AGGREGATIBACTER ACTINOMYCETEMCOMITANS

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By
Dr. Resham Pakhmode

V.S.P.M. Dental College and Research Centre

Branch - II
PERIODONTICS

MAHARASHTRA UNIVERSITY OF HEALTH SCIENCES NASHIK

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Certificate

This is to certify that, the Library Dissertation titled "**Aggregatibacter Actinomycetemcomitans**" submitted by **Dr. Resham Pakhmode** in the Department of Periodontics, Vidya Shikshan Prasarak Mandal's Dental College and Research Centre, Nagpur, has been completed under my guidance and supervision, and to my complete satisfaction.

DR. USHA RADKE

Dean

DR. ABHAY KOLTE

Professor, Guide and HOD

Department of Periodontics

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Introduction

Aggregatibacter actinomycetemcomitans (Aa) is a part of normal flora found in human oral cavity. It is commensal of human mouth, which can be retrieved and cultured in about 20% of healthy population. It is especially found in individuals affected with localised aggressive periodontitis (LAP). This disease affects only certain teeth (incisors and molars) and causes rapid loss of the alveolar bone of the jaw leading to tooth loss.[1] Periodontitis is a chronic infectious inflammatory disease characterized by the destruction of tooth-supporting structures.[2]

Aggressive periodontitis differentiates from chronic periodontitis in its rapid progression of attachment and bone loss. Early microbiologic studies of Localised Aggressive Periodontitis provide clear evidence of a strong association between disease and a unique bacterial microbiota predominated by a microorganism later identified as Aggregatibacter actinomycetemcomitans. Research in the past has provided support for a primary etiologic role of Aa in LAP.[3]

These findings are summarized as follows:

1. The prevalence of a humoral immune response to this organism is elevated in patients with LAP. A. actinomycetemcomitans has been isolated in up to 97% of LAP patients, compared with 21% of adult periodontitis patients and 17% of healthy subjects. Not only is the prevalence of Aa six times greater in LAP than in healthy patients, but its proportion of the cultivable subgingival flora also is elevated. Among the three serotypes, serotype B is the most common, followed by serotype A.
2. The incidence of Aa is greater in younger LAP patients than in older LAP patients. If age is considered relative to the duration of the disease, younger patients have more destructive disease developing within a shorter period. This suggests that the presence of this organism correlates with disease activity.
3. A large number of Aa organisms occur in lesions in LAP patients, but such organisms are absent or occur in low numbers in healthy sites.
4. Aa can be identified by electron microscopy, immunofluorescence, and culture from LAP lesions within the gingival connective tissues.
5. Aa is quite virulent, producing a leukotoxin, collagenase, phosphatases, and bone resorbing factors, as well as other factors important in invasion of host tissue cells, evasion of host defences, immunosuppression, and destruction of periodontal tissues.
6. A positive correlation exists between the elimination of this organism from the subgingival flora and successful clinical treatment of LAP.

It forms a part of HACEK group, comprising of **H**aemophilus species (Haemophilus aphrophilus and Haemophilus paraphrophilus), **A**ggregatibacter actinomycetemcomitans, , Aggregatibacter segnis, **C**ardiobacterium species (Cardiobacterium hominis, Cardiobacterium valvarum), **E**ikenella corrodens and **K**ingella species. These are group of small, fastidious, pleomorphic gram-negative bacteria and are commensals of the oropharyngeal / respiratory tract.

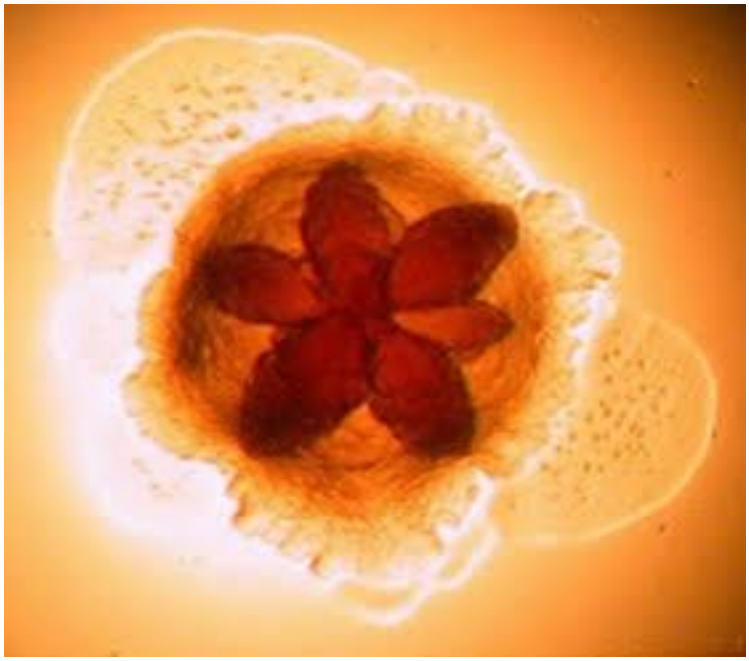

Fig 1.1 Morphology of Aggregatibacter actinomycetemcomitans

Epidemiology

The prevalence of this bacterium shows great variation depending on the geographic origin, age and life style of the examined population.[2] A literature review for non-oral A.

actinomycetemcomitans infections revealed that less than 200 cases were reported during the last 30 years.[4]

In reports from different parts of the world, the prevalence of Aa in subgingival plaque samples from young individuals with localized aggressive periodontitis is seemingly high, varying from 70% to 90%.[5, 6] Numerous studies have examined the prevalence of Aa in populations in Europe and the United States.[7] Variations in the global distribution of periodontopathic bacteria is important in the epidemiology and the treatment of periodontal diseases.

In a study, performed in 160 Chilean adolescents, investigations were done for the presence of 18 bacterial species using checkerboard DNA–DNA hybridization, no association was found between periodontitis and the presence of Aa in subgingival plaque, whereas species of the red and orange complexes were common in individuals with periodontitis, unlike in their controls. Aa virulence factors interact with host cells to initiate an aberrant inflammatory response in the periodontal gingival tissues.[8]

History

Aggregatibacter actinomycetemcomitans was first isolated in 1902 by Lignières and Spitz. This genus contains 11 species with Aa being the most important causative factor in periodontal disease. Its role in periodontal infections was first discovered by Jorgen Slots.[9]

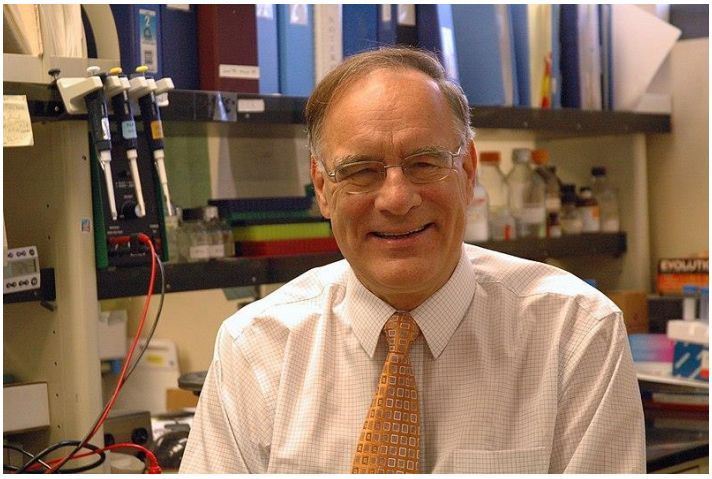

Fig 2.1: Jorgen Slots

Aggregatibacter actinomyctemcomitans was first described by Klinger and was first named Bacterium a. It belongs to the FAMILY Pasteurellaceae and GENUS Aggregatibacter. The

name Actinobacillus a was given by Topley and Wilson**.** The name is derived from it having been first isolated in association with Actinomyces israeli from patients with cervico-facial actinomycosis.[9]

Fig 2.2: Mogens Kilian and Nørskov-Lauritsen

In 2006, Nørskov-Lauritsen and Kilian proposed that Actinobacillus actinomycetemcomitans be reclassified as Aggregatibacter actinomycetemcomitans.[9]

Microbiology

Aggregatibacter actinomycetemcomitans is a fastidious, non-motile, non-encapsulated, slow growing, capnophilic, Gram-negative cocco-bacillus. It has a size of approximately 0.4 x 1.0 μm. It is smooth, dome-shaped and translucent for first few days and grows to become corrugated and star-shaped.[9]

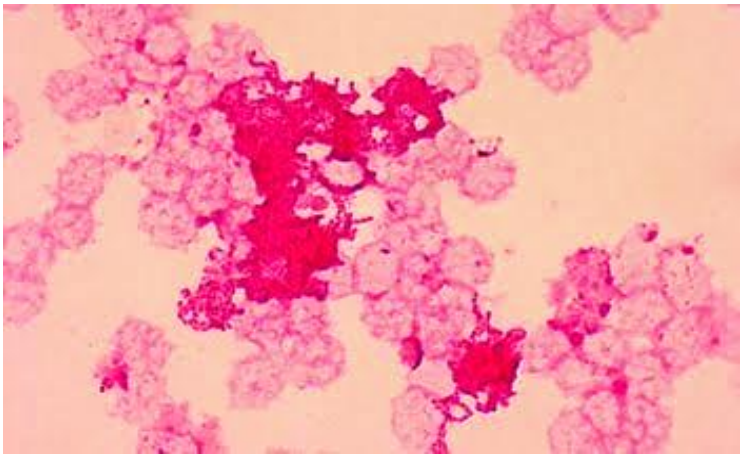

Fig 3.1: Smooth, dome-shaped bacterium, which grows to become corrugated and star-shaped.

This bacterium finds major ecological niches in the oral mucosa, dental plaque and periodontal pockets.

