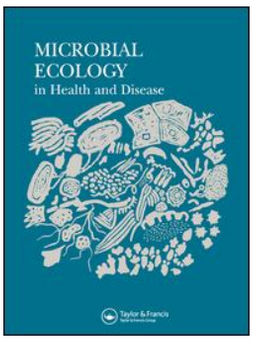

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Immunomodulating Activities of Sodium-dodecyl-sulphate-extracted Antigens from *Actinobacillus actinomycetemcomitans* Serotype b

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Actinobacillus actinomycetemcomitans (serotype b) whole lysates, LPS-free cell wall polymers (CWP) and CWP fractions obtained by immunoblot were assayed for immunomodulating activity. Whole cell lysates and purified CWP stimulated mouse splenocytes to proliferate *in vitro*. Five CWP fractions, ranging from 15 to 209 kDa, suppressed *in vitro* splenocyte proliferation. Fraction-induced inhibition of proliferation was not due to cytotoxicity. Purified *A. actinomycetemcomitans* CWP stimulated murine splenocyte interleukin-1 release during 72 h of *in vitro* culture. Two CWP fractions preferentially stimulated splenocyte interleukin-4 release during *in vitro* culture. This mixed response of CWP-induced splenocyte stimulation and suppression, and IL-1/IL-4 release, is dependent on fragment size. Therefore, *A. actinomycetemcomitans* CWP may play a role in the inflammatory process of periodontal disease as large bacterial fragments stimulate inflammation and smaller, degraded fragments promote anti-inflammatory activities.

KEY WORDS—*Actinobacillus actinomycetemcomitans*; Cell wall polymers; Periodontal disease; Cytokines.

INTRODUCTION

Periodontal disease is a chronic, erosive inflammation of the gingiva, periodontal ligament, alveolar bone and cementum. The gram-negative, coccobacillus, *Actinobacillus actinomycetemcomitans*, is implicated in the aetiopathology of human periodontal disease[2] and integral in an experimental rat model.[35] The mechanism by which *A. actinomycetemcomitans* facilitates periodontal disease is not known. However, several theories have been put forth and include secretion of a leukotoxin,[13] inhibition of host neutrophil chemotaxis[4] and promotion of inflammatory cytokines responding to bacterial antigens.[16] While these are credible mechanisms for acute disease, there is currently no comprehensive study which defines a mechanism for chronic inflammation induced by this bacterium. Hunter and colleagues[11] have shown that periodontitis can be induced experimentally by the injection of streptococcal cell wall polymers (CWP) into rat gingiva. Two other reports suggested a role for bacterial CWP in periodontal disease,[21,33] however, neither study evaluated the CWP role in disease induction.

Several chronic inflammatory diseases of humans have been postulated to have their aetiology in pathogenic bacteria.[20,25,28,29,32] While exact mechanisms are not completely defined in each model, the common factor is the peptidoglycan-containing bacterial CWP. Peptidoglycan (PG) with its species-specific polysaccharide is a ubiquitous bacterial biopolymer. Comparable PG-containing CWP is found in nearly all species of eubacteria. Not only do CWP resist biodegradation but they also induce inflammation of varying chronicity in relation to subtle structural differences.[26] These data suggest that biological activities for CWP resulting in chronic inflammation are correlated with persistance of the antigen.[27] Whole cell lysates of *A. actinomycetemcomitans*-containing CWP and homologous lipopolysaccharide (LPS) have been reported to stimulate

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immunological activity.[15] Other studies have reported the synthesis and secretion of pro-inflammatory cytokines responding to *A. actinomycetemcomitans* antigens *in vivo*[14] and *in vitro*.[19]

Inflammation is orchestrated by opposing up- and down-regulating signals whose relative contribution results in a cyclic process. The normal mechanism is viewed as a dynamic interaction between cellular and humoral factors. Once an inflammatory stimulus is recognised, up-regulating (pro-inflammatory) cytokines (e.g. IL-1, IL-6, TNF, etc.) communicate their presence to the host.[14,34] Cellular degradation of the stimulus with presentation of its processed fragments results in the production of down-regulating (anti-inflammatory) cytokines (e.g. IL-4, IL-10, TGF-β, etc.), which mediate homeostasis.[31,34] This present study describes the immunomodulating activity of LPS-free CWP from *A. actinomycetemcomitans* and addresses its possible role in the chronic inflammation of periodontal disease as mediated by opposing IL-1 and IL-4 cytokines.

