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Oral endotoxin in healthy adults

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This article presents a study that measured oral endotoxin levels in healthy persons with the Limulus amoebocyte lysate microassay. Only young nonsmoking adults with a healthy dentition measured with the plaque index and a good level of oral hygiene based on a twice-daily (morning and evening) tooth-brushing regimen were admitted to the baseline study. A total of 15 healthy adults with a median age of 29 years (range, 25 to 43 years) were included in the trial. The mean plaque score of the group was 1.2 ± 0.1. They all maintained a twice-daily tooth-brushing regimen unaltered throughout the sampling period. A total of 30 mouth rinses were studied. None of the samples yielded potential pathogens including aerobic gram-negative bacilli, Staphylococcus aureus and yeasts; a culturing technique based on preenrichment in nutrient medium was used. Data showed mean oral endotoxin levels of 20 ng per ml of mouth rinse; the aerobic E. coli endotoxin was used as the classical standard. This is equivalent to 1 mg of anaerobic endotoxin per ml of undiluted saliva after correcting for the 10 to 102 dilution factor of the mouth rinse itself and for the 103 times less sensitivity of anaerobic endotoxin in the Limulus amoebocyte lysate-assay. The discussion includes the physiologic and clinical benefit of the low endotoxicity of anaerobic gram-negative flora apart from the technical aspects of both culture and endotoxin assays used in the study. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1996;82:637-43)

Severity of underlying disease is thought to determine the oropharyngeal carriage of “normal” versus “abnormal” potential pathogens. Both chronic and acute underlying conditions promote the acquisition and subsequent oral carriage of “abnormal” aerobic gram-negative bacilli (AGNB). Apart from the indigenous anaerobic (e.g., Bacteroides sp) and aerobic (e.g., viridans streptococci) mouth flora, healthy people may carry “normal” potential pathogens such as Streptococcus pneumoniae and Staphylococcus aureus. Oral carriage of AGNB is uncommon in healthy persons. A glycoprotein fibronectin that covers the oropharyngeal mucosa has been shown to possess attachment sites for gram-positive microorganisms including S. pneumoniae and S. aureus, whereas receptor sites for AGNB are thought to emerge after denudement of the oral mucosa from fibronectin caused by underlying disease.

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The microbiologic observation that surveillance samples of the oropharynx of healthy persons in general do not contain AGNB has been clinically used to distinguish subsets of patients at high risk of infection from patients at low risk. In the same population of immunocompromised patients, AGNB carriage has been associated with an endotoxin pool thought to contribute to the systemic inflammatory response syndrome (SIRS) after absorption through the alimentary mucosal lining. Most work has been done on the intestinal endotoxin pool with fecal samples. However, microbiologically and immunologically the oropharynx is an integral part of the digestive tract. For example, persons who do not carry AGNB in the oropharynx, only harbor the indigenous Escherichia coli in the gut. Patients who carry Pseudomonas aeruginosa in the mouth are generally fecal carriers of the same strain. Much of the difficulty in developing protocols for oral immunization has involved the delivery of sufficient antigen to effectively immunize the host without development of oral tolerance.

Before embarking on studying different homogeneous patient populations, we believe that the establishment of baseline values of oral endotoxin in a healthy group is mandatory. A MEDLINE search of the literature from 1985 to 1995 that used the terms oral endotoxin failed to reveal any studies that addressed the levels of oral endotoxin in healthy per-
sions. This prompted us to apply the existing Limulus gelation microassay for the detection of endotoxin in oral washings of 15 healthy persons without oral AGNB carriage.

VOLUNTEERS AND METHODS

Subjects

The inclusion criteria were as follows: (1) older than 18 years and nonsmoking; (2) in good general health; (3) no antibiotic intake for at least 3 months before the study; (4) healthy dentition evaluated by the plaque index; (5) a good level of oral hygiene; and (6) free of AGNB in the oropharynx.

