Virulence factors of *Actinobacillus actinomycetemcomitans* relevant to the pathogenesis of inflammatory periodontal diseases

M. Wilson a,*, B. Henderson b

a Department of Microbiology Eastman Dental Institute for Oral Health Care Sciences, University of London, 256 Grays Inn Road, London WC1X 8LD, UK

b Maxillofacial Surgery Research Unit, Eastman Dental Institute for Oral Health Care Sciences, University of London, 256 Grays Inn Road, London WC1X 8LD, UK

Received 10 January 1995; revised 6 June 1995; accepted 15 June 1995

Abstract

There is strong evidence implicating *Actinobacillus actinomycetemcomitans* as the causative agent of localised juvenile periodontitis (LJP), a disease characterised by rapid destruction of the tooth-supporting tissues. This organism possesses a large number of virulence factors with a wide range of activities which enable it to colonise the oral cavity, invade periodontal tissues, evade host defences, initiate connective tissue destruction and interfere with tissue repair. Adhesion to epithelial and tooth surfaces is dependent on the presence of surface proteins and structures such as microvesicles and fimbriae. Invasion has been demonstrated in vivo and in vitro although the mechanisms involved are poorly understood. The organism has a number of means of evading host defences which include: (i) inhibiting polymorphonuclear leukocyte (PMN) chemotaxis; (ii) killing PMNs and monocytes; (iii) producing immunosuppressive factors; (iv) secreting proteases capable of cleaving IgG; and (v) producing Fc-binding proteins. Surface components of *A. actinomycetemcomitans* are potent stimulators of bone resorption and can induce the release of a range of cytokines which can initiate tissue destruction. A number of surface components can also inhibit the proliferation of fibroblasts and their production of components of the extracellular matrix. Little is known, however, regarding the way in which these factors operate in vivo to produce the pathological features of the disease.

Keywords: *Actinobacillus actinomycetemcomitans*; Virulence factors; Periodontitis

Contents

1. Introduction ............................................................... 366
   1.1. Morphology ............................................................ 366
   1.2. Physiology ............................................................ 367
   1.3. Habitat ............................................................... 367
   1.4. Association with disease .................................................... 367

* Corresponding author. Tel.: +44 (171) 915 1231; Fax: +44 (171) 915 1127.
2. Colonisation and invasion ...................................................... 368
   2.1. Colonisation .................................................................. 368
   2.2. Invasion ..................................................................... 369

3. Interference with host defences ................................................... 369
   3.1. Effects on polymorphonuclear leukocytes (PMNs) .................. 369
      3.1.1. Inhibition of PMN chemotaxis .................................. 369
      3.1.2. Resistance to killing ............................................. 370
      3.1.3. Production of Fc-binding proteins ............................... 370
      3.1.4. Leukotoxin ......................................................... 370
   3.2. Production of immunoglobulin-degrading proteases ............... 371
   3.3. Immunosuppressive effects ............................................. 371

4. Destruction of host tissues ...................................................... 372
   4.1. Bone loss .................................................................... 372
   4.2. Collagenase production .................................................. 373
   4.3. Acid/alkaline phosphatases ............................................. 373
   4.4. Release of inflammatory mediators ................................... 373
      4.4.1. Monocytes/macrophages ......................................... 373
      4.4.2. Fibroblasts ......................................................... 374

5. Inhibition of tissue repair ....................................................... 374
   5.1. Inhibition of cell proliferation ......................................... 374
   5.2. Inhibition of macromolecule synthesis by fibroblasts ............. 375
   5.3. Inhibition of bone formation .......................................... 375

6. Conclusions .......................................................................... 375

References ............................................................................. 376

1. Introduction

Actinobacillus actinomycetemcomitans is a Gram-negative, non-spore forming, non-motile, facultatively anaerobic coccobacillus. It was first described by Klinger in 1912 and named Bacterium actinomycetem-comitans because it was frequently found with Actinomyces israelii in actinomycotic lesions.

1.1. Morphology

When freshly isolated, colonies are small (1.0 mm diameter), convex, grey, translucent, smooth and non-haemolytic. The colonies also have a characteristic crossed-cigar appearance internally and adhere to the agar surface. The cells are straight or curved rods with rounded ends (1.0–1.5 μm by 0.4–0.5 μm) and occur singly, in pairs or in small clumps. Electron microscopy reveals cells with a characteristic Gram-negative cell wall structure, Ruthenium red-staining surface-associated material, surface fibrils and outer membrane-bound vesicles [1]. These vesicles can be shed from the cell surface and appear to be derived from the outer membrane by evagination as they have been shown to contain lipopolysaccharide and outer membrane proteins [2]. Among the surface polymers are the serotype-determining polysaccharides which has enabled the differentiation of the species into 5 serotypes (a–e). The chemical structures of some of these polysaccharides have been determined [3] and have been shown to consist of deoxy-D-talan (serotype a), a rhamnose/fucose disaccharide repeating unit (serotype b) and deoxy-L-talan (serotype c). Freshly isolated strains also have been shown to have fimbriae (consisting of 54-kDa protein subunits) which are generally lost on sub-culture [4,5].
1.2. Physiology