MATERIALS AND METHODS

Bacterial conditions and cell wall polymer isolation

Actinobacillus actinomycetemcomitans serotype b, ATCC no. 29522, was cultured in fluid thioglycolate medium as described by Shenker *et al.*[22] Whole cell lysates were prepared from lyophilised bacteria as 10 mg (dry weight) per ml distilled water.[24] Bacterial CWP were obtained according to previously published methods[24] with modifications. Briefly, cell lysates were boiled in 10 mM sterile carbonate buffer (pH 8) containing 1 per cent sodium dodecyl sulphate for 30 min, sonicated (Blackstone Ultrasonicator, Sheffield, PA, USA) at 50 per cent power for 10 min (30 s bursts), and centrifuged at 40 000 *g* for 1 h to isolate the PG-containing cell walls. The pellet was washed twice with sterile phosphate-buffered saline (PBS) and extracted twice into chloroform/methanol (2:1). The CWP were then washed twice with sterile distilled water and lyophilised. CWP suspensions were prepared as approximately 150 mg (dry weight)/ml, which correlated to rhamnose concentrations[5] of 150 μg rhamnose/ml. *A. actinomycetemcomitans* CWP and isolated fractions were tested for LPS by the *Limulus* ameobocyte lysate assay (sensitivity to 0·125 EU/ml; BioWhittaker Inc., Walkersville, MD, USA) and found to be LPS free.

SDS–PAGE and immunoblots

Components of *A. actinomycetemcomitans* CWP were separated by SDS–PAGE according to the method of Laemmli.[12] CWP fragments were electrophoretically transferred to nitrocellulose overnight at 4°C at 16 V as described by Towbin *et al.*[30] Blots were stained with colloidal gold (AuroDye Forte, Amersham International, Amersham, UK) to document protein transfer, or probed with antibody (Western blot) to document CWP transfer. Briefly, Western blots were probed with a polyclonal rabbit anti-CWP (generously provided by Dr R. B. Sartor, University of North Carolina at Chapel Hill, NC, USA), with specificity for *N*-acetyl glucosamine and *N*-acetyl muramic acid.[7] Blots were incubated with a biotin-labelled, goat anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA, USA), avidin-HRP (Zymed) and developed with the hydrogen peroxide substrate containing the 3,3′-diaminobenzidine tetrahydrochloride chromogen (Zymed).

Antigen-bearing nitrocellulose particles

The method of Abou-Zeid *et al.*[1] was used to prepare the CWP fractions. Antigen-bearing nitrocellulose particles were obtained by precipitating DMSO-dissolved blot cut-outs into 0·05 M carbonate buffer (pH 9·6). Non-antigen-bearing nitrocellulose cut-outs derived from blank gels were prepared in the same manner to serve as controls. Precipitates were washed three times with PBS and resuspended into tissue culture medium or PBS for respective bioassays.

Splenocyte proliferation

The method was that of Paquet *et al.*[18] Twenty microlitres per well of *A. actinomycetemcomitans* whole cell lysates (10 mg/ml), CWP (146 mg/ml) or CWP fractions (containing various protein and rhamnose concentrations) were added to 4×10^5 splenocytes from male C3H/Hen mice in triplicate wells of 96-well plates. Three micrograms per well Con A (Sigma Chemical Co., St Louis, MO, USA), 5 μg/well *Escherichia coli* 0127:B8 LPS (Sigma) or tissue culture medium was added to triplicate wells as controls. LPS was boiled for 1 h to remove any contaminating endotoxin-protein. Tritiated thymidine (1 μCi/well, specific activity 20 Ci/mM) was added during the last 6 h of the 72 h incubation. The cells were then harvested onto glass fibre filters via a cell harvester (Brandel; Rockville MD, USA), and radioactivity measured

by liquid scintillation spectrophotometry. A stimulation index was determined by dividing the average counts per min (CPM) of the experimental wells by the average CPM of cells incubated in medium alone. Statistical significance was determined by nested ANOVA and the Kruskal–Wallis test.

