The Journal of Laryngology & Otology
April 2002, Vol. **116,** pp. 264–268

Immunocytochemical localization of lysozyme and lactoferrin attached to surface bacteria of the palatine tonsils during infectious mononucleosis

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Abstract
Bacterial samples were obtained from the tonsillar surfaces of seven patients (four males, three females; median age 18 years, range 15 to 21 years) suffering from acute infectious mononucleosis with concomitant pharyngotonsillitis, and from five healthy controls. By using gold-labelled antiserum to human lysozyme and lactoferrin, micro-organisms on the tonsillar surfaces coated with these antibacterial substances could be identified by tracing the gold particles in the transmission electron microscope. In healthy individuals, most of the bacteria were coated with lysozyme and significantly more bacteria were coated with lysozyme than with lactoferrin ($p<0.01$). In patients there was a non-significant reduction in lyoszyme-coating of the bacteria, whereas lactoferrin-coating was significantly increased ($p<0.01$). Changes in the lysozyme and/or lactoferrin coating of the tonsillar surface bacteria on the palatine tonsils during infectious mononucleosis cannot explain the tendency to immense local bacterial colonization with commensals and proneness to bacterial penetration into the epithelial cells.

Key words: Epstein-Barr Virus Infection; Bacterial Infections; Tonsillitis; Lactoferrin; Muramidase

Introduction

The oral microbiota of humans is highly complex and diverse. It consists of more than 300 bacterial species, to which can be added protozoa, yeasts and mycoplasms.[1] The human oral and pharyngeal mucosa is protected against infection by both adaptive and innate immune systems, that seem to keep the bacterial flora in check under normal conditions. The mucosal surfaces are constantly bathed by important physiological fluids originating from the salivary glands, secretory cells of the nasal cavities, and the gingival crevices. In addition, the palatine and pharyngeal tonsils add immunologically competent cells to the mucosal surfaces.[2]

The fluid covering the pharyngeal mucosa contains several antimicrobial factors, including immunoglobulins, lysozyme, lactoferrin, and secretary leukocyte protease inhibitor, as well as antimicrobial peptides.[3] Lysozyme and lactoferrin are the most abundant antimicrobial factors in the upper digestive and respiratory airways, present in sputum at roughly 0.4 g/l and 0.7 g/l, respectively.[4]

It is generally agreed that infectious mononucleosis (IM) is caused in most cases by the Epstein-Barr virus (EBV), a member of the Herpesviridae family. A pathognomonic sign of the disease is the simultaneous appearance of a membranous pharyngotonsillitis, which is, in fact, no more than intense bacterial colonization with commensals in addition to cell detritus covering the palatine tonsils.[5] The bacteria are both aerobes and anaerobes, frequently intermingled with bacterial pathogens such as β-hemolytic streptococci, *Fusobacterium nucleatum* and *Prevotella intermedia*, which can be found in upper respiratory tract infections.[6] During IM infection, it is evident that the normal steady-state established between the mucosal lining and bacterial flora is profoundly disturbed, as many bacteria are seen to penetrate into the tonsillar epithelium.[7]

Recently we showed that in the early stage of the membranous tonsillitis phase, attachment of immunoglobulins IgG and secretory IgA to the surface bacteria on the palatine tonsils was markedly suppressed, compared with conditions in healthy controls.[8,9] This finding could at least, in part, explain the massive bacterial colonization on the palatine tonsils. As lysozyme and lactoferrin are present together in high concentrations in mucosal secretions and neutrophil granules, it is evident that these substances may also contribute to the mucosal defence of the palatine tonsils.

The purpose of the present study was to establish whether or not lysozyme and lactoferrin attach to the bacteria harboured on tonsillar surfaces in health

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Accepted for publication: 12 November 2001.

and disease. In this study we used specific immunoglobulins to human lysozyme and lactoferrin, to which gold particles of uniform size were labelled. These particles could in turn be traced in the transmission electron microscope (TEM), thus facilitating the study of bacterial coating with various substances at a high magnification.