*Actinomycetemcomitans* has been variously described as being microaerophilic, capnophilic and facultatively anaerobic. However, there is general agreement that it grows best in an aerobic atmosphere enriched with 5–10% CO₂. The nutritional requirements of the organism have not yet been defined, but it grows well on blood or serum-containing media. Sreenivasan et al. [6] have reported that growth is stimulated by L-cystine, thiamine and by various steroid hormones including oestrogen, progesterone and testosterone. These investigators also found that optimum growth occurred over the pH range 7.0–8.5. The optimum temperature for growth is 37°C. *Actinomycetemcomitans* can ferment a number of sugars, including glucose and fructose, and 10 biotypes can be distinguished on the basis of their sugar fermentation patterns [7].

1.3. Habitat

*Actinomycetemcomitans* has been isolated from the oral cavity of man and a number of primates as well as from other mammals. Although its primary habitat within the oral cavity has not been definitively identified, evidence suggests that this is most probably dental plaque from the gingival crevice, i.e. the region where the gum meets the tooth [8]. Hence, it is not found in the oral cavity of edentulous individuals, whether infants or adults [9,10]. Its presence at other sites within the oral cavity, such as the buccal mucosa and tongue, could well be the result of its carriage from the gingival crevice into saliva by an exudate (gingival crevicular fluid) from this region, the organism could then be distributed to all parts of the mouth [11]. Although the organism is detected more frequently, and in higher numbers, in the oral cavity of patients with localised juvenile periodontitis (LJP; see below) than in LJP-free individuals, a large proportion of the latter do harbour the organism [7]. For example, Zambon et al. [12] found that the organism was present in plaque taken from the gingival crevice of 19% of periodontally healthy individuals. Furthermore, Gmur and Guggenhein [13] isolated the organism from the supragingival, interdental plaque of one-third of 21 periodontally healthy individuals. Current information, therefore, tends to support the view that the organism is an amphibiotic opportunistic pathogen.

Little is known about the transmission of the organism between hosts. Evidence of spread among members of the same family has been provided by Zambon et al. [12] who found, in a study involving 5 families, that each infected member within a family had the same *Actinomycetemcomitans* serotype and biotype. The existence of infected and non-infected members within a family implies that the organism is not easily transmissible. Such studies will be aided by some of the newer typing methods being developed. For example, using the arbitrarily primed polymerase chain reaction, 17 genotypes have been recognised among 73 clinical isolates of the organism [14], while 24 ribotypes have been identified among 32 strains [15].

1.4. Association with disease

*Actinomycetemcomitans* has been reported to be the causative agent of a number of infections including bacterial endocarditis, meningitis, septicaemia, abscesses and osteomyelitis [16,17]. However, the most prevalent diseases with which this organism is associated are those affecting the oral cavity: LJP and adult periodontitis. As the name implies, LJP is a disease affecting the periodontal tissues which consist of the gingiva (i.e. the gums), the periodontal ligament (which attaches the gingiva to the roots of the teeth) and the alveolar bone (which supports the teeth). The disease involves inflammation of the gingiva and destruction of the periodontal ligament and alveolar bone, resulting in tooth loss. This description could apply to any of the many forms of inflammatory periodontal disease, but LJP is distinguished from these by three additional characteristics: only the permanent incisors and first molars are affected, the age of onset is between 10 and 14 years and the inflammation is usually only mild. The prevalence of the disease shows marked geographical variations, with 0.1% and 17.6% of adolescents affected in Scandinavian countries and in Bombay, respectively [18]. Disease prevalence also shows racial variation, with one study in the USA reporting that 2.05% of black children and 0.13% of white children were affected [19].

Although there is considerable evidence suggest-
ing that *A. actinomycetemcomitans* is the main causative organism of LJP, other bacteria have also been implicated in the aetiology of the disease, including *Eikenella corrodens*, *Capnocytophaga ochracea* and *Prevotella intermedia* [18,20].

Apart from its involvement in LJP, there is also increasing evidence to support an aetiological role for *A. actinomycetemcomitans* in other forms of periodontal disease, particularly adult periodontitis [20,21].

