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# Humoral immune response to *Aggregatibacter actinomycetemcomitans* leukotoxin

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*Background and Objective:* Periodontal disease is an inflammatory condition caused by bacterial infections that result in loss of the tooth supporting tissue. The periodontal pathogens produce virulence factors with capacity to affect the host immune response. *Aggregatibacter actinomycetemcomitans* is a periodontal pathogen that produces a leukotoxin that specifically affects human leukocytes. The aims of the present study were to examine the presence and function of systemic antibodies to the leukotoxin.

*Material and Methods:* One hundred and ninety-seven middle-aged (57 ± 5 years) Swedes with well-documented periodontal status and medical factors related to cardiovascular diseases were studied. These data have been published previously. The serum samples were examined for the presence of leukotoxin antibodies by western blot and the capacity to neutralize leukotoxicity in an activity assay with leukotoxin and cultured leukemic cells.

*Results:* The results showed a high prevalence (57%) of antibodies against *A. actinomycetemcomitans* leukotoxin in the analyzed population. These antibodies were correlated with leukotoxin neutralizing capacity as well as with the ELISA titers of *A. actinomycetemcomitans*-specific IgA and IgG. In addition, high levels of leukotoxin antibodies were correlated with increasing age, but not with periodontal disease parameters or cardiovascular risk factors.

*Conclusion:* Systemic antibodies against *A. actionmycetemcomitans* leukotoxin were common in this adult Swedish population. These antibodies might contribute to limit the systemic effects of the infection.

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Bacteria that adhere to and develop biofilm on teeth and the surrounding tissues are crucial in the pathogenesis of periodontitis, an inflammatory disease (1,2). Pathogens located in the subgingival biofilm release inflammatory components, including collagen- and matrix-degrading proteins, which result in loss of the tooth supporting tissues (3). To maintain homeostasis in the periodontal microflora, the immune system controls colonization and invasion of the different bacteria (4). Local and systemic release of antibodies against antigens of the microorganisms localized in the biofilm or in the surrounding tissues contributes to maintainance of this homeostasis (5). How the presence and levels of systemic antibodies against periodontal pathogens reflect the inflammatory disease activity in the periodontium is not clear (4).

More than 700 different bacterial species can be detected in samples from the subgingival plaque biofilm and other sites of the oral cavity (6). The

majority of these species can be isolated from samples of both healthy and periodontally diseased subjects. Some of these species increase in number and proportion in plaque samples from diseased subjects and have the capacity to express unique virulent factors associated with pathogenic mechanisms (3). Among the periodontal pathogens, *Aggregatibacter actinomycetemcomitans* is often found in high numbers of plaque samples from subjects with periodontitis, specifically in the localized aggressive form (7–10). This gram-negative, capnophilic coccobacillus possesses a number of important virulent factors (8). One of them is a leukotoxin, which is a large pore-forming protein of the repeat in toxin (RTX) family that specifically activates and lyses human leukocytes (11,12). A specific clone (JP2) of *A. actinomycetemcomitans* has a significantly enhanced expression of the leukotoxin and is strongly associated with localized aggressive periodontitis in adolescents of African descent (13).

Serum antibody titers to *A. actinomycetemcomitans* have been shown to correlate with the presence of this bacterium in the oral cavity (4,14–18). These antibodies have usually been quantified by the ELISA technique or by the capacity to neutralize leukotoxin-induced leukocyte lyses (14,16, 17,19–23). In the present study, systemic immunoreactivity to the leukotoxin has been determined by western blot analyses that specifically detect antibodies which bind to the toxin (24). It is still not known whether the presence and levels of systemic leukotoxin neutralizing capacity, leukotoxin specific antibodies and ELISA titers to *A. actinomycetemcomitans* reflect infection with this bacterium in a similar manner. In addition, it has been shown that antibody levels to some periodontal pathogens are associated with enhanced levels of inflammatory markers and incidence of cardiovascular diseases (19,25).

The aims of the present study were to determine the presence and function of serum antibodies to *A. actinomycetemcomitans* leukotoxin in relation to periodontal status and history of myocardial infarction, as well as to serum levels of inflammatory markers.

