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Short communication

# Antigenic cross-reactivity and sequence homology between *Actinobacillus actinomycetemcomitans* GroEL protein and human fibronectin

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The immunologic cross-reactivity between human fibronectin and *Actinobacillus actinomycetemcomitans* GroEL was examined. Analyses by SDS-PAGE/Western immunoblotting and ELISA showed that a polyclonal antibody directed against the purified GroEL protein of *A. actinomycetemcomitans*, but not against the *Escherichia coli* GroEL, cross-reacts with human fibronectin. No antigenic cross-reactivity was observed between anti-*A. actinomycetemcomitans* GroEL antibody and type IV collagen, another important constituent of the basement membrane. A comparative analysis of the amino acid sequences of *A. actinomycetemcomitans* GroEL and human fibronectin revealed eight instances of four-amino acid sequence homology between the two proteins. Six of these tetrapeptide sequences were also shared with *E. coli* GroEL, suggesting that the remaining two tetrapeptides, GQLI (Glycine-Glutamine-Leucine-Isoleucine) and TGLE (Threonine-Glycine-Leucine-Glutamic acid), may be associated with the epitope that the anti-*A. actinomycetemcomitans* GroEL antibody specifically recognizes. Reactivity between TGLE, but not GQLI, with anti-*A. actinomycetemcomitans* GroEL antibody was confirmed by a biospecific interaction analysis using a biosensor technology. Although additional investigations are required, the observed phenomenon may lead to an autoimmune response and thus contribute to tissue destruction during periodontitis.

Key words: *Actinobacillus actinomycetemcomitans*; fibronectin; GroEL; heat shock protein; periodontitis

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Heat shock proteins are families of proteins constitutively expressed by eukaryotic and prokaryotic cells that have important biological functions in cell physiology and survival (16, 20). These proteins are overexpressed under stressful conditions such as temperature increase or nutrient limitation. They act as molecular chaperons in the assembly and folding of proteins, or as proteases when damaged or toxic proteins have to be degraded (16, 20). Heat shock proteins are highly conserved and the genes coding for these stress proteins are the most conserved genes ever identified in nature (16). It has been suggested that the high degree of homology between heat shock proteins of the host and those expressed by pathogens may lead to an autoimmune response in the host (20). In a previous study, we reported that the GroEL protein (heat shock protein 60 family) of the periodontopathogen *Actinobacillus actinomycetemcomitans* is found on the cell surface and extracellular membrane vesicles and possesses a strong cytotoxic activity (8). Interestingly, Koga et al. (14) reported that sera from patients with localized juvenile periodontitis and rapidly progressive periodontitis react strongly to *A. actinomycetemcomitans* GroEL, suggesting that this protein is expressed *in vivo* and is highly immunogenic. In this study, we investigated the immunologic cross-reactivity between human fibronectin and *A. actinomycetemcomitans* GroEL.

## Material and methods

### Reagents

*Escherichia coli* GroEL (#SPP-610) and rabbit anti-*E. coli* GroEL polyclonal antibody (pAb-EcGroEL; #SPA-875) were obtained from StressGen (Victoria, BC, Canada). Human plasma fibronectin (#F-2006), type IV collagen from human placenta (#C-7521), and rabbit anti-human fibronectin polyclonal antibody (pAb-HuFn; #F-3648) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Human fibroblast fibronectin (#341633) was obtained from Calbiochem- Novabiochem Co. (San Diego, CA). The synthetic peptides GQLI (Glycine-Glutamine-Leucine-Isoleucine) and TGLE (Threonine-Glycine-Leucine-Glutamic acid) were prepared by the Eastern Quebec Peptide Synthesis Facility (CHUL, Quebec, QC, Canada). The BIACORE 1000 analytical instrument, the sensor chip CM5, the amine coupling kit (*N*-hydroxysuccinimide, *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide and ethanolamine hydrochloride), and the running buffer were provided by Biacore International AB (Uppsala, Sweden).

### Purification of *A. actinomycetemcomitans* GroEL protein and preparation of anti-GroEL antibody

The GroEL protein was purified from an *A. actinomycetemcomitans* ATCC 29522 cell extract by affinity chromatography on adenosine 5'-triphosphate-agarose as previously described (10). Purified GroEL (10 μg) was mixed with complete Freund's adjuvant and injected intracutaneously into a New Zealand white rabbit. Subsequent intramuscular injections (10 μg in incomplete Freund's adjuvant) were done at days 8, 19, and 40. The rabbit was bled via the marginal ear vein at days 25 and 33, and via the heart at day 48. The antisera (pAb-AaGroEL) were pooled and stored at −20°C until used.