Now that *A. actinomycetemcomitans* has been recognised as a major periodontopathogenic organism, there is great interest in determining and characterising those attributes of the organism which enable it to colonise the oral cavity, invade the periodontal tissues, overcome the host’s defences, induce tissue destruction and be transmitted to a new host. Although studies have shown that *A. actinomycetemcomitans* can be transmitted between human hosts [12], nothing is known of the properties of the organism that aid in such transmission. This review will, therefore, be confined to describing those virulence factors of the organism involved in colonisation, invasion, combatting host defences, inducing tissue destruction and inhibiting tissue repair.

## 2. Colonisation and invasion

### 2.1. Colonisation

The first stage in any infectious process involves adhesion of the microorganism to some host tissue, either directly or via some intermediary microorganism. In the case of *A. actinomycetemcomitans* this could involve adhesion to any of a variety of epithelial surfaces, to the tooth surface or to bacteria already colonising these surfaces.

Meyer and Fives-Taylor [22] have investigated the role of extracellular components on the adherence of *A. actinomycetemcomitans* to a human oral epithelial cell line. Certain strains of the organism were found to have copious surface-associated material and when this was removed (by washing with phosphate-buffered saline; PBS) adherence to the epithelial cells was reduced by 50%. When this PBS extract, which consisted mainly of proteins, was added to strains of the organism which were ordinarily devoid of surface-associated material their adherence to epithelial cells was enhanced. As mentioned previously, certain strains of *A. actinomycetemcomitans* produce extracellular microvesicles. When these microvesicles were harvested and added to non-vesicle-producing strains, there was a 4.5-fold increase in the adherence of the bacteria to epithelial cells. That proteins are involved in adhesion to epithelial cells is supported by the finding of Mintz and Fives-Taylor [23] that pre-treatment of the organism with trypsin reduced the number of bacteria able to adhere to KB cells (derived from a human oral epidermoid carcinoma) by approximately 50%. As further incubation with trypsin did not reduce the numbers of adherent bacteria, adherence of these cells may have been mediated by either a protease-insensitive protein(s) or by non-proteinaceous molecules. Alternatively, there may be phase variation in the adhesive structures of the organism. Fimbriae, detected on four of five clinical isolates [24], may also aid in adhesion to epithelial cells, and Rosan et al. [25] have shown that a fimbriated strain has a greater ability to adhere to a mouse epithelial cell line than does a non-fimbriated strain. Interestingly, treatment of human buccal epithelial cells with enzymes likely to be present as a result of poor oral hygiene (e.g. lysosomal enzymes from polymorphonuclear leukocytes; PMNs) enhanced attachment of *A. actinomycetemcomitans* but diminished adhesion of *Streptococcus mitis*, an organism associated with gingival health [26].

*A. actinomycetemcomitans* has also been shown to adhere to saliva-coated human enamel to a greater extent than nine other Gram-positive and Gram-negative oral species [27]. Such attachment may be mediated by fimbriae, as Rosan et al. [28] showed that fimbriated clinical isolates exhibit greater ability to adhere to saliva-coated hydroxyapatite than non-fimbriated variants.

The basement membrane beneath the sulcular epithelium is the last barrier to bacterial translocation into the underlying connective tissue; therefore, the ability of periodontopathogenic bacteria to adhere to, and penetrate, this membrane, is of obvious interest. Winkler et al. [29] have investigated the adherence of *A. actinomycetemcomitans* to a basement membrane-like matrix. The matrix was obtained by growing embryonic carcinoma cells (PF HR-9) in plastic wells
pre-coated with bovine plasma fibronectin and then lysing and removing the cells. *A. actinomycetemcomitans* was able to bind to this matrix in high numbers and also to the isolated components of the matrix: fibronectin, laminin and type IV collagen. No definitive conclusions could be drawn regarding the identity of the component to which the organism adhered in the intact matrix, although it was found that anti-fibronectin antibodies did not inhibit binding of the bacteria to the intact matrix. The ability of the organism to actually penetrate the matrix was not determined.

No studies have been reported concerning the ability of *A. actinomycetemcomitans* to adhere to other oral bacteria, which is unfortunate as this is known to be an important means by which other organisms maintain themselves in the oral cavity [30].

### 2.2. Invasion

Once colonisation has taken place, there is some evidence to suggest that *A. actinomycetemcomitans* can invade the tissues of the periodontium. In 1982, Saglie et al. [31] detected the presence of antigens from the organism in gingival tissues. Subsequently, using polyclonal rabbit antibodies against the three known serotypes of the organism, bacterial antigens were detected by immunofluorescence in 11 of 12 gingival biopsies from LJP patients. Positive staining, both intra- and extra-cellularly, was found in subepithelial tissue, whereas no staining was found in two biopsies from healthy controls and one from a case of adult periodontitis [32]. Furthermore, the organism was cultured from 8 of the 11 biopsies following surface disinfection and mincing of the tissues [33].

