SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 14-134481- -2 **FECHA**: 2015-06-04 14:36:31
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 8
ACT: 430 REQUERSOLICITA

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
SILVANA MARÍA LÓPEZ DÍAZ

Asunto: Radicación: 14-134481- -2
Trámite: 11
Evento: 378
Actuación: 430
Oficio: 6258
Folios: 8

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/066078				
Fecha de Presentación Internacional	20/12/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 14-134481-00000-0000	20/junio/2014
Reivindicaciones:	Radicado No. 14-134481-00000-0000	20/junio/2014

2. Análisis de la Prioridad

La solicitante no reivindica prioridad.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso, se tiene en cuenta la totalidad del nuevo capítulo reivindicatorio (Radicado No. 14-134481-00000-0000) para el examen de patentabilidad.

Título de la solicitud: “MÉTODOS DE DIAGNÓSTICO”.

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El problema planteado en esta solicitud consiste en la necesidad de suministrar un método que permita determinar la susceptibilidad a la erosión del esmalte.

Para resolver este problema técnico la solicitud en estudio proporciona un método que consiste en medir ciertos biomarcadores en una muestra de saliva de un mamífero.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): G01N33/68, C07K16/18; C07K16/40; G01N33/6893.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 7 a 9 y 18 a 20 no es patentable de acuerdo con el Art citado por estar relacionadas con métodos de tratamiento.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 10 a 14 y 21 no es patentable, de acuerdo con el Art 14.

6. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicaciones 1 y 15 carecen de claridad y concisión ya que no se presentan los valores control específicos que permiten determinar que un mamífero posee una susceptibilidad aumentada a la erosión del esmalte. La solicitante establece en la etapa c) que el valor control es la concentración del biomarcador en una muestra de la saliva de la boca de" un mamífero que no padece de erosión", esta condición es ambigua y no limita el alcance de la solicitud, ya que no se establecen de manera clara las características que permiten determinar que un mamífero no padece de erosión. Se requiere a la solicitante para que establezca valores o rangos puntuales que permitan determinar de manera concreta si el mamífero tiene susceptibilidad aumentada a la erosión dentro de las etapas del método reivindicado, debido a que estos valores son esenciales para que el método sea realmente útil.

Las reivindicaciones 1 y 15 utilizan las expresiones "o más de los siguientes biomarcadores", esta le resta claridad y concisión a la solicitud ya que no se divulgan cuáles son los otros biomarcadores, ni las concentraciones de estos que permiten el diagnóstico.

Las reivindicaciones 1, 15, 4, 5 y 17 contemplan una actividad intelectual de la persona que va a diagnosticar el nivel de erosión del esmalte dental que consiste en comparar la concentración del biomarcador frente a un valor control para que esta persona con fundamento en su experticia y sus conocimientos determine si existe susceptibilidad a la erosión, en consecuencia esta actividad de diagnósitico corresponde al experto (odontólogo) quien con fundamento en sus conocimientos desarrolla una conclusión. Por lo tanto, este método no es reproducible, toda vez que la etapa de comparación depende del criterio de la persona que está realizando el análisis y obteniendo los resultados y no de actividades técnicas del método. Esta comparación no es patentable ya que obedece a un trabajo intelectual y no a un método de diagnóstico fundamentado en técnicas analíticas.

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Las reivindicaciones 2 y 16 caracterizan el método porque la erosión es de "etiología dietética". Esto no se considera una característica técnica del método reivindicado ya que no es una etapa, actividad o condición que permita llevar a cabo dicho método. La causa de la enfermedad o problema no se considera una característica técnica esencial de un método de diagnóstico. Adicionalmente, no es clara la manera como se puede determinar que esa erosión del esmalte dental es exclusivamente de origen dietético y que no está relacionada con otros factores, tales como características genéticas o hábitos que den lugar a esta condición, la descripción no provee información que permita determinar que una erosión es de manera inequívoca de origen exclusivamente dietético.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud internacional: 20 de diciembre de 2011 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Archives of Medical Research 33 (2002) 499–505	"Electrophoretic Analysis of Whole Saliva and Prevalence of Dental Caries. A Study in Mexican Dental Students"	2002
D2	Glycobiology vol. 9 no. 3 pp. 293-302, 1999	"Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product"	1999
D3	Oral Microbiol. Immunol 2001:16: 345-352.	"Lactoferrin, amylase and mucin MUC5B and their relation to the oral microflora in hyposalivation of different origins"	2001

D1[1] divulga la composición proteica de la saliva humana separada por electroforesis y su correlación con el índice de dientes cariados perdidos y obturados (Resumen).

D2 enseña que el producto del gen MUC5B corresponde a la especie de mucina predominante de MG1[2] (Pág. 295, col. derecha)

D3 divulga las concentraciones de MUC5B medidas mediante el ensayo de unión de la enzima a un inmunosorbente. La concentración fue determinada con relación a un estándar de saliva que provenía de una persona sana (Pág. 347, Col. 2, Párr. 3).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un método para identificar un mamífero que tiene una susceptibilidad aumentada a la erosión del esmalte.

Características esenciales	D1
Un método para identificar un mamífero que tiene una susceptibilidad aumentada a la erosión del esmalte, caracterizado porque comprende: a) Obtener una muestra de la saliva entera de la boca de la cavidad oral del mamífero; b) Medir la concentración de uno o más del biomarcador mucina 5B	Establece mediante un análisis por electroforesis que personas con un alto índice de dientes cariados, perdidos y obturados presentan una reducción significativa o ausencia de glicoproteínas de mucina de alto peso molecular (MG1).

1http://www.ncbi.nlm.nih.gov/pubmed/12459324

2 Glycobiology vol. 9 no. 3 pp. 293–302, 1999 (Pág. 293, segunda columna, segundo párrafo)

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	Para esta medición se sigue este procedimiento: Recolección de saliva a sujetos luego de 2 horas del cepillado de dientes, evitando comer, beber o fumar. (Pág. 500, *Recolección de saliva).* Cuantificación de proteína total y electroforesis en gel de poliacrilamida utilizando estándares de peso molecular. (Págs. 500 y 501, *polyacryamide gel electrophoresis)*. Identificación de proteínas de saliva. Análisis estadístico de los valores de cada proteína en la saliva. (Pág. 501, Tabla 1)

Las características esenciales del método de cuantificación de proteínas (marcadores biológicos) en muestras de saliva ya se había divulgado en el documento D1 que enseña un proceso para determinar el contenido de proteínas en la saliva por electroforesis en gel de poliacrilamida que tras un análisis estadístico permite determinar la intensidad de las principales proteínas con fundamento en diversos factores y estos resultados se comparan frente al índice de dientes cariados, perdidos y obturados (DMFT) con lo que se pudo determinar una significativa reducción o incluso la ausencia de MG1 cuando se presentan daños dentales (Págs. 500 a 5001, Tabla 1). Como es conocido, MG1 corresponde a la mucina 5B ya que el producto del gen MUC5B corresponde a la especie de mucina predominante de MG1[3]. En consecuencia, el documento D1 ya había divulgado el efecto asociado con una mayor DMFT con respecto a la concentración de biomarcadores como MG1 (Pág. 503, Col. 2), este documento igualmente indica que la caries es una consecuencia de la desmineralización inducida por la producción de ácido del metabolismo de las bacterias siendo la caries un producto de la erosión del esmalte dental (Pág. 504, col. 1).

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 – 6 no mencionan alguna característica que haga nuevo el método de la reivindicación 1, por lo cual se considera que no son nuevas.

La reivindicación 1 consiste en un método para identificar un mamífero que tiene una susceptibilidad aumentada a la erosión del esmalte.

Características esenciales	**D3**
Un método para identificar un mamífero que tiene una susceptibilidad aumentada a la erosión del esmalte, caracterizado porque comprende: a) Obtener una muestra de la saliva entera de la boca de la cavidad oral del mamífero; b) Medir la concentración de uno o más del biomarcador mucina 5B	Divulga un método para cuantificar las concentraciones de MUC5B medidas mediante el ensayo de unión de la enzima a un inmunosorbente. La concentración fue determinada con relación a un estándar de saliva que provenía de una persona sana (Pág.

3 Glycobiology vol. 9 no. 3 pp. 293–302, 1999 (Pág. 293, segunda columna, segundo párrafo)

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	347, Col. 2, Párr. 3).

Las características esenciales del método de cuantificación de proteínas (marcadores biológicos) en muestras de saliva ya se habían divulgado en el documento D que enseña las concentraciones de MUC5B medidas mediante el ensayo de unión de la enzima a un inmunosorbente. La concentración fue determinada con relación a un estándar de saliva que provenía de una persona sana (Pág. 347, Col. 2, Párr. 3). El documento D3 enseña que en comparación con los controles los tres grupos de hiposalivación mostraron bajas concentraciones y menor secreción por minuto de MUC5B en comparación con los controles (Pág. 349, Col. 1, Párr. 1, figuras 2 y 3). El método de D3 anticipa el método de cuantificación de MUC5B de la presente solicitud.

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 – 6 no mencionan alguna característica que haga nuevo el método de la reivindicación 1, por lo cual se considera que no son nuevas.

La reivindicación 14 consiste en anticuerpos para uno o más de mucina 5B, anhidrasa carbónica 6, y estaterina.

Características esenciales	**D2**
Anticuerpos para uno o más de mucina 5B, anhidrasa carbónica 6 y estaterina.	Divulga anticuerpos para MUC5B (Pág. 300, col. derecha).

Las características esenciales de los anticuerpos de la reivindicación 14 ya se habían divulgado en D2 que enseña los anticuerpos policlonales específicos para mucinas que reconocen las subunidades reducidas de MUC5B (Pág. 300, col. derecha).

En consecuencia, la reivindicación 14 no es nueva, según lo divulgado en el documento D2.

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en un método para identificar un mamífero que tiene una susceptibilidad aumentada a la erosión del esmalte.

Características esenciales	**D1**	**D2**
Un método para identificar un mamífero que tiene una susceptibilidad aumentada a la erosión del esmalte, caracterizado porque comprende: a) Obtener una muestra de la saliva entera de la boca de la cavidad oral del mamífero; b) Medir la concentración de uno o más del biomarcador mucina 5B	Establece mediante un análisis por electroforesis que personas con un alto índice de dientes cariados, perdidos y obturados presentan una reducción significativa o ausencia de glicoproteínas de mucina de alto peso molecular (MG1). Para esta medición se sigue este procedimiento:	MG1 corresponde a la mucina 5B ya que el producto del gen MUC5B corresponde a la especie de mucina predominante de MG1 (Pág. 293, Col. derecha, Párr. 2).

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	Recolección de saliva a sujetos luego de 2 horas del cepillado de dientes, evitando comer, beber o fumar. (Pág. 500, *Recolección de saliva).* Cuantificación de proteína total y electroforesis en gel de poliacrilamida utilizando estándares de peso molecular. (Págs. 500 y 501, *polyacryamide gel electrophoresis)*. Identificación de proteínas de saliva. Análisis estadístico de los valores de cada proteína en la saliva. (Pág. 501, Tabla 1)	

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 enseña los índices de sujetos con alto deterioro, pérdida y dientes obturados (DMFT), estos individuos presentaron una reducción significativa o ausencia de glicoproteína-1 de mucina de alto peso molecular (MG1). La mucina MG1 es de manera inherente mucina 5B ya que esta es la de mayor predominancia en la saliva. D1 igualmente enseña un método que consiste en la recolección de saliva de sujetos que no han consumido alimentos, bebido o fumado por lo menos durante dos horas previo a la recolección de saliva (Pág. 500, col. 2, párr. 2). Las proteínas de la saliva son posteriormente separadas por cromatografía en gel y los patrones de las bandas se obtienen de acuerdo con los métodos conocidos y se cuantificó la cantidad de MG1 de acuerdo al tamaño de la banda y la intensidad de la misma. Posteriormente se realizan los cálculos para determinar la concentración de proteína salivar (Pág. 501, Col. 1 y 2 y tabla 2). El documento D2 indica que la pérdida de MG1 está asociada con un mayor DMFT al comparar los valores de MG1 en pacientes con bajos índices de DMFT (Pág. 503, Col. 2, Párrs. 1 y 2). El documento D1 concluye que la caries dental es una consecuencia de la desmineralización inducida por producción de ácidos por el metabolismo bacteriano que se genera en la placa dental y en consecuencia la caries dental es un producto de la erosión del esmalte dental (Pág. 504, Col. 1).

La diferencia entre la invención, definida en la reivindicación 1 y método de D1 consiste en que especifica que el biomarcador considerado es la mucina 5B.

Estas diferencias no parecen conceder un efecto técnico inesperado o una ventaja al método reivindicado porque se fundamenta en el mismo proceso de cuantificación de concentración de glicoproteínas de alto peso molecular presentes en la saliva y determinar así la susceptibilidad del individuo a la erosión del esmalte y la aparición de caries dental.

El problema técnico objetivo de esta solicitud consiste en la necesidad de un método de identificación de la susceptibilidad a la erosión del esmalte dental en un individuo con fundamento en las enseñanzas del documento D1.

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Sin embargo, el documento D2 enseña que MG1 está conformado esencialmente por la mucina 5B, ya que el producto del gen MUC5B es la especie predominante de las mucinas MG1 (Pág. 293, Col. derecha, Párr. 2).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría como biomarcador la mucina 5B la cual corresponde a la mucina predomínate de la población de mucinas MG1 como lo enseña el documento D2 en el procedimiento divulgado en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y en las reivindicaciones 2 – 6 y 14 a 17 es obvia y no tiene nivel inventivo.

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Archives of Medical Research 33 (2002) 499–505	1 – 6 1-6 y 14 a 17	Novedad Nivel Inventivo
D2	Glycobiology vol. 9 no. 3 pp. 293-302, 1999	14 1-6 y 14 a 17	Novedad Nivel Inventivo
D3	Oral Microbiol. Immunol 2001:16: 345-352.	1 – 6	Novedad

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En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JESUS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-06-09

Elaboró: Emilse Cano Urrego
Revisó: Gloria Jacqueline Alfonso Rozo

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ELSEVIER

Archives of Medical Research 33 (2002) 499–505

Archives of Medical Research

ORIGINAL ARTICLE

Electrophoretic Analysis of Whole Saliva and Prevalence of Dental Caries. A Study in Mexican Dental Students

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Received for publication October 23, 2001; accepted March 6, 2002 (01/172).

Background. Variability in salivary proteins and their posttranslational modifications may play an important role in determining their protective features against dental caries. Knowledge of molecular content of saliva in different populations is important for a better understanding of protective properties of this biological fluid. Aims of this study were to analyze electrophoretic pattern and protein composition in resting human whole saliva (HWS) of a Mexican population and to correlate these data with decayed, missing, and filled teeth (DMFT) index in these subjects.

Methods. Resting human whole saliva samples were collected from 120 healthy Mexican dental students. Salivary flow rate, protein concentration, and electrophoretic profile analyzed qualitatively by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were correlated with DMFT index. Gels were successively triple-stained with Coomassie brilliant blue R250, periodic acid Schiff (PAS), silver stain, and salivary molecules were scored as absent (−), present (±), and high intensity and size (+).

Results. These showed no substantial differences in number of bands between males and females; however, a slight correlation between total protein concentration and sex was found ($p \leq 0.05$). With regard to salivary proteins and DMFT index, subjects with higher DMFT indices presented significant reduction or absence of high-molecular-weight mucin glycoprotein-1 (MG1), low-molecular-weight mucin glycoprotein-2 (MG2), and acidic proline-rich protein-1 (PRP-1), differing from subjects with lower DMFT indices ($p \leq 0.001$).

Conclusions. This study concludes that genetic phenotypic polymorphism is present in the population studied and has correlations with oral health. We found specific characteristics and individual variability in number, intensity, and apparent molecular weight of band features in this Mexican population. These studies provide the initial step for creating an HWS database in this population. © 2002 IMSS. Published by Elsevier Science Inc.

Key Words: Saliva, Electrophoresis, Proteins, Dental caries.

Introduction

Molecules present in saliva include a characteristic multigenic group of proteins and polypeptides grouped into single or polymorphic families that have an important biological function in maintenance of oral health (1–3). Saliva has protective effects against dental caries (1,4–8). Genetically determined variations in salivary protein composition may play an important role in dental caries etiology and other oral diseases (5,9–13). However, most of these studies have been performed with parotid saliva, whose proteins exhibit strong genetic polymorphism (6,9,14–16).

Recent studies using human whole saliva (HWS) have shown individual differences in salivary protein patterns (4,17,65). Because HWS use has become widespread in oral and systemic disease diagnosis (4,19,20), it is important to know its biological and physiologic characteristics and vari-

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ability among population groups. These data may be useful in understanding salivary proteins and their potential use in diagnosis of specific diseases. HWS contains, among other proteins, high-molecular-weight salivary mucin glycoprotein-1 (MG1) (20–22), low-molecular-weight salivary mucin glucoprotein-2 (MG2) (23,24), and proline-rich protein-1 (PRP-1) (24–26). These proteins play an important role in protecting oral surfaces and as precursors of acquired enamel pellicle (27–29); therefore, they can modulate and influence the enamel demineralization-remineralization process and dental caries formation (20,30). Furthermore, it has been demonstrated that functional formation of heterotypic complexes between salivary molecules such as MG-1, amylase, PRPs, and statherin are determinant in plaque formation and dental caries (31). In addition, we cannot exclude genetic factors associated with phenotypic expression of these proteins (32–34) in mixed saliva, which may contribute to oral disease etiology. Thus, presence or absence of these inherited markers will be of great value for evaluating dental caries risk in each human being.

In a previous study, we reported slight variability in salivary flow rate and total protein concentration in Mexican youth when compared to individuals from developed countries (35,36,65). The present work was conducted to enhance our understanding of HWS protective role and protein profile in this population. The purpose of the study was to analyze electrophoretic pattern and protein composition in resting HWS of a Mexican population and to correlate these data with decayed, missing, and filled teeth (DMFT) index in these subjects.

Materials and Methods

A Coomassie blue protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL, USA) and sodium chloride, isobutyl alcohol, methanol, and acetic acid were acquired from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA), while bovine serum albumin [A-7906], methyl green, trizma-base, and 2-mercaptoethanol were procured from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecylsulfate (SDS), glycine, Coomassie brilliant blue R-250, silver stain, TEMED, ammonium persulfate, N,N^1-methyl-bis-acrylamide, acrylamide, and low-molecular-weight markers were obtained from Bio-Rad (Richmond, CA, USA). All additional reagents including 2δ-deionized water (18.3 MΩ, System Milli Q-II, Millipore, Bedford, MA, USA) were high-quality analytical grade.

