

Salivary lactoferrin in a selected group of subjects with exceptional extrinsic dental staining

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Some people exhibit an exceptional tendency to develop brownish extrinsic staining on their teeth and previous studies indicate that iron may be involved in certain types. The object of this investigation was to find out whether the level of salivary lactoferrin, which is an iron-binding glycoprotein, is elevated in persons exhibiting extreme staining tendency. Subjects who developed dark brownish discoloration on the facial surfaces of their anterior teeth during a 3-week period following professional cleaning of the teeth were selected for study. Salivary lactoferrin was measured by the enzyme-linked immunosorbent assay (ELISA). The results showed that this group of persons exhibited a markedly higher concentration of salivary lactoferrin compared with non-stainers. It was also demonstrated in an *in vitro* study that combinations of lactoferrin, iron, and tannic acid produced stain on slabs of enamel and dentin.

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Certain types of brownish discolored dental integuments contain high amounts of iron (1, 2). The chemical form in which the metal is bound to these integuments still remains unclear although some findings indicate an involvement of sulphides (1, 3, 4). It is not known how the iron enters dental pellicles. None of the major constituents of the pellicle is known to have a strong affinity for iron. One hypothesis is that an iron-chelating protein is involved in the iron deposition.

Lactoferrin is a glycoprotein present in most bodily secretions, including saliva (5). It has also been found in dental pellicles (6). The protein has a molecular weight of 76000 and a pI of 9, and can bind 2 atoms of iron, and simultaneously 2 molecules of bicarbonate (7, 8). Lactoferrin has a very high affinity for iron and retains this property over a wide pH range which extends below pH 4. The bactericidal properties of lactoferrin are due at least partially to its iron-binding capacity (9-13). Lactoferrin may bind firmly to acidic macromolecules (14). Phosphorylated acidic glycoproteins are known to constitute the major part of the acquired pellicle (15,

16) and may, thus, contribute to the binding of lactoferrin to the pellicle.

The purpose of the present investigation was to estimate the concentration of salivary lactoferrin in selected groups of stainers and non-stainers.

The development of extrinsic discoloration has been related to a high consumption of beverages such as tea, coffee, and wine (17-19). These beverages contain considerable amounts of tannic acid, which has also been shown to produce staining *in vivo* (20). For this reason, the combined effect of lactoferrin, iron, and tannic acid on slabs of enamel and dentin was studied.

Material and methods

Lactoferrin (LF) was obtained from Sigma Chem. Comp., U.S.A., or purified from human milk to apparent homogeneity as described (21). Goat anti-lactoferrin and rabbit anti-lactoferrin were prepared as described elsewhere (22, 23). Sheep anti-rabbit immunoglobulin was kindly provided by Dr. T. Michaelsen, National Institute of Public Health, Oslo, Norway. Alkaline phosphatase, p-nitrophenylphosphate,

and tannic acid (TA) were purchased from Sigma Chem. Comp. and hydroxyapatite (Bio-Gel HTP) from Bio-Rad Lab., U.S.A. Immune plates were from Dynatech, U.S.A., while iron ammonium citrate was obtained through Norsk Medisinaldepot, Oslo, Norway.

Patients attending the University Dental Hospital, Oslo, were screened for extrinsic dental discoloration and their teeth subsequently professionally cleaned and polished. Twelve patients exhibited extreme dark-brownish staining on the labial surfaces of their anterior teeth after 3 weeks as assessed by the method of Lamb et al. (24) and they were selected for the study (selected stainer group). Saliva samples from these subjects and from 22 subjects without staining tendency, even after rinsing with 0.2% chlorhexidine twice daily for 14 days (14) (selected non-stainers) were examined for lactoferrin content by enzyme-linked immunosorbent assay (ELISA) as described by Kolstø Otnaess et al. (23). Saliva samples from a whole class of dental students (104 persons) were included as a control population. Dietary habits were recorded for all subjects.

Table 1. Salivary lactoferrin

No of persons	Group	Concentration of lactoferrin ($\mu\text{g/mL}$)	
		Mean	S.D.
104	Population	$2.0 \pm$	1.9
12	Selected group of stainers	$23.4 \pm$	19.6*
22	Selected non-stainers	$1.5 \pm$	1.0

*Significance level: $p < 0.05$ when comparing stainer group with selected non-stainers. The stainer group showed significantly higher lactoferrin concentration than the non-stainer group.

Smokers were excluded from all groups. Non-stimulated whole saliva was always used and it was collected by expectoration at the same hour each time (10 a.m.), chilled on ice and stored frozen between the time of collection and assay. The flow rate of saliva was considered to be within physiological limits in all persons. Prior to freezing, an aliquot of each sample was diluted 10 and 100 times with phosphate buffered saline (pH 7.4) containing 0.1% Tween for the measurement of lactoferrin with ELISA.