The results of several in vitro studies also demonstrate that *A. actinomycetemcomitans* has invasive capabilities. Meyer et al. [34] reported that 24% of 42 strains of the organism could invade KB cells (without affecting their viability) and, to a lesser extent, human laryngeal and intestinal epithelial cells. The ability to invade was strain-dependent with the most invasive being as invasive as entero-invasive *Escherichia coli*. Subsequently, these workers reported that invasion was prevented by inhibition of bacterial protein synthesis, bacterial energy production and receptor-mediated endocytosis [35]. Another epithelial cell line susceptible to invasion by *A. actinomycetemcomitans* is HeP-2 (derived from an epidermoid carcinoma of the larynx) from which viable bacteria could be recovered [36].

The ability of the organism to invade the tissues of the periodontium may explain, in part, the difficulties encountered in eradicating it from diseased sites by mechanical means alone — the mainstay of conventional therapy for the periodontal diseases [37].

### 3. Interference with host defences

#### 3.1. Effects on polymorphonuclear leukocytes (PMNs)

Phagocytic cells constitute the host’s first line of parenteral defence against bacteria and the outcome of the phagocyte/bacterium interaction is of great importance in the establishment of an infection. Bacteria have evolved a number of strategies for combating this threat to their survival and many of these are exhibited by *A. actinomycetemcomitans*.

**3.1.1. Inhibition of PMN chemotaxis**

PMN chemotaxis in vivo involves: (i) binding of chemotactic signals (e.g. interleukin (IL)-8 or other chemokines); (ii) upregulation (phosphorylation) of β2 integrin adhesion receptors; and (iii) binding to vascular endothelium via intercellular adhesion molecule (ICAM) or E-selectin receptors and the eventual diapedesis of cells into the underlying tissue. Many cellular biochemical activation processes occur during this process to prime the cell for dealing with bacteria. Van Dyke et al. [38] reported that a low molecular mass compound secreted into the culture supernatant (but not a sonic extract of the organism) inhibited PMN chemotaxis towards the bacterial chemotactic peptide formyl-Met-Leu-Phe (FMLP) at a concentration of about 1 μg/ml. This bacterial component did not interfere with phagocytosis and was not cytotoxic, but functioned by inhibiting binding of FMLP to PMN receptors. Pretreatment of PMNs with the supernatant from sonicated *A. actinomycetemcomitans* was found to decrease PMN chemotaxis to FMLP by 38%. Unfortu-
nately, the lowest concentration tested was 15 μg/ml — a concentration unlikely to be achieved in the periodontal pocket or within the periodontal tissues. The activity was attributable to a protein, because of abrogation of activity by treatment with proteinase K [39]. The mechanism did not involve a decrease in FMLP receptors, inhibition of F-actin polymerisation or a reduction in the expression of CD11b/CD18 or the selectin Gp110.

3.1.2. Resistance to killing

Lysosomes of PMNs contain a variety of antibacterial agents including hydrogen peroxide, defensins, lysozyme, elastase, azurocidin and proteinase 3. Interfering with the bactericidal activity of PMNs could be achieved by inhibition of the ability of the PMNs to produce such compounds or by an intrinsic resistance to them. There is evidence to suggest that both mechanisms are operative in the case of A. actinomycetemcomitans. Ashkenazi et al. [40] reported that a heat-stable protein from the organism inhibited the protein kinase C-dependent production of H₂O₂ by PMNs stimulated with phorbol myristate. The protein had a molecular mass greater than 10 kDa and was active at between 1.5 and 15 μg/ml.

Sela [41] showed that the organism was only partially susceptible to lysis by extracts of peripheral blood leukocytes even at concentrations as high as 100 μg/ml, while Miyasaki et al. [42] demonstrated that many strains were resistant to H₂O₂ concentrations ten-fold greater than those found within phagolysosomes.

Non-oxidative killing of bacteria by neutrophils is mediated, in part, by cationic peptides known as defensins which comprise 30–50% of the azurophil granule content of human neutrophils. A. actinomycetemcomitans has been shown to be resistant to a mixture of the three defensins (HNP-1 –2 and -3) found in human neutrophils and also to the individual peptides at concentrations as high as 500 μg/ml [43]. This group has also reported that the organism is resistant to other components of neutrophil granules, including proteinase 3, azurocidin, lysozyme and elastase [44]. The mechanism(s) responsible for this resistance to phagocyte products is not clear.

3.1.3. Production of Fc-binding proteins

Phagocytosis of antibody-coated bacteria is an important means by which the host eliminates infecting bacteria and involves binding of the Fc region of the antibody to specific PMN receptors. Tolo and Helgeland [45] have shown that A. actinomycetemcomitans releases proteins capable of binding to the Fc region of human IgG. These could bind to the Fc region of opsonising antibodies, preventing attachment to PMN Fc receptors, thereby inhibiting phagocytosis. However, the authors failed to demonstrate that this occurred in vitro, possibly because of the low concentrations of the Fc-binding proteins in the preparations used.