## Material and methods

### Study population

The study population consisted of 197 Swedish adults with a mean age of 57 ± 5 years. One hundred of these individuals were recruited after being admitted to Gävle County Hospital, Sweden, because of acute myocardial infarction.The myocardial infarction was verified by typical changes in the electrocardiogram in combination with elevation of biochemical markers in serum, such as creatinine kinase isoenzyme and troponin T. The other 97 subjects were systemically healthy, age- and sex-matched individuals randomized from the general population of the same geographical area. The periodontal status as well as serum levels of inflammatory markers and antibodies to the periodontopathogens *A. actinomycetemcomitans* and *Porphyromonas gingivalis* of this study population have been documented and published previously (25). Titers IgA and IgG against *P. gingivalis* and *A. actinomycetemcomitans* were quantified by multi-serotype ELISA. The periodontal bone loss in this population documented by X-ray was scored and distributed as follows: $<0.9$, no/minor bone loss (56%); 0.9–1.4, moderate bone loss (24%); and $>1.4$, severe bone loss (10%). Hypertension in the control group was defined as a systolic blood pressure $>139$ mmHg and diastolic blood pressure $>89$ mmHg. Since β-blocking agents with blood pressure-lowering activity are given routinely following myocardial infarction, it is not appropriate to define hypertension following myocardial infarction in a similar way to the control group. Therefore, hypertension in the myocardial infarction group was defined as use of antihypertensive medication prior to the myocardial infarction. Levels of triglycerides, high-density lipoproteins, high-sensitivity C-reactive protein and interleukin-6 were measured from blood samples collected after an overnight fast. All participants gave their written conformed consent, and the study was approved by the Ethics Committee of Uppsala University, Sweden.

### Detection of serum antibodies against *A. actinomycetemcomitans* leukotoxin

Serum antibodies with specific reactivity to *A. actinomycetemcomitans* leukotoxin were detected by western blot analyses as described previously (26). Briefly, purified leukotoxin (0.4 μg per lane) was separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the leukotoxin was then transferred to a polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA, USA) by western blot technique. Each membrane was blocked by 5% skim milk in phosphate-buffered saline (PBS), washed in PBS and cut into strips for each line. The strips were placed into 5 mL tubes and exposed 1 h for serum samples diluted 1:1000 in PBS with 0.1% Tween (PBS-T) and 5% skim milk. Each strip was washed in PBS-T and exposed to horseradish peroxidase-labeled rabbit anti-human IgA, IgG and IgM (Dako A/S, Glostrup, Denmark) diluted 1:2000 in PBS-T with 5% skim milk. The immunoreactive protein bands were enhanced by chemiluminescence technique (Pierce, Rockford, IL, USA) and visualized using ChemiDoc™ XRS (BioRad Laboratories AB, Sundbyberg, Sweden). The leukotoxin band intensity was classified as weak or strong, and the quantitative aspects of this assay were demonstrated by analyses of selected serially diluted samples. A human serum with known immunoreactivity to leukotoxin was used as an internal standard in each blot. Sera that showed similar or stronger reactivity than this serum were classified as strong, while sera with detectable reactivity but weaker than the internal standard were classified as weak.

### Detection of *A. actinomycetemcomitans* leukotoxin neutralizing capacity in serum

The *A. actinomycetemcomitans* leukotoxin neutralizing capacity in the sera was detected as a reduction of

leukocyte damage and subsequent leakage of lactate dehydrogenase upon exposure to purified leukotoxin, as described previously (20).

Briefly, cells of the human acute monocytic leukemia cell line THP-1 (ATCC 16) were cultured in RPMI-1640 (Sigma-Aldrich, St Louis, MI, USA) with 10% fetal bovine serum (Sigma-Aldrich) at 37°C in an atmosphere of air enriched with 5% $CO_2$. Before determination of leukotoxic activity, the THP-1 cells were seeded in 96-well cell culture plates at a cell density of $5 \times 10^5$ cells/mL in 100 μL culture medium supplemented with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and incubated for 72 h. The PMA-activated THP-1 cells exhibited adherent properties and enhanced sensitivity to the leukotoxin. Twenty four hours before leukotoxin exposure, the culture medium was discarded, and 50 μL fresh medium without PMA was added to each well of the THP-1 monolayer.

For detection of leukotoxin neutralizing capacity, purified leukotoxin (25 ng/mL; 26) was mixed with each serum sample (10%) in RPMI-1640. After incubation for 15 min at room temperature, 50 μL of the serum–leukotoxin mixtures was added in triplicate to culture wells with THP-1 cells, and the mixtures were incubated for 120 min at 37°C. The released lactate dehydrogenase activity into the culture supernatant was quantified as described previously (26). Serum samples that inhibited the lactate dehydrogenase release caused by the leukotoxin with ≥50% were classified as positive and were further analyzed in the assay diluted to 1% of the final volume.

### Control analyses of selected serum samples

Immunoreactivity to egg albumin, protein A and *Staphylococcus aureus* α-toxin (all from Sigma-Aldrich) was analyzed by western blot as described.