Subject selection. Protocol approval was obtained from the Universidad Autónoma del Edo. de México (UAEM) Institutional Review Board Committee (April 2, 1998). One hundred twenty clinically healthy dental students of both sexes (60 males and 60 females), between 17 and 24 years of age (average = 19 years) enrolled in the first university academic year were recruited from the School of Dentistry at the UAEM in Toluca, Mexico. All subjects were selected by means of random number table after obtaining the student list. Students provided information and written consent and agreed to participate on a voluntary basis as part of a large prospective salivary research project. No donor was taking prescription medication or drugs at the time. Sample selection has been described in previous works (35,36).

Clinical examination. Caries status was determined with decayed, missing, or filled teeth (DMFT) index by a single examiner (MG) and calibrated according to World Health Organization (WHO) diagnostic criteria (37,38). Based on previous epidemiologic data of our population (38), two scores were utilized to correlate presence or absence of proteins with DMFT: subjects with low DMFT index (≤4.0), and those with high DMFT index (>10.0).

Saliva collection. After brushing their teeth, subjects refrained from eating, drinking, smoking, and oral hygiene for at least 2 h prior to saliva collection. Resting (unstimulated) human whole saliva (HWS) samples were collected between 8 a.m. and 10 a.m. prior to clinical examination to reduce possible circadian contributions under the same conditions and by the same examiner (JAB). After rinsing their mouths with water and 1 min of becoming comfortable, subjects were asked to swallow all remaining saliva. Saliva from each individual was collected over a single 5-min period by spitting into chilled, pre-weighted disposable polypropylene sterile tubes with adapted conic-disposable cup. Resting HWS flow rates were determined gravimetrically and expressed as milliliter per minute (mL/min) (39,40).

General chemical and analytical methods for total protein quantitation. After salivary flow rate determination, saliva samples were clarified by centrifugation at 4,000 rpm for 40 min at 4°C in a Kubota KR702 refrigerated centrifuge (Kubota Co., Tokyo, Japan). Total protein determinations were done by Coomassie blue method described by Bradford (41); a commercial kit was used following manufacturer instructions with slight modifications. Fifty microliters of each HWS sample was suspended in 2.45 mL 0.80% NaCl. Coomassie reagent (0.5 mL) was then added and samples were vortexed for 5 sec and set to incubate for 2 min at room temperature. BSA was used to generate standard curve (0–150 μg protein). A Jasco-Ubest 30 UV/LV spectrophotometer (Jasco, Tokyo, Japan) with integrated computer interfaced to a thermal plotter was set to read each saliva sample in a cycle of three times/min at OD_{595}. Values were expressed as milligram per milliliter (mg/mL); all experiments were run in duplicate. Remaining saliva samples were either frozen and stored at −20°C or lyophilized and dried (Labconco freeze-dry lyophilizer, Kansas City, MO, USA) for gel electrophoresis and subsequent analysis.

Polyacrylamide gel electrophoresis (PAGE). After initial centrifugation, 500 μL of each saliva sample was desalted immediately by means of a Centricon ($3{,}000_{MW}$ cut-off) ul-

tra-filtration microfuge tube (Amicon Co., Beverly, MA, USA) at 10,000 rpm for 5 min, lyophilized, and dried. Protein concentration for saliva standardization was determined as previously described by method of Bradford. Samples were then reconstituted to a concentration 10-μg lyophilized residue/1.0 μL in buffer under denaturing conditions (0.125 M Tris-HCl, 4% SDS, 20% glycerol, pH 6.8, 10% β-mercaptoethanol, and 0.002% bromophenol blue) and boiled at 100°C for 3 min prior to running gels. Individual saliva samples (35 μg protein/dry weight) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at room temperature in discontinuous gel system according to Laemmli method (42) using 3% stacking gel and 12.5% separating gel. Molecular-weight standards were used in all gels. Electrophoresis was carried out at 100 V constant current until front dye of the gel reached the chamber bottom; a minigel apparatus (Mighty Small II vertical slab unit, Hoefer Scientific Institute, San Francisco, CA, USA) was used for this purpose.

Gel staining. Gels were fixed and stained successively with 0.1% Coomassie brilliant blue R250 in methanol-water-acetic acid (1:8:1), periodic acid-Schiff (PAS) reagent, and silver stain. To enhance and obtain a good pattern of blue- and pink-violet-staining bands, gels were initially stained with Coomassie blue solution for 1 h and destained gradually with several changes of destaining solution (methanol-water-acetic acid, 1:8:1) for approximately 60 min until bands showed the appropriate color. Due to differences in glycosylation pattern of mucins that bestow different staining intensity with comparable amount of protein molecules, gels were stained with PAS and silver reagent to obtain a brown, stained pattern.

Identification of salivary proteins. Proteins were identified according to their relative mobility in gel and stain patterns following criteria described by Azen (15), Aguirre et al. (24), Schwartz et al. (17), and Sreebny and Zhu (4). Salivary molecules MG1, MG2, and PRP-1 were scored according to band size and stain intensity as absent (–), present (±), and high intensity and size (+). However, due to subjectivity inherent in these parameters, salivary molecules were additionally scored only as present and absent.

Statistical analyses. Salivary protein values were calculated from standard-derived regression line derived at different optical density (OD) (Conc = a * Abs + b). For statistical analysis, means, standard deviations (SDs), and frequency distributions were computed for each variable. ANOVA one-way statistical analyses were used to verify whether there was any influence among phenotype distribution, protein concentration, and salivary flow rate. Non-paired *t* test was utilized to assess any statistical difference between a) sex and total protein concentration, b) sex and salivary flow rate, and c) sex and DMFT index. Pearson correlation coefficients with significance level of $p \leq 0.05$ were carried out to determine any correlation among age, flow rate, and protein concentration. Finally, associations between higher and lower DMFT indexes with presence or absence of salivary proteins were estimated using chi-square (χ^2) test ($p \leq 0.001$). StatView 512+™ (BrainPower, Inc., Calabasas, CA, USA) and SPSS (SPSS Inc., Mexico) statistical software for personal computers were used for statistical analyses.

Results

Sialometry. Average salivary flow rate from total population showed mean (mL/min SD) in resting HWS of 0.397 ± 0.26. A difference between males and females was observed, females having lower flow rate (0.326 ± 0.24 [$p \leq 0.003$]) in comparison to males (0.464 ± 0.25). Average salivary flow rates in subjects scored according to presence or absence of salivary molecules are shown in Tables 1 and 2. A significant difference in flow rate was observed associated with presence and absence of bands ($p \leq 0.05$). Increases in flow rate were associated with decreased total protein concentrations and absence of MG1, MG2, and PRP-1.

Salivary protein content. Mean protein concentration (mg/mL ± SD) in the population was 1.374 ± 0.45 in resting HWS. Females had more protein concentration (1.406 ±

Table 1. Intercorrelations between baseline salivary proteins findings and DMFT index, salivary flow rates, total protein concentration, and sex

	Distribution of MG1, MG2, and PRP-1 salivary proteins								
	MG1			MG2			PRP-1		
Molecule scored[a]	+	±	−	+	±	−	+	±	−
n (%)	40 (33.3)	72 (60.0)	8 (6.6)	23 (19.1)	81 (67.5)	16 (13.3)	20 (16.6)	94 (78.3)	6 (5.0)
Sex (male/female)	20/20	36/36	5/3	9/14	42/39	7/9	12/8	4 4/50	4/2
Proteins[b]	1.425	1.359	1.300	1.369	1.384	1.296	1.560	1.352[d]	1.094
Flow rate[c]	0.333	0.405	0.615	0.281	0.388	0.551	0.328	0.383	0.850
DMFT	8.9	10.23	11.87	9.7	9.8	11.87	9.3	9.6	10.0

Values are means for number of subjects indicated in each case. Percent of molecular distribution is indicated in parentheses. [a]Molecule scored according to size and stain intensity as described in Materials and Methods; [b]mean of HWS total protein concentration of subjects indicated (mg/mL); [c]mean of HWS salivary flow rates of subjects indicated (mL/min.); [d]$p \leq 0.05$.

Table 2. Salivary flow rates and total protein concentration related to distribution of salivary molecules scored only as absent and present in HWS

	Sialometry (mL/min ± SD)			Total protein (mg/mL ± SD)		
Molecular distribution	MG1	MG2	PRP-1	MG1	MG2	PRP-1
Present[a]	0.381 ± 0.25	0.373 ± 0.24	0.373 ± 0.23	1.379 ± 0.46	1.386 ± 0.47	1.389 ± 0.45
Range	0.04–1.28	0.04–1.19	0.04–1.28	0.01–3.74	0.01–3.74	0.01–3.74
	(n = 112)	(n = 104)	(n = 114)	(n = 112)	(n = 104)	(n = 114)
	(93.3%)	(86.6%)	(95.0%)	(93.3%)	(86.6%)	(95.0%)
M(ale):F(emale)	56:56	51:53	56:58	56:56	51:53	56:58
Absent[b]	0.615 ± 0.30	0.551 ± 0.31	0.850 ± 0.30	1.300 ± 0.29	1.296 ± 0.27	1.094 ± 0.28
Range	0.22–1.20	0.15–1.28	0.40–1.20	0.80–1.70	0.80–1.74	0.70–1.40
	(n = 8)	(n = 16)	(n = 6)	(n =8)	(n = 16)	(n = 6)
	(6.6%)	(13.3%)	(5.0%)	(6.6%)	(13.3%)	(5.0%)
M(ale):F(emale)	5:3	7:9	4:2	5:3	7:9	4:2

Values are means for number and percent of subjects indicated in parentheses; [a]Subjects with presence of bands scored [+] and [±] as described in Materials and Methods; [b]subjects without bands scored as [−] as described in Materials and Methods; M(ale)/F(emale), number of subjects by sex with and without these proteins.

0.49) than males (1.342 ± 0.41); however, this difference was not statistically significant. Tables 1 and 2 show mean protein concentrations by Bradford method in relation to observed bands. Despite differences in presence or absence of these bands, only presence of PRP-1 band and total salivary protein concentration was statistically significant ($p \leq 0.05$). Due to relative subjectivity in scoring bands, Table 2 shows mean salivary flow rates and total protein concentration scored only as absent and present. Results do not differ substantially from those in Table 1.

Electrophoresis. Representative polyacrylamide slab gels (Figures 1–3) of human whole saliva samples show distribution and molecular content from different subjects. Electrophoretic separation revealed considerable variation in patterns of different individuals as shown by substantial differences in number, intensity, and size of observed bands (Table 1). These features represent a polymorphic phenotype in each individual.

High- and low-molecular-weight salivary mucins. High-molecular-weight glycoproteins were characterized and analyzed with PAS stain, which shows molecular pattern of both high-molecular-weight mucin (MG1 >200 KDa) and low-molecular-weight mucin (MG2 ∼110 KDa). Homogeneity in protein pattern of bands was present in MG1, observed in 93.3% of subjects and absent in 6.6%. In contrast, MG2 showed great heterogeneity in both stain intensity and band size (Figure 2). This protein was present in 86.7 and absent in 13.3% of individuals (Table 2).

Proline-rich protein-1 and α-amylase. α-Amylase and proline-rich protein-1 (PRP-1) showed relative homogeneity. A valid method for PRP measurement in whole saliva (in contrast to selective collected parotid saliva) is difficult to achieve due to proteolytic activity. However, genetic variants of PRP-1 were observed in most subjects. This molecule was absent in 5% of subjects. Despite no attempts to score α-amylase, a strong genetic polymorphism was observed in number and intensity of corresponding bands, this polymorphism related to different isozymes of this molecule. Two bands were observed in the majority of cases (Figure 1).

Salivary proteins by sex. Comparing saliva samples by sex, gels stained with Coomassie brilliant blue R250 showed that females have more bands (mean = 25.50) than males (mean = 23.75) (not statistically significant). However, there was a significant correlation ($p \leq 0.05$) between total protein concentrations in saliva and number of bands. Females appeared to have more bands in the lower range of ≤21.5 KDa that corresponds to the families of proteins including cystatins, histatins, and statherins.

DMFT and salivary proteins. DMFT index is very high to date in our population. Subjects with caries index of 11.87 had less MG1 and MG2, while subjects with DMFT index of 10.0 presented lower PRP-1 (Table 1). Statistical analyses by chi-square test between subjects with lower DMFT index (n = 24) and those with higher DMFT index (n = 40) showed significant correlation to presence and absence of MG1, MG2, and PRP-1. Lower number of bands was observed in 25% of subjects with higher caries index ($p \leq 0.001$) (Table 3) (Figure 3).

Discussion

Resting whole saliva flow rate observed in our population was similar to that reported by some (43,44) but not all (45–47) investigators. Differences with the latter studies are the likely result of factors such as age and nutritional status (47–50) and genetic differences among subjects. Our study demonstrated inverse correlation between flow rates and total protein concentrations. Increase in salivary flow rates results in decreased protein concentrations in saliva, possibly due to protein dilution levels in saliva. Additionally, females had higher protein concentrations and lower salivary flow rates than males, as previously shown (35,36,51).

In the present study, subjects with highest DMFT also have highest unstimulated flow rates. From previous studies of patients with hyposalivation, it is well known that these

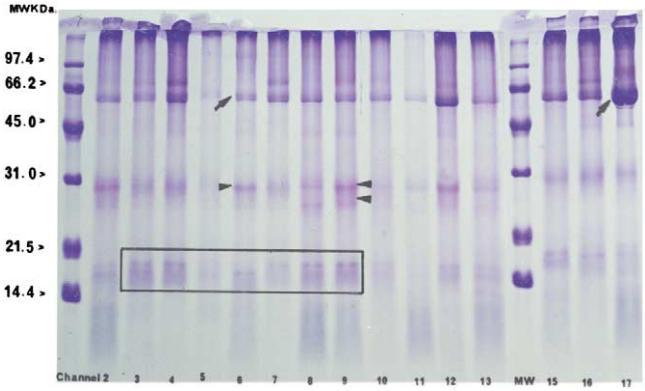

Figure 1. Genetic polymorphism determination of salivary phenotypes in a representative SDS-PAGE stained with Coomassie brilliant blue R250. Differences and variability in molecular pattern (arrows) among different subjects (channels 2–13, 15–17) can be observed. α-Amylase and its isoenzymes are stained with Coomassie blue as a deep-blue band (arrow). In comparison, proline-rich proteins are stained as purple bands (head of arrow). Inside lined rectangle, phenotypic molecular patterns of cystatins with extensive heterogeneity are present. MW = molecular weight standards.

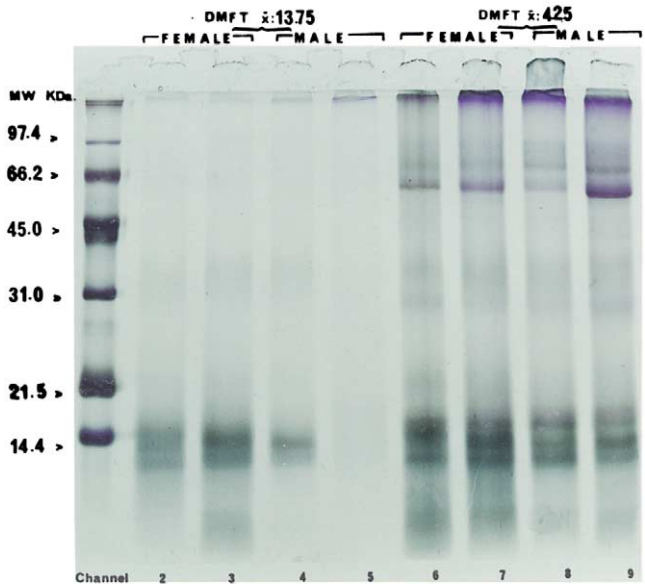

Figure 3. Representative SDS polyacrylamide slab gel analysis of HWS stained with Coomassie blue and silver stain. Separation pattern and content of different salivary molecules between subjects with higher caries index (channels 2–5) and lower caries index (channels 6–9) are shown. MW = molecular weight standards.

patients are at high risk for dental caries. It may be possible that lower flow rates in our Mexican population influence presence and incidence of dental caries related to additional factors that need to be studied.

Our findings indicate presence of genetic polymorphism in salivary proteins of human whole saliva. Variability in protein profiles among individuals in our study population is similar to that reported by Schwartz et al. (17). These findings also revealed presence of a smaller number of bands than those reported by Schwartz et al. (17) but a similar number to that reported by Shiba et al. (52). Only two amylase bands were observed in our population, but 95% of subjects studied had PRP-1. In contrast, three isozymes of amylase were reported in parotid saliva of Caucasian and American Indians with presence of two proline-rich proteins in 96%, which included 45% of PRP-1 (14). Our results show significant correlation ($p \leq 0.05$) between total protein content and number of bands present and additionally with stain intensity of PRPs.

Regarding apparent homogeneity in molecular pattern by sex, females appear to have more qualitative and quantitative differences in the range of low-molecular-weight components (<21.5 KDa). Low-molecular-weight components observed in gels could be not only salivary molecules but also degraded products of higher components susceptible to proteolytic breakdown (6,53).

There appears to be a correlation among MG1 and MG2 and DMFT: lack of these mucins in 6–13% of study population was associated with higher DMFT, indicating importance of saliva quality per se, reflected in oral health status of these subjects. Salivary mucins play an important role in protection of oral surfaces (20,24,27,28) as do PRPs (54,55). In addition, these salivary proteins participate in acquired enamel pellicle formation, thus increasing protective features of saliva (29,56,57).

Absence of these proteins in portions of our population is associated with increased prevalence of dental caries. Previous studies have focused on individual salivas and results were inconclusive (9,10), or studies were conducted to quantify specific proteins (53,58–62). This is important considering that salivary protein interaction or heterotypic complexing formation between molecules occurs in whole saliva (5,17,56), reflected in their protective properties in the mouth against dental caries. Dental enamel is formed by relatively insoluble minerals (calcium-phosphate) that con-

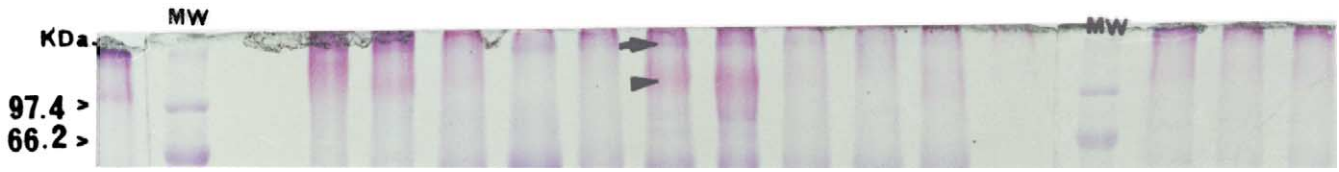

Figure 2. Specific biochemical characterization of glycoproteins in HWS by PAS stain. MG1 (arrow) and MG2 (head of arrow) from different subjects can be seen. Differences are present in molecular pattern of separation, intensity, and band size.