3.1.4. Leukotoxin

The most extensively studied virulence factor of A. actinomycetemcomitans is its leukotoxin. Zambon et al. [46] reported that 55% of A. actinomycetemcomitans strains from LJP patients (and also 16% from periodontally healthy controls) produce a leukotoxin able to lyse human neutrophils, monocytes and a subpopulation of lymphocytes. It is not released into culture supernatants but remains associated with the outer membrane (and in membranous vesicles) by means of a hydrophobic domain at its carboxyl end [47,48]. The toxin is a 116-kDa pore-forming, basic protein that destroys target cells by osmotic lysis [49] and its ED₉₀ is 4 ng/ml for human PMNs [50].

The leukotoxin is encoded by an operon consisting of four genes, ItxC, ItxA, ItxB and ItxD. The ItxA gene encodes the leukotoxin itself, the ItxC gene may encode for an acyl carrier protein involved in acylation of the protein while the remaining two genes are probably involved in the transport of the toxin and its insertion into the membrane [51]. Non-leukotoxic strains have been found to contain all four genes of the Itx gene cluster. Leukotoxin-positive strains produce two mRNA transcripts encoding ItxCABD and ItxCA, whereas leukotoxin-negative strains produce lower levels of ItxCA and no ItxCABD [52]. Therefore, toxin regulation appears to be at the level of mRNA transcription or stability. More recently, in an analysis of 17 strains of the organism, Brogan et al. [51] have shown that the structure of the Itx promoter region of highly leukotoxic strains is similar and differs from that of non-leukotoxic strains. There is also evidence suggesting that leukotoxin production is under the control of environmental factors as it has been shown that the levels of Itx
mRNAs are reduced in cells approaching the stationary phase of growth [52].

The leukotoxin is known to form pores in the membrane of target cells and it is known that this is dependent on the binding of the toxin to the target cell membrane [53]. Although binding of the toxin to cells can be inhibited by phospholipids, the nature of the interaction between the toxin and the target cell membrane remains to be elucidated [54]. The leukotoxin has been shown to be a calcium-binding protein and it has been suggested that Ca\(^{2+}\) ions might link negatively charged regions of the molecule to a cell surface receptor, or that the Ca\(^{2+}\) ions bind to the toxin and induce a conformational change enabling it to bind to the surface of the target cell [55,56].

In addition to the leukotoxin, there is evidence to suggest that other components of *A. actinomycetemcomitans* are able to lyse PMNs. Brogan et al. [51,52] have reported that the ED\(_{50}\) for human PMNs of a ‘non-leukotoxic’ strain of the organism (NCTC 9710) is lower than that of a leukotoxic strain (Y4). This supports our finding that material obtained by saline extraction of strain NCTC 9710 was highly cytotoxic (ED\(_{50}\) < 1.0 ng/ml) to human PMNs [57]. Electron microscopic examination of the cells prior to, and after, extraction with saline revealed that the extract contained material from the surface of the cells and so was designated surface-associated material (SAM). This material had a high protein content (70%), very little LPS (< 10 ng/mg) and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the presence of more than 40 Coomassie brilliant blue-stained spots.

As well as interfering with the host defence system, killing of phagocytic cells will release a variety of enzymes and reactive molecules which can result in tissue damage.

### 3.2. Production of immunoglobulin-degrading proteases

IgG, IgM and IgA antibodies can exert a protective function by neutralising toxins and enzymes, by inducing complement-mediated cytolysis, agglutination and opsonisation and by preventing attachment of bacteria to host tissues.

Gregory et al. [58] showed that the supernatant from cultured *A. actinomycetemcomitans* cleaved IgG, serum (but not secretory) IgA and IgM, but not IgD and IgE. They also found that saliva from LJP patients had significantly higher IgG and IgA proteolytic activity than healthy controls. Bacterial Ig proteases, therefore, are important virulence factors which act by cleaving protective antibodies in crevicular fluid and saliva. Furthermore, the cleaved antibodies may retain antigen-binding ability which, by binding to the bacteria, could prevent attachment of any fully functional, intact antibodies that may be present, hence reducing opsonisation and phagocytosis.