Biochemical properties

Aa grows slowly at 37°C, as a facultative anaerobic organism in standard broth media or on non-inhibitory solid media in an atmosphere of 5% CO2. Aa does not need the X (or) V factor for growth**.** In liquid medium, the micro-organism grows in the form of granules and adheres to the wall of the storage bottle. Thus, it is given the name Aggregatibacter. On agar media, the colonies, at least 3 mm in diameter, become visible within 24 hours. Following the reclassification by Nørskov-Lauritsen and Kilian, the species of the genus Aggregatibacter were stated to be independent of X factor and variably dependent on V factor for growth in vitro.[9]

Aa is a non-haemolytic, catalase positive and oxidase negative organism, that reduces nitrates to nitrites. It decomposes hydrogen peroxide. It can ferment carbohydrates like glucose, fructose, maltose, mannose, xylose and mannitol. It cannot ferment galactose, lactose, sorbitol, sucrose and glycerol**.** It produces strong alkaline and acid phosphatases.

Chemo taxonomic tests based on the following are used to differentiate Aa from closely related taxa: cellular and vesicular fatty acids, cellular proteins, cellular sugars, vesicular and whole cell enzymes, bacteriolysis, metabolic enzymes, respiratory quinones – menaquinones. DNA – DNA hybridization, DNA – rRNA hybridization, genetic transformation, ribotyping and analysis of r RNA.[9]

Serotypes

A. actinomycetemcomitans was initially divided into 24 groups by Pulverer and Ko based on tube agglutination assays, which were further divided into 6 agglutinating antigens.[9] Six serotypes (a, b, c, d, e and f) of the 24 groups have been described based on the composition of structurally and antigenically distinct O-polysaccharides of their lipopolysaccharides.[10] In addition, a novel serotype g has recently been proposed.[11]

In Asian and American populations, serotype c is most common and is present in both healthy and diseased conditions[12-14] whilst in Caucasian populations in Europe, serotype b is also relatively common.[15, 13, 16]

There also exist phenotypically non-serotypeable strains of Aa that lack expression of serotype-specific polysaccharide antigen.[17,18] Non-oral Aa was divided into 3 serogroups by King and Tatum based on a heat stable component. Zambon divided Aa into three serotypes − a, b and c.[9]

Serotypes a and b are common in the oral cavity and serotype c is found in 10% of human isolates and is of importance in extra oral infections. Serotype b is commonly implicated in Localized Aggressive Periodontitis.

A highly leukotoxic clonal type of Aa serotype b was first isolated, in the early 1980s, from an 8-year-old male child with localized aggressive periodontitis.[19] This clonal type was characterized as the Aa JP2 clone and it has been implicated in the pathogenesis of aggressive periodontitis in Moroccan adolescents.[20,21]

In the context of periodontal destruction, serotype b in particular[22, 23] but also serotype c, are viewed as having increased virulence properties[24, 25] and have been associated with

aggressive forms of periodontitis in various populations.[26, 27] Serotypes a and/or c have been linked to Aa - positive subjects without signs of periodontitis.

The number of serotypes has now been extended to 6 – a, b, c, d, e and f based on the differences in the carbohydrate moiety of the cell surface lipopolysaccharide.[28, 29]

Aa is differentiated into 8 biotypes based on fermentation reactions with galactose, mannitol and xylose.[30, 31] It is divided into 10 biotypes based on fermentation of dextrin, maltose, mannitol and xylose.[9] Serotype a does not ferment xylose while serotype b ferments xylose. Serotype c has both xylose positive and xylose negative strains.

Aa population is genetically heterogenous. Spontaneous (or) treatment induced change in oral strains is extremely rare and the same strain and biotype seem to be remarkably persistent. Individuals within a family with Localized Aggressive Periodontitis harbour the same biotype and serotype of A. actinomyctemcomitans. In individuals with periodontal disease, elevated antibodies to multiple serotypes were found. However, serotype b was found to be the most consistent feature.[31]

Antibody reactive to Aa serotype b lipopolysaccharide was found to be protective in generalized early onset periodontitis. Intra familial transmission is also found in Aa.[32] Prevalence of Aa serotypes in different populations shows three predominant serotypes – a,b,c and a lesser frequency of the other two serotypes – d,e.[9] Proportion of Aa serotype b is significantly greater in culture positive patients with aggressive periodontitis than those with chronic periodontitis. Serotype antigens of A. actinomycetemcomitans have high molecular weight and are heat stable. They have primary carbohydrate moieties. The serotype specific antigens are the most immunodominant antigens of Aa.

JP2 strain of serotype b

JP2 strain is a 530-base-pair deletion in the leukotoxin operon, leading to a highly enhanced leukotoxic activity.[33] Based on mutation patterns, it has been hypothesized that the virulent JP2 clone first emerged as a distinct genotype of Aa only 2,400 years ago from the Mediterranean part of Africa, spreading to West Africa and then to the Americas during the transatlantic slave trade.[34]

In Brazilian adolescents infected with this highly toxic clone of Aa, the probability of having aggressive periodontitis was close to 100%, whereas being colonized with other clonal types was associated with a less-destructive outcome of the disease.[35]

The JP2 strain has been mainly recovered from adolescents living in north-western Africa, but its carriage rate among 500 adolescents in Ghana (i.e. in a sub-Saharan area) was recently shown to be only 8.8%.[36] The hypertoxic clone is common in African-American and Brazilian adolescents with aggressive periodontitis.[37] The hypertoxic Aa is infrequently detected in European (Caucasian) and Asian populations.[38-42]

Other strains

Among potential virulence factors, cytolethal distending toxin, which seems to be characteristic for Aa.[43] It was suggested that outer membrane vesicles of Aa may play a role in the transfer of cytolethal distending toxin, as well as of other virulence agents, from the bacterium to the host tissue.[44]

Pathogenesis of A actinomycetemcomitans mediated periodontal infection

The pathogenesis of periodontitis is characterized by microbial challenge and the host's response to it.[45] A. actinomycetemcomitans appears to play an important role in certain types of periodontal disease. Although the pathogenic mechanism by which this bacterial species acts to cause periodontal disease is not known, Aa has clearly adapted well to its environment; its armamentarium of virulence factors ensures its survival in the oral cavity and enables it to promote disease. Aa may constitute exogenous species because of the rare occurrence in periodontally healthy individuals.

The designation of Aa as a periodontal pathogen presupposes that destructive periodontal disease is more prevalent in periodontal sites exposed to the organisms than in non-exposed periodontal sites.[45] This organism is able to produce a variety of virulence factors capable of facilitating the colonization, invasion and destruction of the periodontal tissues and interfere with tissue repair.[46]

This organism has been associated with a variety of infectious disease processes in man, including infective endocarditis, brain abscesses, osteomyelitis, subcutaneous abscesses, and several forms of periodontal disease.[47] Adhesion to epithelial and tooth surfaces is dependent on the presence of surface proteins and structures such as microvesicles and fimbriae.

Penetration of epithelial cells

The bacterial invasion process includes entry of the bacterium into epithelial cells, as well as subsequent events, such as the fate of the internalized organism. Surface components which play an important role include adhesins and fimbriae. Adhesins are proteinaceous in

nature and mediate the attachment of bacteria to specific receptors on epithelial cells. Fimbriae gives the bacteria a rough morphology, the morphological type associated with fresh isolates adhesion of Aa to epithelial cells involves multiple determinants.[45]

All Aa strains do not invade to the same extent. In general, those strains which exhibit a smooth colonial morphology are more invasive than those with a rough morphotype. Invasion occurs by receptor mediated endocytosis, as evidenced by inhibition of invasion by cytochalasin D.[48] Other requirements for Aa invasion include active metabolism and De novo protein synthesis by both the bacterium and mammalian cell and attachment of Aa to the host cell.

Cell biology of the entry process

Invasiveness of A. actinomycetemcomitans strains varies, with some relation between the extent of invasion and colony morphology that is smooth. Invasion by Aa is the greatest when the cells are in early-exponential growth phase. Cytochalasin-D, which blocks polymerization of actin and represses phagocytosis, inhibits Aa entry into epithelial cells Microfilaments are demonstrated, which are a major component of the cell cytoskeleton participate in the Aa entry process.[45]

Molecular biology of A. actinomycetemcomitans entry

Studies have shown that Aa can attach to, and enter into, the host epithelial cells and multiply, virulence attributes also provide it with the capacity to cause disease. Aa attaches to the epithelial cell.[49, 50] The bacteria are transported through the projections and enter adjacent cells.

Virulence of A. actinomycetemcomitans

Virulence is recognized depending on: coordinated expression of several genes whose products mediate attachment, ability to escape host defences and production of tissue destroying toxins and enzymes etc.[51] Circulating antibodies against Aa leukotoxin recovered in most subjects with juvenile periodontitis is reported to interfere with leukotoxin – mediated cell killing. Periodontopathic bacterial invasion of periodontal tissues is seen as an important component of virulence, which contributes to periodontal tissue breakdown.[45]

Transmission studies and vigorous host response to periodontal infection by this organism and the ability of appropriate therapies to eradicate the organism completely from the oral cavity (which is not the case for indigenous pathogens) also appear to confirm this fact.[9]

Host response

Aa infection induces a supra-physiological immune-inflammatory response state, which disrupts normal periodontal tissue homeostasis in the gingiva, periodontal ligament (PDL), cementum, and alveolar bone, ultimately promoting tooth loss.[8] Periodontopathogenic potential of some bacteria may be due to their ability to manipulate the immune response of the host.[52]

T lymphocytes are the regulators of immune response. The best characterized immunomodulatory bacterial products are the lipopolysaccharides of gram negative bacteria, which can activate B cells, monocytes, macrophages and polymorphonuclear neutrophils.[32,53] Aa contains proteinaceous products that have the ability to selectively stimulate T suppressor cells and also potentially suppress immunoglobulin production.