Table 3. Salivary flow rate and total protein concentration in group of subjects with the lower and higher DMFT index

	Sialometry		Total protein	
	(mL/min ± SD) Higher[a]	(mg/mL ± SD) Lower[b]	Higher[a]	Lower[b]
DMFT	15.7	2.4	15.7	2.4
	0.387 ± 0.27	0.415 ± 0.28	1.321 ± 0.40	1.422 ± 0.35
Range	0.05–1.20	0.13–1.19	0.01–1.92	0.67–1.92
	(n = 40)	(n = 24)	(n = 40)	(n = 24)
	(32.5%)	(4.1%)	(32.5%)	(4.1%)
M(ale):F(emale)	19:21	16:8	19:21	16:8

Values are the means for the number of subjects indicated in parentheses; percent indicates subjects without one or more scored molecules; [a]Higher DMFT index considered as >10.0; [b]lower DMFT index considered as ≤4.0; M(ale)/F(emale), number of subjects by sex.

stitute the hydroxyapatite (HAP). Under normal conditions, these minerals are dissolved in saliva with a lower content of salivary proteins, increasing demineralization of HAP (63,64). Dental caries is a consequence of demineralization induced by acidic production from bacterial metabolism in dental plaque. Furthermore, mucins and PRPs contribute to formation of the biofilm covering teeth. Thus, biofilm serves as an attachment for bacteria as well as permeability barrier for organic acid challenge. It may be possible that less output of these salivary proteins may also modulate microbial dental plaque composition and affect the permeability barrier.

To our knowledge this is the first attempt to correlate salivary protein pattern in whole saliva with DMFT. Although these findings are preliminary, they suggest differences in electrophoretic patterns of subjects with high and low caries index. Additional studies including other salivary proteins can provide further evidence concerning the role of these various molecules in modifying risk for dental caries. Further information on molecular epidemiology of salivary proteins can support use of this methodology as a diagnostic tool in dental caries and other oral health problems.

At this time, we are attempting to establish a standard baseline for whole saliva for future studies. Further studies on salivary research need to be effected to understand and compare biological characteristics in Mexican population.

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References

1. Sreebny LM, Banoczy J, Baum BJ, Edgar WM, Epstein JB, Fox PC, Larmas M. Saliva: its role in health and disease. Int Dent J 1992;42(Suppl 4):291–304.
2. Levine MJ. Salivary macromolecules. A structure/function synopsis. Ann NY Acad Sci 1993;694:11–16.
3. Levine MJ. Development of artificial salivas. Crit Rev Oral Biol Med 1993;4:279–286.
4. Sreebny LM, Zhu WX. The use of whole saliva in the differential diagnosis of Sjögren's syndrome. Adv Dent Res 1996;10:17–24.
5. Rudney JD. Does variability in salivary protein concentrations influence oral microbial ecology and oral health? Crit Rev Oral Biol Med 1995;6:343–367.
6. VanWuyckhuyse BC, Perinpanayagam HER, Bevacqua D, Billings RJ, Bowen WH, Tabak LA. Association of free arginine and lysine concentrations in human parotid saliva with caries experience. J Dent Res 1995;74:686–690.
7. Gaubenstock LM. Dental caries and the secretory activity of human labial minor salivary glands. Arch Oral Biol 1995;40:525–528.
8. Mazengo CM, Tenovuo J, Hausen H. Dental caries in relation to diet, saliva and cariogenic microorganisms in Tanzanians of selected age groups. Community Dent Oral Epidemiol 1996;24:169–174.
9. Anderson LC, Mandel ID. Salivary protein polymorphisms in caries-free and caries-active adults. J Dent Res 1982;61:393–396.
10. Anderson LC, Lamberts BL, Bruton WF. Salivary protein polymorphisms in caries-resistant adults. J Dent Res 1982;61:1167–1168.
11. Sreebny LM, Valdini A. Xerostomia. I. Relationship to other oral symptoms, drugs and diseases. Oral Surg Oral Med Oral Pathol 1988;66:451–458.
12. Sreebny LM, Valdini A, Yu A. Xerostomia. II. Relationship to nonoral symptoms, drugs and diseases. Oral Surg Oral Med Oral Pathol 1989;68:419–427.
13. Atkinson JC, Fox PC. Sjögren's syndrome: oral and dental considerations. J Am Dent Assoc 1993;124:74–86.
14. Smith QT, Shapiro BL, Hamilton MJ. Polyacrylamide gel patterns of parotid saliva proteins in Caucasoids and Amerindians. Arch Oral Biol 1975;30:369–373.
15. Azen EA. Genetic protein polymorphisms in human saliva: an interpretative review. Biochem Genet 1978;16:79–82.
16. Hay DI, Ahern JM, Schluckebier SK, Schlesinger DH. Human salivary acidic proline-rich protein polymorphisms and biosynthesis studied by high-performance liquid chromatography. J Dent Res 1994;73;1717–1726.
17. Schwartz SS, Zhu WX, Sreebny LM. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human whole saliva. Arch Oral Biol 1995;40:949–958.
18. Banderas JA, González M. Genetic salivary protein polymorphism in Mexican population. Bol Estud Med Biol 1996;44:20–24.
19. Mâkinen KK, Chen C-Y, Mâkinen P-L, Bennett CA, Isokangas PJ, Isotupa KP, Pape HR Jr. Properties of whole saliva and dental plaque in relation to 40-month consumption of chewing gums containing xylitol, sorbitol or sucrose. Caries Res 1996;30:180–188.
20. Vehkalahti M, Nikula-Sarakorpi E, Paunio I. Evaluation of salivary tests and dental status in the prediction of caries increment in caries-susceptible teenagers. Caries Res 1996;30:22–28.
21. Tabak LA, Levine MJ, Mandel ID, Ellison SA. Role of salivary

mucins in the protection of the oral cavity. J Oral Pathol 1982; 11:11–17.
22. Loomis RE, Prakobphol A, Levine MJ, Reddy MS, Jones PC. Biochemical and biophysical comparison of two mucins from human submandibular-sublingual saliva. Arch Biochem Biophys 1987;258:452–464.
23. Levine MJ, Jones PC, Loomis RE, Reddy MS, Al-Hashimi I, Bergey EJ, Bradway SD, Cohen RE, Tabak LA. Functions of human saliva and salivary mucins: an overview. In: Mackensie IC, Squier CA, Dabelsteen E, editors. Oral mucosal diseases: biology, etiology and therapy. Proceedings of 2nd Downs Symposium. Copenhagen, Denmark: Laegeforeningens Forlag;1987. pp. 24–27.
24. Prakobphol A, Levine MJ, Tabak LA, Reddy MS. Purification of a low-molecular weight, mucin-type glycoprotein from human submandibular-sublingual saliva. Carbohydr Res 1982;108:111–122.
25. Aguirre A, Testa-Weintraub LA, Banderas JA, Haraszthy GG, Reddy MS, Levine MJ. Sialochemistry: a diagnostic tool? Crit Rev Oral Biol Med 1993;4:343–350.
26. Bennick A. Salivary proline-rich proteins. Mol Cell Biochem 1982;45:83–99.
27. Robinson R, Kauffman DL, Wayne MM, Blum M, Bennick A, Keller PJ. Primary structure and possible origin of the non-glycosylated basic proline-rich protein of human submandibular-sublingual saliva. Biochem J 1989;263:497–503.
28. Levine MJ, Tabak LA, Reddy MS, Mandel ID. Nature of salivary pellicle in microbial adherence: role of salivary mucins. In: Mergenhagen S, Rosan B, editors. Molecular basis of oral microbial adhesion. Washington, D.C., USA: American Society for Microbiology;1985. pp. 125–130.
29. Levine MJ, Reddy MS, Tabak LA, Loomis RE, Bergey EJ, Jones PC, Cohen RE, Stinson MW, Al-Hashimi I. Structural aspects of salivary glycoproteins. J Dent Res 1987;66:436–441.
30. Al-Hashimi I, Levine MJ. Characterization of *in vivo* salivary-derived enamel pellicle. Arch Oral Biol 1989;34:289–295.
31. Nieuw Amerongen AV, Oderkerk CH, Driessen AA. Role of mucins from human whole saliva in the protection of tooth enamel against demineralization *in vitro*. Caries Res 1987;21:297–309.
32. Iontcheva I, Oppenheim FG, Troxler RF. Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. J Dent Res 1977;76:734–743.
33. Nielsen PA, Mandel U, Therkildsen MH, Clausen H. Differential expression of human high-molecular-weight salivary mucin (MG1) and low-molecular-weight salivary mucin (MG2). J Dent Res 1996;75:1820–1826.
34. Cohen RE, Aguirre A, Neiders ME, Levine MJ, Jones PC, Reddy MS, Haar JG. Immunochemistry of high-molecular-weight human salivary mucin. Arch Oral Biol 1990;35:127–136.
35. Cohen RE, Aguirre A, Neiders ME, Levine MJ, Jones PC, Reddy MS, Haar JG. Immunochemistry and immunogenicity of low-molecular-weight human salivary mucin. Arch Oral Biol 1991;36:347–356.
36. Banderas JA, Nava J, González M. Salivary flow rate and protein concentration from human whole saliva in Mexican young population. J Dent Res 1995;74:503 (abstract no. 823).
37. Banderas JA, González M, Sánchez M, Millán E, López A, Vilchis A. Flujo y concentración de proteínas en saliva total humana. Salud Publica Mex 1997;39:433–441.
38. World Health Organization. Oral health surveys: basic methods. 3rd ed. Geneva, Switzerland: WHO;1986.
39. González M, Cabrera R, Grossi SG, Franco F, Aguirre A. Prevalence of dental caries and gingivitis in a population of Mexican schoolchildren. Community Dent Oral Epidemiol 1993;21:11–14.
40. Mandel ID, Wotman S. The salivary secretions in health and disease. In: Rankow RM, Polayes IM, editors. Diseases of the salivary glands. Philadelphia, PA, USA: Saunders;1976. pp. 32–35.
41. Narhi TO, Meurman JH, Ainamo A, Nevalainem JM, Schmidt-Kaunisaho KG, Sinkosaari P. Association between salivary flow rate and the use of systemic medication among 76-, 81- and 86-year-old inhabitants in Helsinski, Finland. J Dent Res 1992;71:1875–1880.
42. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
43. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;277:680–688.
44. Sreebny LM, Broich G. Xerostomia, dry mouth. In: Sreebny LM, editor. The salivary system. Boca Raton, FL, USA: CRC Press;1987.
45. Soderling E, Pienihakkinen K, Alanen M-L, Hietaoja M, Alanen P. Salivary flow rate, buffer effect, sodium and amylase in adolescents: a longitudinal study. J Dent Res 1993;101:98–102.
46. Ben-Aryeh H, Miron R, Szargel R, Gutman D. Whole salivary secretion rates in old and young healthy subjects. J Dent Res 1984;63:1147–1148.
47. Navazesh M, Christensen CM, Brightam V. Clinical criteria for the diagnosis of salivary gland hypofunction. J Dent Res 1992;71:1363–1369.
48. Johansson I, Saellstrom A-K, Rajan BP, Parameswaran A. Salivary flow and dental caries in Indian children suffering from chronic malnutrition. Caries Res 1992;26:38–43.
49. Navia JM, DiOrio LP, Menaker L, Miller S. Effect of undernutrition during the perinatal period on caries development in the rat. J Dent Res 1970;49:1091–1098.
50. Menaker L, Navia JM. Effect of undernutrition during the perinatal period on caries development in the rat. V. Changes in whole saliva volume and protein content. J Dent Res 1974;53:592–597.
51. Johnson DA, López H, Navia JM. Effects of protein deficiency and diet consistency on the parotid gland and parotid saliva of rats. J Dent Res 1995;74:1444–1452.
52. Dodds MW, Johnson DA, Mobley CC, Hattaway KM. Parotid saliva protein profiles in caries-free and caries-active adults. Oral surgery, oral medicine, oral pathology, oral radiology and endodontics. Oral Surg Oral Med Oral Pathol 1997;83:244–251.
53. Shiba A, Shiba KS, Suzuki K. Analysis of salivary proteins by thin layer sodium dodecylsulphate polyacrylamide gel electrophoresis. J Oral Rehab 1986;13:263–271.
54. Cowman RA, Schaefer SJ, Fitzgerald RJ, Rosner D, Shklair IL, Walter RG. Differential utilization of proteins in saliva from caries-active and caries-free subjects as growth substrates by plaque-forming *Streptococci*. J Dent Res 1979;58:2019–2027.
55. Clark WB, Beem J, Nesbitt WE, Cisar JO, Tseng CC, Levine MJ. Pellicle receptors for *Actinomyces viscosus* type 1 fimbriae *in vitro.* Infect Immun 1989;57:3003–3008.
56. Gibbons RJ, Hay DI, Schlesinger DH. Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of *Streptococcus gordonii* to apatitic surfaces. Infect Immun 1991;59:2948–2954.
57. Kousvelari EE, Baratz RS, Burke B, Oppenheim FG. Immunochemical identification and determination of proline-rich proteins in salivary secretions, enamel pellicle, and glandular tissue specimens. J Dent Res 1980;59:1430–1438.
58. Banderas JA, Levine MJ. *In vivo* characterization of the acquired enamel pellicle: a time course study. J Dent Res 1992;71:163 (abstract no. 463).
59. Everhart DL, Grigsby WR, Carter WH Jr. Evaluation of dental caries experience and salivary immunoglobulins in whole saliva. J Dent Res 1972;51:1487–1491.
60. Bolton RW. Naturally-occurring IgA antibodies to glycerol-teichoic acid in human saliva. Correlation with caries activity. J Dent Res 1981;60:878–882.
61. Bolton RW, Hlava GL. Evaluation of salivary IgA antibodies to cariogenic microorganisms in children. Correlation with dental caries activity. J Dent Res 1982;61:1225–1228.
62. Rose PT, Gregory RL, Gfell LE, Hughes CV. IgA antibodies to *Streptococcus mutans* in caries-resistant and -susceptible children. J Dent Res 1994;16:272–275.
63. Rudney JD, Michalowicz BS, Krig MA, Kane PK, Pihlstrom BL. Genetic contributions to saliva protein concentrations in adult human twins. Arch Oral Biol 1994;39:513–517.
64. Mandel ID. The functions of saliva. J Dent Res 1987;66:623–627.
65. Bradway SD, Levine MJ. Salivary glands and saliva. In: Encyclopedia of human biology. Vol. 6. New York: Academic Press;1991. pp. 689–700.

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Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product

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The MG1 population of mucins was isolated from human whole salivas by gel chromatography followed by isopycnic density gradient centrifugation. The reduced and alkylated MG1 mucins, separated by anion exchange chromatography, were of similar size (radius of gyration 55–64 nm) and molecular weight (2.5–2.9 $\times$ 10^6 Da). Two differently-charged populations of MG1 subunits were observed which showed different reactivity with monoclonal antibodies to glycan epitopes. Monosaccharide and amino acid compositional analyses indicated that the MG1 subunits had similar glycan structures on the same polypeptide. An antiserum recognizing the MUC5B mucin was reactive across the entire distribution, whereas antisera raised against the MUC2 and MUC5AC mucins showed no reactivity. Western blots of agarose gel electrophoresis of fractions across the anion exchange distribution indicated that the polypeptide underlying the mucins was the product of the *MUC5B* gene. Amino acid analysis and peptide mapping performed on the fragments produced by trypsin digestion of the two MG1 populations yielded data similar to that obtained for MUC5B mucin subunits prepared from respiratory mucus (Thornton *et al.*, 1997) and confirmed that the MUC5B gene product was the predominant mucin polypeptide present. Isolation of the MG1 mucins from the secretions of the individual salivary glands (palatal, sublingual, and submandibular) indicate that the palatal gland is the source of the highly charged population of the MUC5B mucin.

Key words: mucin/ MUC5B/saliva/salivary glands

Introduction

Saliva is a specialized, dilute, aqueous secretion produced by three major exocrine glands—the parotid, submandibular and sublingual—and several minor glands in the mouth. Functions of saliva include lubrication of the oral cavity and non-immune defense against pathogens in the environment (Wu *et al*., 1994; Veerman *et al*., 1995). Human saliva contains several components implicated in the protection of the oral cavity, including secretory IgA, lysozyme, proline-rich proteins, histatins, and mucins (Iontcheva *et al*., 1997). These latter macromolecules are large glycoproteins with O-linked glycans, and human saliva has been shown to contain at least two structurally and functionally distinct populations of mucins which are divided into the high molecular weight (M_r > 10^6 Da) oligomeric, gel-forming MG1 population and the lower molecular weight (M_r 1.2–1.5 $\times$ 10^5 Da) monomeric MG2 mucins (Loomis *et al*., 1987; Ramasubba *et al*., 1991; Veerman *et al*., 1992). The polypeptide chain of the MG2 population has been identified as the product of the *MUC7* gene (Bobek *et al*., 1993). The MG1 population is heterogeneous (Thornton *et al*., 1995) and it has been demonstrated that the different salivary glands secrete distinct populations of MG1, which vary in buoyant density (Veerman *et al*., 1992) and the degree of sulfation and sialylation of their glycan chains (Bolscher *et al*., 1995). cDNA cloning studies and Northern blotting data indicate that the MUC5B gene product forms a major constituent of MG1 (Troxler *et al*., 1995, 1997; Nielsen *et al*., 1997). However, at present it is unclear whether one or a number of gene products constitute the MG1 mucins.

In this study we have attempted to identify the polypeptide(s) underlying the MG1 population of mucins. We have demonstrated the presence of two distinct populations of MG1 mucins, which vary in their carbohydrate content and their charge density. Amino acid compositional analysis of the glycopeptides and MALDI-TOF MS analysis of tryptic peptides from both populations indicate they are differently glycosylated products of the *MUC5B* gene, and therefore we propose that the MUC5B gene product is the predominant mucin species of MG1.

Results

Mucin purification

Saliva contains two families of mucin termed MG1 and MG2 (MUC7) which can be distinguished on the basis of their molecular size. Gel chromatography on Sepharose CL-4B (data not shown) separated the high-M_r mucins, corresponding to MG1, which were eluted in the void volume of the column from the smaller MG2 mucins which were more included (Mehrotra *et al*., 1998). The MG1-containing fractions were further purified by density-gradient centrifugation, first in CsCl/4 M guanidinium chloride to remove proteins and then in CsCl/0.2 M guanidinium chloride to remove nucleic acids. Typical profiles from such a purification are shown in Figure 1 and the final MG1 mucin preparation was pooled as shown in Figure 1b. The MG1 mucins eluted in the void volume of Sepharose CL-2B and after reduction a single more included peak was observed (data not shown). The reduced mucin subunits were used for further study.