### 3.3. Immunosuppressive effects

*A. actinomycetemcomitans* has been shown to exert an immunosuppressive effect in vivo. Injection of the organism into mice resulted in decreased lymphoproliferative responses to *E. coli* LPS and no antibodies against *A. actinomycetemcomitans* could be detected in the serum for up to 20 days following administration of bacteria [59]. Injection of mice with a sonic extract of the organism suppressed the IgG response to sheep red blood cells and resulted in a low CD4/CD8 ratio [60]. Low CD4/CD8 ratios have been reported in patients with early-onset forms of periodontitis [61]. *A. actinomycetemcomitans*-injected mice also demonstrated low secretion of IL-2 and IL-6 when their spleen cells were exposed to concanavalin A, and this may account for the poor antibody response as both of these cytokines stimulate antigen-activated B cells to differentiate into antibody-secreting cells.

Shenker et al. [62] have isolated a 60-kDa protein from the organism that inhibits (ID\(_{50}\) = 50 ng/ml) IgG and IgM synthesis by human peripheral blood lymphocytes (HPBL) in response to mitogens. The protein is claimed to exert its effect by activating a regulatory subpopulation of B cells which downregulates B and T cell responsiveness via ‘suppressor’ T cells. Apart from its cytotoxic actions, *A. actinomycetemcomitans* leukotoxin also impairs lymphocyte responsiveness to mitogens and antigens without affecting lymphocyte viability. At a concentration of 1 \(\mu\)g/ml, leukotoxin inhibits DNA, RNA and protein as well as IgG and IgM synthesis [63].

The ways in which *A. actinomycetemcomitans*
may overcome host defence systems are summarised in Fig. 1.

4. Destruction of host tissues

The means by which *A. actinomycetemcomitans* can promote the tissue destruction associated with LJP continues to be an extremely active area of research. The organism possesses a variety of components and releases a number of products which, if active in vivo, could account for the observed tissue pathology. These are summarised in Fig. 2.

4.1. Bone loss

A characteristic feature of LJP is loss of alveolar bone and the ability of *A. actinomycetemcomitans* to induce such bone destruction has been demonstrated in rats mono-infected with the organism [64] and in mice following oral inoculation with the organism [65].

Several in vitro studies have demonstrated the ability of various components and extracts of *A. actinomycetemcomitans* to stimulate bone resorption in vitro. Kiley and Holt [66] showed that LPS was active at a concentration of 0.3 μg/ml and Nowotny et al. [67] demonstrated the presence of a proteolysis-sensitive bone resorbing factor, active at a concentration of 1.0 μg/ml, in the microvesicles of this organism. We have shown that a saline extract containing surface-associated material (SAM) from the organism can stimulate bone resorption at concentrations as low as 1.0 ng/ml [68,69]. We have recently identified the active component as a 62-kDa protein, N-terminal sequencing of which has shown it to have 95% homology (over the first 38 residues) with the molecular chaperone GroEL (hsp60) of *Escherichia coli* [70]. Furthermore, electron microscopic examination of the protein using negative staining revealed that it formed barrel-shaped aggregates very similar to those formed by the *E. coli* GroEL. As far as we are aware, this is the first demonstration that a molecular chaperone has tissue-degrading activity. Other bone resorbing agents present on the surface of the organism include a group of outer membrane proteins (lipid A-associated proteins; LAP) which can be co-extracted with LPS. These have been shown to be more potent osteolytic agents than LPS, exhibiting activity at a concentration of 10 ng/ml [71].

LPS-stimulated bone resorption is partially inhibited by either indomethacin or anti-IL-1 antibodies and totally inhibited by dexamethasone, suggesting the involvement of both prostaglandin (PG)E₂ and
IL-1 in bone resorption [72]. In contrast, SAM-induced bone resorption was only slightly inhibited by indomethacin and IL-1 receptor antagonist protein while anti-tumor necrosis factor (TNF) antibodies had no effect [69]. This suggests that the molecular chaperone of this bacterium (the osteolytic component of SAM) acts by a different mechanism, possibly involving a direct interaction with the major bone-resorbing cell population, the osteoclast. Indirect evidence that SAM-induced bone resorption may play a role in vivo is provided by the finding that the sera of some patients with LJP can neutralise the osteolytic activity of this extract [73].

4.2. Collagenase production

Another characteristic feature of LJP is a reduction in gingival collagen fibre density. This could be accounted for, in part, by collagenase production by *A. actinomycetemcomitans* although there has been only a single report of its ability to degrade collagen and this was a property of only one of two strains tested [74].

4.3. Acid/alkaline phosphatases

*A. actinomycetemcomitans* exhibits both acid and alkaline phosphatase activity and this could contribute to bone destruction if the enzymes were to reach the alveolar bone and if they were active under the conditions existing there [75]. We have found that the phosphatase activity of *A. actinomycetemcomitans* is greater under anaerobic than aerobic conditions [76] and this may be of significance in view of the decreased oxygen content of inflamed gingiva [77].