Health status of the subjects

Subjects with a chronic underlying disease such as diabetes, alcoholism, chronic obstructive pulmonary disease, or liver, kidney, or heart disease were excluded. All these conditions promote oropharyngeal AGNB carriage. Young adults with viral illnesses of the respiratory tract that are always associated with oral mucositis were not allowed to participate because of increased adherence of AGNB to infected mucosal cells.

Oral health status

Neither carious lesions nor prosthetic appliances were allowed. All volunteers were examined by the same clinical-dental hygienist team led by one of us (L.T.S.). All 15 persons received an oral examination that included an assessment of the amount of dental plaque present on six teeth: the upper right first molar, lower left first molar, lower left central incisor, lower right central incisor, upper right central incisor, upper left first premolar. The buccal and lingual surfaces at the gingival margin of the teeth were scored according to a simplified modification of the semiquantitative plaque index first described by Silness and Loe. The amount of plaque seen on the tooth was given a score of 0 to 3: (0 = no plaque noted; 1 = plaque seen only on the tip of an explorer passed over the tooth surface; 2 = plaque obvious with the naked eye; 3 = gross deposits of plaque present over the entire tooth). For those persons missing any of the index teeth, the teeth remaining closest to those missing index teeth were scored. A single score was given for each tooth that represented the surface harboring the most plaque. This oral examination was performed only once, at the time of the first gargling.

Oral hygiene

Only nonsmoking subjects with a good level of oral hygiene, (i.e., who brushed their teeth minimally two times daily) were enrolled in the open study. The subjects were instructed to maintain a twice-daily (morning and evening) tooth-brushing regimen unaltered throughout the sampling period and to avoid any kind of mouth rinses, lozenges, or antimicrobial agents. The possible variations as a result of eating and drinking were minimized by sampling at 11 AM approximately 3 hours after breakfast.

Oral washings

The volunteers rinsed and gargled with 10 ml of sterile pyrogen-free saline (SPFS) solution for 30 seconds. The oral washings were collected in sterile vials (Cordis Laboratories, Roden, The Netherlands). One week later the second sample was collected in the same way.

Microbiologic techniques

Samples were transported to the laboratory and processed by an experienced senior technician within 1 hour of collection. One milliliter of sample was added to 9 ml of brain heart infusion (BHI) broth (Lab M, Salford, England), to make serial tenfold dilutions. Dilution series were made in trays of 64 (8 x 8) cups of 1.5 ml (Thovadec, Ede, The Netherlands). Each cup was filled with 0.45 ml of BHI. A 0.05 ml sample from the 1:10 diluted mouth rinse was mixed with 0.45 ml BHI in the first cup, resulting in a 1:100 oral washing. All dilution steps from $10^{-2}$ through $10^{-9}$ were prepared in BHI with 0.05 ml microdiluters (Dynatech AG, Zug, Switzerland). The samples were incubated at 37°C for 18 hours. The number of cups that showed turbidity from the growth of microorganisms indicated the logarithm of the concentration of microorganisms per millimeter of mouth rinse (quantitative determination). Thereafter, all the dilutions with growth were inoculated on MacConkey agar (BBL, Heidelberg, Germany), yeast morphology agar (Merck, Darmstadt, Germany), and blood agar (BBL) for qualitative determination. AGNB were evaluated on MacConkey agar, yeasts on yeast morphology agar, and streptococci and staphylococci on blood agar. Morphologically distinct colonies were isolated in pure culture. The identification of AGNB was performed by means of the biochemical test of API 20E (API B.V.'s, Hertogenbosch, The Netherlands). Staphylococcus aureus was distinguished from coagulase-negative staphylococci by means of the slide agglutination test to detect clumping factor and protein A. If the results were inconclusive, a tube-coagulase test was done. All results were expressed by $\log_{10}$ of colony-forming units per ml of mouth rinse.
Determination of the endotoxin concentration in oral washings