Materials and methods

Patients

Seven patients with IM and concomitant membranous tonsillitis referred to our out-patient Department comprised the study material. The patients (four males, three females) were aged 15 to 21 years (median 18 years). All had lymphadenopathy, lymphocytosis with >10 per cent atypical McKinley cells, a positive heterophil antibody reaction (Monosticon Dri-Dot slide test, Organon Diagnostics) and a Paul-Bunnell test with titres ⩾40. All patients showed positive IgG and/or IgM antibody titres to capsid antigen EBV.[10] Moreover, the IgG avidity was very weak, hinting at a primary EBV infection of recent date.[11] None of the patients had a serum immunoglobulin concentration below normal, as judged by an immuno-turbidimetric assay.

Aerobic culturing

Throat swab specimens were obtained from both palatine tonsils by rotating a cotton wool tipped wooden swab, which had been dipped in physiological saline, twice on the tonsillar surface. Samples were smeared over blood-agar and chocolate-agar plates for standard aerobic culturing according to routine laboratory procedures. The specimens were kept incubated in five per cent CO_2 at 37°C for 48 hours.

Immunocytochemical assay

The method is described in detail elsewhere.[12,13] Briefly, swabbed cell material was obtained from both palatine tonsils and loosened from the swab by rinsing it in physiological saline. The cell mixture was given a light centrifugation for 10 minutes at 1500 rpm to harvest the cells at the bottom of the tubes. Supernatant was carefully discharged using a Pasteur pipette and the precipitate carefully adjusted to 0.5 ml with physiological saline. To this cell mixture, 0.5 ml eight per cent formaldehyde buffered in 200 mM HEPES fixative was added to give a working concentration of four per cent formaldehyde. After fixation, the mixture was centrifugated at 16 000 rpm for one to two minutes. The supernatant was pipetted away and the cells diluted in 100 µl ten per cent gelatin. The samples were centrifuged briefly (15 seconds) and kept on ice for two hours. The cell pellet was excised, immersed in 2.3 M sucrose for one hour, placed on aluminium specimen pins and frozen by immersion in liquid nitrogen.

Ultrathin sections were prepared using Reichert Ultracut S ultramicrotome with a FCS cryochamber (Leica, Deerfield, IL, USA). The sections (approximately 30–60 nm thick) were cut with diamond knives and retrieved from the knife surface with a drop of methyl cellulose mixed with sucrose. The specimens were then mounted on carbon-coated grids, which were placed in one per cent cold fish-skin gelatin (FSG) for 15 minutes. After two washes in phosphate buffered saline (PBS), the grids were placed on 15 µl droplets of rabbit antiserum to human lysozyme (dilution in FSG 1:150) or rabbit antiserum to human lactoferrin (dilution in FSG 1:200) for 20 minutes. After renewed washing in PBS (five times), the grids were placed on protein A-gold diluted in one per cent FSG for 15 minutes (10 nm gold particles) and then washed with PBS (five times). Further washing with distilled water (four times) for 10 minutes was followed by drying in the presence of 0.3 per cent uranyl acetate in two per cent methyl cellulose.

The sections were examined using a JEOL JEM 1010 transmission electron microscope operated at 80 kV. From each patient, three grids with lysozyme-coated bacteria and three grids with lactoferrin-coated bacteria were evaluated. From each grid, between 300 and 3000 bacteria were scrutinized by one of the authors (H-M. B.) without any knowledge of the origin of the sample.

Prior to investigation, the antisera were titrated to minimize background attachment of the gold particles. Purified immunoglobulin fraction of rabbit antiserum to human lysozyme (DAKO A/S, DK-2600 Glostrup, Denmark; Code no. A 0099, Lot 056) and to human lactoferrin (DAKO A/S; Code no. A 0186, Lot 116) was used. The antiserum to lysozyme showed positive staining when tested against paraffin-embedded granulocytes and macrophages of human palatine tonsils. The antiserum to lactoferrin tested positively to paraffin-embedded epithelial cells of human breast tissue. No reaction was noted when tested against lactoferrin of cow's milk or fermented products of cow's milk. As negative controls we used plain cultures of *Streptococcus pyogenes*, *Streptococcus mitis* or *Escherichia coli*. Treating the specimens with one per cent FSG and protein-A gold without antisera or with antisera absorbed with excess antigen proved negative. All samples were treated in exactly the same way.