Super antigens are T cell modulating components of bacteria and viruses. These super antigens, though potent T cell stimulators are ultimately immunosuppressive. This feature is also thought to be present in Aa.[52] Despite the high antibody levels, A. actinomycetemcomitans persists in the periodontal pockets.

The anaerobic environment of the periodontal pocket can impair the bactericidal effect of polymorphonuclear leukocytes. Aggregatibacter actinomycetemcomitans demonstrates resistance to complement mediated killing.[32] Higher concentrations of leukotoxin, a surface-associated and secreted protein by the bacteria, may enhance Aa pathogenesis with the potential to exacerbate periodontal disease progression. The virulence factor of Aa interacts with host cells to initiate inflammatory response in the periodontal tissues.[8]

Gingival fibroblasts and epithelium cells (non-hematopoietic resident cells) are the first responders. A. actinomycetemcomitans stimulates the host responses via exotoxic and endotoxic virulence factors, activating superficial epithelial cells and underlying fibroblast cells.[8] The bacterium bypasses initial barriers, and a host inflammatory response is initiated. As the bacteria penetrates deeper into the subgingival tissues, a broader host immune response is activated.

Following the initial colonization, innate immune cells derived from myeloid hematopoietic origin such as monocytes, neutrophils, and dendritic cells (DCs) are recruited into the periodontal microenvironment. Leukotoxin and cytolethal distending toxin (CDT) are Aa exotoxins that contribute to its pathogenesis.

Leukotoxin is thought to be the main Aa constituent that causes cell death of monocytes and facilitates Aa evasion of host defence mechanisms.[8]

Aggregatibacter actinomycetemcomitans perpetuation of the host immune system promotes adaptive immune responses by T and B cells (derived from lymphoid progenitors). Initial responding resident tissue macrophages and DCs secrete cytokines and chemokines to promote activation and recruitment of B and T cells.[8]

Patients with localized aggressive periodontitis had circulating opsonic IgG antibodies, which were secreted by mature B cells (plasma cells) against the bacteria. Aa also induces cytokines that polarize T helper like Th1, Th2, regulatory T (Treg), and Th17 cells.

A. actinomycetemcomitans stimulates interferon-c (IFN-c) and interleukin-12 (IL-12) production.[8]

The stimulation of immune system in the periodontal microenvironment elicits a pathophysiologic pro-inflammatory state, which disrupts normal periodontal tissue remodelling processes ultimately promoting collateral tissue damage.

In periodontal disease, the level of proinflammatory and pro-resorptive cytokines favours alveolar bone resorption by monocyte or defined osteoclast progenitor (dOCP) derived osteoclasts, versus alveolar bone formation by mesenchymal derived osteoblast cells. When bone resorption exceeds bone formation, an unbalanced bone remodelling process with catabolic effects on alveolar bone homeostasis ultimately results in net alveolar bone loss.[8]

Response of initial non-hematopoietic host barriers to A. actinomycetemcomitans

Non-hematopoietic barriers to Aa include gingival epithelial cells, resident gingival fibroblasts, and PDL fibroblast. Aa has the ability to adhere to mucosal and gingival epithelial cells.

Fibroblasts originating from a mesenchymal stem cell are extracellular matrix collagen building cells capable of responding to pathogenic insult. Resident gingival and PDL fibroblasts elaborate proinflammatory mediators consistent with the early immune-inflammatory response during Aa elicited perio-pathogenesis.

Response of cells of hematopoietic origin to A. actinomycetemcomitans

Circulating immune cells possess surface integrins and chemokine receptors that cross the vascular endothelium in response to local chemokines present in the periodontal tissue matrix. Once inflammatory cells are recruited to the local site of infection, they employ overlapping and unique effector defence functions.

Inflammatory cells identified, involved in Aa infection include, but are not limited to, myeloid-derived macrophages, neutrophils, and DCs, and B and T lymphocyte populations.

Neutrophil interactions with the host and A. actinomycetemcomitans

Neutrophils (polymorphonuclear leukocytes/PMNs) are first responders to microbial pathogenic insult, critical in this process. PMNs isolated from patients with aggressive periodontal disease with IgG titers against Aa demonstrated greater PMN phagocytosis and killing compared with PMNs isolated from periodontally healthy control subjects. Relative to healthy control subjects, PMNs from patients with aggressive periodontal disease were characterized by a significant increase in basal activity of human neutrophil elastase.

With regards to the oxidative pathway, neutrophil phagocytosis of Aa is associated with reactive oxygen species (ROS), as indicated by the ability of Aa to induce neutrophil activity by promoting ROS production.[8]

It was suggested that self-aggregating Aa, possessing a rough fimbriated surface, impaired PMN phagocytosis, which perhaps is simply secondary to PMN limitations in engulfing large foreign bodies.

Based on the destructive nature of Aa, studies have been conducted to determine the effects of antibiotics on the ability of PMNs to clear Aa. Neutrophils with intracellular azithromycin accumulation demonstrated decreased Aa survival.

Aggregatibacter actinomycetemcomitans ligands induce Toll-like receptor signalling in macrophages

Macrophages are proven key players in Aa pathogenesis. Monocytes enter the local tissue site of infection by diapedesis from the circulation where they differentiate into activated macrophages or osteoclasts. Surface toll-like receptors (TLRs) recognize pathogenic constituents and have been extensively studied in macrophage function.

Aggregatibacter actinomycetemcomitans induces inflammasome pathways in monocytes. Aa has a dual function in host evasion via virulence actions causing cell cycle arrest and promoting apoptosis. Leukotoxin is considered the main component that induces monocyte cell death.

Monocyte surface receptors, LFA-1 and CD14, mediate Aa internalization, which ultimately may result in monocyte apoptosis. Lymphocytes have been implicated to play an integral role in adaptive immune responses directly associated with Aa pathogenesis .[8]

Virulence factors

Virulence is the ability of an organism to cause infection, which includes the ability to enter the host, find unique ecological niche, subvert the host's normal defences, replicate in the new environment and express specialized pathogenic traits. The genetic diversity among different isolates of Aa is great and its ability to express and release virulence factors varies.[2]

A. actinomycetemcomitans induces infection at the periodontal sites by attaching to epithelial cells, existing microbes, tooth surface. It competes with the resident flora in an effective manner and overcomes the cellular and humoral host defence mechanisms.

The different adhesins and fimbria expressed by this bacterium have been shown to be important factors that promote colonization at the various ecological niches of the human oral cavity.[54] Bacterial adhesion which facilitates colonization is the key virulence mechanism.[55] Adhesins are proteinaceous structures found on cell surfaces, responsible for adhesion. They bind with specific receptors in the saliva, tooth, extra cellular matrix and epithelial cells. Aa binds to collagen I, II, III and V but not IV. It also binds to fibronectin but not fibrinogen.[56]

The tight auto-adhesion of Aa has been described is due to the expression of long, bundled fibrils composed of a 6.5-kDa subunit protein, Flp-1 (fimbrial low-mol. wt protein) which has been reported to be glycosylated.[57-59]

Surface ultrastructure of A. actinomycetemcomitans are

i) Fimbriae: Bacterial cell surface appendages associated with bacterial colonization of host tissue. These are peritrichous, > 2 μm in length and 5 nm diameter.[60] Strains with fimbriae adhere three to four folds better.[56]

ii) Vesicles: A prominent feature of Aa, these are oligopolysaccharide units, exhibiting leukotoxic activity. Leukotoxic strains have abundant extracellular membranous vesicles. These surface entities mediate aggregation. These membranous vesicles also have endotoxins, adhesions, bacteriocins and bone resorption activity. Bacteria in contact with cell surface have been found to exhibit vesicles. The role of vesicles as a virulence factor is yet to be conclusively determined.[9]

iii) Extracellular amorphous material: The production of this material is based on culture conditions, which increases adhesion of the microbe.

Aa penetrate and survive within eukaryotic cells and penetrate gingival epithelium. They occur in specific intracellular locations like the epithelial wall, enlarged intracellular pocket spaces and the epithelial side of basal lamina in connective tissue and alveolar bone.[56] It has been suggested that the transferrin and integrin receptors are involved in the adhesion of the bacteria to host cells.[61]

Unique aspects of the behaviour of Aa are its rapid exit from cells after invasion, its ability to move from one cell to another and its capacity to divide rapidly within host cells.[62] It has been observed that microfilaments and microtubules for intracellular movement.

Tissue destruction potential

The tissue destruction potential of A. actinomycetemcomitans may be by toxin production, enzyme production or induction of immunopathological reactions.[32,52]

Factors	Functions
Colonization and persistence in oral cavity.	Adhesins Invasions Bacteriocins Antibiotic resistance
Host defence mechanisms	Leukotoxin Chemotactic inhibitors Fc binding proteins Immunosuppressive proteins
Host tissue destruction	Cytotoxins Collagenase Bone resorption factors Stimulation of inflammatory mediators
Host tissue repair	Inhibitors of fibroblast proliferation Inhibitors of bone formation

Table 3.1 Factors affecting tissue destruction potential of Aggregatibacter actinomycetemcomitans

Leukotoxin

Leukotoxin Production

Aa expresses two exotoxins, a cytolethal distending toxin (Cdt) and a leukotoxin. Cdt's are expressed by a number of gram-negative bacteria and cause death of the host cells by blocking their proliferation.[63] The leukotoxin selectively affects human cells of hematopoetic origin by binding to the lymphocyte function associated receptor 1 (LFA-1) and disrupting membrane integrity.[2]

Leukotoxin belongs to the Repeat in Toxin family (RTX) and shares genome organization and molecular structures with RTX proteins produced by a number of other gram-negative bacteria.[64] The Repeats-in-Toxin (RTX) exoprotein, which exhibits clear protein sequence homology, is produced by several gram-negative bacteria.[65] These toxins have a varying number of glycine rich Ca^{2}++ binding tandem repeats in the N-terminal end of the structural toxin molecule.