The endotoxin concentration in the oral washings was determined by a microtechnique of the Limulus amebocyte lysate (LAL) test. The equipment and materials used included pyrogen-free glass test tubes, slides, micropipettes (1, 2, 3, 4, and 5), hand pipettes with sterile tips (Finnpipette, Helsinki, Finland), an incubator at 37°C ± 1°C, and a colored solution containing 0.1% bromophenol blue (Merck, Darmstadt, Germany) in 96% ethanol diluted to 20% with phosphate-buffered saline without Ca2+. Sterile pyrogen-free saline (SPFS) solution was used to dissolve all reagents and used as a negative control. It was also used for the preparation of standards and predilution of the samples. All materials coming in contact with specimen or test material were rendered pyrogen-free by heating to 180°C for 4 hours.

The lyophilized LAL (Whittaker, Walkersville, West Virginia) was reconstituted with SPFS according to the manufacturer’s instructions. The reconstituted LAL was swirled gently but thoroughly without foaming for at least 30 seconds. The solution was dispensed into small sterile pyrogen-free tubes, depending on the quantity necessary each day, and stored at −20°C. Immediately before use, the frozen LAL tube was thawed at 37°C and kept on ice during the test.

Lyophilized endotoxin from Escherichia coli 055:B5 (10 ng/vial) was used (Whittaker) as a standard. It was reconstituted by the addition of 5 ml SPFS followed by vigorous shaking for at least 5 minutes with a vortex mixer. This solution had a concentration of 2 ng/ml and was guaranteed by the manufacturer to be stable for 4 weeks at 4°C. Before use, the solution was warmed to room temperature and mixed vigorously for 1 minute.

To determine the potency of the LAL and to verify the reproducibility of the test, duplicate twofold dilution series were made with a 100 pg/ml endotoxin solution. The 100 pg/ml endotoxin solution was made by diluting the stock solution. Two microliter samples of each endotoxin solution were picked up in a micropipette. To mix endotoxin and LAL, the contents of each micropipette were blown out onto a slide (4 μl = 2 μl LAL + 2 μl endotoxin dilution). Of the mixture, 3 μl were reaspirated into the same micropipette. To circumvent penetration of air bubbles and evaporation of the mixture, the capillary was incubated in a metal box (16 x 13 x 3 cm) in a horizontally sloping position and in a humidified atmosphere. When all the endotoxin dilutions had been treated in the same manner, the metal boxes were closed and placed for 60 minutes in an incubator at 37°C. Formation of the gel was detected by dipping the micropipettes one by one for 5 seconds vertically in a tube of the colored solution. If a firm gel was present the micropipette remained colorless, and the test was positive. A negative test was characterized by the absence of a gel, the micropipette becoming colored after immersion in the colored solution.

The oral washings were stored at −20°C. Before measuring, the test samples were warmed to room temperature. The samples were centrifuged (10,000 g, 10 minutes) in polypropylene tubes (Eppendorf, Hamburg, Germany) and the supernatants were collected. A 1:10 dilution series of up to 10−6 of the supernatant in SPFS was made and tested with the LAL in the manner described for the LAL potency assay. The endotoxin concentration was estimated by multiplying the maximum dilution producing a positive test against the sensitivity of the LAL, which was verified daily with fresh endotoxin standard. Each first sample was centrifuged, diluted, and tested twice to determine the reproducibility of the method.

The lysate potency verification assay consisted of a minimum of four up to a maximum of eight dilution assays to determine that the geometric mean (GM) sensitivity value and the standard deviation (SD) of the endpoints were within acceptable ranges (6 pg to 250 pg/ml). A value within this range was considered equivalent to the value stated on the lysate vial label that was determined with the current U.S. Standard Endotoxin.