Reduced MG1 mucin subunit fractionation

Reduced MG1 mucin subunit preparations from three individuals were fractionated by analytical anion-exchange chromatography

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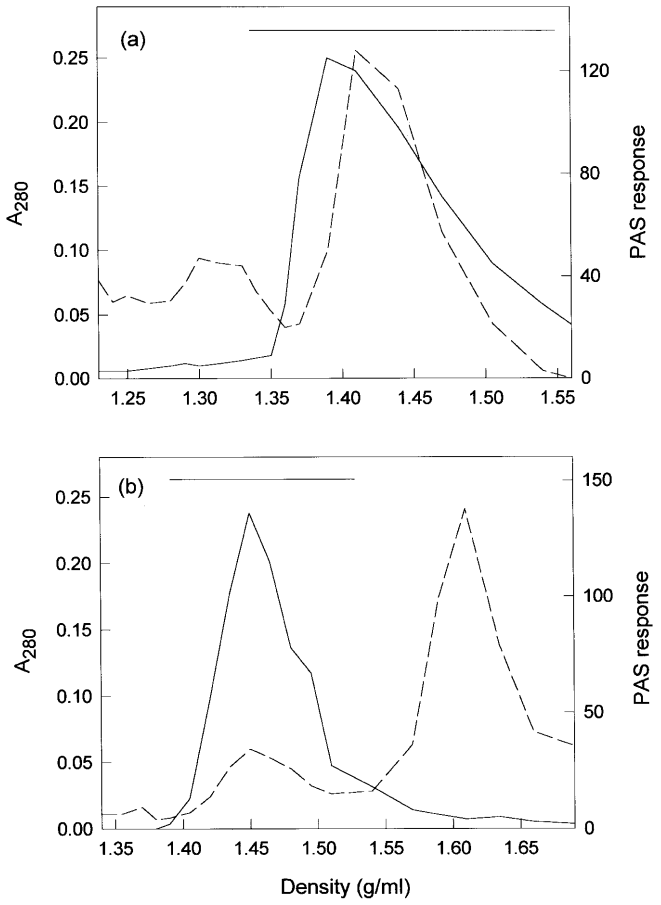

Fig. 1. Density gradient centrifugation of MG1 mucins from Sepharose CL-4B in (**a**) CsCl/ 4M guanidinium chloride and (**b**) CsCl/ 0.2M guanidinium chloride. (**a**) Density-gradient centrifugation in CsCl/ 4M guanidinium chloride was performed as described in the experimental section. Tubes were emptied from the top and fractions (5 ml) were assayed for carbohydrate with the PAS assay (solid line), nucleic acids and proteins by absorbance measurements at 280 nm (broken line) and the density determined by using a Hamilton syringe as a pycnometer. Mucin-containing fractions were pooled as indicated prior to (**b**) centrifugation in CsCl/0.2 M guanidinium chloride. Tubes were emptied and assayed as above. Mucin containing fractions were pooled as indicated with the horizontal bars.

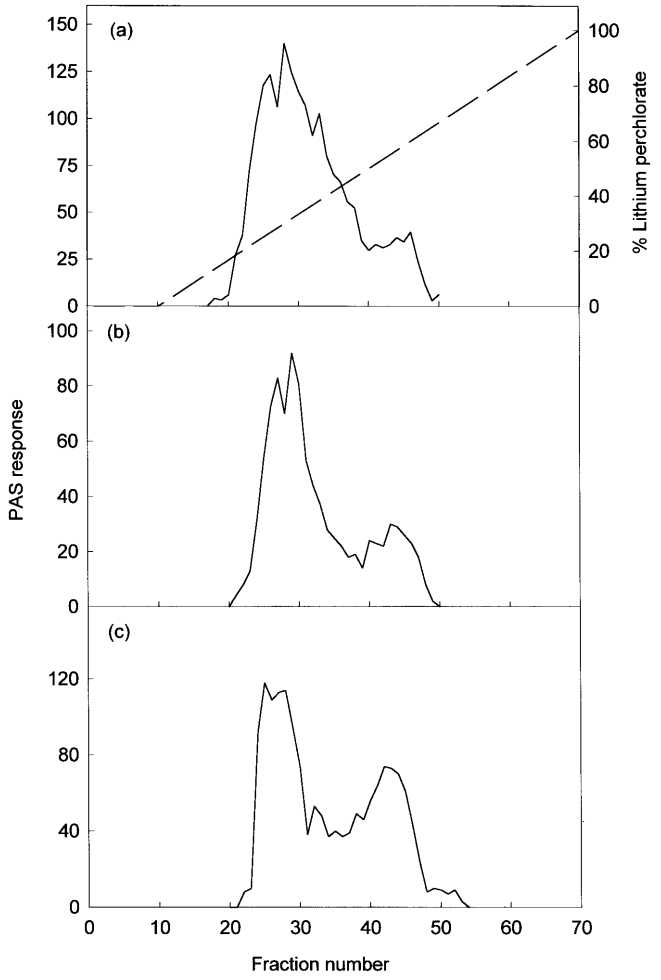

Fig. 2. Anion-exchange chromatography of reduced MG1 mucin subunit preparations. Reduced mucin subunits from three individuals (**a**–**c**) were chromatographed on a Mono Q HR5/5 column as described in *Materials and methods*. Fractions (0.5 ml) were assayed after immobilization onto nitrocellulose with the PAS-reagent (solid line) and the nominal elution gradient is shown (dashed line).

and the carbohydrate-profiles, as monitored with a PAS-assay, are presented in Figure 2. In general, two differently charged populations of molecules are present with the lower charged glycoprotein as the predominant component (Figure 2). The molecular weight (M_r) and radius of gyration (R_G) of the reduced mucin subunits in fractions across the anion exchange distribution (Figure 2a) were determined by in-line laser light scattering after chromatography on a Superose 6 column (data not shown) and the values are presented in Table I. The data show little evidence of a difference in M_r across the molecular charge distribution indicating the reduced MG1 mucin subunits are of similar mass. However, there is a slight increase in the size (R_G) of the more charged species. The refractive index profiles from the Superose 6 chromatography indicate that no smaller proteins/glycoproteins were present in the fractions (data not shown).

Table I. Molecular weight and size data for the reduced mucin subunits across the molecular charge distribution

Mono Q fraction	M_r ($\times 10^{-6}$ Da)	R_G (nm)
28	2.8	53
30	2.5	53
32	2.6	56
34	2.8	58
36	2.8	59
38	2.6	60
40	2.9	64
42	2.6	61
44	2.5	59

Reduced MG1 mucin carbohydrate analysis

The neutral sugar compositions of the reduced MG1 subunits in the fractions across the anion exchange distribution were determined and a graphical representation of the data (Figure 3a) yields a profile similar to that observed with the PAS assay (Figure 2a). The anion exchange distribution was also analyzed with two

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monoclonal antibodies, INES and F2, with glycan epitopes. The antibodies showed differential reactivity (Figure 3b,c); INES highlighted the less acidic population of molecules, whereas F2 was reactive with the more acidic MG1 subunits. The reactivity of the monoclonal antibody INES was greatly enhanced by neuraminidase treatment of the molecules (data not shown). The relative ratios of the monosaccharides in each fraction were similar when normalized to GalNAc, although there was a greater proportion of fucose in the structures associated with the early eluting subunits (Figure 3d). Fractions 22–37 and 38–49 corresponding to the two populations of MG1 subunits were pooled and designated A and B, respectively, and their total monosaccharide compositions were determined (Table II). More neutral sugars are associated with the lower charged population A, but normalized to GalNAc the molecules have a similar average composition. Again these data indicate that the overall glycan structures are similar on all the MG1 subunits and that the lower charged population A may have higher fucosylation. The sialic acid content of the mucins is similar, whereas the sulfate content is at least 2-fold higher for population B (Table II) and explains the observed differences in their charge density. The compositional and antibody data suggest that two different mucin species are present in the MG1 preparation; therefore, experiments were performed to determine the identity of their mucin polypeptide(s).

Table II. Monosaccharide composition of the two mucin populations

Monosaccharide	Mucin population[a]			
	A	B	A	B
	(nmol/5 µg mucin)		(normalized to GalNAc)	
Fuc	4.1	1.1	2.6	1.8
GalNAc	1.6	0.6	1.0	1.0
GlcNAc	4.7	1.5	2.9	2.5
Gal	6.8	1.9	4.2	3.2
Man	2.8	2.8	1.8	4.7
Total neutral Sugars	19.9	7.9		
Sialic acid	0.3	0.15	0.2	0.25
Sulfate	0.05	0.12		

[a]Fractions from anion exchange chromatogram shown in Figure 2a, corresponding to the two populations of MG1 mucin subunits A and B, were pooled as follows: A, 22–37; and B, 38–49.

Reduced MG1 mucin polypeptide identification

The amino acid composition of selected fractions from the anion exchange column was determined and the serine, threonine and proline contents of the MG1 subunits are shown in Figure 3e. The data indicate that the molecules have a similar total proportion of these amino acids suggesting they all contain the same polypeptide. The proportion of the other amino acids was also similar (data not shown). Fractions from the anion exchange column were also probed with antisera targeted to the polypeptide portion of MUC2, MUC5AC, and MUC5B mucins. Of these, only the MUC5B antiserum gave a positive response, which followed the carbohydrate-profile (Figure 3f). A similar profile was observed after aliquots of fractions were assayed with a polyclonal antiserum (MAN-SUBS) that recognizes a range of human reduced mucin subunits (data not shown). To further analyze the MG1 subunits in these fractions, electrophoresis was performed in 1% agarose gels and Western blots of the gels were probed with either the MUC5B mucin-specific antiserum (Figure 4a) or the general mucin reduced subunit antiserum (Figure 4b). The MUC5B-reactive bands coincide with those seen with general mucin subunit antiserum. Furthermore, the data show that there is an increase in migration of the reduced MG1 subunits with increasing charge density as assessed by their elution position from the anion exchange column (Figure 2a).

The antibody data above suggests MUC5B is a major mucin in the MG1 preparation; however, it does not tell us whether it is the only mucin present. To ascertain what proportion of the MG1 preparation was accounted for by the MUC5B mucin, tryptic mapping and further amino acid analyses were performed. Prior to this it was necessary to prepare larger quantities of reduced MG1 subunits and this was accomplished by preparative anion exchange chromatography (data not shown). Fractions from the column were pooled to yield two groups of molecules corresponding to the two differently charged mucin populations A and B, and these were subjected to trypsin treatment. The degradation products gave similar elution profiles after gel chromatography on Superose 12 (Figure 5), and this resulted in the separation of the high-M_r mucin glycopeptides (A-I and B-I) from lower-M_r glycosylated and unglycosylated peptides (A-II and B-II). The high-M_r mucin glycopeptides (A-I and B-I) were subjected to amino acid analysis and the data are presented in Table III. Both samples appear to have the same composition, which is similar to that for high-M_r mucin glycopeptides derived from MUC5B reduced mucin subunits purified from respiratory secretions (Thornton *et al*., 1997). MALDI-TOF MS analysis of the lower-M_r peptides in pools A-II and B-II yielded similar mass fingerprints (Figure 6), which are similar to those observed after trypsin digestion of the respiratory MUC5B mucins (Thornton *et al*., 1997). The four peptides identified and sequenced from the respiratory mucin sample were also present in these samples (Table IV). The data from these analyses suggest the majority of the mucins in the MG1 preparation are products of the *MUC5B* gene.

Table III. Amino acid composition of high-M_r mucin glycopeptides (A-I and B-I)

	High M_r-mucin glycopeptide fraction		
Amino acid	A-I	B-I	MUC5B mucin glycopeptide[a]
Asx	10	13	10
Glx	34	34	34
Ser	178	168	172
Gly	64	64	56
Thr	290	295	290
Ala	98	103	129
Pro	187	169	180
Arg	18	19	19
Tyr	5	5	4
Val	38	40	35
Ileu	20	20	16
Leu	46	45	39
Phe	nd	7	14
Lys	12	17	2

Values are expressed per 1000 residues; nd, not determined.
[a]Data from Thornton *et al*., 1997.

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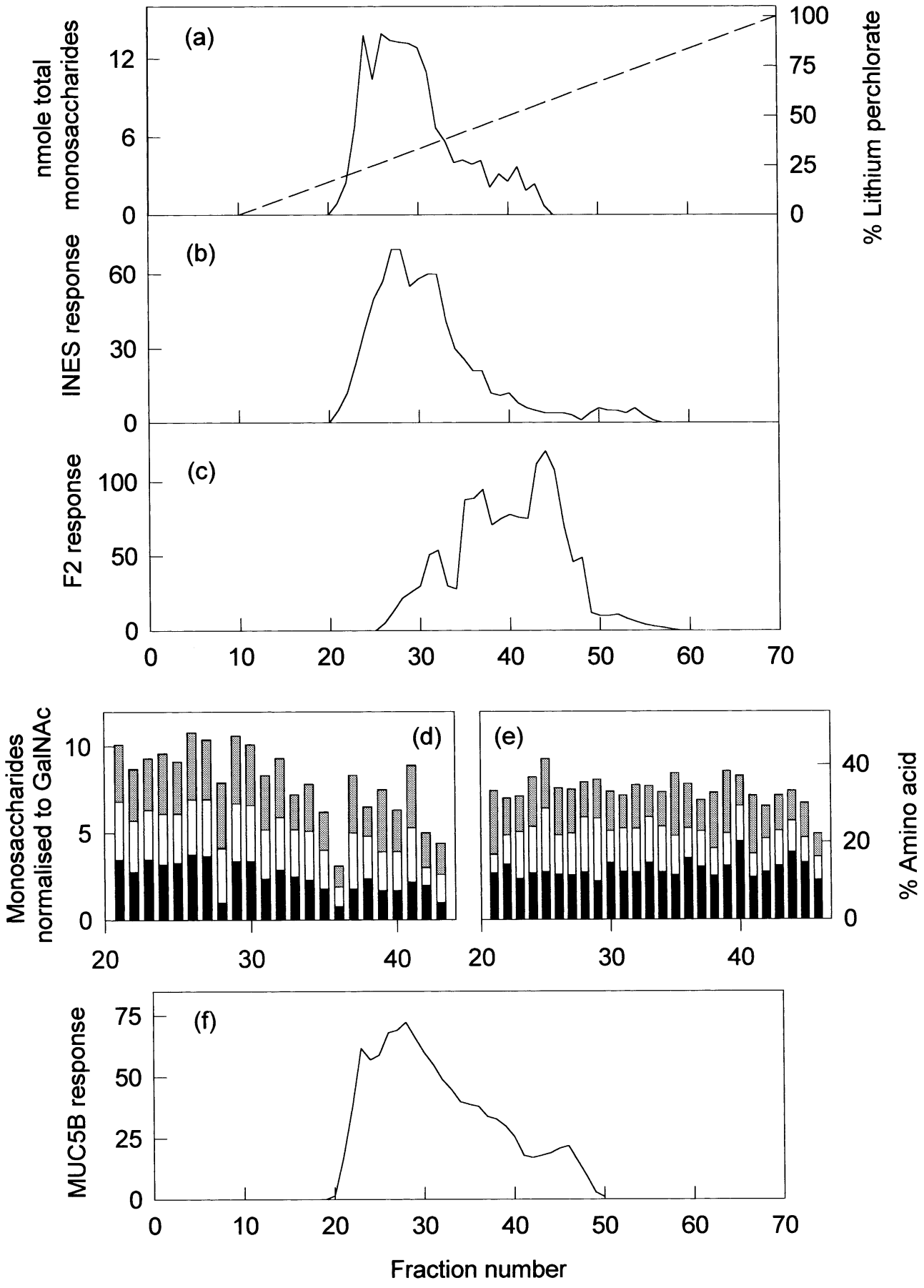

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Table IV. Comparison of the masses (Da) of tryptic peptides with those from the MUC5B mucin isolated from respiratory mucus

A-II	B-II	MUC5B mucin[a]
1032	1035	1038
1128	1132	1129
1683	1688	1685
1976	1978	1975

[a]Data from Thornton *et al*., 1997.

Table V. Molecular weight and size data for the intact mucins isolated from different salivary glands

Gland	M_r ($\times 10^{-6}$ Da)	R_G (nm)
Palatal	19	222
Sublingual	32	271
Submandibular	20	191
Whole saliva	25	230

Mucins produced by individual salivary glands

Mucins, purified as above from whole saliva and from the secretions isolated from sublingual, submandibular, and palatal glands, were reduced and alkylated, and the resulting reduced subunits were subjected to anion exchange chromatography (Figure 7). The carbohydrate-profiles of the three distributions encompass the range of charge density observed for the reduced mucin subunits from whole saliva (Figure 7a), with those from the palatal glands accounting for the most charged molecules in the total distribution (Figure 7b). The mucins from the sublingual and submandibular glands were of similar, but lower relative charge density (Figure 7c,d) and these two glands can be seen to contribute the majority of the MG1 mucins. The three distributions were probed with the MUC5B antiserum, and in each case the MUC5B mucin subunit profile followed that of the carbohydrate.

The average molecular weight and size of the intact mucins from whole saliva and the three glands was determined by in-line laser light scattering after chromatography on Sephacryl S-1000 (data not shown) and the values are presented in Table V. The MUC5B mucins purified from whole saliva had M_r in the range $14–40 \times 10^6$ Da with an average M_r of 25×10^6 Da. The mucins from the palatal and submandibular glands were lower in molecular weight than the total population whereas the sublingual gland mucins were larger. The size distribution of the MUC5B mucins produced by each of the salivary glands was assessed by rate zonal centrifugation (Figure 8). The MUC5B mucins from the different glands were polydisperse in size, but after reduction a single more homogeneous peak is observed with a lower apparent mass (data not shown). In agreement with the light scattering data, the MUC5B mucins from the sublingual glands are the most massive while the palatal MUC5B glycoproteins contain more smaller species.

Discussion

In the present study we have isolated the high-M_r MG1 salivary mucins by exploiting their large hydrodynamic volume (void volume CL-4B) and high buoyant density (1.35–1.55 g/ml in 4 M guanidinium chloride/CsCl and 1.40–1.52 g/ml in 0.2 M guanidinium chloride/CsCl). In common with the gel-forming mucins isolated from both respiratory tracts (Thornton *et al*., 1990) and cervical tracts (Carlstedt *et al*., 1983), the mucins are oligomeric and can be fragmented into their constituent subunits (M_r 2.5–2.9 $\times 10^6$) by reduction of disulfide bonds. The MG1 mucin preparations contain two populations of molecules that differ with respect to their charge density. This is in agreement with previous studies which have shown that the high molecular weight MG1 mucins are heterogeneous and that the major salivary glands secrete differently charged forms of these mucins (Veerman *et al*., 1991, 1992; Bolscher *et al*., 1995). The identity of the polypeptides underlying these mucins has not previously been elucidated. Recent cDNA cloning data indicates that the MUC5B mucin is present in the MG1 population (Nielsen *et al*., 1997; Troxler *et al*., 1997), but its proportion is undetermined. Using a combination of antibody reactivity, peptide mapping and amino acid analysis, we have demonstrated that the polypeptide of the majority of the mucins in the MG1 preparation are encoded for by the *MUC5B* gene. While it may be argued that antibody reactivity alone cannot give quantitative information on the relative abundance of the MUC5B mucin, this is not so for the two chemical analyses. Both peptide mapping and amino acid analysis indicate that MUC5B is the predominant mucin, and we are unable to find evidence for significant quantities of other mucins in the MG1 preparation. There is a remarkable similarity in the amino acid composition between the MG1 high-M_r glycopeptides and those derived from the MUC5B mucin from the respiratory tract (Thornton *et al*., 1997). Furthermore, the mass fingerprints of the peptides derived from the MG1 mucins are very similar to those derived from the respiratory MUC5B mucins (Thornton *et al*., 1997). The peptides identified in the previous study were found to be present within a contiguous 51 amino acid sequence, and we proposed on the basis of their abundance that the sequence would be repeated numerous times throughout the mucin polypeptide. Indeed, recent cDNA cloning data has confirmed the presence of this repetitive motif within the MUC5B mucin polypeptide (Desseyn *et al*., 1997).