Antigen cytotoxicity assay

To evaluate the cytotoxicity of *A. actinomycetemcomitans* CWP, a modification of a mouse fibroblast lysis assay was used.[9] L929 fibroblasts are anchorage-dependent cells and viable cells adhere to the plastic well. Toxicity by these bacterial products would result in a measurable loss of fibroblast adherence as measured by dye absorbance after 18 h of culture. Sterile distilled water served as the positive lysis control and tissue culture medium was the negative lysis control.

Cytokine assays

Splenocytes from male C3H/Hen mice were aseptically isolated and cultured as 8×10^5 cells in wells of 24-well plates containing tissue culture medium. *A. actinomycetemcomitans* CWP, CWP fractions (antigen-bearing nitrocellulose) or controls were added as 200 µl/well in a final volume of 1 ml and cultured for 24, 48 or 72 h at 37°C in 5 per cent CO_2. Murine IL-1 and IL-4 were measured by ELISA as described by the manufacturers using kits purchased from Genzyme Inc. (Cambridge, MA, USA) and Endogen Inc. (Boston, MA, USA), respectively. ELISAs were specific for the measurement of murine IL-1 and IL-4, with sensitivities of 15 pg/ml and 5 pg/ml, respectively.

RESULTS

Splenocyte proliferation

Whole cell lysates and purified CWP from *A. actinomycetemcomitans* were found to induce significant proliferation of mouse splenocytes after 3 d of incubation, i.e. stimulation indices demonstrated that *A. actinomycetemcomitans* whole cell lysates and LPS-free CWP were mitogenic to murine splenocytes, to the same degree as commercial *E. coli* LPS (Table 1).

Table 1. Proliferation of splenocytes from C3H/Hen mice responding to mitogens or cell wall polymers (CWP) from *A. actinomycetemcomitans*

Agent	CPM (mean ± SD)*	SI†
Medium control	2 639 ± 319	1·00
Con A	81 809 ± 4154‡	31·00
E. coli LPS	31 950 ± 1241‡	12·11
Whole cell lysate	35 492 ± 3158‡	13·45
Purified CWP	27 477 ± 38·37‡	10·41

*Mean ± 1 standard deviation for triplicate samples.
†Stimulation index (see methods for derivation).
‡$P<0·001$ by the Kruskal–Wallis test compared with medium control.

Isolation of CWP fragments

Separation of CWP and preparative analysis of the fragments was accomplished with SDS–PAGE and immunoblot. Figure 1 shows the protein-stained immunoblot (Lane A) and the peroxidase-stained Western blot (Lane B). Of note are the many bands which stain with colloidal gold (Lane A) but not with antibody to CWP (Lane B). We excised five fractions from unstained blots (indicated as numbers 1–5 next to Lane B of Figure 1) to evaluate the immunomodulating abilities of these specific molecular weight fragments. The molecular weights of the five fractions were 209, 59, 50, 17 and 15 kDa, respectively.

Antigen-bearing nitrocellulose particles

Antigen-bearing nitrocellulose particles from the five excised fractions were cultured for 3 d (as above) with splenocytes from C3H/Hen mice. Table 2 shows the marked inhibition of splenocyte proliferation due to these *A. actinomycetemcomitans* antigen-containing particles compared with non-antigen-bearing nitrocellulose particles ($P<0·05$).

Cytotoxicity of CWP

The data evaluating immunoblot fractions for cytotoxicity are reported in Table 3. These data indicate that the fractions were not toxic in this assay as compared with water controls ($P<0·05$); in fact, the data suggest that the fractions stimulated L929 growth as compared with medium controls ($P<0·05$). In separate experiments, non-antigen-bearing nitrocellulose particles were not toxic and not stimulatory to L929 cells in culture, yielding staining profiles similar to cells exposed to culture medium alone (data not shown).