Statistical analysis

Wilcoxon's rank sum test was used for comparison of the groups, p-values below 0.05 being considered statistically significant.

Results

Controls

Aerobic culturing of bacterial samples obtained from the palatine tonsils of the healthy control individuals revealed growth of both Gram positive and Gram negative species. In particular, α-haemolytic viridans streptococci, *Corynebacterium* spp., *Actinomyces spp., Neisseria* spp., *Lactobacillus* spp., and coagulase-negative staphylococci were noted. In two controls, upper respiratory tract pathogens *Haemo-*

https://doi.org/10.1258/0022215021910717 Published online by Cambridge University Press

TABLE I

SEX, AGE AND PROPORTION (MEAN VALUES WHEN THREE GRIDS FROM EACH INDIVIDUAL WERE EVALUATED) OF LYSOZYME AND LACTOFERRIN COATED BACTERIA ON THE PALATINE TONSILS IN HEALTHY CONTROLS (NOS. 1–5) AND IM PATIENTS (NOS. 6–12)

			Proportions (%) of coated bacteria	
Individual no.	Sex	Age (years)	Lysozyme	Lactoferrin
1 (LE)	male	21	74	8
2 (AS)	male	20	85	7
3 (AZ)	female	18	48	12
4 (MA)	female	16	70	12
5 (NS)	male	19	52	11
Mean values		19 +/− 2 SD	66 +/− 15 SD	10 +/− 2 SD
6 (97–856)	male	16	54	20
7 (98–54)	male	17	47	27
8 (98–362)	female	18	57	35
9 (00–210)	female	19	54	28
10 (98–255)	male	21	49	28
11 (97–198)	male	20	57	33
12 (99–146)	female	15	82	19
Mean values		18 +/− 2 SD	57 +/− 12 SD p = NS	27 +/− 7 SD $p<0.01$

Differences in lysozyme and lactoferrin coated bacteria on the tonsillar surfaces within and between the healthy controls and IM patients were calculated using Wilcoxon's rank sum test.

philus influenzae and *Staphylococcus aureus* could be identified.

The findings of lysozyme and lactoferrin coated bacteria are summarized in Table I and Figure 1. Generally speaking, most of the tonsillar surface bacteria were coated with lysozyme (range 48–85 per cent; mean value 66 ± 15 per cent; Figure 2). In contrast, only a few of the bacteria were coated with lactoferrin (range seven to 12 per cent; mean value 10 ± two per cent; Figure 3). Significantly fewer bacteria were coated with lactoferrin than with lysozyme ($p<0.01$).

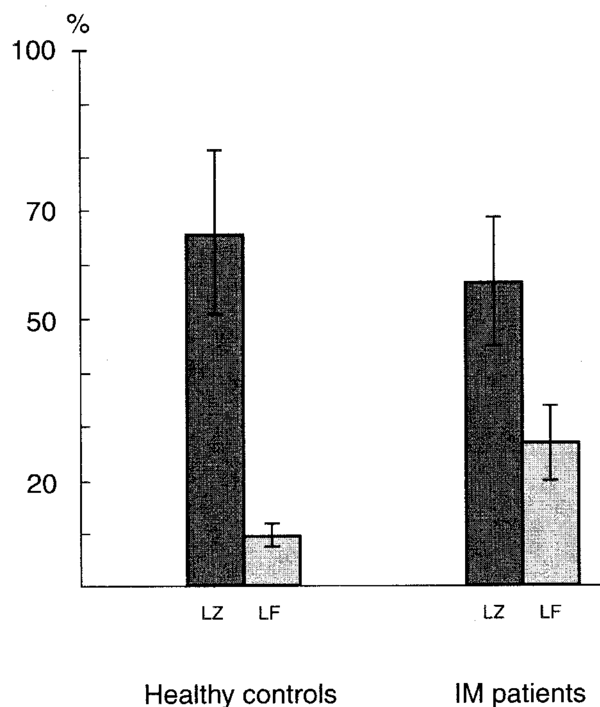

FIG. 1

Lysozyme (LZ) and lactoferrin (LF) coating of tonsillar surface bacteria (mean values ± SD) in healthy individuals (n = 5) and infectious mononucleosis (IM) patients (n = 7). Differences between groups were calculated using Wilcoxon's rank sum test. Healthy controls: LZ *vs.* LF $p<0.01$; IM patients: LZ *vs.* LF $p<0.01$; healthy controls *vs.* IM patients: LZ non-significant, LF $p<0.01$.