Recently, mucin gene expression has been studied in the sublingual and submandibular glands by Northern blot analyses (Troxler *et al*., 1997). The data indicated that MUC5B is the major known mucin expressed in these tissues. MUC4 RNA was shown to be expressed weakly by the two glands, but MUC2, MUC3, MUC5AC, and MUC6 expression were not detected. As far as the MUC2 and MUC5AC mucins are concerned, our antibody data are consistent with these observations. On the basis of the

Fig. 3. Analysis of the reduced MG1 mucin subunits after anion-exchange separation. The data presented are on the anion exchange distribution shown in Figure 2a. Aliquots of fractions were taken for determination of (**a**) their total neutral sugar composition and their reactivity with anti-carbohydrate monoclonal antibodies (**b**) INES and (**c**) F2. In (**b**) the membrane was treated with neuraminidase prior to probing with the antibody. In addition the contents of (**d**) the monosaccharides Fuc (black bars), GlcNAc (white bars) and Gal (gray bars) normalized to GalNAc and (**e**) the percentage of the amino acids Ser (black bars), Thr (white bars), and Pro (gray bars) are shown for the mucin subunit-containing fractions. (**f**) Fractions were also analyzed for their reactivity with the MUC5B-mucin specific antiserum (MAN-5B-I). The nominal elution gradient is shown (dashed line).

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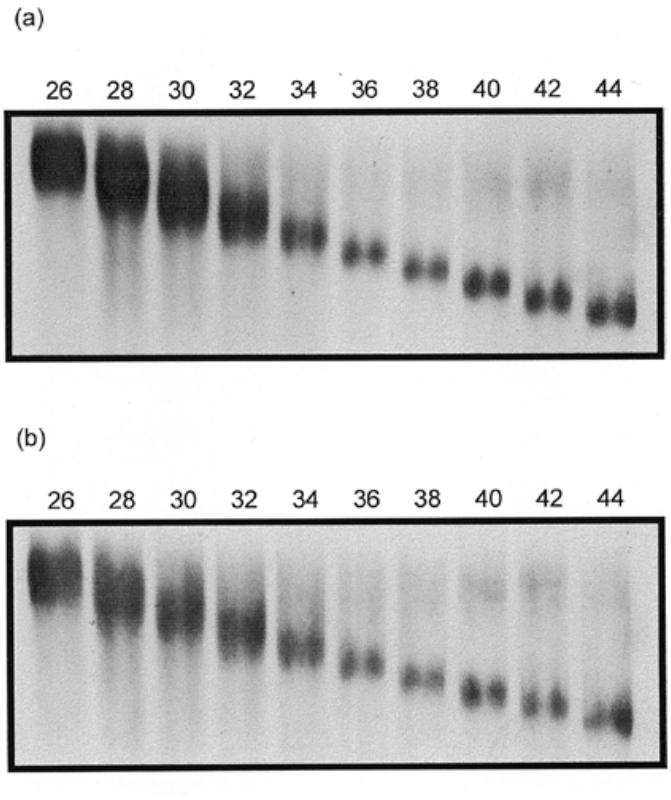

Fig. 4. Agarose gel electrophoresis of the fractionated reduced MG1 mucin subunits. Aliquots of selected fractions from the Mono Q separation of the reduced mucin subunits were electrophoresed on a 1% (w/v) agarose gel and then Western blotted onto nitrocellulose as described in *Materials and methods*. The blot was probed (**a**) with the MUC5B-mucin specific antiserum (MAN-5B-I) and then stripped and reprobed (**b**) with the general mucin subunit antiserum (MAN-SUBS).

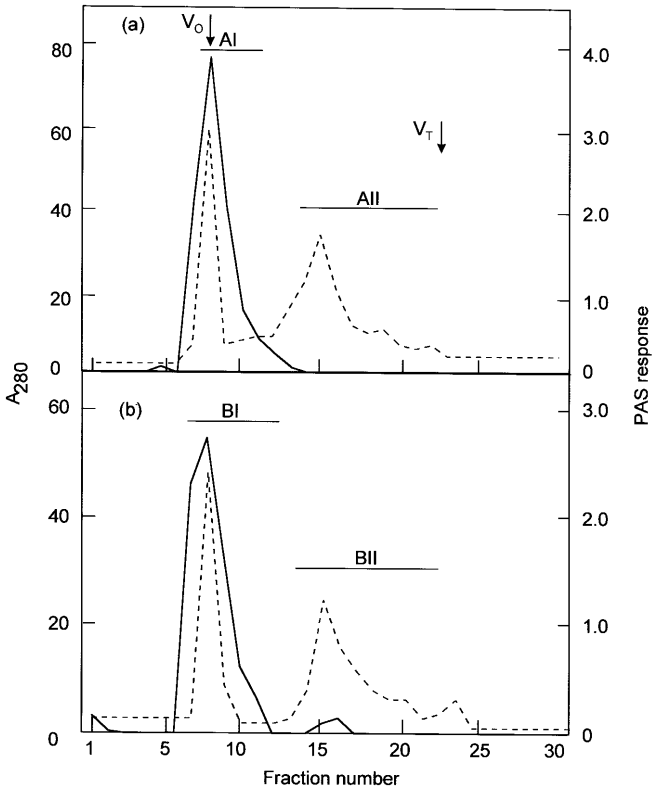

Fig. 5. Superose 12 chromatography of trypsin digestion products of reduced MG1 mucin subunit populations A and B. The reduced mucin subunit populations (**a**) A and (**b**) B were treated with trypsin and subsequently chromatographed on a Superose 12 HR 10/30 column. The column eluent was monitored for absorbance at 280 nm (broken line) and with the PAS-reagent (solid line). Fractions from the column runs were pooled as follows: A-I 6–12, A-II 13–22, B-I 6–12, and B-II 13–22. These poolings are indicated by the bars.

biosynthetic capacity of the submandibular glands, Troxler *et al.* (1997) suggest that other as yet unidentified mucins are secreted from these glands and contribute to the MG1 population. Our data suggests that if other mucins are secreted they do not form a significant part of the MG1 preparation studied here. However, we do not rule out that either smaller mucins or mucins with a different density are produced from the submandibular glands, which would be excluded from our preparation.

The MUC5B reduced mucin subunits are heterogeneous in their charge distribution and two major populations of molecules can be observed. We have previously noted differently glycosylated populations of this mucin in respiratory tract secretions (Thornton *et al.*, 1997). The higher charge population in saliva appears to originate from the palatal glands, and the proportion of the MUC5B mucins in whole salivas contributed to by the palatal glands can vary. It would appear that the palatal gland MUC5B mucin is a minor component compared to the mucins from the sublingual and submandibular glands, which are of similar but lower charge density. The two populations of MUC5B mucins can be distinguished on the basis of glycan-epitope specific monoclonal antibody, and there is evidence that the lower charged form has a higher fucose content. However, carbohydrate compositional data indicates the two populations may have similar glycan structures.

The heterogeneity in charge density of the MUC5B mucin subunits is not accompanied by any significant change in their molecular weights. Furthermore, the sialic acid content of the mucins is similar. However, the high charged subunit population B has a 2- to 3-fold increase in sulfate content compared to the less acidic population A. Thus, the increasing acidity observed by anion exchange chromatography and the increasing electrophoretic mobility in agarose gels of the mucin subunits must be due to their increasing levels of sulfation. The sulfate is probably present as SO_3-3Galβ1-3GlcNAc moieties since the more charged molecules are reactive with the monoclonal antibody F2 which recognizes this portion of the sulfo-Lewis^a antigen. (Veerman *et al.*, 1997).

The MUC5B mucins isolated from whole saliva and the different salivary glands are polydisperse in size. This polydispersity must arise from a variable number of subunits in the intact oligomeric mucins. Thus, for example, the mucins in whole saliva (M_r 14–40 × 10^6) are constructed from between 5–16 subunits (M_r 2.5–2.9 × 10^6) and these multi-subunit assemblies are stabilized by disulfide bonds. The MUC5B mucins from sublingual gland are of highest average molecular weight, whereas those from the palatal and submandibular glands are on average smaller. It is interesting to note that the MUC5B mucins in the palatal secretions have the broadest spread of molecular sizes, with a proportion of molecules possibly as small as monomers.

Conclusions

The MUC5B mucin is the predominant mucin in the salivary MG1 preparation and is found in different glycosylated forms.

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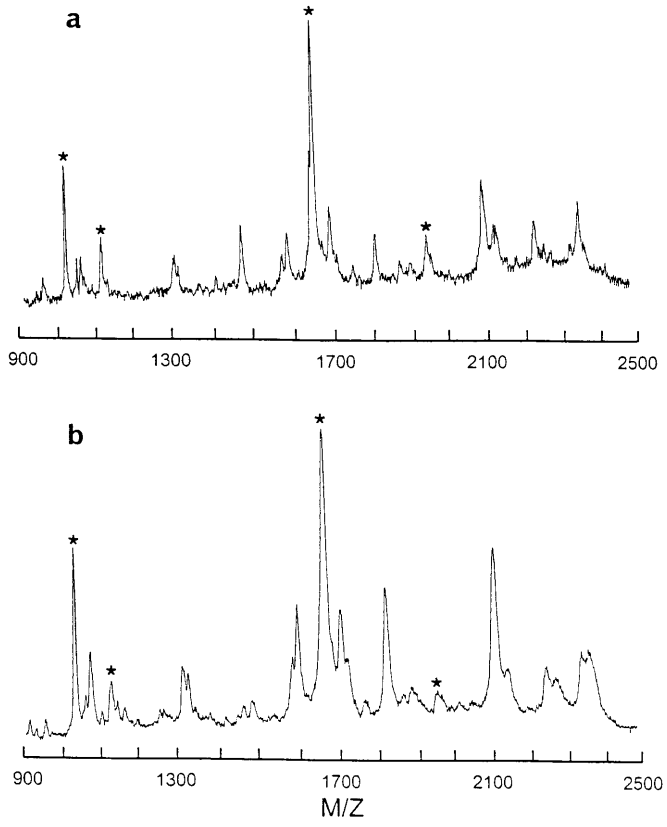

Fig. 6. MALDI-TOF mass spectrometry of the low-M_r tryptic peptides derived from reduced mucin subunit populations A and B. Tryptic peptide-containing fractions (**a**) A-II and (**b**) B-II were analyzed by MALDI-TOF MS. The peak heights represent the relative abundance of each species. Highlighted with asterisks are the major peptides isolated and sequenced after trypsin digestion of the MUC5B mucin isolated from respiratory mucus (Thornton *et al*., 1997).

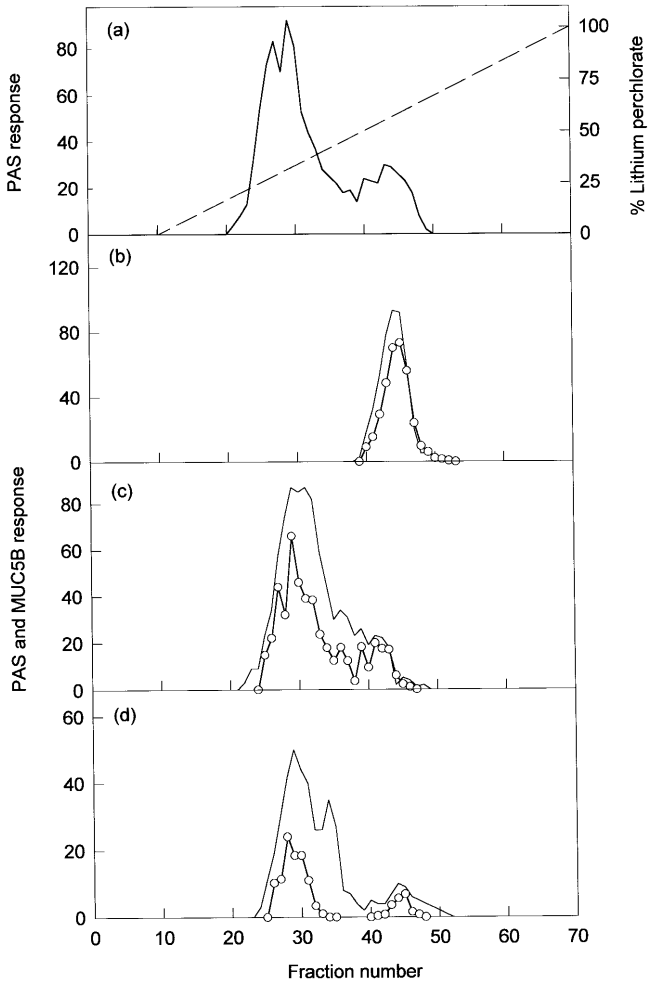

Fig. 7. Anion-exchange chromatography of the reduced mucin subunit preparations from individual salivary glands. Mucins isolated from (**a**) whole saliva and (**b**) palatal, (**c**) sublingual, and (**d**) submandibular glands, were reduced and alkylated and the resulting reduced mucin subunits chromatographed on a Mono Q HR5/5 column as described in *Materials and methods*. Fractions (0.5 ml) were assayed after immobilization onto nitrocellulose with the PAS-reagent (solid line) and for reactivity with the MUC5B-mucin specific (MAN-5B-I) antiserum (open circles). The nominal elution gradient is shown (dashed line).

The mucins differ in their relative levels of sulfation and fucose, the most charged (most sulfated) form of the mucin arises from the palatal glands. The intact MUC5B mucins are on average very large; however, there is evidence that the palatal gland mucins contain a significant proportion of smaller species.

Materials and methods

Agarose UltraPURE (electrophoresis grade) was from BRL (Paisley, UK). The ECL Western detection kit was from Amersham International Plc. (Amersham, UK). Neuraminidase (EC 3.2.1.18; type X from *Clostridium perfringens*), Tween 20, (3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate) or CHAPS, Schiff's reagent, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, α-cyano-4-hydroxy cinnamic acid, Substance P, insulin (bovine pancreas) and guanidinium chloride (practical grade) were purchased from Sigma Chemical Co. (Poole, UK). Stock solutions of guanidinium chloride (~8 M) were treated with charcoal before use. Trypsin, modified by reductive alkylation to reduce autolysis, was purchased from Promega (Southampton, UK).

Saliva collection

Glandular secretions were collected separately using custom fitted devices as described previously (Veerman *et al*., 1996). Secretions were collected in ice-cooled vessels containing 8 M GuHCl and a cocktail of protease inhibitors (final concentrations 10 mM EDTA, 5 mM benzamidine hydrochloride, and 100 mM ε-aminocaproic acid). Whole saliva was stimulated by chewing on parafilm and collected directly into the extraction buffer.

Preparation of mucins

Mucins were extracted from saliva (2 days, gentle stirring, 4°C) with 6 M guanidinium chloride/10 mM sodium phosphate buffer/0.05% (w/v) CHAPS, pH 6.5 containing proteinase inhibitors (10 mM EDTA/10 mM N-ethylmaleimide/100 mM ε-amino-n-caproic acid/ 5mM benzamidine hydrochloride) and purified by Sepharose CL-2B chromatography and density-gradient centrifugation. Briefly, the extract was spun at 4400 × *g* for 10 min at 4°C and the supernatant chromatographed on a column of Sepharose CL-4B (48 cm × 5cm) eluted with 4 M guanidinium chloride/10 mM sodium phosphate buffer/0.1% (w/v) CHAPS, pH 6.5, at a flow rate of 40 ml/h. The mucins eluted in the void

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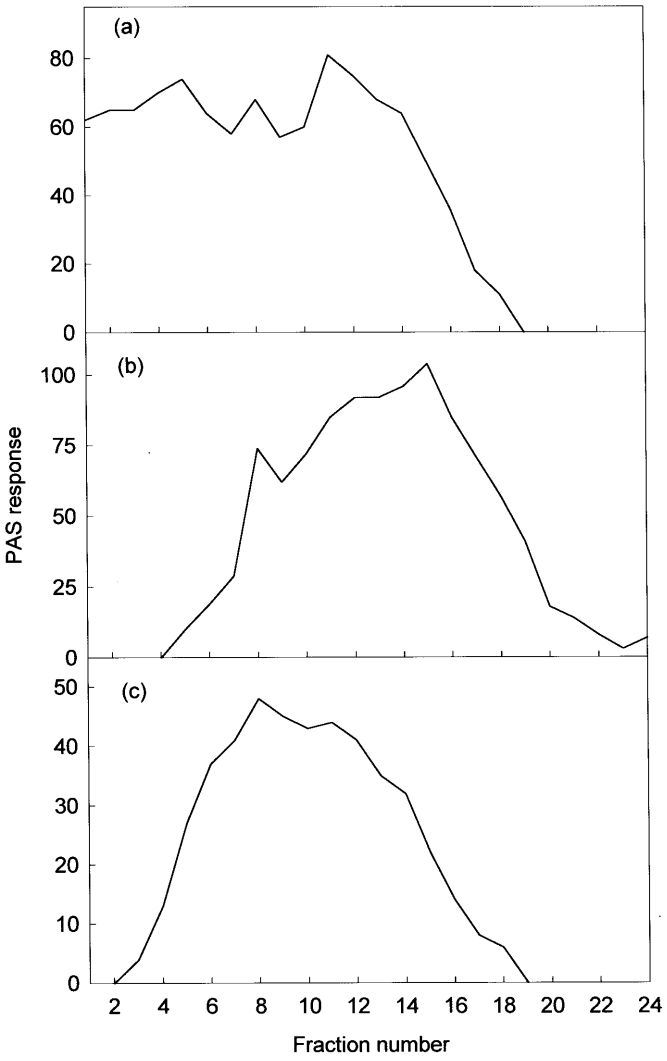

Fig. 8. Rate-zonal centrifugation of the MUC5B mucins isolated from different salivary glands. Mucins purified from (a) palatal, (b) sublingual, and (c) submandibular gland secretions were centrifuged on 6–8 M guanidinium chloride gradients as described in *Materials and methods*. Gradients were emptied from the top into 0.5 ml fractions and aliquots blotted onto nitrocellulose and analyzed for carbohydrate with a PAS assay.

volume of the column (MG1) were then subjected to CsCl/4 M guanidinium chloride density-gradient centrifugation at a starting density of 1.4 g/ml in a Beckman Ti45 angle rotor at 40,000 r.p.m. for 67 h at 15°C. The mucin (MG1) containing fractions were pooled as shown in Figure 1a and subjected to CsCl/0.2 M guanidinium chloride density-gradient centrifugation at a starting density of 1.5g/ml in a Beckman Ti45 angle rotor at 40,000 r.p.m. for 68 h at 15°C. Mucins were finally pooled as shown in Figure 1b and dialyzed into 6 M guanidinium chloride/0.1 M Tris pH 8.0.