### Electrophoresis and Western immunoblotting

Protein concentrations in the samples to be analyzed were determined by the method of Lowry et al. (17). Prior to loading, purified proteins were treated at 100°C for 15 min in the presence of electrophoresis sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 0.01% bromophenol blue). SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 10% polyacrylamide slab gels (Mini PROTEAN II, Bio-Rad Laboratories, Mississavga, ON, Canada) and the Laemmli buffer system (15). The migrated proteins were electrophoretically transferred (100 V for 1 h) onto a nitrocellulose membrane. After blocking the unreactive sites with 3% gelatin, the membrane was incubated with either pAb-AaGroEL (1 : 500 dilution), pAb-EcGroEL (1 : 500 dilution), or pAb-HuFn (1 : 2,000 dilution) for 2 h as the first antibody. Preliminary assays revealed that these concentrations were optimal. The membrane was washed and incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (1 : 3,000 dilution). The membrane was then washed and immersed in development buffer (0.3 mg/ml nitro blue tetrazolium, 0.15 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt in 50 mM carbonate buffer, pH 9.8). To ensure that the tissue-damaging effect of the immunization process itself did not induce the production of anti-fibronectin antibodies, a polyclonal antibody to *Porphyromonas gingivalis* whole cells, prepared similarly to pAb-AaGroEL, was used as control.

### ELISA analysis

*A. actinomycetemcomitans* GroEL (20 μg/ml) and human fibronectin (20 μg/ml) were serially diluted in 50 mM phosphate buffered saline pH 7.2 (PBS). Antigen (100 μl) was added to the wells of an ELISA plate (Maxisorp™ Nalge Nunc International, Maperville, IL), which was incubated at 37°C for 2 h. The wells were then washed three times with PBS containing 0.05% (v/v) Tween-20 (PBST) and unreacted sites were blocked for 1 h at 37°C with 100 μl of PBST containing 2.5% skim milk. After washing three times with PBST, pAb-HuFn or pAb-AaGroEL diluted 1 : 4,000 and 1 : 250, respectively, in PBST containing 1% skim milk was added and the plate was incubated at 37°C for 2 h. These dilutions were found to be optimal in preliminary assays. After washing the wells three times with PBST, alkaline phosphatase-conjugated goat anti rabbit IgG (100 μl) diluted 1 : 3,000 in PBST, was added and the plate was incubated for a further 2 h at 37°C. Wells were washed three times with PBST and 100 μl of *p*-nitrophenylphosphate (1 mg/ml) was added to each well. The plate was incubated at room temperature in the dark overnight. Absorbance at 405 nm was determined with an ELISA plate reader. In one experiment, the ELISA assay was carried out by incorporating the tetrapeptide TGLE or GQLI (4 mM) during the incubation step with the first antibody. All assays were run in triplicate and the means ± standard deviations were calculated.

### Amino acid sequence comparison

The Wisconsin Package Version 10.2 (Genetics Computer Group, Madison, WI) computer program was used to search for amino acid sequence homologies between *A. actinomycetemcomitans* GroEL (accession number D28817), *E. coli* GroEL (accession number AAA97042) and human fibronectin precursor (accession number P02751). In the case of *A. actinomycetemcomitans* GroEL, hydrophilicity and protein secondary structure profiles (5) were predicted using the Hopp & Woods parameter (12).

### Biospecific interaction analysis by BIACORE 1000

The sensor chip CM5 was activated according to the recommendations of the BIACORE 1000 amine coupling kit and 400 μg/ml of synthetic tetrapeptides (GQLI or TGLE) were then covalently coupled to the sensor chip. Sera (pAb-AaGroEL or pre-immune serum) were diluted (1 : 2,000) in running buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20 and 3 mM EDTA, pH 7.4). The experiments were performed according to the manufacturer's instructions and the resonance signal was recorded continuously during passage of the sample. The amount of bound antibody was detected 30 s after the end of the injection.