Patients

Roughly the same aerobic bacterial flora as present in the healthy controls was found in samples obtained from the patients, viz., viridans streptococci, *Corynebacterium* spp., *Neisseria* spp., *Lactobacillus* spp., and coagulase-negative staphyloccoci. The bacterial colonization was much more pronounced, however.

Table I and Figure 1 show that there was a non-significant reduction in lysozyme coating of the bacteria in the patient group as compared with healthy controls, whereas a significant increase in lactoferrin coating was noted ($p<0.01$). However, the

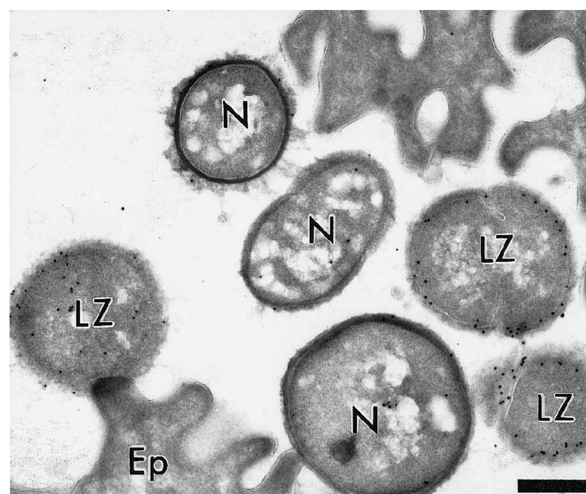

FIG. 2

TEM picture showing coccoid bacteria abundantly coated with lysozyme (LZ; 10 nm particles). Non-coated bacteria (N). (Original magnification ×20 000. Bar 400 nm).

https://doi.org/10.1258/0022215021910717 Published online by Cambridge University Press

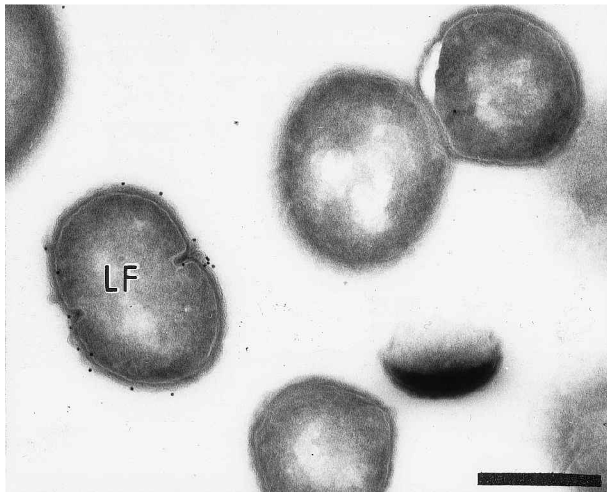

FIG. 3

TEM picture of a coccoid bacterium coated with lactoferrin (LF; 10 nm gold particle) (Original magnification ×20 000. Bar 400 nm)

extent of lactoferrin coating of the bacteria was still significantly under the level of lysozyme coating ($p<0.01$).

Discussion

The growth of oral and pharyngeal micro-organisms is influenced by a variety of factors such as temperature, pH, oxidation-reduction potential, availability of nutrients and water, the anatomy of pharyngeal structures, salivary flow and antimicrobial substances of the oral fluids.[1] In fact, the oral and pharyngeal cavities host many factors having a broad spectrum of antimicrobial activity and which in many ways can influence bacterial colonization.