Total serum protein was quantified with the Micro BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA) in accordance with the manufacturer's protocol.

### Statistical analyses

The correlation between presence of serum antibodies against *A. actinomyctemcomitans* leukotoxin with other parameters of inflammatory markers or clinical status was calculated with nonparametric chi-square test using SPSS software (IBM, Chicago, IL, USA). Values of $p < 0.05$ are indicated as significant differences.

## Results

The quantitative aspects of the western blot method that detected immunoreactivity of leukotoxin-specific antibodies (IgG, IgA and IgM) were demonstrated by serial dilution of serum with weak or strong immunoreactivity (Fig. 1). Of the 197 sera of the present study population tested at a dilution of 1:1000, 85 (45%) showed strong immunoreactivity to the leukotoxin in the western blot assay (Fig. 2). Furthermore, 23 sera (12%) showed weak immunoreactivity to the leukotoxin, and the rest of the tested sera (43%) had no detectable immunoreactivity to the leukotoxin at this dilution.

The leukotoxin neutralizing capacity (function) of the 197 sera was analyzed in an activity assay. Fifty-four per cent ($n = 106$) of these sera neutralized the leukotoxic activity at a concentration of 10% in this activity assay. Further dilution of these 106 sera to a final assay concentration of 1% resulted in 28 of these sera losing detectable leukotoxin neutralizing ability at this higher dilution. In comparison with the western blot assay, all of the 85 sera with strong immunoreactivity to the leukotoxin had leukotoxin neutralizing capacity and only 8% of these sera lost their ability to neutralize leukotoxin when diluted to 1% (Fig. 2). Among the 23 sera with weak immunoreactivity to the leukotoxin, 21 had leukotoxin neutralizing capacity, but 13 of them lost this ability when diluted to 1%. In the group of 89 sera without detectable immunoreactivity to leukotoxin, eight of these samples neutralized the leukotoxicity. Seven of these eight sera lost their leukotoxin neutralizing ability when they were further diluted to a final assay concentration of 1%. The neutralizing capacity of the tested sera were significantly correlated with the immunoreactivity to the leukotoxin ($p < 0.001$).

Analyses of serum antibodies to periopathogens, periodontal condition, general health and systemic risk markers for cardiovascular diseases in this study population have been documented and published previously (25). The correlations between these data and the systemic immunoreactivity to leukotoxin analyzed in the present study have been evaluated here. The specific immunoreactivity to the leukotoxin was correlated with an enhanced ELISA titer against *A. actinomycetemcomitans*-specific IgA and IgG and *P. gingivalis* IgA, but not with an enhanced ELISA titer against *P. gingivalis* IgG (Table 1). None of the parameters used for examination of the periodontal status of this study population was correlated with the presence of leukotoxin-specific immunoreactivity in the serum samples (Table 2). The serum levels of triglycerides and the body mass index were significantly enhanced ($p = 0.012$ and 0.036, respectively) in the group with weak immunoreactivity to the leukotoxin (Table 3). In addition, strong immunoreactivity to leukotoxin was correlated significantly ($p = 0.003$) with increasing age. The proportion of myocardial infarction cases and levels of the other associated risk markers were not correlated with the prevalence

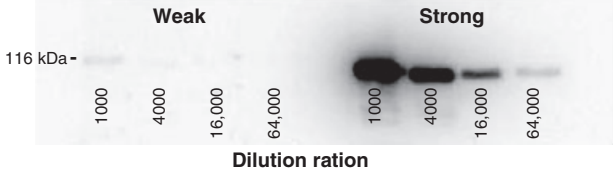


*Fig. 1.* Western blot of two serially diluted selected sera with weak or strong immunoreactivity to *Aggregatibacter actinomycetemcomitans* leukotoxin (0.4 μg per lane).

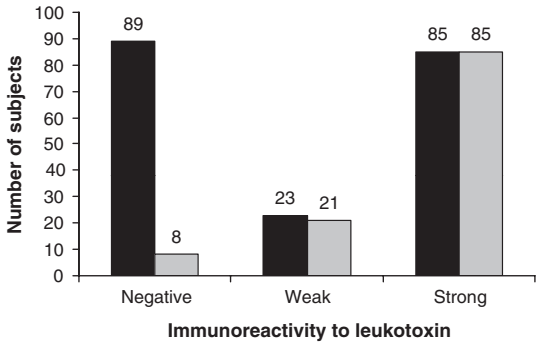


*Fig. 2.* Immunorectivity to *Aggregatibacter actinomycetemcomitans* leukotoxin in serum samples detected with western blot (filled columns) in relation to their leukotoxin neutralizing capacity (shaded columns). Serum samples from 197 subjects were analyzed, and neutralizing capacity was significantly correlated with leukotoxin immunoreactivity ($p < 0.001$). Number of subjects is indicated above each bar.