## Results and discussion

Antigenic cross-reactivity between *A. actinomycetemcomitans* GroEL and human fibronectin was first evaluated by SDS-PAGE/Western immunoblotting analysis. Figure 1 shows that pAb-AaGroEL reacted strongly with plasma fibronectin (lane 2) and to a lesser extent with fibroblast fibronectin (lane 3), whereas no reactivity occurred with human type IV collagen, which was used to confirm the specificity of the reaction (data not shown). The reactivity with plasma fibronectin was less intense when the amount of protein loaded was reduced. *A. actinomycetemcomitans* GroEL was not recognized by pAb-HuFn (data not shown). pAb-EcGroEL did not show any cross-reactivity with human fibronectin (lane 5). The fact that no reaction was observed with anti-*P. gingivalis* antibodies, prepared similarly to pAb-AaGroEL, indicates that the immunization process by

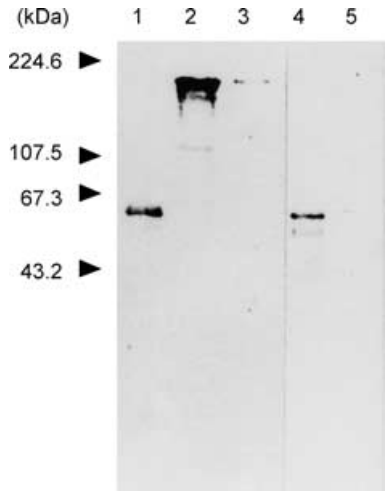


*Fig. 1.* Immunoreactivity of human fibronectin with antibodies directed against bacterial GroELs. Lane 1. *A. actinomycetemcomitans* GroEL (0.02 μg), lane 2. Human plasma fibronectin (1.67 μg), lane 3. Fibroblast fibronectin (1.67 μg), lane 4. *E. coli* GroEL (0.01 μg), lane 5. Human plasma fibronectin (1.67 μg). Lanes 1–3 were probed with pAb-AaGroEL (1 : 500 dilution). Lanes 4 and 5 were probed with pAb-EcGroEL (1 : 500 dilution).

itself does not induce the production of anti-fibronectin antibodies.

To determine whether pAb-AaGroEL recognize only the denatured form of human fibronectin, cross-reactivity using the native form of plasma fibronectin was investigated by ELISA. The data presented in Table 1 show that pAb-AaGroEL also reacted with native fibronectin. No reactivity was noted with the pre-immune serum. pAb-HuFn did not recognize purified *A. actinomycetemcomitans* GroEL in the ELISA analysis, supporting the data obtained by SDS-PAGE/Western immunoblotting.

A search for amino acid sequence homologies between the GroEL proteins (*A. actinomycetemcomitans*, *E. coli*) and human fibronectin was performed. This homology analysis revealed that *A. actinomycetemcomitans* GroEL and fibronectin share eight tetrapeptidic sequences (Table 2). Six of the tetrapeptides were also shared by *E. coli* GroEL. Only two tetrapeptides (GQLI and TGLE) were specific to both *A. actinomycetemcomitans* GroEL and human fibronectin. Based on an analysis of the hydrophilicity and secondary structure profile of *A. actinomycetemcomitans* GroEL, TGLE was located in a hydrophilic region and is thus an epitope that may be recognized by the immune system. When a competitive ELISA assay was performed by adding these tetrapeptides to the first antibody, no significant inhibition of reactivity of pAb-AaGroEL with fibronectin was observed. This may be explained by the fact that the three-dimensional structure of the protein is crucial for a stable immunoreactivity. In addition, the involvement of another epitope should not be excluded.

To further investigate a possible reactivity between tetrapeptides and pAb-AaGroEL, a biospecific interaction analysis using a biosensor technology was performed. In this analysis, tetrapeptides were immobilized on the sensor chip surface and diluted sera (pAb-AaGroEL or pre-immune serum) were injected in the BIACORE 1000 instrument. The sensorgram presented in Fig. 2(A) indicates a low but positive interaction between pAb-AaGroEL and the TGLE sensor chip, as indicated by the resonance signal (absolute response in resonance unit) recorded. No interaction was observed with the GQLI sensor chip (Fig. 2B).