Lysozyme, a low molecular cationic protein, is present in all major body fluids. It is produced in the epithelium and appears in addition to be associated with lysosomes of neutrophils and monocytes, but is absent in lymphocytes. Lysozyme is involved in a variety of defence mechanisms, including bacteriolysis, opsonization, immunopotentiation and anti-tumour activity.[14]

Human lactoferrin is an iron-binding protein present in milk and most exocrine secretions, such as tears, saliva, bile and pancreatic secretions. It is a major component of granules of polymorphonuclear neutrophils[15] but is also present in serous cells of submucosal mixed glands in the nasal cavities[16] and is secreted by the salivary glands.[17] Lactoferrin has a broad spectrum of anti-microbial properties due to its iron-binding,[18] destabilization of the outer membrane of Gram negative bacteria[19] and liberation of bactericidal peptides.[20] As lactoferrin and lysozyme are present together in high concentrations in mucosal secretions and neutrophil granules, these substances probably act synergistically and additively in killing bacteria. Thus, their interaction contributes substantially to host defence.[3,21]

The present study showed that the great majority of the surface tonsillar bacteria were coated with lysozyme under healthy conditions. During IM there was only a slight and non-significant reduction in bacterial coating with this protein. A reduction in lysozyme level has also been noted in tears during an acute attack of eye infection caused by herpes simplex virus.[22] The slight decrease is somewhat confusing, however, as the furry covering of the palatine tonsils during membranous tonsillitis harbours innumerable neutrophils whose granulae are probably filled with lysozyme.

Only 10 per cent of the bacteria on the palatine tonsils were coated with lactoferrin under healthy conditions (Table I, Figure 1). The difference in bacterial coating between these two antibacterial proteins was significant ($p<0.01$). However, lactoferrin coating of the tonsillar surface bacteria was significantly increased during the membranous tonsillitis phase ($p<0.01$). Similar findings have also been reported for *Streptococcus pyogenes* during the course of acute tonsillitis.[23] Lactoferrin is a marker for neutrophils and lactoferrin titres are known to be increased in purulent sputa.[24] It is therefore conceivable that the increased bacterial lactoferrin coating results from an accumulation of polymorphonuclear neutrophils in the inflammatory target area.

In recent studies,[8,9] it was shown that bacteria harboured on the palatine tonsils under normal healthy conditions were abundantly coated with both SIgA and IgG antibodies. In consequence, SIgA, the main immunoglobulin present on mucous membranes, participates in the first line of mucosal defence of the palatine tonsils. This subgroup of immunoglobulins originates from the salivary glands.[25] Several functions have been attributed to SIgA, such as preventing bacterial attachment to epithelial cells, limiting the colonization of microorganisms on mucosal surfaces, neutralizing viruses and toxins, and inhibiting antigen penetration through mucosal surfaces.[26]

IgG antibodies, both serum-derived and locally produced, reach external secretions by passive diffusion or leakage between epithelial cells, especially during mucosal infections.[27] IgG antibodies can cause bacteriolysis directly, opsonize the bacteria for phagocytosis, and activate complement system by the classical pathway.[28] Although IgG antibodies play their major role in tissues, they have an excellent opportunity to participate in the first line of mucosal defence of the palatine tonsils as well.

During the membranous pharyngotonsillitis phase there is an immense bacterial colonization of commensals on the tonsillar surfaces. The present study hinted that this increased bacterial colonization cannot be attributed to suppression of bacterial coating by lysozyme and/or lactoferrin. It seems more probable that the suppression of immunoglobulins IgG and SIgA is more important in this context. However, antimicrobial peptides such as human β-defensins 1 and 2, locally produced in the

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surface tonsillar epithelium, might also play an important role in innate defence of microorganisms.[29]

To conclude, by using an immunocytochemical assay with gold particles that label antibodies to human lysozyme and lactoferrin, it was shown that under healthy conditions bacteria covering the palatine tonsils were heavily coated with lysozyme but only occasionally with lactoferrin. By contrast, during IM with concomitant membranous tonsillitis, a non-significant reduction of lysozyme coating was noted, but a significant increase in lactoferrin coating. These changes cannot explain, however, the immense bacterial colonization of the tonsillar surfaces and the tendency for bacterial penetration into the epithelial cells.

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Professor L-E Stenfors takes responsibility for the integrity of the content of the paper.
Competing interests: None declared

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