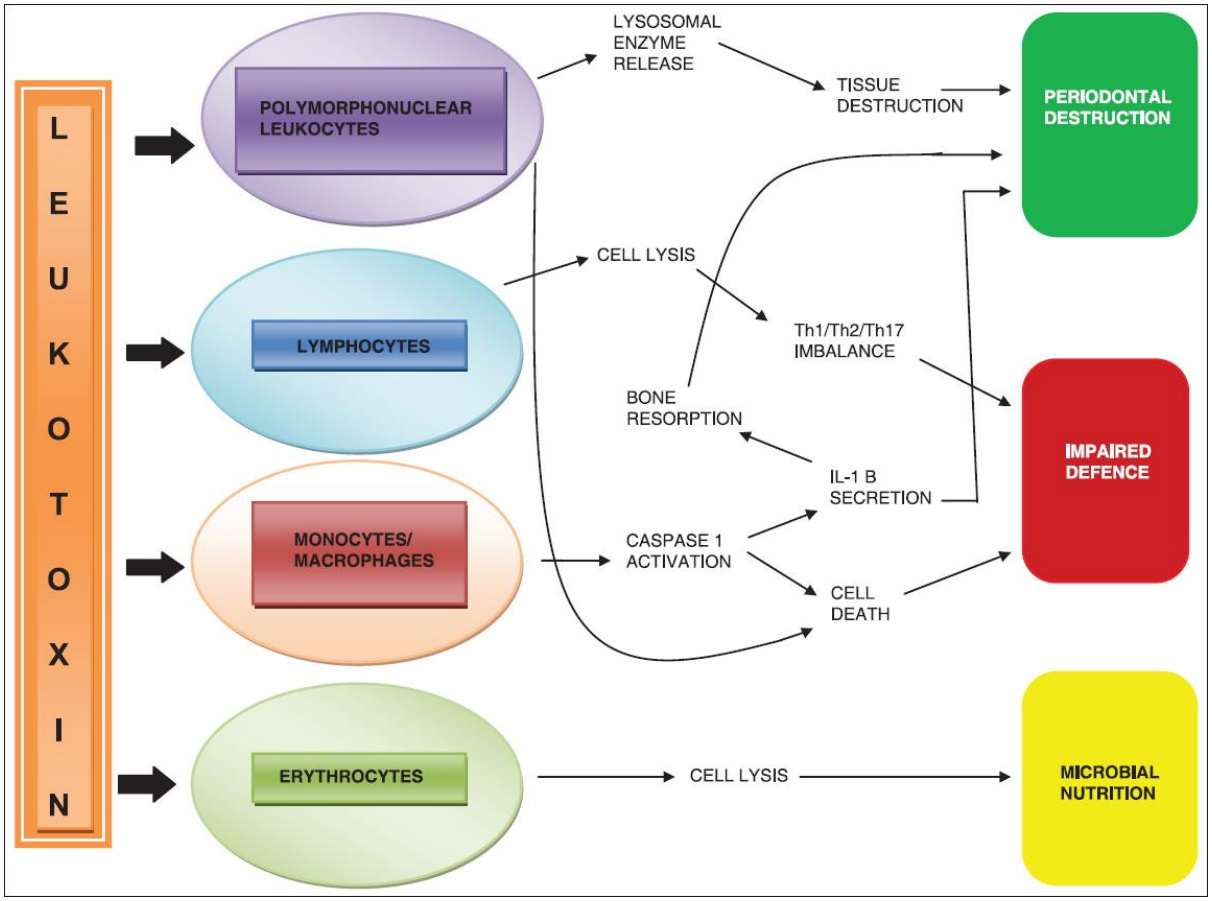

Fig 3.2: Role of Leukotoxin in periodontal pathogenesis.

Leukotoxin is one of the most important virulence factors of Aa. It is an RTX exoprotein that plays an important role in periodontal disease pathogenesis and is lethal to neutrophils.[66] It is a heat labile protease sensitive toxin, secreted outside the periplasmic space, but remains adherent to the nucleic acids that coat the outer surface of Aa. The toxin is associated with the outer cell membrane in contrast to other RTX secreting bacteria, which can kill both lymphoid and myeloid leukocytes.[32]

Target cells include human polymorphonuclear leukocytes, monocytes and macrophages. Target cell susceptibility is due to the cell surface expression of β2 integrin molecule,

lymphocyte function – associated antigen 1 (LFA 1) suggesting that killing is a receptor mediated process.[9]

The expression of leukotoxin varies amongst the various strains of Aa, depending primarily on the structure of the ltx promoter region. Leukotoxic strains are characterized by a 530 base pair deletion within the ltx promoter in aggressive periodontitis patients. Insertion of the transposable DNA element correlates with the high level of leukotoxin expression.[32]

RTX leukotoxin is encoded by an operon of 4 genes – ltx A, ltx B, ltx C, ltx D. The A gene encodes the leukotoxin itself and is produced as an inactive protoxin. LtxA encodes the structure of the toxin, ltxC for components required for post-translational acylation of the toxin and ltxB and D for transport of the toxin to the bacterial outer membrane. The leukotoxin promoter is located upstream of the ltxC gene.[2]

Organization of the A. actinomycetemcomitans leukotoxin operon

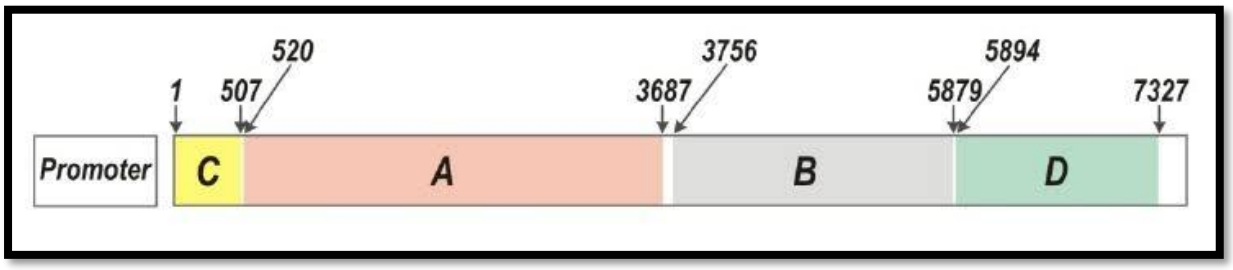

Fig 3.3: The leukotoxin promoter is located upstream of the ltxC gene

A. actionomycetemcomitans exhibits significantly enhanced leukotoxicity in periodontally diseased individuals.

The cellular and molecular mechanisms in which a modified leukotoxin promoter enhances the expression of leukotoxin are not known, but the most well-known phenomenon is the highly leukotoxic JP2 clonal strains of Aa characterized by a 530 bp deletion in the promoter of the leukotoxin operon.[67] Presence of the JP2 clone is highly associated to aggressive forms of periodontitis and has been shown to correlate with disease onset of adolescents in Morocco.[68] Clonal diversity analyses of JP2-like isolates have indicated that all strains of this clone have a common ancestor from Northern Africa, due to strict vertical transmission pattern of this bacterium.[67]

Molecular Structure of the Leukotoxin

The RTX proteins are a highly diverse steadily growing family of toxins secreted from a number of gram negative bacterial species and with some wide range different biological activities.[64] RTX proteins fall into two categories: the haemolysins, which affect a variety of cell types, and the leukotoxins, which are cell-type and species-specific.[69]

LtxA expressed by A. actinomyctemcomitans is a large pore-forming protein that consists of 1055 amino acids encoded by the ltxA gene in the leukotoxin operon.[70,71] The molecule can be divided into four regions based upon analysis of the amino acid sequence: the N-terminal region, the central region, the repeat region and the C-terminal region.[72] The N-terminal region, of LtxA exhibits alternating hydrophobic and hydrophilic clusters and the pore-forming region have been suggested to be mediated by the hydrophobic clusters.[69,72]

The central region of the RTX proteins, contains large hydrophilic domains and the two acylation sites of LtxA is located at $lysine_{562}$ and $lysine_{687}$. [73] The fatty acids at these

positions have been shown necessary for the activity of the toxin and are suggested to contribute to the anchorage at the target cell membrane.[73]

The target cell receptor LFA-1 binds to the repeat region of RTX proteins and this interaction has been shown to be responsible for the host cell specificity of LtxA.[74] Finally, residues at the C-terminal end of the RTX proteins have been shown to be needed for export of the toxin to the bacterial outer membrane by interactions with secretory proteins.

A partial denaturation of the LtxA molecule has been reported to enhance its leukotoxicity, which indicates that conformational changes interact with the activity of the toxin.[75]

Leukotoxin Secretion

Three proteins of A. actinmycetemcomitans, LtxB, LtxD and TdeA, are reported to be required for export of leukotoxin to the bacterial outer membrane: -[76]

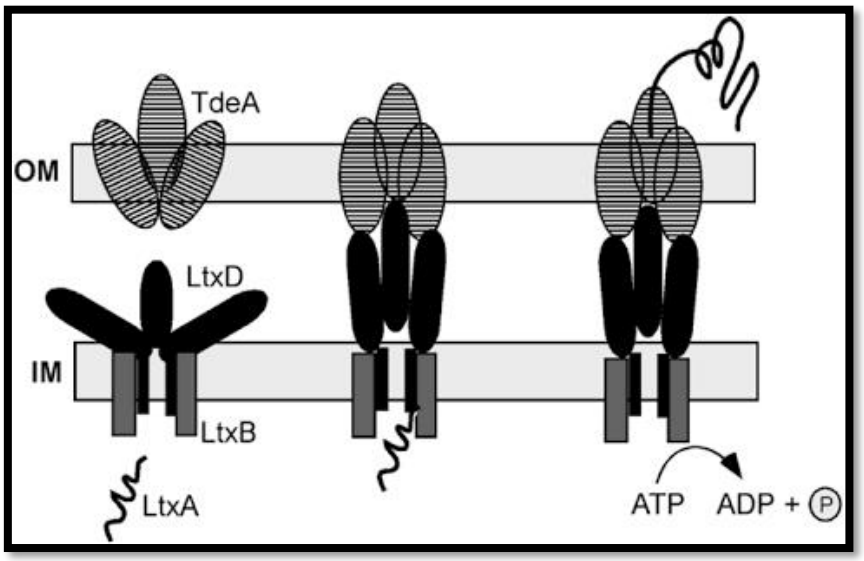

Fig 3.4: Type I secretion system required for export of the expressed A. actinomycetemcomitans leukotoxin to the bacterial outer membrane.

