of plasmid containing the cloned target gene. Under these conditions, 1 fg of plasmid DNA was detected (fig 2). On the basis of a molecular length of 9.7 kb for pNL432 and 9 kb for pATK03, these results correspond to the detection of 10 genomes of equivalent target DNA.⁵⁰ Based on these sensitivity experiments, it was possible to detect approximately one copy of the virus genome in 10 µl equivalents. Thus the comparison of reactivity with viral specific primers makes it possible to approximate the content of viral DNA in an unknown samples.

REVERSE TRANSCRIPTASE ACTIVITY ASSAY

Salivary gland tissues were destructed with lysing buffer (10 mM Tris-HCl buffer, pH 8.0, 0.14 M NaCl, 3 mM MgCl₂, 0.1% Triton X-100, 1 mM dithiothreitol (DTT) and 2 mM phenyl methyl sulphonyl fluoride), and centrifuged at 12 000 rpm for 10 minutes. The supernatant was used as the source of RT, the activity of which was assayed as described below.

To determine the conditions of RT activity measurement and to control the reaction procedure, we employed 0.1 unit of purified HIV RT (Seikagaku Corporation, Tokyo, Japan), with which Mn²⁺ can be replaced to some extent by Mg²⁺.⁵¹⁻⁵⁴ The concentration of MnCl₂ for RT activity assay of enzyme samples from salivary glands was found to be optimal at 0.5 mM.

The reaction mixture (total volume, 50 µl) contained 10 µg/ml polyadenylate (poly(A)); oligo(dT)15 mixture, 50 mM Tris-HCl, pH 8.0, 0.1 mM DTT, 20 mM KCl, 0.5 mM MnCl₂, 15% glycerol, 4 µM cold 2'-deoxynucleoside 5'-triphosphates, 15 pmol [3H]-thymidine 5'-triphosphate (1.11 MBq/mmol, Amersham-Japan, Tokyo, Japan), and the enzyme sample (10 µg protein). The reaction mixture was incubated at 37°C for 60 minutes. The reaction was stopped with 20 µl of 0.2 M ethylenediamine tetra-acetic acid, and the mixture was allowed to stand on ice. A volume of 50 µl was transferred onto DE-81 filter paper and processed for counting radioactivity.⁵⁵

TRANSMISSION ELECTRON MICROSCOPY

Labial salivary gland samples were fixed for one hour with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 1% tannic acid, post-fixed for one hour with 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections counterstained with 2% uranyl acetate and lead citrate were examined using an electron microscope.⁵⁶

Results

IDENTIFICATION OF ANTIBODIES TO RETROVIRAL PROTEINS BY IMMUNOBLOTTING

In the western blot assay, sera from HIV infected subjects may contain antibodies reactive to HIV proteins designated p15, p18, p24, p32, gp41-43, p51, p55, p65, gp120, or gp160. Under the stringent conditions of this assay,

Figure 3 Immunoblot patterns of Sjögren's syndrome and normal sera with HIV (A) or HTLV-I (B) proteins. Strips of the nitrocellulose membrane were exposed to sera from Sjögren's syndrome patients (lanes 1-4), normal subject (Normal), or patients infected with HIV or HTLV-I (Positive control).

Figure 4 Immunoperoxidase staining of labial salivary gland from patient with Sjögren's syndrome using a monoclonal antibody to HIV specific p24 antigen. The antigen expressed cells were observed in the duct and acinar epithelial cells and some mononuclear cells (arrowheads). A, $\times 100$. B, $\times 200$.

sera from most subjects fail to react with any HIV protein. Five of 15 sera (33%) from patients with primary Sjögren's syndrome showed reactivity to p24, whereas 10 normal sera did not react to any of the HIV proteins (fig 3A). None reacted with the other *gag* proteins p15, p18, or p55, *pol* proteins p32, p51, or p65, or *env* proteins gp41-43, gp120, or gp160.

None of the 15 Sjögren's syndrome sera reacted with any HTLV-I specific proteins (fig 3B).

EXPRESSION OF HIV P24 ANTIGEN IN EPITHELIAL CELLS OF SALIVARY GLANDS

Epithelial cells in seven of the 15 salivary gland biopsy specimens from patients with primary Sjögren's syndrome (47%) reacted strongly with the monoclonal antibody that recognises HIV p24 *gag*. The antigen was stained in the duct and acinar epithelial cells, and some mononuclear cells were stained by the monoclonal antibody to HIV p24 *gag* (fig 4A and B). All of the biopsy specimens from 10 healthy controls were negative. No positive staining was noted when a control murine myeloma protein was used in the place of the specific antibodies (data not shown).