It has been suggested that the molecular mimicry between bacterial GroEL proteins and human heat shock protein 60 or constitutive human proteins may lead to autoimmune responses (6). Most studies on such possible autoimmune reactions in periodontitis have been limited to immunogenic mimicry between prokaryotic and human heat shock proteins (11, 18, 19). Very few reports of antigenic cross-reactivity between heat shock proteins and constitutive host proteins are available. Arguas et al. (1) reported that polyclonal antibodies against human lactoferrin react with the 65 kDa heat shock protein (GroEL) of mycobacteria. It was proposed that the molecular mimicry between mycobacterial heat shock protein and human proteins might be involved in the etiology of T-cell-dependent autoimmune diseases such as rheumatoid arthritis. To our knowledge, our study was the first to report immunoreactivity between human fibronectin and an antibody directed against a bacterial heat shock protein. This antigenic cross-reactivity between *A. actinomycetemcomitans* GroEL and human fibronectin appeared to be highly specific since it was not observed with antibodies directed against *E. coli* GroEL. In addition, a second human structural protein (type IV collagen) did not cross-react with antibo-

*Table 2.* Tetrapeptide sequence homologies between *A. actinomycetemcomitans* GroEL and human fibronectin[a]

| Amino acid position in *A. actinomycetemcomitans* GroEL | Shared tetrapeptide | Amino acid position in human fibronectin precursor[b] |
|---|---|---|
| 27 | VKVT[c] | 921 |
| 90 | TTAT[c] | 2064 |
| 126 | SVVA[c] | 1701 |
| 159 | GQLI | 679 |
| 181 | TGLE | 1961 |
| 246 | PLLI[c] | 1438 |
| 404 | RAAV[c] | 290 |
| 406 | AVEE[c] | 882 |

[a]Instances of tetrapeptidic sequence homology between *A. actinomycetemcomitans* GroEL (accession number D28817) and human fibronectin precursor (accession number P02751).
[b]The amino acid sequence of human fibronectin precursor contains a signal region (1st–31s). The mature form of fibronectin thus consists of amino acids 32 through 2386.
[c]Tetrapeptides also found in *E. coli* GroEL (accession number AAA97042).

*Table 1.* ELISA analysis of antigenic cross-reactivity between native human plasma fibronectin and *A. actinomycetemcomitans* GroEL

| Antigen | Concentration (μg/ml)[a] | First antibody | Absorbance at 405 nm |
|---|---|---|---|
| *A. actinomycetemcomitans* GroEL | 20 | pAb-AaGroEL | 1.344 ± 0.069[b] |
| | 10 | | 1.354 ± 0.069 |
| | 5 | | 1.099 ± 0.030 |
| | 20 | pAb-HuFn | 0 |
| Human fibronectin | 20 | pAb-HuFn | 1.043 ± 0.016 |
| | 10 | | 1.068 ± 0.070 |
| | 5 | | 1.017 ± 0.098 |
| | 20 | pAb-AaGroEL | 0.133 ± 0.004 |
| | 10 | | 0.065 ± 0.004 |
| | 5 | | 0.015 ± 0.008 |
| | 20 | Pre-immune serum | 0.012 ± 0.013 |
| | 10 | | 0.017 ± 0.017 |
| | 5 | | 0.001 ± 0.001 |

[a]Protein concentration used for coating wells of the microtiter plates.
[b]Means ± standard deviations obtained from three assays.