An inner membrane protein (M or C) has shown to be necessary for efficient export of the toxin. It affects the outer membrane structure of the bacterial cell.[77] The toxin was found to be localized on the outside of the membrane and in membrane associated vesicles

Ohta and co-workers showed that leukotoxin could be released from the bacterial membrane by DNAase or RNAase treatment, which indicates involvement also of electrostatic forces between the negative charged nucleic acid and the positive charged leukotoxin.[78]

Presence of serum proteins also mediates release of the toxin from the bacterial outer membrane, which indicates involvement of competitive mechanisms. The serum mediated release of the toxin[79] as well as its highly systemic immunogenic response, indicates a release of the toxin from bacteria growing in an oral biofilm in vivo.

In 1981, McArthur and co-workers[80] showed that the activity of leukotoxin in interaction with polymorphonuclear leukocytes (PMNs) was enhanced in the presence of human serum. This phenomenon could be explained by the protective effect of the serum protease inhibitors on leukotoxin degradation caused by lysosomal enzymes released by the affected PMNs.

Mechanism of action of leukotoxin

Two ltx A mediated mechanisms of cell death are known to exist: Necrosis and apoptosis. The ltx A forms pores in the target cell membrane leading to water influx and osmotic lysis in conditions where the ltx A is present in high concentrations. At low concentrations, ltx A mediates cell death via apoptosis. This leukotoxin is thought to be the most important virulence factor in the pathogenesis of localized aggressive periodontitis and other forms

of early onset periodontitis. Some highly leukotoxic strains of Aa produce about 10 – 20 times more leukotoxin than the other minimally leukotoxic strains.[9]

Distribution and clonality, intra-familial transmission of the highly leukotoxic strains of Aa reveals that localized aggressive periodontitis and other forms of early onset periodontitis are primarily associated with the highly leukotoxic clones of Aa.

Interaction of LtxA with the Target Cell Membrane

Leukotoxin A expressed by Aa exhibits a unique specificity against cells of hematopoietic origin from humans and some other primates. This is suggestive of species-specific effect of LtxA, mediated through a unique receptor on the target cells and a precise region in the toxin that recognizes and interacts with the receptor.[81]

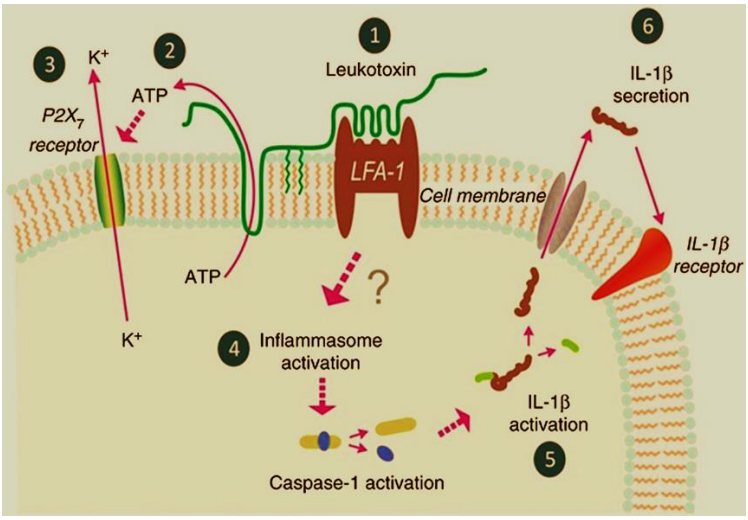

Fig 3.5: Mechanism of interaction of Leukotoxin with target cell membrane

The principal feature of this species recognition region of LtxA is that it contains a series of 14 tandemly repeated amino acid sequences in the repeat region of the toxin.

It has been suggested that the role of LFA-1 in LtxA mediated cell lysis is to help the protein to have a correct orientation on the target cell membrane.

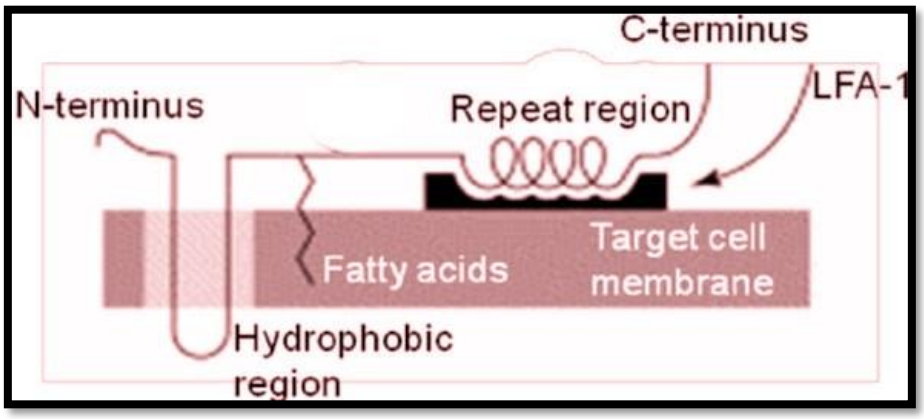

Fig 3.6: Molecular structure of the interaction between leukotoxin and the target cell membrane.

Low concentrations of the toxins might induce apoptosis through loss of membrane integrity caused by the small pores and that higher concentrations of the toxin allows oligomerizerization of LtxA-LFA-1 complexes on the target cell membrane, mediating a rapid and complete membrane collapse.[69] LtxA has been shown to require lipid rafts for target cytotoxicity, which also indicates the importance of a high mobility on the target cell membrane.[82] The LFA-1 molecule identified as the LtxA target cell receptor is a heterodimer consisting of the αL (CD11a) and α2 (CD18) subunits.

The extracellular region of human CD18 has been shown to be critical for conferring susceptibility to LtxA-induced cell lysis.[81] Three different affinity states (low, intermediate, high) of LFA-1 that interfere with ligand binding have been described.[83]

Virulence Mechanisms of the Leukotoxin (LtxA)

The ability of Aa extracts to cause death of leukocytes was first shown more than 30 years ago. Leukotoxin was said to affect human lymphocytes and erythrocytes from human and animal origin and lyse PMNs and monocytes at higher concentrations of the toxin.[2]

Polymorphonuclear Leukocytes

PMNs are the first defense cells to be recruited in the acute phase of an inflammation, as in a periodontal infection.[84] PMNs in the periodontium have been described as a "double-edged sword", capable of producing periodontal disease as well as protecting against such disease. LtxA, as well as leukotoxic bacteria, have been shown to efficiently cause death of human PMNs, and consequently LtxA is assumed to protect Aa against phagocytic killing.[2] Transmission electron microscopy pictures of the exposed PMNs showed a peripheral translocation of the granules in cells exposed to the highly leukotoxic bacteria.

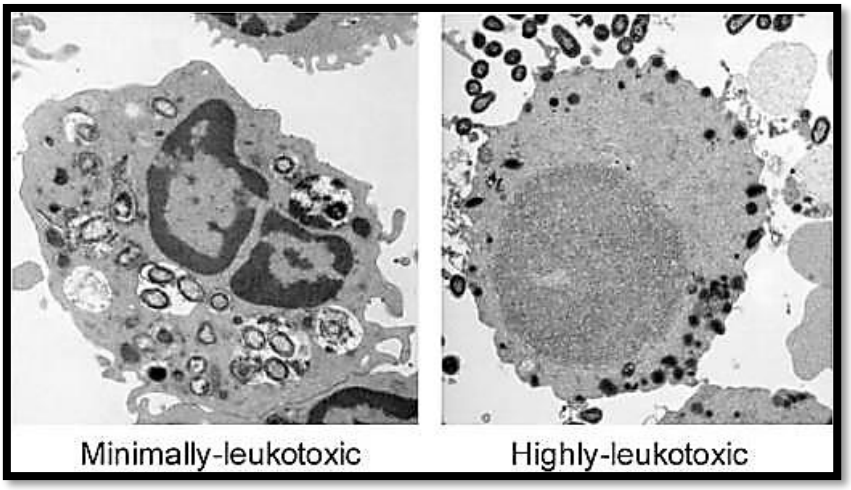

Fig 3.7: The low leukotoxic bacteria phogocytized and killed by the PMN (left); the highly leukotoxic bacteria (JP2) resists PMN phagocytosis and causes extracellular release of lysosomal components (right).

Moreover, the interaction between LtxA and PMNs mediates activation and release of matrix metalloproteinase-8 (MMP-8).[2] Thus, LtxA induces activation and release of proteolytic enzymes from these cells, which might contribute to the disease progression. The presence of serum proteins and the relatively high pH (≈8) in the pocket indicates that LtxA is released from the bacterial surface in this ecological niche.[80] Highly leukotoxic strains of A. actinomycetemcomitans are likely to cause an imbalance of neutrophil-mediated defences, whereas strains with a lower leukotoxic profile may lead to an enhanced inflammatory response without causing excessive neutrophil lysis.[85] The released toxin makes it to an easy target for inactivation by several of the components present in the periodontal pocket, such as superoxide radicals and proteinases released from the host defense cells or the colonizers of the oral subgingival biofilm.[86]

The great variation over time in the balance between host serum proteinase inhibitors and bacterial SOD expressed by A. actinomycetemcomitans, probably affects the activity of LtxA at the infected site and the burst periods observed in the pathogenesis of periodontitis might depend on such a phenomenon.