*Table 1.* ELISA antibody titers against *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in relation to the immunoreactivity to *A. actinomycetemcomitans* leukotoxin detected by western blot

| | Immunoreactivity to *A. actinomycetemcomitans* leukotoxin | | |
|---|---|---|---|
| ELISA titer | Not detectable | Weak | Strong |
| *A. actinomycetemcomitans* IgA | 1.34 ± 0.84 | 5.52 ± 1.42* | 3.41 ± 1.82* |
| *A. actinomycetemcomitans* IgG | 2.16 ± 1.36 | 2.52 ± 1.51 | 5.15 ± 2.31* |
| *P. gingivalis* IgA | 1.66 ± 2.77 | 1.81 ± 2.37* | 1.98 ± 2.96 |
| *P. gingivalis* IgG | 4.67 ± 4.27 | 4.47 ± 3.81 | 5.41 ± 4.49 |

Values are means ± SD; $n = 197$.
* Significantly different from the immunoreactivity negative samples ($p < 0.05$).

*Table 2.* Clinical parameters of oral conditions correlated with immunoreactivity against *A. actinomycetemcomitans* leukotoxin and detected by western blot

| | Immunoreactivity to *A. actinomycetemcomitans* leukotoxin | | |
|---|---|---|---|
| Oral registration | Not detectable | Weak | Strong |
| Plaque index (%) | 28.0 ± 16.1 | 28.6 ± 14.6 | 25.0 ± 14.9 |
| Gingival index (%) | 18.9 ± 15.8 | 21.1 ± 15.9 | 18.1 ± 11.5 |
| Number of teeth | 24.4 ± 4.3 | 23.9 ± 4.6 | 23.9 ± 5.5 |
| Number of pockets >4 mm | 5.1 ± 7.7 | 5.2 ± 7.9 | 6.0 ± 8.2 |
| Bone loss score | 0.72 ± 0.56 | 0.75 ± 0.51 | 0.76 ± 0.48 |

Values are means ± SD; $n = 197$. No significant differences were seen between parameters of oral registration and prevalence of immunoreactivity to leukotoxin.

of these leukotoxin antibodies (Table 3). It has previously been shown that enhanced serum IgG levels against *P. gingivalis* in the examined study population were related to both myocardial infarction and parameters of periodontal status (25). This relationship was not found for the other tested periodontal pathogen, *A. actinomycetemcomitans*.

Control analyses of randomly selected serum samples with and without leukotoxin antibodies showed normal values of total protein without significant differences between the positive and negative samples (data not shown). In addition, all of the selected samples showed binding to protein A, but not to egg albumin, in the western blot assay (data not shown). Furthermore, immunoreactivity to leukotoxin was not correlated with immunoreactivity to *S. aureus* α-toxin (data not shown). Taken together, these control analyses indicate functional immunoglobulins in the serum samples, as well as specificity for the leukotoxin in the western blot assay.

## Discussion

In the present study, we analyzed sera from a population of Swedish adults regarding the presence of *A. actinomycetemcomitans* leukotoxin antibodies and whether these sera could neutralize leukotoxic activity. The periodontal status and the systemic risk markers associated to cardiovascular diseases (CVD) were well documented in these middle-aged Swedish subjects (25). Antibodies against leukotoxin, are commonly detected in sera of patients infected with *A. actinomycetemcomitans* (14,16,17,27), indicating that the leukotoxin antibodies are useful as a systemic marker for *A. actinomyctemcomitans* infections. Our results showed that antibodies to *A. actinomycetemcomitans* leukotoxin could be detected in 57% of the sera by western blot analyses of the present population, which was a surprisingly high proportion. Previous reports have found *A. actinomycetemcomitans* in the subgingival plaque of about 10% of periodontally healthy adult European subjects, with a slightly increased prevalence in periodontally diseased subjects (8,18,28). In a recently published paper, it was shown that enhanced IgG titers against *A. actinomycetemcomitans* occurred in about 10% of the subjects in a representative adult US population (29). In the present study, presence of leukotoxin antibodies was correlated with leukotoxin neutralizing capacity and ELISA titers against *A. actinomycetemcomitans*. The strong correlation between these methods supports the specificity of the leukotoxin antibodies and that western blot is a sensitive, direct method for detection of these antibodies in serum samples. The periodontal status of the subjects was not correlated with the presence of systemic leukotoxin antibodies. This is