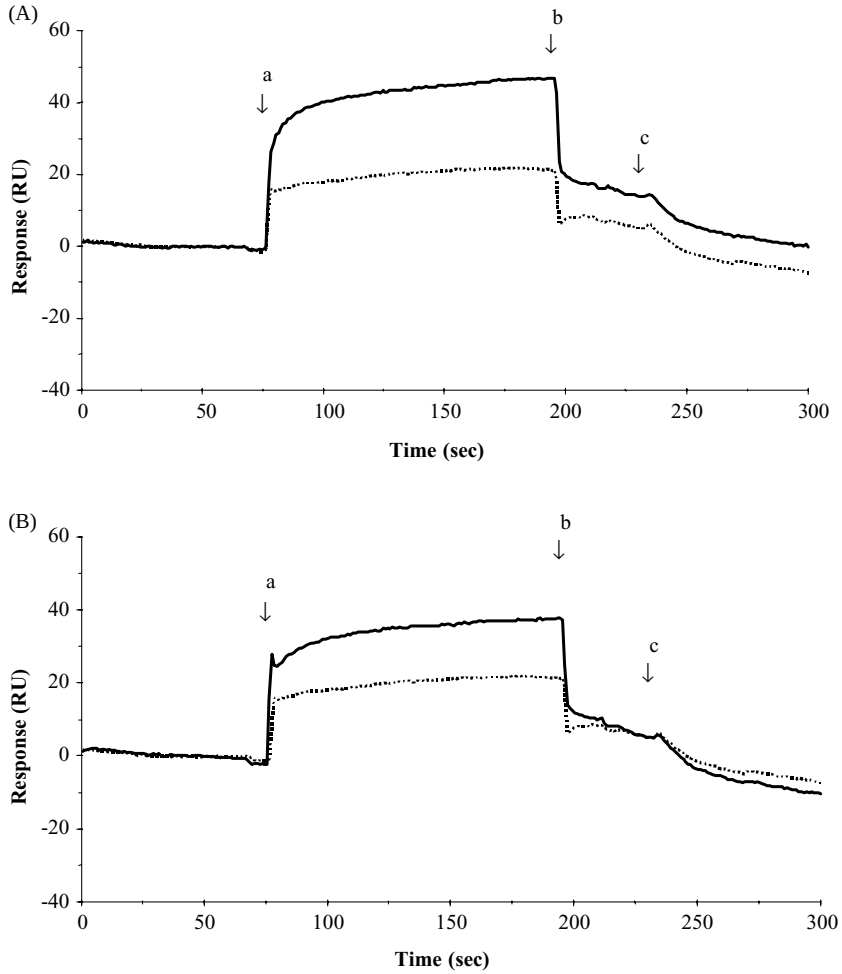


*Fig. 2.* Sensorgrams obtained when pAb-AaGroEL (continuous line) or pre-immune serum (broken line) were injected over a sensor chip with immobilized TGLE (A) or GQLI (B). Phase a corresponds to the injection of sera. Phase b corresponds to the dissociation of antibodies after the end of the injection. Phase c corresponds to the amount of bound antibody detected 30 s after the end of the injection.

dies directed against *A. actinomycetemcomitans* GroEL. The absence of cross-reactivity between pAb-HuFn and *A. actinomycetemcomitans* GroEL may be related to the fact that fibronectin contains a great variety of antigenic determinants with high immunogenicity and that most antibodies against fibronectin are directed against antigenic determinants different from the epitope that pAb-AaGroEL recognizes.

Fibronectin is a multifunctional extracellular matrix protein whose biological effects are mediated through the affinity of its domains for various molecules. Consequently, the reactivity of fibronectin with antibodies could interfere with its natural biological functions. Evidence has been brought forward to support the presence of anti-*A. actinomycetemcomitans* GroEL antibody during periodontitis (2, 14). For instance, Koga et al. (14) reported that sera from 10 healthy individuals did not react with *A. actinomycetemcomitans* GroEL, whereas sera from nine of 29 patients with periodontitis reacted strongly. However, the reactivity of periodontitis serum with human fibronectin needs to be demonstrated. Interestingly, antibodies reacting with fibronectin have been detected in a number of diseases, including systemic lupus erythematosus, rheumatoid arthritis and various bacterial infections (4, 9). These antibodies were found to inhibit collagen/fibronectin and cell/fibronectin interactions (3), a phenomenon that may reduce tissue integrity and affect the repair of tissues damaged by the inflammatory reaction.

On comparing the published amino acid sequence of human fibronectin with that of *A. actinomycetemcomitans* GroEL, eight homologous tetrapeptides were found, including two not present in *E. coli* GroEL, which suggests that these two peptides are likely involved in the cross-reactivity with pAb-AaGroEL. Based on the predicted secondary structure of *A. actinomycetemcomitans* GroEL, the tetrapeptide TGLE was probably the antigenic component. The biospecific interaction analysis performed confirmed an interaction between TGLE and pAb-AaGroEL. Interestingly, small peptides are known to be long enough to induce the production of specific antibodies (7). When they analyzed the amino acid homology of the *P. gingivalis* GroES and GroEL proteins, Hotokezawa et al. (13) found seven homologous regions in type I and type III collagens. Although the homologous amino acid sequences may not necessarily correspond to epitopes recognized by specific antibodies, the homologous sequences may be involved in immunologic reactions that lead to tissue destruction. Additional studies are required to determine how immunoreactivity between tissue proteins and periodontopathogen GroELs contributes to the etiopathogenesis of periodontitis.

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