Impaired PMN function is closely associated with periodontitis and functional PMNs seems to be of certain importance when Aa is present in the oral subgingival biofilm. Individuals with Papillon-Lefèvre syndrome have impaired PMN serine proteases maturation, which causes an enhanced LtxA sensitivity due to decreased capacity to degrade extracellular LtxA by the released lysosomal PMN enzymes.[2]

Lymphocytes

LtxA has shown to suppress the function of peripheral blood lymphocytes. Human lymphocytes show a great heterogeneity in regard to LtxA sensitivity and a subgroup of these cells has been shown to be lysed at approximately the same concentrations as human PMNs. It has been shown that low concentrations of LtxA cause apoptosis, and in higher concentrations necrosis, in cultures of a human carcinoma cell line of myeloid origin (HL-60).[87]

It has been known for >30 years that the onset of periodontitis involves a switch from a T cell lesion to one involving large numbers of B-cells and plasma cells. A shift occurs in the balance between the so-called Th1 and Th2 subsets of T-cells, with Th2 cells associated to chronic periodontitis. T regulatory (Treg) and Th17 cells have been detected in periodontal tissues, indicating that these cells also are of importance in the host response and pathogenesis of periodontal disease.[2]

The ability of LtxA to induce apoptosis in lymphocytes might contribute to a locally impaired acquired immune response in periodontal infections. The ability of LtxA to affect also the lymphocytes indicates a possible role of this molecule in Th1/Th2/Th17 differentiation, a process that seems to be of great importance in the pathogenesis of inflammatory diseases, such as periodontitis.[2]

Monocytes/Macrophages

Characterization of the LtxA induced monocyte killing has described three different phases:[88]

i) Cessation of the membrane undulating folding and an accumulation of granulae in the perinuclear area;

ii) Abnormal membrane movement and strings of cytoplasm projecting from the cell;

iii) Explosive release of cytoplasmic material from the cell.

Analyses of different subsets of leukocytes separated from peripheral blood of a single donor showed that monocytes have an enhanced sensitivity to LtxA compared to PMNs and lymphocytes.

The LtxA-induced monocyte lysis was shown to involve activation of caspase-1, cytosolic cysteine proteinase that specifically induces activation and secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18). This is indicative of involvement of proinflammatory intracellular signalling.

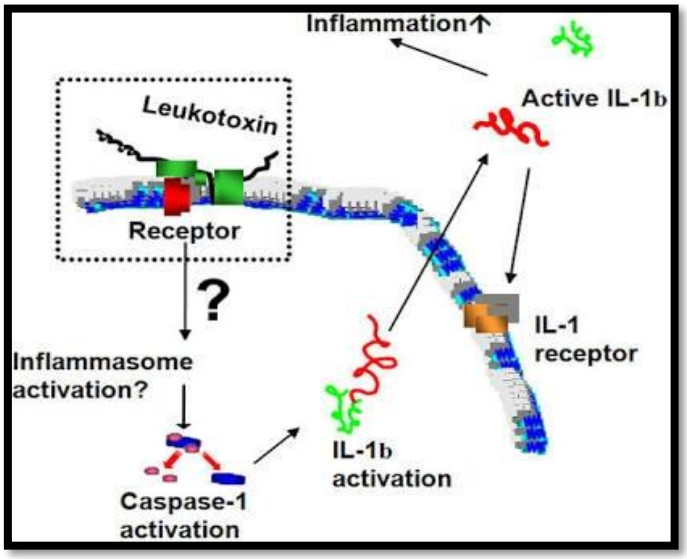

Fig 3.8: Cellular mechanisms involved in LtxA-induced monocyte/macrophage death.

Human macrophages (adherent blood monocytes) exposed to LtxA activate a rapid and abundant secretion of bioactive IL-1β. Macrophages are rare cells in a healthy periodontium but are often found in high numbers in tissues from periodontal lesions.

The monocytes during diapedesis, differentiate into macrophages and the inflammatory machinery is up-regulated during this process and further during the migration towards the infected site.[89] This process involves an accumulation of proinflammatory precursor molecules, such as IL-1β and IL-18, in the migrating macrophages.

In the case of an infection containing A. actinomycetemcomitans, the gradient of bioactive components in the connective tissue will contain LtxA, and the migrating macrophages will sooner or later meet concentrations of LtxA that activates secretion of these proinflammatory cytokines into the surrounding tissues.

Initial analyses of gingival crevicular fluid indicate an association between enhanced IL-1β levels and high number of A. actinomycetemcomitans in the periodontal pocket.[2]

The enhanced LtxA-sensitivity of human macrophages indicates that these antigens presenting cells might be affected during a primary infection with leukotoxic Aa, which might cause a delayed acquired immune response.

Erythrocytes

Hemolysis of red blood cells of human and animal origin that is caused by A. actinomycetemcomitans involve interaction with LtxA. Red blood cells lack expression of the LtxA receptor LFA-1, which has been shown to be a prerequisite for LtxA-induced leukocyte lysis**.**[90]

Acquired Humoral Immune Response to LtxA

It has clearly been shown that leukotoxin specific antibodies are present in the peripheral circulation of both periodontally healthy and diseased subjects. Plasma samples from the subjects with specific immunoreactivity against LtxA have been shown to neutralize LtxA activity and contain enhanced antibody titres against whole cells of Aa in comparison with samples from subjects without immunoreactivity to LtxA.

Systemic leukotoxin neutralization is correlated to the presence of this bacterium in the oral subgingival biofilm.[91] Systemic LtxA antibodies have been shown to be present in >50% of samples from adults and with a similar prevalence in periodontally healthy and periodontally diseased subjects. Systemic LtxA neutralizing capacity correlates to decreased risk of the incidence of stroke in women. A role of LtxA in the association seen between peridontitis and cardiovascular diseases.[2]

The strong correlation between prevalence of highly leukotoxic Aa and the development of attachment loss indicates a minor role of neutralizing antibodies in the infected periodontal pocket.

Systemic LtxA neutralizing antibodies are an important protection against the systemic side effects that are associated with periodontitis, such as increased risk for diabetes and cardiovascular diseases. Thus, ability of LtxA to specifically affect the immune cells, in particular the antigen presenting monocytes/macrophages, causes a delayed acquired immune response in the primary Aa infection.[2]

Bacteriocins

These are proteins produced by bacteria, that are lethal for other strains and species of bacteria. Actinobacillin is a bacteriocin which is active against S. sanguis, S. uberis and Actinomyces viscosus, which is associated with both the bacterial cell surface and the extracellular vesicles. It acts by increasing the permeability of the cell membrane to the target bacillus and may be responsible for the reciprocal relationship between Aa and S.sanguis / Actinomyces viscosus in plaque and in patients with localized aggressive periodontitis.[9]

Collagenase

Collagenase activity is commonly seen in A. actinomycetemcomitans and P. gingivalis. This may cause reduction in collagen density, which is a common feature of periodontal disease.

Cytotoxins

Aa produces a heat labile cytotoxin which exhibits virulence by its impact on fibroblast activity. Most strains of A. a produce a 115 KDa heat labile protein that specifically lyses human polymorphonuclear leukocytes and macrophages.[9]

Immunosuppressive factors

Aa produces an immunosuppressive factor that affects both B lymphocytes and T regulatory cells. The factor is a protein capable of inhibiting DNA, RNA and protein T cells activated by mitogens (or) antigens.

Surface associated materials

The surface associated material of A. actinomycetemcomitans has several putative virulence factors. This material, which has potent osteolytic activity, contains a protein which blocks cell cycle progression. It also has potent proinflammatory cytokine stimulating activity with extremely potent induction of IL – 6 and IL – 8 syntheses by monocytes and fibroblasts.

Prostaglandin E2 is also involved in the mechanism of formation of osteoclast – like cells, mediated by the Y4 capsular polysaccharide antigen of Aa. This mechanism is said to play an important role in inflammatory bone resorption by promoting osteoclast formation in periodontal disease. Aa surface associated material at very low concentrations inhibits fibroblast proliferation. The active component of this surface associated material is termed gapstein. [9]

Capsular polysaccharide from Aa Y4 completely inhibits IL – 6 and IL – 8 productions from human gingival fibroblast, suggestive of Y4 modulating the inflammatory response in periodontitis. This inhibitory effect has been found to be reversed by specific anti Aa Y4 capsular polysaccharide, suggesting an important relationship between the organism and humoral immune response.

Lipopolysaccharide

Lipopolysaccharide (LPS) is an important bacterial virulent factor that may actively intervene in the pathogenesis of the connective tissue destruction and alveolar bone resorption that occurs during periodontitis.[92] Chemically, the LPS consist of a hydrophilic polysaccharide covalently linked to a hydrophobic lipid portion, termed lipid A, which serves as an anchor for the molecule in the outer membrane.