*Table 3.* Incidence and risk markers of myocardial infarction in relation to the immunoreactivity against *A. actinomycetemcomitans* leukotoxin detected by Western blot

| Myocardial infarction and risk markers | Immunoreactivity to *A. actinomycetemcomitans* leukotoxin | | |
|---|---|---|---|
| | Not detectable | Weak | Strong |
| Myocardial infarction (%) | 50.6 | 43.5 | 52.9 |
| Hypertension (%) | 22.5 | 30.4 | 15.3 |
| Smoked (%) | 52.3 | 73.9 | 50.6 |
| Body mass index | 26.2 ± 3.5 | 27.6 ± 3.5* | 27.4 ± 5.1 |
| Age (years) | 56.5 ± 5.6 | 55.6 ± 5.7 | 59.0 ± 4.6* |
| Fasting plasma glucose (g/L) | 5.8 ± 2.2 | 6.0 ± 1.7 | 5.7 ± 1.8 |
| Serum cholesterol (mM) | 5.7 ± 1.3 | 5,9 ± 1.1 | 5.6 ± 1.0 |
| Serum triglycerides (mM) | 1.6 ± 0.9 | 2.1 ± 1.0* | 1.7 ± 1.1 |
| High-denisity lipoprotein-cholesterol (mM) | 1.4 ± 0.4 | 1.3 ± 0.4 | 1.5 ± 0.5 |
| High-sensitivity C-reactive protein (mg/L) | 1.7 ± 2.0 | 3.0 ± 5.4 | 2.1 ± 2.9 |
| Serum interleukin-6 (pg/mL) | 2.8 ± 2.5 | 3.2 ± 2.5 | 3.5 ± 3.6 |

Values are expressed as proportions or means ± SD; $n = 197$.
* Significantly different from the immunoreactivity negative samples ($p < 0.05$).

in line with previous findings from adult populations with periodontitis (21), while the presence of systemic leukotoxin antibodies is strongly associated with adolescents with periodontitis (22,24,30–32). The protective role of these antibodies in the pathogenesis of periodontal diseases is still not clear, and conflicting data exist in the literature (24,31,32). The localization of the infection and the physiological conditions in the periodontal pocket (proteolytic, anaerobic) might affect antibody function. In addition, cross-reactive systemic antibodies that bind to the leukotoxin and neutralize its activity might exist. In the present study, eight of the sera without reactivity to leukotoxin in western blot had the capacity to neutralize leukotoxic activity, indicating the presence of blocking antibodies without reactivity to the blotted protein or that other blocking serum components exist.

The *A. actinomycetemcomitans* leukotoxin is an efficient virulence factor strongly associated with the pathogenesis of periodontitis (8,12). Besides the ability to kill leukocytes, it causes massive release of proteases from neutrophils and has a certain ability to activate and release interleukin-1β from monocytes/macrophages (11,33, 34). These inflammatory mechanisms induced by the leukotoxin are the same as those involved in atherosclerosis (35). Serum antibodies against *A. actinomycetemcomitans* leukotoxin might therefore provide important protection against the systemic effects associated with periodontitis, such as atherosclerosis, myocardial infarction and stroke (36). It has previously been shown that subjects with systemic leukotoxin neutralizing capacity have a decreased risk for stroke (20). The mechanism behind this protection is not known, but it was suggested that a primary *A. actinomycetemcomitans* infection late in life might contribute to this result. The present study showed that the presence of strong systemic immunoreactivity to leukotoxin increased with increasing age of the subjects. A previous study found a correlation between systemic immunoreactivity and increased age in women (20). Furthermore, the results of the present study indicate that primary infections with *A. actinomycetemcomitans* also could occur in adults. Specific for the action of *A. actinomycetemcomitans* is that the leukotoxin has the capacity to cause massive death among the leukocytes in the vicinity of the infection, which might cause a delayed antibody response. This suggested delay might increase the risk for systemic complications at a primary infection in adults with this bacterium.

Neither the incidence of myocardial infarction nor the different risk markers for CVD were associated with the presence of systemic antibodies against *A. actinomycetemcomitans* leukotoxin in the present study population. This is in line with previous analyses of sera in this study population, where ELISA titers to *A. actinomycetemcomitans* not were correlated with myocardial infarction or the associated systemic biomarkers (25).

In conclusion, the presence of antibodies against *A. actinomycetemcomitans* leukotoxin in sera from the studied population was high (57%). Furthermore, the presence of leukotoxin antibodies was not correlated with the incidence of periodontitis or myocardial infarction, but might, however, have a protective function against systemic effects that could be induced by the infection.

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