RESULTS

Fifteen healthy nonsmoking adults (eight women and 7 men) met the criteria for enrollment in the study. The median age was 29 years (range, 25 to 43 years). The mean plaque score of the healthy adult group was 1.2 ± 0.1. None of the subjects carried AGNB in the oropharynx. In addition, all volunteers were shown not to have yeasts and S. aureus in their mouth rinses. The mean concentration of endotoxin found in the 30 oral mouth rinses was 20 ng/ml of mouth rinse (SD 10) (Table I). The first series of 15 washes was tested twice. Identical endotoxin concentrations were found. There was no difference in the oral endotoxin levels between the two sets of samples. The LAL sensitivity value was found to be within the range (3 pg/ml versus 6 pg/ml) and considered to be equivalent to the value stated on the lysate vial label; this confirms that the lysate used in the study was as sensitive as claimed by the manufacturer. After the addition of endotoxin to the diluted samples, no inhibition was observed. The mean oral endotoxin concentration of 20 ng/ml...
Table I. Oral endotoxin in two mouth rinses obtained from 15 healthy adults 1 week apart. Oral endotoxin concentrations are expressed in nanogram (ng) per ml of mouth wash.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>ng Endotoxin/ml Week 1 (in duplicate)</th>
<th>ng Endotoxin/ml Week 2</th>
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<td>SD</td>
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of oral washing was obtained with the aerobic *E. coli* endotoxin used as the classical standard. This is equivalent to 1 mg of anaerobic endotoxin per milliliter of undiluted saliva after correcting for the 10⁻² dilution factor of the mouth rinse itself and for the 10⁻³ x less sensitivity of anaerobic endotoxin in the LAL assay.

DISCUSSION

In healthy adults, 1 mg of anaerobic endotoxin per milliliter of undiluted saliva was found after correcting for the dilution factor of the mouth rinse itself and for the less sensitivity of anaerobic endotoxin in the LAL assay with *E. coli* endotoxin used as the classical standard.

Six inclusion criteria were thought to guarantee the homogeneity of the healthy population studied. The healthy status of the 15 young nonsmoking adults (median age, 29 years) was confirmed clinically by the absence of any chronic or acute underlying disease that is associated with the oral AGNB carrier state. They had not taken any antimicrobial nor had they suffered any viral illness causing oral mucositis that promotes adherence of AGNB to oropharyngeal mucosae. Their healthy dentition was confirmed by the low semiquantitative plaque index of 1.2. Finally, the sensitive and reproducible mouth rinse method based on pre-enrichment in nutrient medium showed the 30 mouth rinses to be negative for AGNB, yeasts and *S. aureus*, reflecting the healthy status of the subjects enrolled in the baseline study.

The value of 1 mg of endotoxin per milliliter of undiluted saliva is highly likely to be generated by the indigenous anaerobic gram-negative bacilli such as *Bacteroides sp* carried in the oropharynx. This assumption is based on the indirect proof that healthy persons do not carry AGNB in their oropharynx, and, hence that these absent abnormal bacteria were impossible to be responsible for this level of oral endotoxin. Moreover, the culture methods used in the study were unable to show AGNB. Of course, the sensitivity of the microbiologic techniques is the determining factor.

Mouth rinses have been shown to be superior compared with throat swabs for three reasons: (1) gargling permits sampling of surfaces of the entire oropharyngeal cavity including tonsillar crypts and possibly otherwise inaccessible areas; (2) with the use of a defined volume of saline solution and with the subject gargling for a specified period of time, there is a minimal variation of sampling; (3) the mean concentrations of microorganisms received from gargle samples are higher than the mean concentrations of microorganisms obtained by the swab technique.¹⁷