The polysaccharide portion of LPS consists of a core oligosaccharide region and an O-antigen presenting a chain of O-PS repeating units that stimulate a wide range of immunological responses in the host, which may have important pathological consequences. The O-PS from the LPS produced by serotype b of Aa is structurally distinct from the O-PS produced by the other serotypes. The O-PS from serotype b consists of a repeating trisaccharide unit composed of a-D-fructose, a-L-rhamnose and b-D-N-acetyl-galactosamine residues and the O-PS from serotypes a and c is composed of 6-deoxy-a-D-talose and 6deoxy-a-L-talose, respectively.[93] These structural differences in the LPS among the different Aa serotypes could imply differences in the TLR recognition and signalling and, hence, differences in the DC response and T-lymphocyte polarization and function.[94]

Aa LPS has been shown both in vitro and in vivo to promote inflammation and osteoclastic differentiation resulting in alveolar bone resorption and these pathological events were mediated by production of the Th1 and Th17 type of cytokines at the local site of the periodontal lesion. It has been established that T-lymphocyte activity is a key determinant in the pathogenesis of periodontitis and a Th1/Th17-dominated immune response has been associated with destructive periodontal disease.[95] During periodontitis, Th1- and Th17-associated cytokines have the capacity to induce differentiation and activation of osteoclasts by indirectly and directly acting on their precursors and the mature osteoclasts.[94]

The lipopolysaccharide of Aa contains 30% carbohydrate, 30% lipid A, 10 – 12% hexosamine, 03 – 10% phosphate, heptose. Differences in the sugar composition and structure of LPS among the different Aa serotypes could be involved in the detected differential immuno-stimulatory potential on DCs and T lymphocytes. It is known to inhibit collagen and DNA synthesis as well as stimulation of bone resorption in a dose dependent manner. It stimulates interleukin 1 inhibitor (interleukin -1ra) released by macrophages. This plays an important meditative role in the development of periodontal disease.

In particular, IL-1b, IL-6, IL-17A and TNF-α may be able to facilitate local inflammation by recruiting and activating immune cells, which leads to an abundance of inflammatory cytokines and enhancement in the levels of RANKL and the activity of RANKL-induced osteoclasts. RANKL/ RANK signalling makes osteoclast precursors refractory to the inhibition of IFN-c, even at maximum anti-osteoclastogenic doses, and these IFN-resistant pre-osteoclasts will produce low levels of nitric oxide upon IFN-c stimulation and will also be resistant to morphological changes, suggesting that early exposure of osteoclast precursors to RANKL induces a broad resistance to the cellular effects of IFN-c.[94]

The lipopolysaccharide of Aa stimulates macrophages to produce interleukin 1α, interleukin 1β and tumour necrosis factor, mRNA and proteins involved in tissue inflammation and bone resorption. This lipopolysaccharide is cytotoxic to fibroblasts. It is a potent inhibitor of fibroblast proliferation.[9]

Fc binding protein

The Fc region of antibody is important in the binding of antibody to specific receptors on polymorphonuclear leukocytes. Any competing proteins for this region inhibits antibody

binding, and hence phagocytosis is inhibited. A heat modifiable membrane protein of Aa has been identified to serve this purpose. Biotinylated Fc molecules can inhibit binding of Fc molecules to Aa and inhibit complement activation.

Bone resorption

Periodontitis is a chronic inflammatory disease characterized by the destruction of tooth-supporting alveolar bone that if untreated may lead to tooth loss.[96] Proinflammatory cytokines with potent pro-resorptive actions, including Tumor Necrosis Factor-a (TNF-α), IL-1, and IL-6 are highly upregulated by Aa and so promote osteoclast formation and bone resorption.[8]

The Th17 lymphocyte pathway has been reported as key in the increased production of receptor activator of nuclear factor-B ligand (RANKL), which is involved in the osteoclast differentiation and activation.[96]

A. a stimulates bone resorption by lipopolysaccharide mediated mechanisms, proteolysis sensitive factor in microvesicles, surface associated material (molecular chaperone GroEL). The LPS from Aa has been shown to promote osteoclastic differentiation and activation, resulting in alveolar bone resorption.[96]

Aggregatibacter actinomycetemcomitans increases RANKL expression in the local inflammatory microenvironment by indirect mechanisms. Aa serotype b-primed dendritic cells, when used to stimulate autologous T lymphocytes, will induce a higher Th17-associated RANKL response. This will subsequently differentiate and activate RANKL-induced osteoclasts and bone resorption.[96]

Several well-known osteotropic factors, including IL-1b, IL-6, IL-17, and tumor necrosis factor-a (TNF-α), exert their osteoclastogenic activity by enhancing the production of Th17-associated RANKL and the activity of RANKL-induced osteoclasts. Lin et al[96] in 2011 suggested that, RANKL has a role in the periodontal bone resorption during Aa - specific Th17-lymphocyte induced periodontitis.

IL-1RA, which regulates the activity of IL-1b and reduces the production of IL-6 and TNF-α, has been shown to block the alveolar bone resorption induced by Aa, and hence it has been proposed as a potential drug to treat periodontitis. Early studies assessing the effects of Aa on bone formation revealed that Aa is a potent inhibitor of bone collagen synthesis**.**

Inhibition of neutrophil function

Aggregatibacter actinomycetemcomitans secretes a low molecular weight compound that inhibits polymorphonuclear leukocytes' chemotaxis, which is abrogated by treatment with proteinase K, suggesting that the compound is proteinaceous in nature. It is capable of inhibiting neutrophils from producing antibacterial agents. It produces a heat stable protein that inhibits hydrogen peroxide production by leukocytes. Aa is also resistant to defensins (cationic peptides found in neutrophils).[9]

Penetration of epithelial cells

A. actinomycetemcomitans can penetrate the gingival epithelium. It is found on the epithelial wall, enlarged intracellular spaces of the pocket epithelial surface, epithelial side

of the basal lamina, connective tissue and the alveolar bone. The primary receptor of Aa invasion is transferrin.[9]

The phospholipase C present in this microbe is implicated in vacuole lysis. Aa shows rapid intracellular replication and this is attributed to the Aggregatibacter – microtubule interaction. It can invade the oral epithelial cells and spread from cell to cell by endocytosis and colonize the sub epithelial gingival tissue. The adherence ability of Aa to titanium implant surfaces is dependent on the strain.[97]

Serotype a has the highest affinity while serotype e has the least adherence capability. Significant associations between the periodontal status and several health conditions were found in the adult population, including gender, smoking habit, diastolic blood pressure, white blood cell counts, C-reactive protein and serum IgG antibodies to IgG of A. actinomycetemcomitans whole cell titres.[9]

Factors influencing growth of Aa

Following is the list of factors affecting the growth of Aa: -[9]

a. Appropriate culture media – microbes need a suitable culture medium to support their nutritional needs. MGB – utilizes trypticase soy broth with malachite green and bacitracin (inhibitory to other indigenous flora.
b. TSBV – trypticase soy agar with serum, bacitracin and vancomycin.
c. "A" medium – TSBV with spiramycin, fusidic acid and carbenicillin.
d. Defined media like RPMI – 1640 and Dulbecco's modified eagle medium.
e. Supplements
f. Yeast extract added to trypticase soy broth

g. Cysteine

h. Thiamine

i. Steroid hormones

j. Iron compounds

k. pH – the optimum pH for growth of A. actinomycetemcomitans is 7.0 – 8.0. Environmental pH is a critical physiological parameter that determines the growth and metabolism of microbes.

l. Salt concentration – optimal growth of Aa is in the salt concentration between 85.1 mEq/l – 170.0 mEq/l.

Implication in disease

Aa along with Haemophilus, Cardiobacterium, Eikenella and Kingella are associated with bacterial endocarditis. It is an important pathogen in severe and recurrent forms of periodontitis. Aa is frequently associated with rapidly progressive periodontitis. Serotype b of this microbe is most commonly implicated in localized aggressive periodontitis.

Role of Aa in Localised Aggressive Periodontitis

Localized aggressive periodontitis, previously termed localized juvenile periodontitis, is a periodontal condition in adolescents that exhibits rapid destruction of periodontal tissue, which slows with time. It shows molar – incisor localization and burn out phenomenon.

Periodontal bone loss resembles a “mirror image” pattern. A. actinomycetemcomitans has been implicated as the organism causing localized juvenile periodontitis.[9]

Large numbers of Aa are routinely isolated from lesions of localized aggressive periodontitis, whereas isolation of the bacterium from healthy sites is low. Isolation is positive in 97 % of aggressive periodontitis cases. Eradication of the organism from diseased sites is usually correlated with a significant humoral immune response.

Presence of large numbers of the bacterium in the periodontal pocket is correlated with a significant humoral immune response. Aa produces a wide variety of potent, cell bound and secreted virulence factors that are implicated in the pathogenesis of aggressive periodontitis. Rapidly progressing periodontitis is characterized by severe and rapid bone loss.[9]

The organism commonly implicated is A. actinomycetemcomitans, either alone or in association with Porphyromonas gingivalis, Campylobacter, Prevotella intermedia, Eikenella corrodens. A. actinomycetemcomitans is also associated with refractory periodontitis as it is more difficult to be eradicated from the subgingival area than other bacteria because of its invasive capability. It can also cause re-infection from other sites in the mouth.

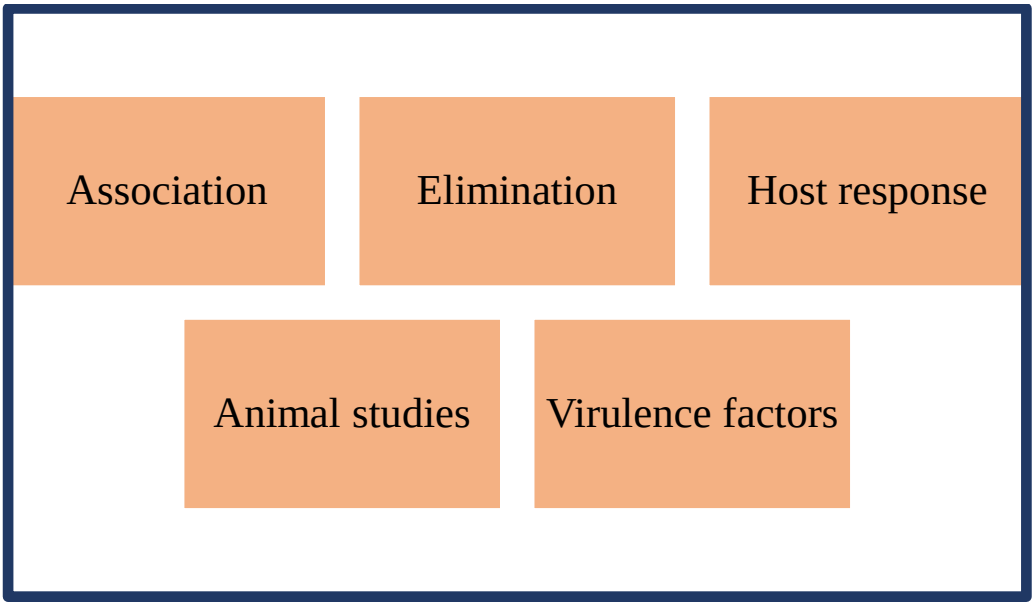

Fig 4.1 Socransky's criteria.