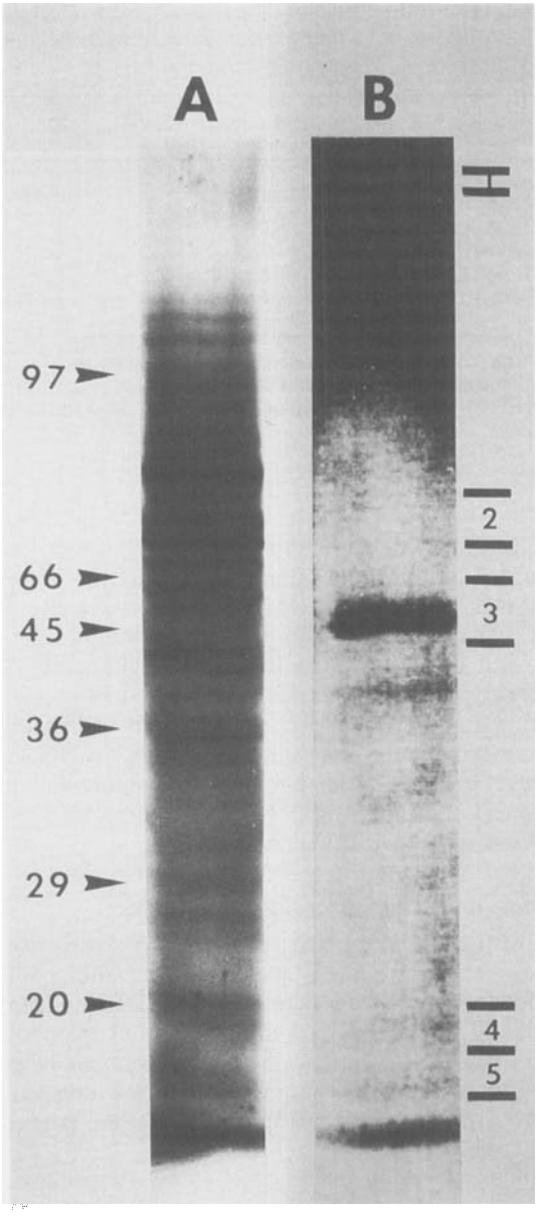

Figure 1. Immunoblots obtained from an SDS–PAGE gel separating *A. actinomycetemcomitans* CWP. Lane A is a blot stained with colloidal gold to visualise proteins. Lane B is a blot stained with peroxidase-conjugated rabbit anti-CWP to visualise cell wall fragments. Molecular weight markers are at the left with units in kilodaltons (kDa). Fractions extracted for bio- and immuno-assays are indicated by numbers on the right

Table 2. Proliferation of splenocytes from C3H/Hen mice responding to cell wall polymer fraction particles from *A. actinomycetemcomitans*

Agent	CPM (mean ± SD)*	SI†
Nitrocellulose control	2623 ± 437	1·00
Medium control	2521 ± 272	0·96
Fraction		
1	1889 ± 320‡	0·72
2	1919 ± 156‡	0·73
3	1452 ± 91‡	0·55
4	1600 ± 192‡	0·61
5	1308 ± 129‡	0·50

*Mean ± 1 standard deviation for triplicate samples.
†Stimulation index (see Methods for derivation).
‡$P<0·05$ by the Kruskal–Wallis test compared to nitrocellulose control.

Table 3. Effect of *A. actinomycetemcomitans* cell wall polymer fractions on toxicity of L929 fibroblasts measured by dye absorption

Fraction	OD_{590}*
1	0·280 ± 0·044†‡
2	0·102 ± 0·005†‡
3	0·196 ± 0·029†‡
4	0·263 ± 0·051†‡
5	0·338 ± 0·115†‡
Medium control	0·090 ± 0·018‡
Water control	0·008 ± 0·004†

*Mean value of triplicate samples ± 1 standard deviation.
†$P<0·05$ by Student's *t*-test compared with medium control.
‡$P<0·05$ by Student's *t*-test compared with water control.

Cytokine release by splenocytes

Figure 2 shows the release of IL-1 from cultured C3H/Hen splenocytes over 3 d of incubation. Only the intact *A. actinomycetemcomitans* CWP product stimulated a constant release of IL-1 over the 72 h. Fraction 1 stimulated IL-1 release only after 72 h. Nitrocellulose controls and other fractions did not stimulate IL-1 release at any of the time points. Fractions 1 and 3 were the only products which stimulated the release of IL-4 from cultured C3H/Hen splenocytes, and only at the 72 h time point (Figure 3).