To enhance the sensitivity of the saline gargle method, nine tenfold dilution steps in broth were made and immediately incubated at 37°C for 18 hours; the traditional culturing methods only include three steps 10⁻¹, 10⁻³, and 10⁻⁵ in sterile phosphate-buffered saline solution from which 0.1 ml of each dilution is inoculated on blood agar followed by 24 hours and 48 hours incubation. The sensitivity of the method used in this study is further increased by the broth incubation immediately after collecting the sample (enrichment step) that permits detection of very low concentrations of microorganisms. This technique of pre-enrichment followed by the use of selective solid media has been described for the isolation of small numbers of salmonellae and antibiotic-resistant bacteria from feces. The traditional technique without overnight culture is based on the concept that the dynamics of microbial growth in a mixed culture system are unpredictable. AGNB are reported to proliferate more rapidly than, for example, gram-positive cocci. The enrichment method for this study basically relies on that latter observation. Detection of oropharyngeal carriage of AGNB in low concentrations was the major aim of the use of the mouth rinse culture technique with overnight broth culture, because oral AGNB carriage is a sensitive marker of severity of underlying disease.⁵

The second technical issue is the sensitivity of the classical existing LAL assay using aerobic *E. coli*
endotoxin as control, for the detection of endotoxin of anaerobic gram-negative bacilli. Endotoxin or lipopolysaccharide (LPS) is released by gram-negative aerobic and anaerobic bacteria. LPS or endotoxin can be detected by means of the Limulus gelation assay. The assay is based on the principle that bacterial LPS serves as control for the detection of endotoxin of LPS differs from the classical aerobic endotoxin of Escherichia coli, Bacteroides species endotoxicity of LPS of anaerobic gram-negative bacteria such as Bacteroides species is low compared with the endotoxin potency associated with AGNB such as E. coli. Bacteroides LPS causes gelation of the LAL assay at a concentration 1000 times higher than that for the aerobic endotoxin of E. coli. Bacteroides LPS differs from the classical E. coli LPS in that LPS of the anaerobic bacterium lacks two essential 2-keto-3-deoxyoctonate (KDO) and heptose. These in vitro findings are confirmed by the clinical observations that course and outcome of a septicemia caused by Bacteroides species is much more favorable than a blood infection caused by E. coli. There are no LAL tests commercially available with anaerobic LPS used as the standard. All manufactured LAL kits use E. coli LPS as control to detect AGNB-LPS. If one assumes that the assay used in our study underestimates gram-negative anaerobic LPS levels by 1000 times, then the oral endotoxin level is probably 20 μg/ml of mouth rinse. If the resting saliva levels are in the range of 0.1 to 1 ml/minute and 10 ml of the rinse is gargled, this represents a 1:10 to 1:100 dilution factor of the mouth rinse itself. The actual anaerobic LPS concentration in undilated saliva may range from 0.1 to 1 mg/ml. Assuming that anaerobic LPS is 10^-3 less sensitive, the absolute concentration of oral endotoxin does not represent a significant difference from the fecal endotoxin pool of 1 mg/gm of feces in healthy adults. What it does represent is perhaps the gram-negative anaerobe's adaption to an environment that it wishes to colonize.