Preparation of reduced mucin subunits

Reduced mucin subunits were obtained by treatment of the purified mucins in 6 M guanidinium chloride/0.1 M Tris, pH 8.0, with 10 mM dithiothreitol for 5 h at 37°C. Iodoacetamide was then added to a final concentration of 25 mM, and the mixture was left in the dark overnight at room temperature. Reduced mucin subunits were dialyzed into 6 M guanidinium chloride and stored at 4°C.

Anion-exchange chromatography

Reduced mucins were transferred from 6M guanidinium chloride into 6 M urea/10 mM piperazine pH 5.0 containing 0.02% CHAPS by chromatography on a Pharmacia PD-10 column prior to separation by anion-exchange chromatography. Reduced mucins were then chromatographed on a Pharmacia Mono Q HR 5/5 column eluted with a linear gradient of 0–0.4 M lithium perchlorate/10 mM piperazine pH 5.0 in 6M urea containing 0.02% CHAPS (Thornton *et al*., 1995).

Agarose gel electrophoresis

Reduced mucin subunits were electrophoresed in 1% (w/v) agarose gels in 40 mM Tris-acetate/1 mM EDTA pH 8.0 containing 0.1% (w/v) SDS, for 30 V and 18 h at room temperature. After electrophoresis, subunits were transferred to nitrocellulose by vacuum blotting prior to detection of mucins using antibodies (Thornton *et al*., 1995). Bands were visualized using horseradish peroxidase–labeled secondary antibodies in conjunction with an ECL Western detection kit.

Antibodies

As a general nonspecific mucin probe, a polyclonal antiserum raised against cervical mucin reduced subunits (MAN-SUBS) that recognizes protein epitopes on a range of reduced mucins was employed (Sheehan *et al*., 1991). Mucin-specific polyclonal antisera that recognize the reduced subunits of the MUC2 (Sheehan *et al*., 1996), MUC5AC (Thornton *et al*., 1996), and MUC5B mucins (Thornton *et al*., 1997) were also used. Monoclonal antibody F2 recognizes the SO_3-3Galβ1-3GlcNAc moiety of the sulfo-Lewis^a antigen. (Veerman *et al*., 1997). Monoclonal antibody INES was elicited against a purified high-M_r salivary mucin species (Rathman *et al*., 1990). This monoclonal antibody recognizes a periodate sensitive epitope on salivary mucins and removal of sialic acid enhances its binding to antigen.

Rate-zonal centrifugation

Rate zonal centrifugation was performed essentially as described previously (Thornton *et al*., 1990). In brief mucins were loaded onto preformed 6–8 M guanidinium chloride gradients and centrifuged in a Beckman SW 40Ti swing out rotor at 40,000 r.p.m. for 1.5 h at 15°C.

Light scattering

Intact mucins were chromatographed on a Sephacryl S-1000 column, and reduced mucin subunits were chromatographed on a Pharmacia Superose 6 HR 10/30 column. Both columns were eluted with phosphate-buffered saline, pH 7.4, at a flow rate of 0.5 ml/min. The column eluate were passed through an in-line Dawn DSP laser photometer and a Wyatt/Optilab 903 interferometric refractometer (Optichem, Clywd, UK) to measure light scattering and sample concentration, respectively. Light scattering measurements were taken continuously at 18 angles between 15° and 151° and the data analyzed according to Zimm (1947).

Preparation of high-M_r glycopeptides and tryptic peptides

Reduced mucin subunits were dissolved in 0.1 M ammonium hydrogen carbonate, pH 8.0, and digested with trypsin for 24 h at

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37°C. The digest was chromatographed on a Superose 12 HR 10/30 column in 0.1 M ammonium hydrogen carbonate, pH 8.0, at a flow rate of 0.4 ml/min and fractions from the column were combined into two pools (Figure 5) which were further analyzed.

MALDI-TOF mass spectrometry

Samples (1 μl) in 0.1% TFA were mixed with an equal volume of 50 mM α-cyano-4-hydroxy cinnamic acid, applied to a TOFspec target and analyzed by MALDI-TOF MS in linear positive ion mode using a VG TOFSpec-E with Substance-P (M^+ 1348.7 Da) and bovine insulin (M^+ 5734.5 Da) as internal standards. The data generated were processed using the OPUS peak detection program.

Amino acid analysis of high-M_r mucin glycopeptides

Samples were hydrolyzed under nitrogen in 3M HCl at 105°C for 16 h, and the resulting amino acids were derivatized with phenyl isothiocyanate and then separated by reverse-phase chromatography using a 3 μm ODS2 column.

Compositional analysis of MG1 reduced mucin subunits separated by anion exchange chromatography

Reduced MG1 mucin species separated by Mono Q anion exchange chromatography were immobilized on PVDF membrane and were hydrolyzed and analyzed by HPAEC-PAD for monosaccharide composition as described by Packer *et al*. (1996). Amino acid composition of PVDF blotted mucin fractions was determined by pre-column FMOC derivatization essentially as in Yan *et al*. (1996) except that the mucins were acid hydrolyzed in 6N HCl at 105°C for 24 h to prevent charring.

Sulfate analysis

Samples (20 μg) were hydrolyzed in 4 M HCl for 4 h at 100°C. The samples were dried in a Speed-Vac concentrator and washed once with water. The sulfate was separated from other ions by HPLC on an AS11 (4 × 250 mm) ion-exchange column (Dionex) using an AMMS II post-column ion suppressor (Dionex) with conductivity detection. A gradient of 5 mM to 30 mM NaOH over 10 min, followed by 5 min at 30 mM NaOH, at a flow rate of 1 ml/min was used to elute the anions. Sulfuric acid (50 mM) was used as the neutralizing counter-current in the ion suppressor at a flow rate of about 3 ml/min. Quantitation was by external calibration with 1 nmol sodium sulfate.

Analytical methods

Slot blotting of chromatographic fractions and PAS staining was carried out as previously described (Thornton *et al*., 1989). Antibody detection of slot blotted fractions was as described by Thornton *et al*. (1994). Blots were visualized using horse radish peroxidase–labeled secondary antibodies in conjunction with an ECL Western detection kit or alkaline phosphatase-labeled secondary antibodies using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the chromogenic substrate. Color yields were quantitated essentially by reflectance densitometry as described by Thornton *et al*. (1989).

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Abbreviations

MG1, high-M_r salivary mucins; MG2, low-M_r salivary mucins; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PAS, periodate Schiff's; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine, Fuc, fucose; Gal, galactose; Man, mannose; ECL, enhanced chemiluminescence; HPAEC-PAD, high-pH anion exchange chromatography with pulsed amperometric detection. CHAPS, (3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate).

References

Bobek,L.A., Tsai,H., Biesbrock,A.R. and Levine,M.J. (1993) Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J. Biol. Chem*., **268,** 20563–20569.

Bolscher,J., Veerman,E., Nieuw Amerongen,A.V., Tulp,A. and Verwoerd,D. (1995) Distinct populations of high-M_r mucins secreted by different human salivary glands discriminated by density-gradient electrophoresis. *Biochem. J*., **309,** 801–806.

Carlstedt,I., Lindgren,H. and Sheehan,J.K. (1983) The macromolecular structure of human cervical mucus glycoproteins. Studies on fragments obtained after reduction of disulfide bonds and after subsequent trypsin digestion. *Biochem. J*., **213,** 427–435.

Desseyn,J.L., Guyonnet-Duperat,V., Porchet,N., Aubert,J.P. and Laine,A. (1997) Human mucin gene MUC5B, the 10.7-kb large central exon encodes various alternate subdomains resulting in a super-repeat. Structural evidence for a 11p15.5 gene family. *J. Biol. Chem*., **272,** 3168–3178.

Iontcheva,I., Oppenheim,F.G. and Troxler,R.F. (1997) Human salivary mucin MG1 seletively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. *J. Dent. Res*., **76,** 734–743.

Loomis,R.E., Prakobphol,A., Levine,M.J., Reddy,M.S. and Jones,P.C. (1987) Biochemical and biophysical comparison of two mucins from human submandibular-sublingual saliva. *Arch. Biochem. Biophys*., **258,** 452–464.

Mehrotra,R., Thornton,D.J. and Sheehan,J.K. (1998) The isolation and physical characterisation of the MUC7 (MG2) mucin from saliva: evidence for self-association. *Biochem. J*., **334**, 415–422.

Nielsen,P.A., Bennett,E.P., Wandall,H.H., Therkildsen,M.H., Hannibal,J. and Clausen,H. (1997) Identification of a major human high molecular weight salivary mucin (MG1) as tracheobronchial mucin MUC5B. *Glycobiology,* **7,** 413–419.

Packer,N.H., Wilkins,M.R., Golaz,O., Lawson,M.A., Gooley,A.A., Hochstrasser,D.F., Redmond,J.W. and Williams,K.L. (1996) Characterization of human plasma glycoproteins separated by two-dimensional gel electrophoresis. *Bio/Technology,* **14,** 66–70.

Ramasubbu,N., Reddy,M.S., Bergey,E.J., Haraszthy,G.G., Soni,S.D. and Levine,M.J. (1991) Large-scale purification and characterisation of the major phosphoproteins and mucins of human submandibular–sublingual saliva. *Biochem. J*., **280,** 341–352.

Rathman,W.M., van den Keijbus,P.A.M., van Zeyl,M.J., Dopp,E.A., Veerman,E.C.I. and Nieuw Amerongen,A.V. (1990) Characterization of monoclonal antibodies to human salivary (glyco)proteins. Cellular localization of mucin, cystatin-like 14 kDa protein and 20kDa glycoprotein in the human SM gland. *J. Biol. Buccale*, **18,** 19–27.

Sheehan,J.K., Boot-Handford,R.P., Chantler,E., Carlstedt,I. and Thornton,D.J. (1991) Evidence for shared epitopes within the 'naked' protein domains of human mucus glycoproteins. A study performed by using polyclonal antibodies and electron microscopy. *Biochem. J*., **274,** 293–296.

Sheehan,J.K., Thornton,D.J., Howard,M., Carlstedt,I., Corfield,A.P. and Paraskeva,C. (1996) Biosynthesis of the MUC2 mucin: evidence for a slow assembly of fully glycosylated units. *Biochem. J*., **315,** 1055–1060.

Thornton,D.J., Holmes,D.F., Sheehan,J.K. and Carlstedt,I. (1989) Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. *Anal. Biochem*., **182,** 160–164.

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Thornton,D.J., Davies,J.R., Kraayenbrink,M., Richardson,P.S., Sheehan,J.K. and Carlstedt,I. (1990) Mucus glycoproteins from normal human tracheobronchial secretions. *Biochem. J.*, **265,** 179–186.

Thornton,D.J., Carlstedt,I. and Sheehan,J.K. (1994) Identification of glycoproteins on nitrocellulose membranes and gels. *Methods Mol. Biol.*, **32,** 119–128.

Thornton,D.J., Howard,M., Devine,P.L. and Sheehan,J.K. (1995) Methods for separation and identification of mucin subunits. *Anal. Biochem.*, **227,** 162–167.

Thornton,D.J., Carlstedt,I., Howard,M., Devine,P.L., Price,M.R. and Sheehan,J.K. (1996) Respiratory mucins: identification of core proteins and glycoforms. *Biochem. J.*, **316,** 967–975.

Thornton,D.J., Howard,M., Khan,N. and Sheehan,J.K. (1997) Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. *J. Biol. Chem.*, **272,** 9561–9566.

Troxler,R.F., Offner,G.D., Zhang,F., Iontcheva,I. and Oppenheim,F.G. (1995) Molecular cloning of a novel high molecular weight mucin (MG1) from human sublingual gland. *Biochem. Biophys. Res. Commun.*, **217,** 1112–1119.

Troxler,R.F., Iontcheva,I., Oppenheim,F.G., Nunes,D.P. and Offner,G.D. (1997) Molecular characterisation of a high molecular weight mucin from sublingual gland. *Glycobiology*, **7,** 965–973.

Veerman,E.C.I., Valentin-Benz,M., van den Keybus,P.A.M., Rathman,W.M., Sheehan,J.K. and Nieuw Amerongen,A.V. (1991) Immunochemical analysis of high molecular-weight salivary mucins (MG1) using monoclonal antibodies. *Arch. Oral Biol.*, **36**, 923–932.

Veerman,E.C.I., Ligtenberg,A.J.M., Schenkels,L.C.P.M., Walgreen-Weterings,E. and Nieuw Amerongen,A.V. (1995) Binding of human high-molecular weight salivary mucins (MG1) to *Hemophilus parainfluenzae*. *J. Dent. Res.*, **74,** 351–357.

Veerman,E.C.I., van den Keybus,P.A.M., Valentijn-Benz,M. and Nieuw Amerongen,A.V. (1992) Isolation of different high-M_r mucin species from human whole saliva. *Biochem. J.*, **283,** 807–811.

Veerman,E.C.I., van den Keybus,P.A.M., Vissink,A. and Nieuw Amerongen,A.V. (1996) Human glandular salivas: their separate collection and analysis. *Eur. J. Oral Sci.*, **104,** 346–352.

Veerman,E.C.I., Bolscher,J.G.M., Appelmelk,B.J., Bloemena,E., van den Berg,T.K. and Nieuw Amerongen,A.V. (1997) A monoclonal antibody directed against high M_r salivary mucins recognizes the SO_3Galβ1–3GlcNAc moiety of sulfo-Lewis^a. A histochemical survey of human and rat tissue. *Glycobiology*, **7,** 37–44.

Wu,A.M., Csako,G. and Herp,A. (1994) Structure, biosynthesis, and function of salivary mucins. *Mol. Cell. Biochem.*, **137,** 39–55.

Yan,J.X., Packer,N.H., Tonella,L., Ou,K., Wilkins,M.R., Sanchez,J.-C., Gooley,A.A., Hochstrasser,D.F. and Williams,K.L. (1996) High Sample throughput phosphoamino acid analysis of proteins separated by one-and two-dimensional gel electrophoresis. *J. Chromatogr. A.*, **764,** 201–210.

Zimm,B.H. The scattering of light and the radial distribution function of high polymer solutions. (1948) *J. Chem. Phys.*, **16,** 1099–1116.

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Lactoferrin, amylase and mucin MUC5B and their relation to the oral microflora in hyposalivation of different origins

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There are several reasons for hyposalivation, each affecting the salivary composition in different ways. The aim of this study was to analyze and compare lactoferrin, amylase and mucin MUC5B in stimulated whole saliva collected from subjects with hyposalivation of different origins and to relate the results to the presence of some microbial species associated with oral disorders. Albumin was determined as a marker of serum leakage. The characteristic feature for subjects with radiation-induced hyposalivation was a large increase in lactoferrin, probably due to leakage through inflamed mucosal tissues, while it was a high albumin content for the group with primary Sjögren's syndrome, probably due to disruption of the fragile mucosa. The saliva composition in subjects with hyposalivation of unknown origin or due to medicines was close to that in the healthy controls. All three hyposalivation groups tended to display a decrease in the concentrations of MUC5B and amylase. None of the microbial species analyzed (streptococci, mutans streptococci, *Lactobacillus* spp., *Fusobacterium nucleatum*, *Prevotella intermedia*/*Prevotella nigrescens*, *Candida albicans*, *Staphylococcus aureus* and enterics) correlated with concentration of MUC5B in saliva. The RT group, having the highest concentration of lactoferrin, had the lowest median number of *F. nucleatum* and was the only group in which median number of *P. intermedia*/*P. nigrescens* was zero.

Key words: hyposalivation; saliva; lactoferrin; amylase; mucin; MUC5B

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Saliva, consisting of 99% water and 1% organic and inorganic components, has several important functions in the oral cavity. One of them is to modulate the oral microflora by favoring the attachment and proliferation of certain microorganisms and promoting the clearance of others (36). Saliva also protects the oral tissues from desiccation and exogenous insult from acids and degradative enzymes (22, 25, 40).

Subjects with radiation-induced hyposalivation frequently have almost no salivary secretion due to the destruction of the salivary glands. In subjects with Sjögren's syndrome, there is an inflammation in the salivary glands, resulting in decreased salivary flow. In addition, several medicines, alone or in combination, affect the nerve signals to the salivary glands, also reducing the flow of saliva. Different reasons for hyposalivation will affect the salivary composition in different ways (14, 41, 44).

The parotid glands consist mostly of serous cells, whereas the minor glands consist of mucous cells. The submandibular and sublingual glands contain of both serous and mucous cells. The serous and the mucous cells are dissimilar in their secretion of water and saliva components (47). Lactoferrin is mainly secreted by the interductal cells of serous acini, amylase by serous cells (11, 34) and mucins by mucous cells (11).

Lactoferrin binds iron, which is an important nutrient factor for many microbial species (19). It also displays bacteriostatic and/or bactericidal activity towards several microbial species (5, 6, 31, 38). Lactoferrin has been found in increased concentrations in subjects with radiation-induced hyposalivation (42) and in subjects with Sjögren's syn-

drome (7, 20, 21, 39). A high lactoferrin concentration in saliva can result from damage to the salivary glands (42), gingival inflammation or leakage of serum through inflamed mucosal membranes (9, 17). Since saliva collected directly from the glands usually contains only trace amounts of albumin (32), it was therefore used as an indicator of serum leakage.

Amylase one of the most prevalent enzymes in the saliva, is generally considered to be a reliable marker of serous cell function. In addition to enzyme activity amylase coats the oral tissues, binds to streptococci, has antimicrobial activity and is involved in the selective clearance and adherence of microorganisms (11). The amylase concentration in subjects with radiation-induced hyposalivation has been found to be reduced (10, 23). In contrast, in patients with primary Sjögren's syndrome, the amylase concentration is comparable to that in healthy controls (24, 44).

Mucins are glycoproteins with low solubility and high viscosity, elasticity and adhesiveness (40) and have been shown to interact with several microorganisms (8, 18, 28, 46). When free in saliva, mucins agglutinate microorganisms. Their presence on the oral tissues, protect mucosal membranes and favour the attachment and proliferation of certain microorganisms. The carbohydrates, present in mucins, may also be a source of nutrients for some oral microorganisms (40). There are two types of mucin in saliva, MUC5B (formerly MG1), with a molecular weight of >1000 kDa, and MUC7 (formerly MG2), with a molecular weight of 130–150 kDa. The concentration of MUC5B in unstimulated saliva in subjects with primary Sjögren's syndrome has been found to be higher than that of healthy controls (35).