DETECTION OF HIV AND HTLV-I GENOMES BY PCR
PCR failed to detect HIV genes (920 bp: *gag*; 533 bp: LTR (fig 5A)) and HTLV-I genes (240 bp: LTR-*gag* (fig 5B); 158 bp: *tax* (fig 5C)) in any of the 15 salivary gland specimens or PBMC from patients with primary Sjögren's syndrome, in contrast to the HIV or HTLV-I positive controls. These genes were not found in normal salivary gland tissues or PBMC (data not shown).

DETECTION OF REVERSE TRANSCRIPTASE ACTIVITY

One defining characteristic of retroviruses is the presence of RT, a finding that was observed in three of 10 salivary gland specimens from patients with primary Sjögren's syndrome (fig 6A). RT of various retroviruses can often be distinguished by substrate or ionic preferences.

Therefore, the abilities of detergent disrupted preparations from the subjects to synthesise DNA were compared at various concentrations of Mg^{2+} and Mn^{2+} , with poly(A) used as the synthetic template and oligo(dT) as primer. The highest RT activities associated with retrovirus preparations were obtained when Mn^{2+} was used as the divalent cation. RT activity was not obtained when 5 mM Mg^{2+} was used as the divalent cation (fig 6B). Although HIV and HTLV-I associated RT activities were previously reported to be higher with Mg^{2+} than Mn^{2+} ,⁵⁴ this retrovirus associated RT activity was higher with Mn^{2+} than with Mg^{2+} in the salivary gland tissues from patients with primary Sjögren's syndrome. These results show that the retrovirus associated RT differed from that of HIV and HTLV-I. The preparation did not contain sufficient levels of a DNA dependent DNA polymerase or a deoxyribonucleotidyl (terminal) transferase to account for the observed DNA polymerase activity.

OBSERVATION OF THIN SECTIONS OF SALIVARY GLANDS FROM SJÖGREN'S SYNDROME PATIENTS BY TRANSMISSION ELECTRON MICROSCOPY

We ultrastructurally observed A-type-like retroviral particles in some epithelial cells of these RT activity positive salivary glands from three Sjögren's syndrome patients (fig 7A). The morphological features of these particles consisted of a distinct, eccentrically located internal core and an outer surface envelope. Particles were generally found in thin sections through a region of the cell containing cytoplasm, as has been described in a variety of normal and transformed cells from other species.⁵⁶ We selected RT positive and HIV p24 positive specimens by immunohistochemical and the western blot analysis for electron microscopic study, to make sure that there were A-type-like particles in these specimens. Particles with this distinctive morphology were not found in epithelial cells of salivary glands from control subjects, despite an extensive search throughout various levels of the cells. HIV virion including a particle with a typical lentivirus core were found in CEM cells infected with HIV (fig 7B).

Figure 5 PCR analysis of retroviral genes in salivary gland from patients with Sjögren's syndrome. DNA was prepared from Sjögren's syndrome patients (lanes 1-7). Retroviral genes were not found in all tissues by PCR. (A) HIV (920 bp: gag; 533 bp: LTR); MTC = mitochondria (330 bp); (B) HTLV-I (240 bp: LTR-gag), β globin (325 bp); (C) HTLV-I (158 bp: tax).

Discussion

The first part of our study investigated the possibility of retroviral infection in Sjögren's syndrome by western blotting. Our study showed that 33% of sera obtained from patients with primary Sjögren's syndrome contained antibodies that reacted on immunoblot with p24 of the HIV gag protein. There were no antibody reaction gag proteins p15, p18, or p55, or pol or env proteins. We suspect that patients who are seropositive for anti-p24 antibodies are reacting immunologically to a retrovirus different from HIV, or to cross reactive autoantibodies,⁵⁷⁻⁶⁰ although they were not infected with HIV. Non-specific reactivities against p24 have been described previously.³⁴⁻³⁹ Talal *et al*²⁵ reported serum antibodies to HIV

p24 (gag) in 14 of 47 patients with primary Sjögren's syndrome (30%), but no reaction against pol or env proteins on immunoblotting. Two sera also reacted against p17 (gag). Our result was similar, but we did not find anti-p17 antibodies.