4.4. Release of inflammatory mediators

Although periodontopathogenic bacteria possess a number of virulence factors that can contribute directly to connective tissue destruction, there is now increasing interest in the ability of bacterial components to cause tissue destruction by stimulating the release of cytokines and other inflammatory mediators. Many of the biological activities of such mediators are directly relevant to the pathogenesis of periodontitis [78]. Cytokines such as IL-1β, IL-6 and TNFα, for example, can stimulate bone resorption [79-81], IL-1β and TNFα stimulate the release of proteases and PGE₂ from fibroblasts [82-84], while IL-1β and IL-6 stimulate B cell proliferation [85]. The key cells of the periodontium responding to bacterial components in this manner are the monocyte/macrophage and the fibroblast.

4.4.1. Monocytes/macrophages

Lindemann and Economou [86] reported that whole cells of *A. actinomycetemcomitans* can stimulate the release of IL-1β and TNFα from human monocytes and a number of bacterial components have similarly been shown to stimulate such cells to release cytokines and other mediators. LPS, at a concentration of 1.0 ng/ml, stimulates the release of IL-1β and TNFα from human monocytes [87] and similar concentrations also stimulate the synthesis of mRNA coding for IL-1α, IL-1β and TNFα in human macrophages [88]. However, Nishihara et al. [89] have demonstrated that the polysaccharide portion of LPS inhibits the release, but not the synthesis, of IL-1 from *A. actinomycetemcomitans* LPS-stimulated murine macrophages. This group also reported that LPS stimulates the production of IL-1 receptor antagonist (IL-1ra) by murine macrophages [90]. IL-1ra blocks the binding of IL-1 to its receptor so preventing many of its biological activities. The release of this cytokine from macrophages in response to LPS serves to illustrate that not all interactions between monocytes/macrophages and bacterial components result in the production of pro-inflammatory mediators.

In contrast to its ability to stimulate cytokine release, LPS is less potent at stimulating PGE₂ release from human monocytes as a concentration of 0.1 μg/ml is required [91].

Our own studies have shown that SAM from *A. actinomycetemcomitans*, at a concentration of 10 ng/ml, can stimulate the release of IL-1β and TNFα from human monocytes. Furthermore, IL-6 production from these cells can be induced by concentrations as low as 10 pg/ml [92]. In contrast, highly purified LPS from this organism was far less active at stimulating release of these cytokines. Serotype-specific polysaccharides from the organism have also been shown to induce IL-1β release from murine macrophages, the activity of the polysaccharide from
serotype b strains (consisting of an L-rhamnose-D-fucose disaccharide repeating unit) being more active than those from serotypes a and c. However, concentrations of 12.5 μg/ml are required before IL-1 release is detectable [93].

LAP can stimulate the release of IL-1β and IL-6 from human monocytes at a concentration of 10 ng/ml [94]. Release of TNFα from these cells was also detectable but only at a concentration of 1.0 μg/ml.

The range of inflammatory mediators produced by monocytes in response to various components of *A. actinomycetemcomitans* is shown in Fig. 3.

### 4.4.2. Fibroblasts

SAM can induce the release of IL-6 and IL-8, but not IL-1β or TNFα, from human gingival fibroblasts (HGF) at a concentration of 10 ng/ml [94]. However, LAP at a concentration of 10 ng/ml was able to stimulate the release of only IL-6. Release of IL-6 from most cells usually requires prior IL-1 transcription which, in a feedback fashion, acts on the producing cell to induce the transcription of the IL-6 gene [95]. It was of interest, therefore, to find that SAM was unable to stimulate fibroblasts to release IL-1β or TNFα even at concentrations as high as 10 μg/ml. This implies that SAM activates IL-6 gene transcription in these cells by a mechanism distinct from that operating in other inducers of IL-6 release.

### 5. Inhibition of tissue repair

#### 5.1. Inhibition of cell proliferation

Fibroblasts are the predominant cells of gingival connective tissue and are responsible for maintaining tissue integrity. A number of studies have reported on the ability of extracts and components of *A. actinomycetemcomitans* to inhibit fibroblast proliferation. Shenker et al. [96] showed that sonic extracts of *A. actinomycetemcomitans*, at a concentration of 100 μg/ml, inhibited the proliferation of HGF by 90% but had no effect on cell viability. The active component was proteinaceous. A similar effect was described by Stevens et al. [97] although the preparation was considerably more potent with an ID50 of 2 μg/ml. We have demonstrated that a saline extract containing surface-associated material (SAM) from *A. actinomycetemcomitans* demonstrated significant inhibition of fibroblast proliferation at concentrations as low as 10 ng/ml [57,98]. The osteoblast-like cell line MG63 has proved to be particularly sensitive to SAM with an ID50 value of 1-10 ng/ml. The active component of this SAM is an 8-kDa protein, which we have isolated and termed Gapstatin, which exerts its effects by inhibiting cell cycle progression. The possibly unique property of this protein is its capacity to inhibit cells in the G2 phase of the cell cycle [99]. We have recently established that, while cells are blocked in G2, Gapstatin exerts its effect in early S-phase. We think that the mechanism of action of Gapstatin is the inhibition of synthesis of cyclin B or the generation of active cyclin B-Cdc2/28 kinase complexes. Of particular interest was the finding that a proportion of LJP patients have IgG antibodies able to neutralise the anti-proliferative activity of Gapstatin [100]. The role that such antibodies may play in controlling disease progression remains to be determined.