In our previous studies, we found differences in the oral microflora in subjects with hyposalivation of different origins (1, 2). These differences may be associated with altered salivary protein composition in these patients. The aim of this study was to compare lactoferrin, amylase and MUC5B concentrations in stimulated whole saliva collected from subjects with radiation-induced hyposalivation, subjects with pSS and subjects with hyposalivation of unknown origin or due to medicines. In addition, we analyzed the data in relation to the presence of selected microbial species that have been associated with oral disorders.

Material and methods

This study was approved by the ethics committee at Göteborg University. Three groups of subjects with hyposalivation and a healthy control group participated in the study. To be included, the subjects had to be between 35 and 70 years of age and had to have at least ten teeth, no removable prostheses or implants and an unstimulated secretion rate of ≤0.1 ml/min. The study groups comprised ten subjects who had completed radiation therapy in the head and neck region 6 months earlier (radiation therapy group), 20 subjects with primary Sjögren's syndrome, diagnosed according to the Copenhagen criteria (26) 7±5 years previously, 19 subjects with subjective feelings of hyposalivation for 6±8 years but who displayed no signs of salivary gland inflammation on biopsy (unknown group) and 26 healthy subjects with an unstimulated secretion rate of >0.1 ml/min.

The subjects were asked to refrain from eating, drinking, smoking and toothbrushing <2 hours before the appointment. Firstly, the unstimulated secretion rate was measured, after which a rinsing sample for microbial analysis was taken. Subsequently, the stimulated secretion rate was measured and finally the clinical examination took place.

Clinical examination

The clinical examination has been described previously (1, 2). In brief, the clinical parameters that were registered were the status of the mucosal membranes and the number of teeth, fillings, crowns and bridges, presence of plaque along the gingival margin after Diaplac® staining, bleeding following probing and periodontal pocket probing depths of >4 mm at 4 sites per tooth.

Secretion rate, pH and buffer capacity

The determinations of the salivary secretion rates and the measurement of pH and buffer capacity have been described previously (2). In brief, the unstimulated secretion rate was measured for 15 min and the paraffin-wax-stimulated secretion rate for 5–10 min. The stimulated saliva was collected in an ice-chilled test tube. The pH of stimulated saliva was measured using a pH meter (Metrohm 632, Herisau, Switzerland). For the determination of the buffer capacity, one part of the stimulated saliva was mixed with three parts of 0.005 M HCl and the final pH was measured. With the assay used here, "normal" pH ranges between pH 6.3 and 7.2 and "normal" buffer capacity between pH 5.0 and 7.0 (13).

Duplicate samples and determinations of the secretion rates, pH and buffer capacity were performed, 10±7 days apart, and a mean value was calculated. As ten the 50 subjects with hyposalivation had an unstimulated salivary flow rate below detectable levels, the stimulated saliva was used for the protein analysis in all subjects. For the protein analysis, the stimulated saliva was centrifuged at 11,600×*g* for 15 min in an Eppendorf centrifuge (Eppendorf M 5415) at room temperature. The supernatant was divided into aliquots and stored at −70°C pending analysis.

Microbial sampling and analysis

Sampling for microbial analysis was performed on the first appointment. For microbial sampling, the subject rinsed his or her mouth with 10 ml of sterile distilled water for 20 s before spitting into a sterile vessel. One ml of the fluid was transferred to a bottle with 5.7 ml of VMG IIS (29) anaerobically prepared. Dilution of the samples from 10^{-1} to 10^{-5} was performed in VMG I (29), and 0.1 ml was inoculated onto each agar plate. The cultivation and incubation procedures and the identification of microorganisms have been described previously (2). In brief, the total microbial count and the numbers of *Fusobacterium nucleatum* and *Prevotella intermedia*/*Prevotella nigrescens* were determined from their growth on Brucella agar, streptococci on mitis-salivarius agar, mutans streptococci on mitis-salivarius-bacitracin agar, *Lactobacillus* spp. on Rogosa-SL agar, *Staphylococcus aureus* on *Staphylococcus* agar, *Candida albicans* on Saboraud-T agar and enterics on Drigalski agar.

Protein analysis

The total protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a stan-

dard. The saliva supernatant was diluted 2- and 4- fold with distilled water and was analyzed in duplicate. Twenty µl of saliva or BSA in concentrations ranging from 0 to 1500 mg/ml and 180 µl of BCA reagent were added to wells in a microtitre plate (Greiner, Frickenhausen, Germany). The plate was incubated at 37°C for 30 min and was then read at 592 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA).

The concentration of lactoferrin was determined using the capture ELISA technique (43). Polysorb microtiter plates (Nunc, Roskilde, Denmark) were incubated at room temperature overnight with rabbit-anti-human lactoferrin (Dakopatts, Glostrup, Denmark) diluted in phosphate-buffered saline (PBS) to a concentration of 1 µl/ml. The plate was washed three times with PBS with 0.1% Tween 20 (PBS-T). The washing procedure was repeated after each step. A standard curve was prepared using human lactoferrin (Department of Oral Biochemistry, Amsterdam, the Netherlands) diluted in PBS with a start concentration of 250 ng/ml, which was then two-fold serially diluted. Duplicate wells were incubated with 200 µl of saliva supernatant diluted 20 times in PBS-T with 0.1% gelatin, which were then two-fold serially diluted in the plate. The plate was incubated for 2 h at room temperature. A 2000-fold dilution of goat-anti-human-lactoferrin conjugated with horseradish peroxidase (Dakopatts, Denmark) in PBS-T with 0.1% gelatin was then added, 100 µl/well, and the plate was incubated for 1 h at room temperature. After washing, 100 µl of TMB (3,3, 5,5,-tetramethylbenzidine) (ICN, Costa Mesa, CA) diluted in sodium acetate (pH 5.5) with a final concentration of 0.01% and with 0.003% H_2O_2 was added to the plate, 100 µl/well, and the plate was incubated at room temperature. The color reaction was stopped after 15 min by adding 2 M H_2SO_4, 50 µl/well, and the plate was read at 450 nm in the ELISA reader.

A competitive ELISA technique was used for the determination of the albumin concentration (45). Wells in a microtiter plate (Greiner) were coated with 0.1 mg/ml of human albumin diluted in 0.1 M Na_2CO_3 and the plate was incubated over night at 4°C. The washing procedure was the same as in the lactoferrin ELISA. After washing, 200 µl/well of a blocking solution, PBS-T with 1% gelatin, was added and the plate was incubated for 30 min at room temperature. The blocking solution was poured out and 160 µl of the saliva supernatant, diluted 10-fold in PBS-T with 1% gelatin, or 160 µl of human serum albumin with a start concentration of 50 µg/ml were added in duplicate wells in the microtiterplate. The solutions were 2-fold serially diluted in the plate. A mixture of a 1:1500 diluted rabbit anti-human albumin and a 1:3000 diluted goat-anti-rabbit immunoglobulin conjugated with horseradish peroxidase, in PBS-T with 1% gelatin, were added, 120 µl/well. The plate was incubated for 3 h at room temperature. After washing, 200 µl/well of the same color solution as in the lactoferrin ELISA was added. The color reaction was stopped after 5 min by adding 2 M H_2SO_4, 100 µl/well, and the plate was read at 450 nm in the ELISA reader.

The amylase concentration was determined using an amylase activity kit (Sigma Chemical Co., St Louis, MO). The saliva supernatant was diluted 80-fold with 0.85% NaCl and 10 µl was added to triplicate wells in a microtiterplate (Greiner). The multi-enzyme lintrol solution (Sigma Chemical) was used as a standard and was added, 10 µl/well, in concentrations ranging from 0 to 60%. Ninety µl of the amylase reagent was added to each well and the plate was incubated at 37°C. The plate was read after 2 and 4 min at 405 nm in the ELISA reader.

The concentration of MUC5B was determined using an ELISA method (47). The concentration of MUC5B as units/ml was determined relative to standard saliva (from one healthy person). Duplicate wells in a high-affinity microtiter plate (Greiner) were coated with 200 µl of saliva supernatant diluted 200-fold in 0.1 M Na_2CO_3, which were then 2-fold serially diluted in the plate. After incubation at room temperature overnight, the plate was washed 3 times as in the lactoferrin ELISA. The washing procedure was repeated after each step. A blocking solution of PBS-T with 2% BSA was then added in volumes of 150 µl to each well, and the plate was incubated at 36°C for 1 h. An antibody to human MUC5B (Department of Oral Biochemistry, Amsterdam, the Netherlands) was diluted 40-fold in PBS-T with 1% gelatin and was added in volumes of 100 µl/well to the plate. After incubation for 1 h at 36°C, a 1:2000 diluted rabbit-anti-mouse immunoglobulin (Dakopatts, Denmark) was added, 100 µl/well, and the plate was incubated again in the same conditions. One hundred µl of the same colour solution as in the lactoferrin ELISA was added and the plate was incubated at room temperature for 15 min, after which 50 µl/well of 2 M H_2SO_4 was added and the plate was read at 450 nm in the ELISA reader.

Statistical methods

The statistical methods were ANOVA and the Newman-Keul test, the latter at a significance level of 5%. Pearson's correlation was used to measure correlations between pairs of variables.

Results

Clinical recordings

In the radiation therapy group, 90% were men, while the primary Sjögren's syndrome group and the unknown group consisted of 95% and 89% women respectively. In the control group, the gender distribution was 23% men and 77% women. The mean age for all 75 subjects included in the study was 55±8 years with a median of 55 years. The mean unstimulated secretion rate in the hyposalivation groups was ≤0.04 ml/min and in the controls 0.33 ml/min. The mean stimulated secretion in the radiation therapy group was 0.39±0.41 ml/min, in the primary Sjögren's syndrome group 0.47±0.38 ml/min, in the unknown group 0.99±0.50 ml/min and in the controls 2.3±0.95ml/min. Compared with the controls, the pH and buffer capacity were lower in all three hyposalivation groups and lowest in the radiation therapy group. The three hyposalivation groups and the controls displayed similar proportions of surfaces with plaque along the gingival margin and surfaces showing bleeding on probing. Periodontal pocket probing depths of >4 mm were detected in 40% of the subjects in the radiation therapy group, 30% in the primary Sjögren's syndrome group, 37% in the unknown group and 38% of the controls. For subjects with periodontal probing depths of >4 mm, the number of pockets varied between 1 and 8. The mean probing depths of pockets of >4 mm were 5.8±0.3 mm in the radiation therapy group, 5.3±0.4 mm in the primary Sjögren's syndrome group, 5.0±1.2 mm in the unknown group and 5.6±0.6 mm in the controls. Clinically visible open caries lesion were rarely detected.

Table 1. Frequency (%) of microorganisms in subjects with radiation-induced hyposalivation (radiation therapy), subjects with primary Sjögren's syndrome, subjects with hyposalivation of unknown origin or due to medicines (unknown) and in healthy controls

Group	Number of subjects	Mutans streptococci	*Lactobacillus* spp.	*F. nucleatum*	*P. intermedia/ P. nigrescens*	*C. albicans*	*S. aureus*	Enterics
Radiation therapy	10	70	100	60	30	70	30	20
Primary Sjögren's syndrome	20	100	85	80	75	60	20	10
Unknown	19	100	79	74	80	53	21	5
Controls	26	81	35	81	50	19	27	4

Microbial results

The frequencies of microorganisms are presented in Table 1 and the mean±SD and median log 10 numbers in Fig. 1. No significant difference in total microbial count or in number of streptococci could be detected between the groups. Mutans streptococci were frequently detected in all four groups. There was a significant difference in the number of mutans streptococci between the groups (P=0.004), but the Newman-Keul test provided no further information about this difference. All three hyposalivation groups had more *Lactobacillus* spp. than the controls (P=0.0001). The number of *C. albicans* was significantly higher in the radiation therapy and primary Sjögren's syndrome group than in the unknown group and in the controls (P=0.0001). *F. nucleatum* was frequently found in all four groups. There were no significant differences in the numbers of *F. nucleatum* and *P. intermedia/P. nigrescens.* The lowest frequency of *P. intermedia/P. nigrescens* was found in the radiation therapy group, where it was detected in three of the 10 subjects, and the radiation therapy group was the only group in which the median number was zero. *S. aureus* and enterics were only detected in a few subjects in all four groups, and their median numbers were zero.

Protein concentrations

Table 2 presents the concentrations and outputs per min of total proteins, albumin, lactoferrin, amylase and MUC5B in the controls. Fig. 2 presents the concentrations of the salivary components per ml of stimulated whole saliva in the hyposalivation groups as proportions of the concentrations in the controls. There was a significant difference in the total protein concentration between the groups (P=0.0001). The total protein concentration in the radiation therapy group and the primary Sjögren's syndrome group was about twice as high as in the controls, while the concentration in the unknown group was comparable to that in the controls.

The albumin concentration also differ significantly between the groups (P=0.0001). Compared with the controls and the unknown group, the radiation therapy group and the primary Sjögren's syndrome group displayed an albumin concentration that was about five times higher.

The most pronounced difference between the groups was that in the lactoferrin concentration (P=0.0001). The lactoferrin concentration was about 24 times higher in the radiation therapy group. Only one subject in the radiation therapy group displayed a lactoferrin concentration comparable to that of the controls. This subject had a stimulated secretion rate within normal limits, 1.2 ml/min. In the primary Sjögren's syndrome group and unknown group, the lactoferrin concentration was about twice as high as in the controls.

The amylase concentration differed significantly between the groups (P=0.013). The radiation therapy group displayed the lowest amylase concentration, about 30% of the value in the controls (P<0.05). For the primary Sjögren's syndrome group, the amylase concentration was about 70% of that in the controls, while it was about 92% in the unknown group (not significant).

No significant difference in MUC5B concentration could be detected between the groups.

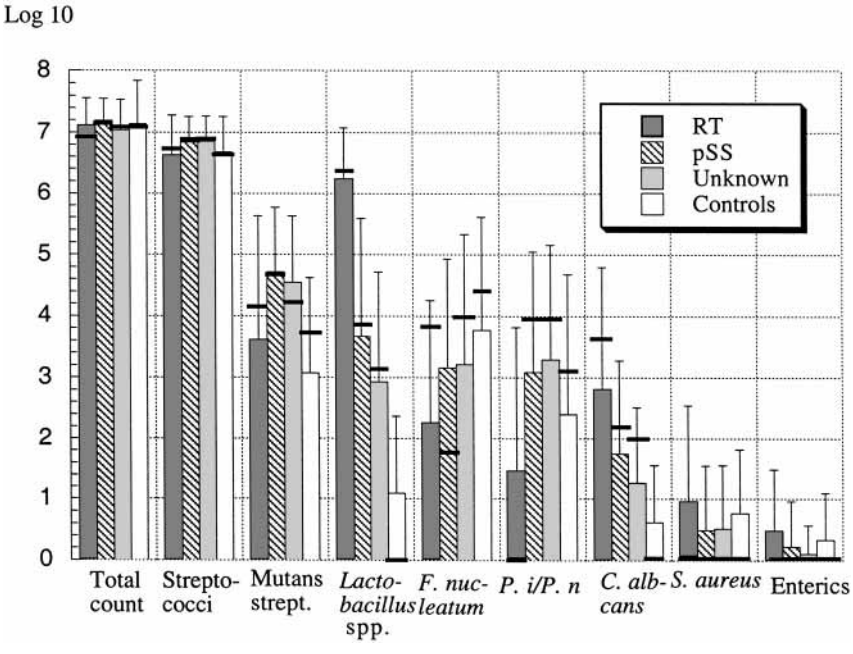

Fig. 1. Mean±SD and median log 10 numbers of microorganisms in the 10 subjects with radiation-induced hyposalivation (RT), the 20 subjects with primary Sjögren's syndrome (pSS), the 19 subjects with hyposalivation of unknown origin or due to medicines (unknown) and the 26 controls.

Proteins in relation to stimulated salivary secretion rate/min

Fig. 3 presents the proteins in relation to the stimulated salivary secretion rate per min (output per min) in the hypo-

salivation groups as proportions of the values in the controls. There were significant differences between the groups in the output per min of total protein, lactoferrin, amylase and MUC5B (P=0.0001 for all). Compared with the controls, all three hyposalivation groups displayed a lower output per min of total protein, amylase and MUC5B. In the radiation therapy group, the median output per min of lactoferrin was 3.5 times higher than in the controls. For albumin, the primary Sjögren's syndrome group showed a slightly higher output per min than the radiation therapy, unknown and control groups.

Correlations between salivary proteins and clinical parameters

Based on calculations for all 75 subjects, there was a weak positive correlation between the albumin concentration and the proportion of surfaces with bleeding on probing (P=0.02). The stimulated salivary secretion rate and the saliva pH were negatively correlated with the total protein and lactoferrin concentrations (P=0.0001 for all). No correlation between the buffer capacity and the proteins was detected.

Correlations between salivary proteins and microorganisms

The lactoferrin concentrations were positively correlated to the numbers of *Lactobacillus* spp. (P=0.0001) r=0.47 and *C. albicans* (P=0.0003) r=0.41. There was a weak negative correlation between amylase activity and the number of *Lactobacillus* spp. (P=0.035) r=−0.24. A weak negative correlation was also revealed between the lactoferrin concentration and the number of *F. nucleatum* (P=0.045) r=−0.24. The radiation therapy group, which had a very high lactoferrin concentration, was the only group in which the median number of *P. intermedia*/*P. nigrescens* was zero. None of the microbial species analyzed correlated with the concentration of MUC5B in saliva.

Discussion

In the healthy controls, the unstimulated and the stimulated salivary secretion rate, pH and buffer capacity as well as the total protein concentration were in accordance with what has previously been reported (16, 27, 33, 44).

The subjects in the radiation therapy

Table 2. Concentrations and outputs of proteins in the controls

	Concentration/ml Mean±SD (median)	Output per min
Total protein (mg)	0.9±0.2 (0.9)	2.0±0.9 (1.8)
Albumin (mg)	0.2±0.1 (0.2)	0.4±0.2 (0.4)
Lactoferrin (μg)	3.7±2.5 (2.7)	8.6±7.3 (5.7)
Namylase (U)	3257±1682 (3160)	7455±5537 (6220)
MUC5B (U)	2.4±1.7 (2.1)	5.4±4.9 (4.0)

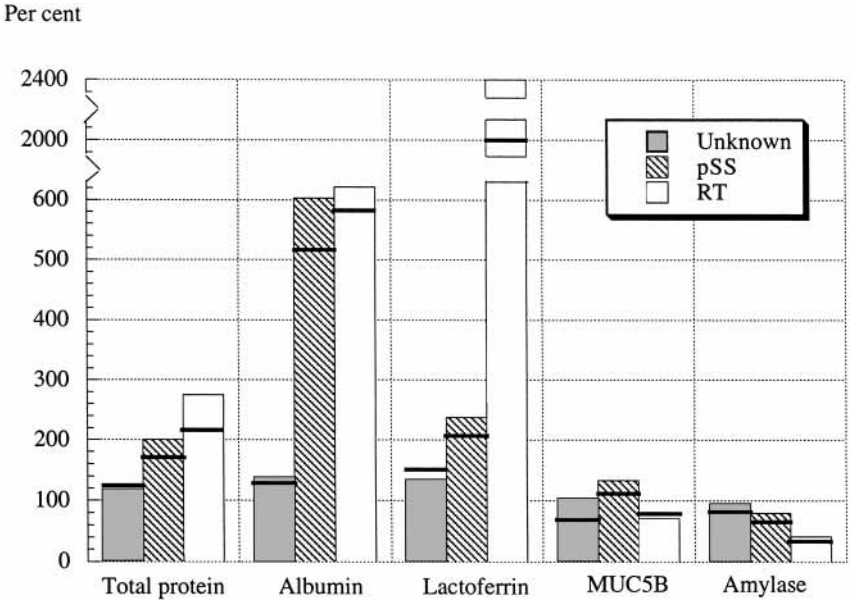

Fig. 2. Mean (bars) and median (lines) concentrations of total protein, albumin, lactoferrin, amylase and MUC5B in the three hyposalivation groups as proportions of the values in the controls. The concentrations in the controls are set at 100%.