For the *in vitro* study slabs of enamel and dentin from surgically removed third molars were washed with saline and pretreated with saliva and/or lactoferrin (5-50 $\mu\text{g/mL}$) and then suspended

in 1-10 mmol/L aqueous iron ammonium citrate containing 10 mmol/L NaHCO_3 (5, 8) at 4°C for 1-18 h. The saliva from stainers contained 10-60 μg LF/mL. The samples were finally exposed to 0.001-0.1% tannic acid at 20°C for various times. Stain intensity was assessed as described previously (24) during periods varying from 2 h and up to 3 weeks.

Data from the different groups were compared by the Student's *t*-test. A probability value equal to or less than 0.05 was accepted as statistically significant.

Results and discussion

In our student population the concen-

tration of salivary lactoferrin was $2.0 \pm 1.9 \mu\text{g/mL}$ (Table 1). This value is comparable to the values cited in the literature (25). None of the subjects examined showed any sign of salivary gland disease. It has been observed that such disease may profoundly influence the lactoferrin secretion (26) as may also the salivary flow rate (27). Stainers exhibited significantly higher lactoferrin concentrations compared to non-stainers (Table 1). The affinity of lactoferrin for acidic macromolecules suggests that a high lactoferrin concentration may contribute to an increased adsorption of iron to the tooth surface in stainers.

A high amount of iron in the pellicle has previously been related to extrinsic discoloration (28). Though speculative, it is possible that large variations in iron content may be partly attributed to differences in the concentration of lactoferrin and other iron-binding components (fx lactoperoxidase) in the pellicle.

No specific differences in dietary habits were found between the groups; however, all subjects in the stainer

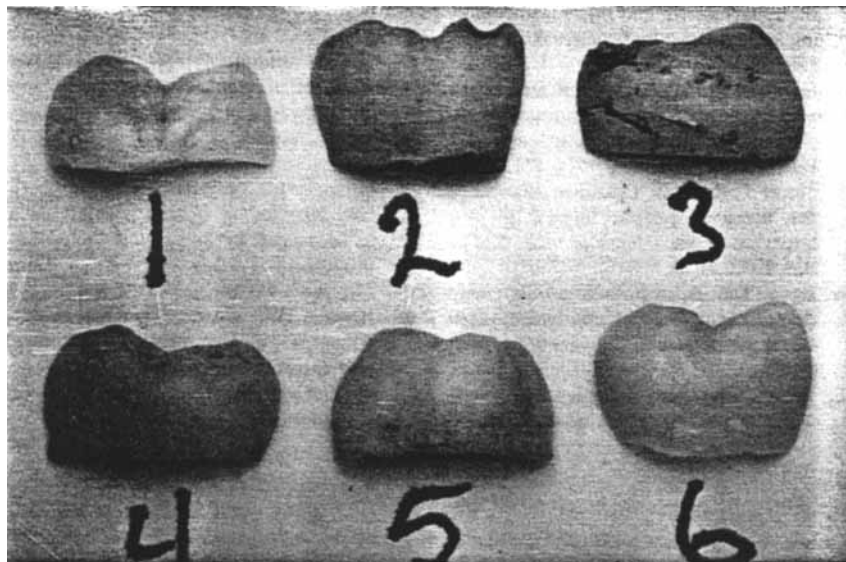


Fig. 1. Staining of enamel slabs *in vitro* in a typical experiment.

The slabs were successively exposed to:

1. Ferric ions (10 mmol/L)
2. Ferric ions (10 mmol/L) and tannic acid (0.1%)
3. Lactoferrin (50 $\mu\text{g/mL}$), ferric ions (10 mmol/L), and tannic acid (0.1%)
4. Saliva from a stainer (containing 31 μg LF/ml), ferric ions (10 mmol/L), and tannic acid (0.1%)
5. Saliva from a non-stainer (containing 1.2 μg LF/ml), lactoferrin (50 $\mu\text{g/mL}$), ferric ions (10 mmol/L), and tannic acid (0.1%)
6. Tannic acid (0.1%)

The slabs having been exposed to lactoferrin, iron, and tannic acid generally showed distinct spots of brown staining.



Fig. 2. Staining of dentin slabs *in vitro* in a typical experiment.

The slabs were successively exposed to:

1. Lactoferrin (50 $\mu\text{g/mL}$), ferric ions (1 mmol/L), and tannic acid (0.01%)
2. Ferric ions (1 mmol/L) and tannic acid (0.01%)

The slabs having been exposed to lactoferrin, iron, and tannic acid generally showed distinct spots of brown staining.

group had a regular intake of tea. Tea consumption was somewhat more irregular in the other groups.

Enamel and dentin slabs were discolored by successive exposures to lactoferrin (either in saliva or simple aqueous solution), iron, and tannic acid (Figs. 1, 2, from a typical experiment). All slabs (5 series) exposed to the heavy stainers' salivas (high in lactoferrin) followed by treatment with iron and tannic acid in succession exhibited staining. Most of the slabs exposed to the heavy stainers' salivas developed distinct brownish spots after a few hours. These spots became gradually darker over the next few days.

In experiments carried out with non-stainers' salivas (very low in lactoferrin) without a successive treatment with lactoferrin no such spots were formed (not shown in Fig. 1).

The discoloring effect of tannic acid observed is in agreement with earlier observations concerning the influence of denaturants on iron staining of dental pellicle (1, 4).

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