DISCUSSION

Several investigators have reported elevated *A. actinomycetemcomitans*-specific antibody titres in sera, saliva and crevicular fluid of periodontitis patients.[6,8,10] Further, Zambon and colleagues[36]

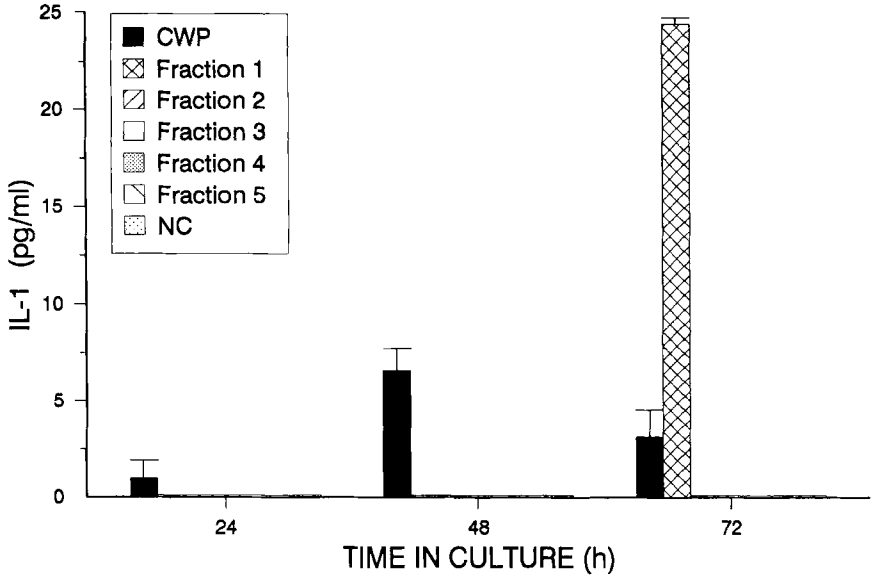

Figure 2. IL-1 released from C3H/Hen splenocytes *in vitro*. *A. actinomycetemcomitans* CWP or homogenously sized fractions obtained from nitrocellulose (see Methods for details) were cultured with splenocytes for 3 d. IL-1 was measured by ELISA at 24, 48 and 72 h. Values represent the mean ± SD of duplicate samples. NC, nitrocellulose control

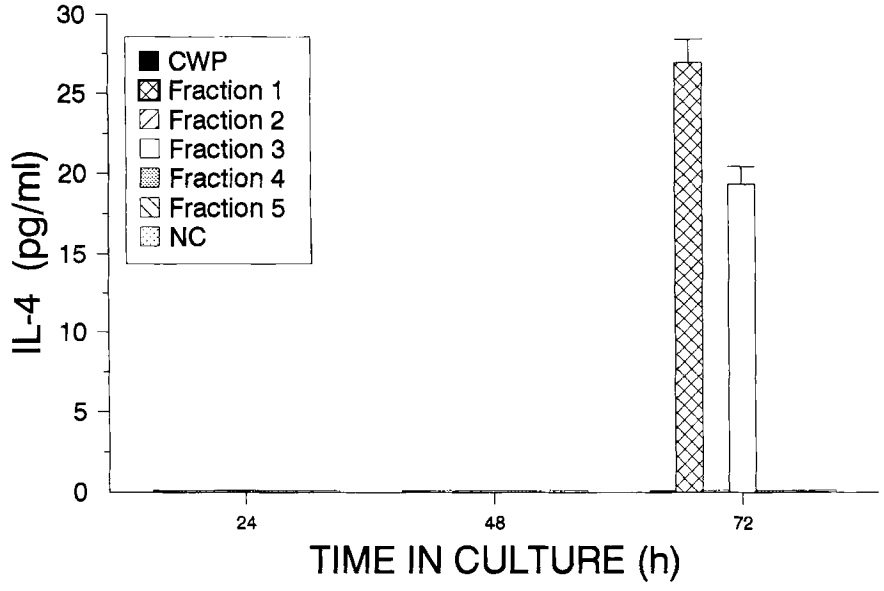

Figure 3. IL-4 release from C3H/Hen splenocytes *in vitro*. *A. actinomycetemcomitans* CWP or homogenously sized fractions obtained from nitrocellulose (see Methods for details) were cultured with splenocytes for 3 d. IL-4 was measured by ELISA at 24, 48 and 72 h. Values represent the mean ± SD of duplicate samples. NC, nitrocellulose control

have shown that sonicated extracts of whole *A. actinomycetemcomitans* cells contain common antigens to which antibodies between *A. actinomycetemcomitans* serotypes and other *Actinobacillus* and *Haemophilus* species react. More recently, several reports have identified carbohydrates in