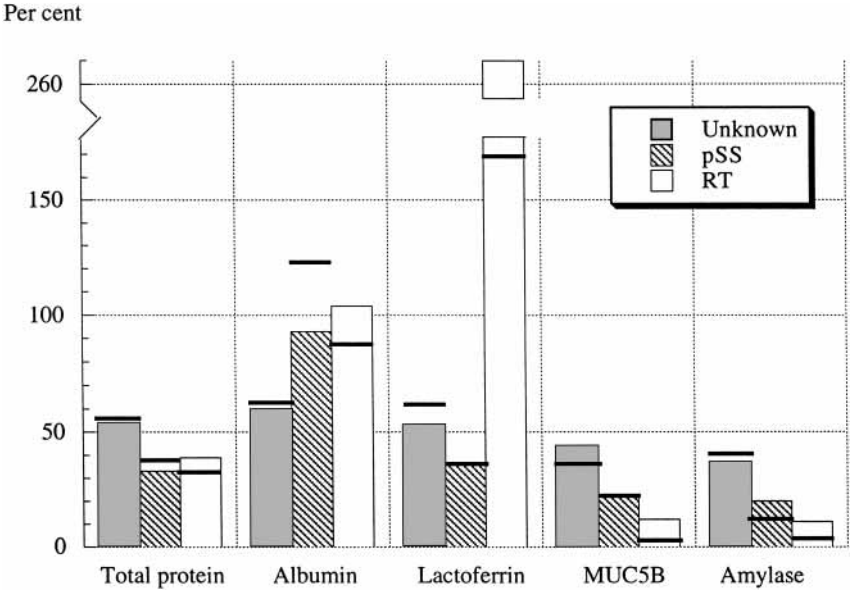

Fig. 3. Mean (bars) and median (lines) output per min of total protein, albumin, lactoferrin, amylase and MUC5B in the three hyposalivation groups as proportions of the values in the controls. The concentrations in the controls are set at 100%.

group had completed their radiation therapy 6 months prior to inclusion in the study. At this time, the clinically visible acute effect of the radiation therapy on the oral tissues was subsided. The subjects of the primary Sjögren's syndrome group and of the unknown group had had a decreased salivary secretion rate for several years. To avoid the effect of aging and of dental status on the salivary composition and the oral microflora, the subjects included in the groups were about the same age and had a similar number of teeth; the median number of the groups ranged from 26 to 28 teeth. The three hyposalivation groups and the controls displayed comparable proportions of surfaces tooth surfaces with plaque along the gingival margin and dental sites showing bleeding on probing, as well as number of periodontal pocket probing depths of >4 mm.

We chose to analyse protein concentrations in stimulated whole saliva in all subjects because several of the subjects in the hyposalivation groups displayed very low or no detectable unstimulated secretion. The results of the protein analysis in the hyposalivation groups are presented as proportions of the values in the controls.

While MUC7 readily interacts with microorganisms (8, 18, 28), the major function of MUC5B is assumed to be the coating and protection of the oral tissues (22, 25, 40). Only a few microorganisms have been identified as binding specifically to MUC5B (30). This could be the reason why no correlations were found between MUC5B and the microorganisms that were analyzed. Both the radiation therapy and the Sjögren's syndrome groups displayed a marked decrease in output per min of MUC5B. Their almost absent unstimulated salivary secretion rate and reduced concentration in stimulated salivary secretion may make the coating of MUC5B to the mucosal surfaces difficult. This could lead to a reduction in the protection of the oral tissues and increased susceptibility to mucosal damage.

We found a reduced amylase concentration in the radiation therapy group, a finding also made by others (10, 23). In primary Sjögren's syndrome subjects, Mandel & Baurmash (24) and Van der Reijden (44) reported that amylase concentration was comparable to that in healthy controls. This finding is supported by the fact that the mean amylase concentration in our primary Sjögren's syndrome group was similar to that in the controls. However, the primary Sjögren's syndrome group displayed a 30% lower median amylase concentration. The median amylase concentration and output per min in the hyposalivation groups suggests a loss of serous cell function that was most severe in the radiation therapy group.

In accordance with the results obtained by Valdez et al. (42), the radiation therapy group in our study displayed a 24 times higher concentration of lactoferrin than the controls. Since the radiation therapy group and the controls displayed a similar periodontal status, this factor is unlikely to explain the difference in lactoferrin concentration. Leakage of serum through the mucosal tissues infiltrated by acute-phase inflammatory cells due to radiation and low salivary secretion could be a more plausible reason. The high concentration of albumin found in the radiation therapy group (Fig. 2) supports this hypothesis. A high concentration of albumin in radiation therapy subjects has also been found by others (9, 23). Furthermore, the preliminary results of our ongoing longitudinal study indicate that the lactoferrin concentration in radiation therapy subjects decreases gradually over time.

The primary Sjögren's syndrome group displayed an albumin concentration close to that in the radiation therapy group (Fig. 2). Their output per min of albumin was even higher than in the radiation therapy group (Fig. 3). The primary Sjögren's syndrome group displayed a higher lactoferrin concentration than the controls. However, while the lactoferrin concentration in the radiation therapy group was 24 times higher than in the controls, it was twice as high in the primary Sjögren's syndrome group (Fig. 2). Konttinen et al. (21) found a similar increase in lactoferrin concentration in unstimulated whole saliva. An increase in the lactoferrin concentration in saliva from the parotid gland was found by Jezequel et al. (20) and by Konttinen et al. (21). However, there are conflicting results when it comes to the correlation between the lactoferrin concentration in stimulated parotid saliva and the degree of inflammation in the salivary glands (20, 21). We think it is reasonable to believe that part of the lactoferrin in the primary Sjögren's syndrome group comes from the inflamed salivary glands and the rest from leakage through disrupted fragile mucosal membranes due to a longstanding low salivary secretion rate.

The ELISA method used to determine lactoferrin in this study does not discriminate between iron-free lactoferrin, iron-saturated lactoferrin and fragments of lactoferrin. In contrast to iron-saturated lactoferrin, iron-free lactoferrin has bacteriostatic effects on streptococci, staphylococci, enterics and gram-negative bacteria for example (4, 12) and fungicidal effects on *C. albicans* and *Candida krusei* (4, 31). Iron-free lactoferrin also has a bactericidal effect on *Streptococcus mutans* (37), inhibits the adhesion of *S. mutans* to hydroxyapatite (49) and inhibits its glucose uptake and lactic acid synthesis (5). The degradation of lactoferrin reduces its antimicrobial function. Since the configuration of the lactoferrin molecule is of importance for its antimicrobial concentration, the lactoferrin in the different study groups will be analyzed further. The results of *in vitro* experiments suggest that the function of lactoferrin is also affected by pH, but in opposite ways on different microorganisms (6, 38).

The negative correlation between the lactoferrin concentration and the number of *F. nucleatum* might indicate an antimicrobial effect by lactoferrin (6). Previously, a negative correlation has been found between lactoferrin and the periodontal pathogen *Actinobacillus actinomycetemcomitans* in redundant diseased patients (15). The very low frequency and number of *P. intermedia*/*P. nigrescens* in the saliva in the radiation therapy group could be explained by both the iron dependence for growth for *P. intermedia*/*P. nigrescens* and an antimicrobial effect by lactoferrin. The pH and buffer capacity were lower in the hyposalivation group than in the controls and significantly lower in the radiation therapy and primary Sjögren's syndrome groups. The positive correlation between the lactoferrin and *C. albicans* could be due to a reduced antifungal function of lactoferrin at an acid pH (38). However, the fact that the acid milieu itself favours growth of both *Lactobacillus* spp. and *C. albicans* in the radiation therapy and Sjögren's syndrome groups is probably a stronger contributory factor (3, unpublished results).

It can be concluded that there are dif-

ferences in whole saliva protein composition in subjects with hyposalivation of different origins. The characteristic feature in the radiation therapy group was a large increase in lactoferrin, probably due to leakage through mucosal tissues, while in the primary Sjögren's syndrome group it was a high albumin content, probably due to the disruption of the fragile mucosa. The saliva composition in the unknown group was close to that in the controls. All three hyposalivation groups tended to have a decrease in the concentration of MUC5B. None of the microbial species analyzed here correlated with the concentration of MUC5B in saliva. The results suggest, however, that lactoferrin contributes to the differences in the oral microflora detected between the hyposalivation groups.

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References

1. Almståhl A, Kroneld U, Tarkowski A, Wikström M. Oral microbial flora in Sjögren's syndrome. J Rheumatol 1999: **26**: 110–114.
2. Almståhl A, Wikström M. Oral microflora in subjects with hyposalivation. J Dent Res 1999: **78**: 1410–1416.
3. Almståhl A, Wikström M, Kroneld U. Microflora in oral ecosystems in primary Sjögren's syndrome. J Rheumatol 2001: **28**: in press.
4. Arnold RR, Brewer M, Gauthier JJ. Bacterial activity of human lactoferrin: sensitivity of a variety of microorganisms. Infect Immun 1980: **28**: 893–898.
5. Arnold RR, Russell JE, Champion WJ, Brewer M, Gauthier JJ. Bactericidal activity of human lactoferrin: differentiation for the stasis of iron deprivation. Infect Immun 1982: **35**: 792–799.
6. Arnold RR, Russell JE, Champion WJ, Gauthier JJ. Bactericidal activity of human lactoferrin: influence of physical conditions and metabolic state of the target microorganisms. Infect Immun 1981: **32**: 655–660.
7. Atkinson J. Major salivary gland function in primary Sjögren's syndrome and its relationship to clinical features. J Rheumatol 1990: **17**: 318–322.
8. Biesbrock AR, Reddy MS, Levine MJ. Interaction of a salivary mucin-secretory immunoglobulin A complex with mucosal pathogens. Infect Immun 1991: **59**: 3492–3497.
9. Brown LR, Dreizen S, Rider LJ, Johnston DA. The effect of radiation induced xerostomia on saliva and serum lysozyme and immunoglobulin levels. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1976: **1**: 83–92.
10. Christensen ME, Hansen HS, Poulsen SS, Bretlau P, Nexø E. Immunohistochemical and quantitative changes in salivary EGF, amylase and haptocorrin following radiotherapy for oral cancer. Acta Otolaryngol (Stockh) 1996: **116**: 137–143.
11. Cohen RE, Levine MJ. Salivary glycoproteins. In: Tenovuo JO, ed. Human saliva: clinical chemistry and microbiology. Vol. I. Boca Raton, FL: CRC Press, 1989: 101–130.
12. Ellisson RT, Giehl TJ, LaForce FM. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun 1988: **56**: 2774–2781.
13. Ericsson Y. Clinical investigations of the salivary buffering action. Acta Odontol Scand 1959: **17**: 131–165.
14. Funegård U, Franzén L, Ericson TH, Henriksson R. Parotid saliva composition during and after irradiation of head and neck cancer. Oral Oncol Eur J Cancer 1994: **30B**: 230–233.
15. Groenink J, Walgreen-Weterings E, Nazmi K, Veerman ECI, van Winkelhoff AJ, Nieuw Amerongen AV. Salivary lactoferrin and low-M_r mucin MG2 in *Actinobacillus actinomycetemcomitans*–associated periodontitis. J Clin Periodontol 1999: **26**: 269–275.
16. Heintze U. Secretion rate, buffer effect and number of lactobacilli and *Streptococcus mutans* of whole saliva of cigarette smokers and non-smokers. Scand J Dent Res 1984: **92**: 294–301.
17. Henskens YMC, Van der Velden U, Veerman ECI, Nieuw Amerongen AV. Protein, albumin and cystatin concentration in saliva of healthy subjects and of patient with gingivitis or periodontitis. J Periodontal Res 1993: **28**: 43–48.
18. Hoffman MP, Haidaris C. Analysis of *Candida albicans* adhesion to salivary mucin. Infect Immun 1993: **61**: 1940–1949.
19. Holt SC. Basic bacteriology. In: Slots J, Taubman MA, eds. Contemporary oral microbiology and immunology. St. Louis: Mosby Year Book, 1992: 3–29.
20. Jezequel N, Depasse F, Jouquan A, Lelong A, Roncin S, Pare G et al. Salivary lactoferrin in primary Sjögren's syndrome. Clin Exp Rheumatol 1989: **7**: 123–125.
21. Konttinen YT, Kulomaa M, Malmström M, Kilpi A, Reitamo S. Lactoferrin in Sjögren's syndrome. Arthritis Rheumatol 1984: **4**: 462–467.
22. Levine MJ, Reddy MS, Tabak LA, Loomis RE, Bergey SJ, Jones PC et al. Structural aspects of salivary glycoproteins. J Dent Res 1987: **66**: 436–441.
23. Makkonen TA, Tenovuo T, Vilja P, Heimdahl A. Changes in the protein composition of whole saliva during radiotherapy in patients with oral or pharyngeal cancer. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1986: **62**: 270–275.
24. Mandel ID, Baurmash H. Sialochemistry in Sjögren's syndrome. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1976: **2**: 182–187.
25. Mandel ID. Functions of saliva. J Dent Res 1987: **66**: 623–627.
26. Manthorpe R, Oxholm P, Prause JU, Schiødt M. The Copenhagen criteria for Sjögren's syndrome. Scand J Rheumatol 1986: **61**: 19–21.
27. Meurman JH, Rantonen P. Salivary flow rate, buffering capacity, and yeast counts in 187 consecutive adult patients from Kuopio, Finland. Scand J Dent Res 1994: **102**: 229–234.
28. Murray PA, Prakobphol A, Lee T, Hoover CI, Fisher SJ. Adherence of oral streptococci to salivary glycoproteins. Infect Immun 1992: **60**: 31–38.
29. Möller ÅJR. Microbiological examination of root canals and periapical tissues of human teeth (dissertation). Odontol Tidskr 1966: **74**: 1–380.
30. Nieuw Amerongen AV, Bolscher JGM, Veerman ECI. Salivary mucins: protective functions in relation to their diversity. Glycobiology 1995: **5**: 733–740.
31. Nikawa H, Samaranayake LP, Tenovuo J, Pang KM, Hamada T. The fungicidal effect of human lactoferrin on *Candida albicans* and *Candida krusei*. Arch Oral Biol 1993: **38**: 1057–1063.
32. Oppenheim FG. Preliminary observations on the presence and origin of serum albumin in human saliva. Helv Odontol Acta 1970: **14**: 10–17.
33. Parvinen T, Larmas M. Age dependency of stimulated salivary flow rate, pH and lactobacillus and yeast. J Dent Res 1982: **9**: 1052–1055.
34. Reitamo S, Konttinen YT, Segerberg-Konttinen M. Distribution of lactoferrin in human salivary glands. Histochemistry 1980: **66**: 285–291.
35. Saari H, Halinen S, Ganlöv K, Sorsa T, Konttinen YT. Salivary mucous glycoprotein MG1 in Sjögren's syndrome. Clin Chim Acta 1997: **259**: 83–96.
36. Scannapieco FA. Saliva-bacterium interactions in oral microbial ecology. Crit Rev Oral Biol Med 1994: **5**: 203–248.
37. Soukka T, Lumikari M, Tenovuo J. Combined inhibitory effect of lactoferrin and lactoperoxidase system on the viability on *Streptococcus mutans*, serotype c. Scand J Dent Res 1991: **99**: 390–396.

38. Soukka T, Tenovuo J, Lenander-Lumikari M. Fungicidal effect of human lactoferrin against *Candida albicans*. FEMS Microbiol Lett 1992: **90**: 223–228.
39. Stuchell RN, Mandel ID, Baurmash H. Clinical utilization of sialochemistry in Sjögren's syndrome. J Oral Pathol 1984: **13**: 303–309.
40. Tabak LA, Levine MJ, Mandel I, Ellison S. Role of salivary mucins in the protection of the oral cavity. J Oral Pathol 1982: **11**: 1–17.
41. Thorn JJ, Prause JU, Oxholm P. Sialochemistry in Sjögren's syndrome: a review. J Oral Pathol Med 1989: **18**: 457–68.
42. Valdez IH, Atkinson JC, Ship JA, Fox PC. Major salivary gland function in patients with radiation-induced xerostomia: flow rates and sialochemistry. Int J Radiation Oncol Biol Phys 1993: **25**: 41–47.
43. Van Berkel PHC, van Veen HA, Geerts MEJ, de Boer HA, Nuijens JH. Heterogeneity in utilization of *N*-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants. Biochem J 1996: **319**: 117–122.
44. Van der Reijden WA, Van der Kwaak JS, Veerman ECI, Nieuw Amerongen AV. Analysis of the concentration and output of whole salivary constituents in patients with Sjögren's syndrome. Eur J Oral Sci 1996: **104**: 335–340.
45. Van Kamp GJ, van Bezu JSM, Mulder CA. A rapid and sensitive immunosorbent assay for urinary albumin. Ann Clin Biochem 1989: **26**: 427–429.
46. Veerman ECI, Ligtenberg AJM, Schenkels LCPM, Walgreen-Weterings E, Nieuw Amerongen AV. Binding of human high-molecular-weight salivary mucins (MG1) to *Hemophilus parainfluenzae*. J Dent Res 1995: **74**: 351–357.
47. Veerman ECI, Valentijn-Benz M, Van den Keybus PA, Rathman WM, Sheehan JK, Nieuw Amerongen AV. Immunochemical analysis of high molecular-weight human salivary mucins (MG1) using monoclonal antibodies. Arch Oral Biol 1991: **36**: 923–932.
48. Veerman ECI, van den Keybus PAM, Vissink A, Nieuw Amerongen AV. Human glandular salivas: their separate collection and analysis. Eur J Oral Sci 1996: **104**: 346–352.
49. Visca P, Berlutti F, Vittorioso P, Dalmastri C, Thaller MC, Valenti P. Growth and adsorbtion of *Streptococcus mutans* 6715–13 to hydroxyapatite in the presence of lactoferrin. Med Microbiol Immunol 1989: **179**: 323–333.