Kataoka et al. [101] have isolated a 65-kDa protein from the cytosol which is capable of inhibiting fibroblast proliferation with an ID50 of 0.2 μg/ml. Helgeland and Nordby [102] reported that a 50-kDa protein from the supernatant of *A. actinomycetemcomitans* cultures could also inhibit proliferation of HGF, although this was less potent with an ID50 of 10 μg/ml.

Kamen [103] has also shown that a sonic extract
(at a concentration of 1–10 μg protein/ml) could inhibit proliferation of human foreskin keratinocytes. The active component was heat-labile.

5.2. Inhibition of macromolecule synthesis by fibroblasts

The extracellular matrix of gingival connective tissue consists of collagen embedded in a non-fibrous, proteoglycan-containing, gel. Any affect on synthesis of collagen or proteoglycan would compromise tissue integrity. However, there have been very few reports of the ability of *A. actinomycetemcomitans* to exert such effects.

SAM, but not LPS, from *A. actinomycetemcomitans* has been shown to inhibit collagen synthesis in HGF with an ID₅₀ of 5 μg/ml [98]. Bartold and Millar [104] reported that LPS decreased proteoglycan synthesis by HGF, but only by 50% at a concentration of 50 μg/ml.

5.3. Inhibition of bone formation

Bone resorption, a characteristic feature of LJP, could result from an increased rate of bone resorption, a decreased rate of bone formation, or a combination of both. Although considerable interest has been shown in determining whether components of *A. actinomycetemcomitans* can stimulate bone resorption, little attention has been directed at deter-

mining whether they can also inhibit bone formation. SAM from the organism has been shown to be a potent inhibitor of DNA and collagen synthesis by cultured murine calvaria and osteoblasts, being active at a concentration of 10 ng/ml [105,106]. In addition, as discussed previously, Gapstatin can inhibit the proliferation of the bone-forming cell: the osteoblast.

The ability of various components of *A. actinomycetemcomitans* to inhibit tissue repair is summarised in Fig. 4.

6. Conclusions

The periodontal diseases are among the most prevalent chronic inflammatory diseases of mankind and are the major cause of tooth loss in those over 30 years of age. The considerable effort devoted to determining the aetiological agents of these diseases has resulted in *A. actinomycetemcomitans* being identified as the major causative organism of LJP and, more recently, as one of the organisms responsible for adult periodontitis.

A variety of extracts and components of *A. actinomycetemcomitans* have been examined for their ability to function as virulence factors. Some of these have been shown to exhibit activities that may be relevant to the pathogenesis of periodontitis. Hence, a number of components of the organism are potent cytokine-inducing and osteolytic agents, some can interfere with the antibacterial activities of PMNs and macrophages while others can inhibit tissue repair. However, the vast majority of these putative virulence factors have been examined only in cell or tissue culture and, quite often, at concentrations unlikely to be achieved in vivo. Further research along two broad fronts is now needed to establish which of these components could function as virulence factors in vivo. Firstly, we need to determine which, if any, of the above components are actually produced in vivo — an area almost totally neglected by oral microbiologists. Application of in vivo expression technology (IVET) to enable the effect of host signals on virulence factor expression to be determined should, however, rectify this and promises to be an extremely exciting area of research [107]. Secondly, appropriate animal models are necessary to deter-
mine the combined effect of those virulence factors expressed in vivo so as to take into account their possible augmentatory and/or competing effects. To take but one example of such an interaction; IL-6 (produced by monocytes and fibroblasts in response to LAP and SAM) is pro-inflammatory but may also act to down-regulate some of the actions of IL-1 and TNF, which are produced by monocytes in response to LPS and SAM. In the absence of the results of such studies it would be premature to attempt to determine how the activities of the putative virulence factors discussed above are orchestrated to account for the pathological features characteristic of JLP.

References


mycetemcomitans and colonisation of the mouth. J. Dent. Res. 67, 179.


[53] Iwase, M., Lally, E.T., Berthold, P., Korchak, H.M. and Taichman, N.S. (1990) Effects of cations and osmotic protectants on the cytolytic activity of Actinobacillus actino-