We examined the possibility of retroviral expression in salivary glands from Sjögren's syndrome patients using anti-HIV p24 monoclonal antibody to the gag regions of human exogenous retroviruses. Epithelial cells in 47% of salivary glands reacted strongly with the monoclonal antibody. The antigen was stained in the duct and acinar epithelial cells and some mononuclear cells by the antibody. Serum of 47% of all affected patients contained antibodies to HIV p24. We therefore speculate that serum antibodies to HIV p24 might be reacting immunologically to p24 antigen in salivary gland from patients with Sjögren's syndrome. The antigen expression might also be part of the inflammatory response in epithelial cells of salivary glands. Shattles *et al*^{62, 63} reported that the human T cell lymphotropic virus (HTLV) related endogenous sequence, HRES-1, could be overexpressed in salivary gland epithelium of patients with Sjögren's syndrome, and HTLV-I p19 (gag) antigen was detected in labial salivary gland epithelial cells from 31% of 39 patients with primary Sjögren's syndrome, but not from control subjects. The authors speculated that this antigen could be a new endogenous retroviral gag sequence distinct from HRES-1. Anti-HRES-1/p28 antibodies, which can cross react with HTLV-I p24 (gag) polypeptide, were found in 10% of specimens from 19 patients with Sjögren's syndrome by western blot analysis.⁶³ Recently, Mariette *et al* reported that in situ hybridisation and PCR detected the tax gene, but not the gag, pol, or env genes, of HTLV-I in labial salivary gland sections from two of nine patients with Sjögren's syndrome but not from any of the control subjects.³² Serum of the two affected patients did not contain antibodies to HTLV-I. The classic target of HIV or HTLV-I is the CD4+ cell or the CD4+ T lymphocyte,⁶⁴ but other types of human epithelial cells can be infected. Genetic variation of HIV in vivo might result in altered cell tropisms and possibly implicate strains of HIV with glial cell tropism in the pathogenesis of some of the neurological disorders of AIDS.⁶⁵ In some cases of polymyositis, HTLV-I DNA and antigens have been found in muscle fibres.⁶⁶

We investigated whether the antigen is related to retroviruses, including HIV and HTLV-I, in the salivary glands by PCR. However, we did not find the HIV or HTLV-I genes in salivary gland tissues or PBMC from Sjögren's syndrome patients. HIV and HTLV-I genes run in PCR under conditions of low stringency did not amplify nucleic acid sequences from these cells, indicating that they were not infected with HIV or HTLV-I. However, in future experiments we shall need to ensure that we use another detection system. At the present time, since there were no typical symptoms of HIV or HTLV-I in these patients,

Figure 6 Reverse transcriptase (RT) activity analysis on the salivary gland (SG) from patients with Sjögren's syndrome and normal subjects. (A) RT activity with Mn^{2+} . (B) RT activity with Mg^{2+} .

we believe that they do not carry HIV or HTLV-I.

We examined whether RT activity specific for retrovirus was detected in salivary gland tissues from Sjögren's syndrome patients. We found Mn^{2+} dependent, Mg^{2+} independent RT activity in three of 10 salivary gland specimens from Sjögren's syndrome patients. However, HIV and HTLV-I associated RT activities are higher with Mg^{2+} than with Mn^{2+} .⁵⁴ These results indicated that the retrovirus associated RT activity is different from that of HIV or HTLV-I. The three patients (fig 6A, Sjögren's syndrome SG Nos 1-3) showed Mn^{2+} depend-

ent RT activity. These patients also had serum antibodies to HIV p24, and reacted with anti-HIV p24 monoclonal antibody to epithelial salivary glands. We therefore speculate that an unknown retrovirus may exist in the epithelial cells of Sjögren's syndrome salivary glands, expressing sequence homology to HIV p24. Garry *et al*⁶⁶ identified human intracisternal A-type retroviral particle (hIAP), which is antigenically related to HIV, in lymphoblastoid cells cocultured with homogenates of salivary glands from Sjögren's syndrome patients. Our study, like this study, indicated that hIAP associated RT activity was higher with Mn^{2+} than with Mg^{2+} . Another possibility is that we were detecting the recently reported human endogenous retrovirus sequences (HERV-K).⁶⁷ We should be able to rule out this possibility in the next experiment.

Since these findings cannot be considered as proof for a causative role of retroviruses in the development of Sjögren's syndrome, future studies need to examine the relevance of these findings. Characterisation of the unknown retrovirus found in salivary glands from Sjögren's syndrome patients must await cloning and sequencing. Such data may prove valuable in elucidating the origins of human retroviruses and understanding the mechanisms by which they induce immune dysfunction.

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Figure 7 Ultrastructure of retroviral particles in salivary gland specimens from patients with Sjögren's syndrome were examined by transmission electron microscopy. (A) Production of mature virions and apical microvilli or plasma membrane with viral particles (arrowheads). (B) HIV infected T cell line (CEM). Arrowheads indicate HIV budding from the plasma membrane.

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