serotype-specific LPS of *A. actinomycetemcomitans* as the immunodominant antigens to which antibodies from periodontitis patients react.[3,17,24] These data suggest that serotype-specific polysaccharides of this organism are potent antigens in periodontal disease. Carbohydrate antigens could also be complexed with the peptidoglycan in the CWP as well as with the LPS. It is therefore quite plausible that serotype-specific CWP are strongly antigenic and also serve as their own adjuvant in the development of chronic inflammation. Our data support this hypothesis by demonstrating that heterogeneous, LPS-free CWP from *A. actinomycetemcomitans* are mitogenic to murine splenocytes. The stimulation indices for whole cell lysate and the purified CWP were comparable to the level of stimulation produced by the B-cell mitogen, LPS. However, this activity must be due to a component other than LPS, since our *A. actinomycetemcomitans* CWP contained no LPS as measured by the *Limulus* test.

A. actinomycetemcomitans CWP fractions were identified by the polyclonal antibody to group A streptococcal CWP. This antibody has specificity to six known epitopes of group A streptococcal CWP including the *N*-acetyl muramic acid, *N*-acetyl glucosamine, and D-Ala-D-Ala peptide side-chain common to most bacterial species.[7] Western analysis revealed several fractions which contained CWP but no protein, e.g. fraction 1. Other fractions contained both CWP and protein (e.g. fractions 3, 4 and 5) in varying amounts. We chose to evaluate five fractions representing the various combinations of protein and CWP content for their ability to stimulate splenocyte proliferation and induce splenocyte production of IL-1 and IL-4. Each fragment appeared to contain a unique combination of protein and CWP, reflecting its 'native' character. The relative ratio of protein and CWP confers to each fragment a specific antigenicity (molecular conformation). Thus, we did not 'standardise' the fractions by adjusting them to a uniform protein or CWP concentration. Rather, we presented the fractions to splenocytes as a uniform volume, preserving antigenicity.

While splenocytes proliferated significantly in response to whole cell lysates of *A. actinomycetemcomitans* and LPS-free CWP, they were inhibited from proliferating by CWP fractions. Responses to Con A and LPS indicated that the cells were capable of responding if stimulated. The lowered proliferative response resulting from incubation with CWP fractions could have resulted from direct fraction-induced cytotoxicity or fraction-induced suppression of splenocyte proliferation. The results of the L929 bioassay show that the CWP fractions were not toxic, but stimulated fibroblast growth. Thus, homogeneously sized, low molecular weight antigens of *A. actinomycetemcomitans* CWP suppressed splenocyte proliferation, consistent with observations that *A. actinomycetemcomitans* antigens can be immunosuppressive.[22,23]

Immunological responses of mammals clearly result from dynamic interactions between cells and their secreted cytokines. While a complete cytokine profile resulting from *A. actinomycetemcomitans* CWP stimulation of immunocytes would help characterise the disease process, this report evaluates the effect of *A. actinomycetemcomitans* CWP on the production and release of only two cytokines. We measured the release of IL-1 and IL-4 from normal mouse splenocytes exposed to *A. actinomycetemcomitans* CWP and CWP fractions to correlate their production with the molecular weight of *A. actinomycetemcomitans* CWP fragments. Of interest is the fact that the heterologous CWP stimulated IL-1 continuously over 72 h in culture. IL-4 was produced between 48 and 72 h and only by two of the smaller molecular weight fragments.

The data herein suggest that a CWP fragment size of approximately 200 kDa is the transition size for stimulating opposing biological activity. For example, heterologous CWP fragments mostly larger than 200 kDa are pro-inflammatory, stimulating splenocyte proliferation and IL-1 release. Fragments less than 200 kDa inhibit splenocyte proliferation and, depending on their CWP content, stimulate IL-4 release. Fragments near this transition size (i.e. fragment 1) have overlapping activities. These results are consistent with, and extend, the data of Stimpson *et al.*[27] correlating CWP degradation with chronicity of inflammation. Further, our results are supported by the recovery of pro- and anti-inflammatory cytokines from patients with periodontal disease.[14]

We conclude that the intact CWP from *A. actinomycetemcomitans* stimulate pro-inflammatory mechanisms, while anti-inflammatory mechanisms are induced by smaller CWP fragments. This suggests that substantial processing and degradation of *A. actinomycetemcomitans* CWP is required for resolution of inflammation induced by this bacterial product.

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