From a microbiologic point of view, the AGNB free carrier state apart from the indigenous E. coli in the gut in a concentration of 10^5-6 CFU/gm of feces reflects the healthy status of a person. From endotoxin point of view, levels of 1 mg in both saliva and feces are found by our group. However, there is a crucial difference between the 1 mg of anaerobic endotoxin per milliliter of undiluted saliva and the 1 mg of aerobic E. coli endotoxin per gram of feces. Anaerobic gram-negative endotoxin has a low endotoxicity, about 1000 times less, compared with the high endotoxic potency of AGNB endotoxin. In normal subjects the intravenous administration of a bolus of E. coli endotoxin (4 ng/Kg) caused a depression of the left ventricular function besides the cytokinemia. Our observations that about 1 mg of endotoxin is carried per milliliter and gram of saliva and feces, respectively, imply potent defense mechanisms to be in place at oropharyngeal and intestinal level to control absorption of endotoxin. Traditionally, three lines of defense are being described. First, the intactness of anatomy and physiology of the oropharynx and gastrointestinal tract is paramount to keep endotoxin intraluminally. Second, in the case of absorption through the intact or damaged digestive tract mucosal lining, one of the functions of the macrophages of both oropharynx- and gut-associated lymphatic tissue (GALT) is the neutralization of oral and gut endotoxin. Third, the blood composed of platelets, proteins, and leucocytes neutralizes the endotoxin in the systemic circulation after spillover. Clearance of AGNB and endotoxin from the body is an important physiologic mechanism mainly based on their mechanical removal from throat and gut. The swallowing reflex, tongue movements, and salivary flow are all involved in the efficient cleansing of the oropharynx. AGNB are cleared from the oropharynx within 3 hours after challenge in human volunteers. Salivary mucins are been described to neutralize endotoxin. In analogy, gut motility and bile are pivotal in the clearance of gut AGNB or endotoxin. The endotoxin barrier is not only physical but also immunologic. The uptake and subsequent detoxification of endotoxin occurs in the mononuclear reticuloendothelial system of the oropharynx-associated lymphoid tissue that contains the ring of Waldeyer including the adenoids, tonsils, and cervical lymph nodes and of the GALT including the liver primarily by Kupffer cells lining the sinusoidal vascular network. Finally, apart from the blood cells scavenging systemic endotoxin in case of endotoxin spillover (platelet consumption by endotoxin), there is recent evidence that supports the beneficial role of the low endotoxicity of anaerobic gram-negative flora in protecting the host from harmful inflammatory events such as SIRS. The major route of oral and gut endotoxin (or alimentary canal-derived cytokines) is thought to be lymphatic drainage into the right lymphatic and thoracic duct. These two lymph vessels return the lymph to the venous blood circulation and via the heart into the lungs. The alveolar macrophages in the lower airways are the target immune cells to be primed by endotoxin or cytokines. Anaerobic endotoxin has been shown to be unable directly to stimulate the macrophage in the release of tumor necrosis factor and procoagulant activity. Moreover, the specificity of molecular interaction of the macrophage with anaerobic
Bacteroides LPS of low endotoxicity was found to block further stimulation by aerobic E. coli endotoxin of high potency.31

Clinically, the presence of a chronic and acute underlying condition such as diabetes, alcoholism, trauma, or surgery is associated with the detection of abnormal AGNB in oropharyngeal and gastrointestinal secretions. The underlying mechanism is not clear. Some people believe that these diseases both chronic and acute are associated with macrophages releasing several inflammatory mediators34 and elastase.7 Elastase is also excreted in saliva, bile, and mucus and hence into oropharynx and gut and denudes the mucosal cells of their protective layer of fibronectin, exposing receptor sites for the AGNB. Increased adherence of the AGNB has been associated with the loss of fibronectin from the surface of the digestive tract mucosae because of the damaging inflammatory response. The indigenous both oropharyngeal and intestinal anaerobic flora outnumber the aerobic bacteria by a factor of 102 to 104. Despite some favorable data the actual relevance of the anaerobic flora in the prevention of the abnormal AGNB carrier state associated with the underlying condition via bacterial interference35 is uncertain in human beings. Translocation of live AGNB does occur at both throat and gut level36,37 in spite of the presence of indigenous anaerobic gram-negative flora. Furthermore, AGNB carriage is likely to increase the LPS concentrations of high endotoxicity and, after absorption through the mucosal lining, to enhance the generalized inflammation state triggered by the underlying condition. Excessive inflammation or SIRS still does occur despite the beneficial effect of low endotoxicity of the anaerobic gram-negative flora via blocking of sensitized macrophages against further stimulation by aerobic endotoxin. If the body’s largest lymphoid organ of GALT38 fails to control microbial translocation and endotoxin absorption in critically ill patients, it is very unlikely that the oropharynx-associated lymphatic tissues that are minor compared with GALT are able to cope with oral AGNB and associated LPS of high endotoxicity. Topical polymyxin and tobramycin has been shown to be effective in eradicating the AGNB carrier state and in neutralizing endotoxin at both oropharyngeal and intestinal level.11,12,39,40

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