Socransky's criteria applied to explain role of A. actinomycetemcomitans in periodontal diseases:[9]

ASSOCIATION	Increased in localized aggressive periodontitis Increased in some lesions of chronic periodontitis Detected in the tissues of localized aggressive periodontitis lesions
ELIMINATION	Suppressed (or) eliminated in successful therapy Found in recurrent lesions
HOST RESPONSE	Increased serum and local antibody levels in localized aggressive periodontitis
ANIMAL STUDIES	Capable of inducing disease in gnotobiotic rats
VIRULENCE FACTORS	Host tissue cell invasion Leukotoxin Collagenase Lipopolysaccharide – endotoxin Epitheliotoxin Fibroblast inhibiting factor Bone resorption inducing factor

Table 4.1 Role of Aggregatibacter actinomycetemcomitans in periodontal disease.

Routes of infection

Saliva is considered to be the most important transport vehicle for Aa, as it can be cultured from salivary samples.[98] It can survive in saliva during transportation to a new host. Mucosal contact or toothbrush sharing may allow implantation of bacteria to potential growth locales. The salivary and subgingival serotypes of Aa are the same in a patient.

Extraoral infections

Periodontal disease is known to influence the systemic condition in various ways and the bacteria and their products such as lipopolysaccharides may spread from the periodontal lesion via the systemic circulation to affect distant organs.

The occurrence of this organism in extra oral sites, therefore, suggests translocation of the organism from oral to non-oral sites.

The dental health of a patient (dental diseases en masse) has a bearing on the general health. Periodontal diseases have been linked to coronary heart disease, including myocardial infarction, ischaemia, coronary atherosclerosis.[9]

The leukotoxin of Aa is a proven virulence factor in periodontal disease. It is most commonly associated with prosthetic valve endocarditis as well as native valve endocarditis. Individuals with a locus minoris resistentiae are at elevated risk. The ability of A. actinomycetemcomitans to reduce the amount of oxygen, is important in the synergism between this organism and Actinomyces in causing infection. It may also be present in atheromatous plaques that expresses molecules that cross react with the antibodies to HSP 60 (Heat Shock Protein).

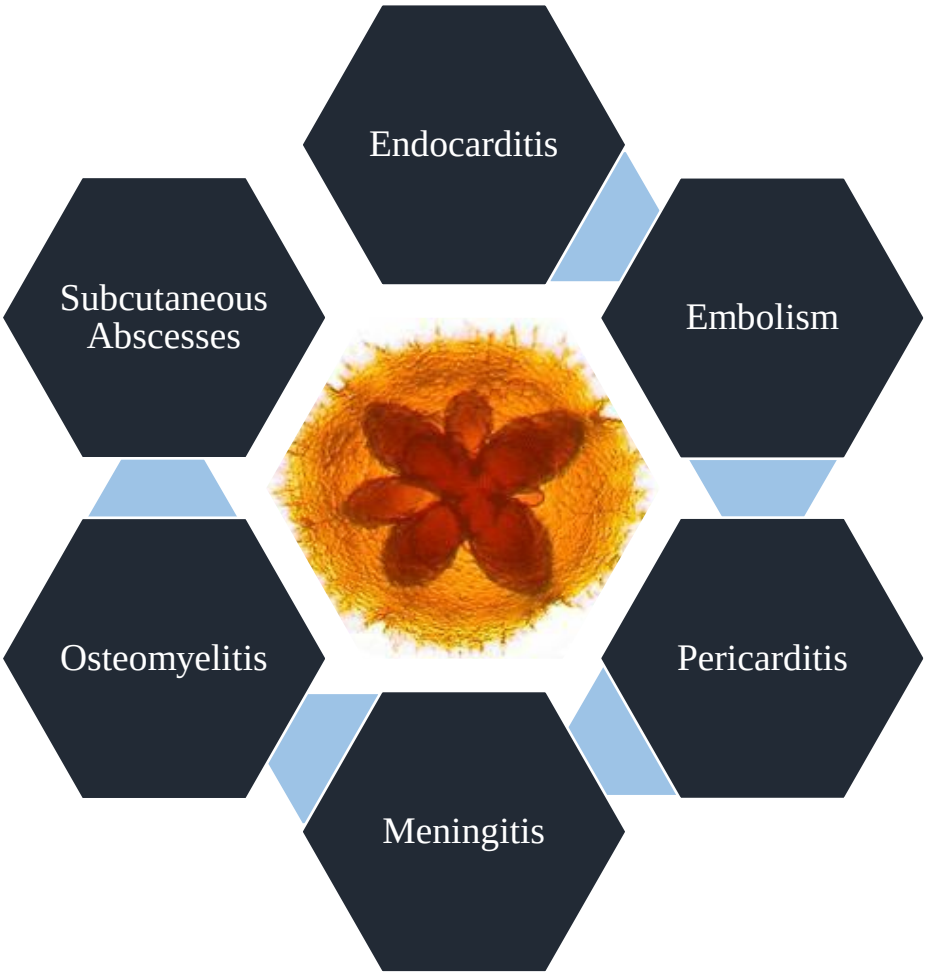

Fig 4.2 The most common extra oral infections mediated by Aggregatibacter actinomycetemcomitans

The level of lipopolysaccharide in plasma from periodontally diseased patients is found to be very low and this low lipopolysaccharide level is suspected to have a priming (or) desensitizing effect. Pre-treatment with 5 pg/ml Aa lipopolysaccharide significantly enhances IL - 1β and IL – 6 productions. A low dose of blood stream lipopolysaccharide found in periodontitis patients appears to prime monocytes and may be capable of affecting the systemic response of the immune and inflammatory cells.[9]

Subgingival prevalence

Subgingival prevalence of Aa is found to be as high as 80 %. Destructive periodontal disease in children is frequently associated with this bacterium. In prepubertal periodontitis and other types of early onset periodontitis, the prevalence is about 40 – 100 %. Aa is also associated with periodontal lesions of Papillon – Lefèvre syndrome. The dynamics of subgingival Aa is the result of a complex bacterium – host interaction.

Aa is also isolated rarely from healthy mucosal sites around integrated dental implants and is detected in failing root formed dental implants. It is a major pathogen in infectious implant failure. Aa is also known to get attached to barrier membranes used in periodontal regeneration and result in failure of regeneration.[9]

Diagnostic tests

Culture

TSBV agar is the medium of choice for culturing A. actinomycetemcomitans. It forms small colonies measuring about 0.5 – 1.0 nm in diameter, which are translucent/transparent, with irregular edges, smooth, circular, convex in shape. Fresh isolates have a “star shaped” (or) “crossed cigar” morphology form, embedding in the agar.[9]

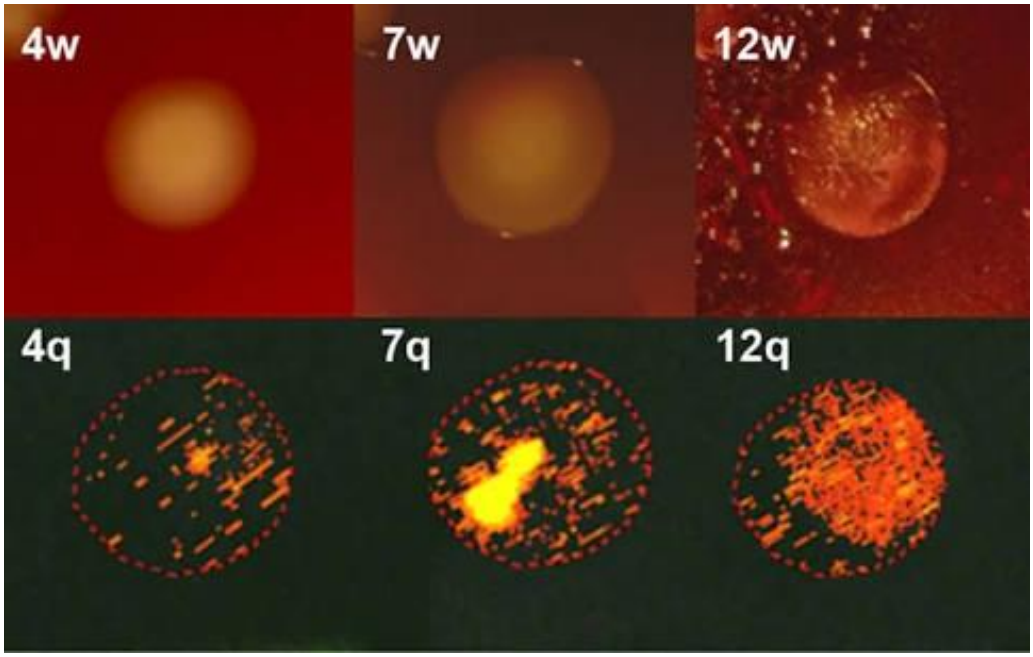

Fig 5.1: A solitary, distinct colony of A. actinomycetemcomiatns after 4, 7 and 12 days of growth.

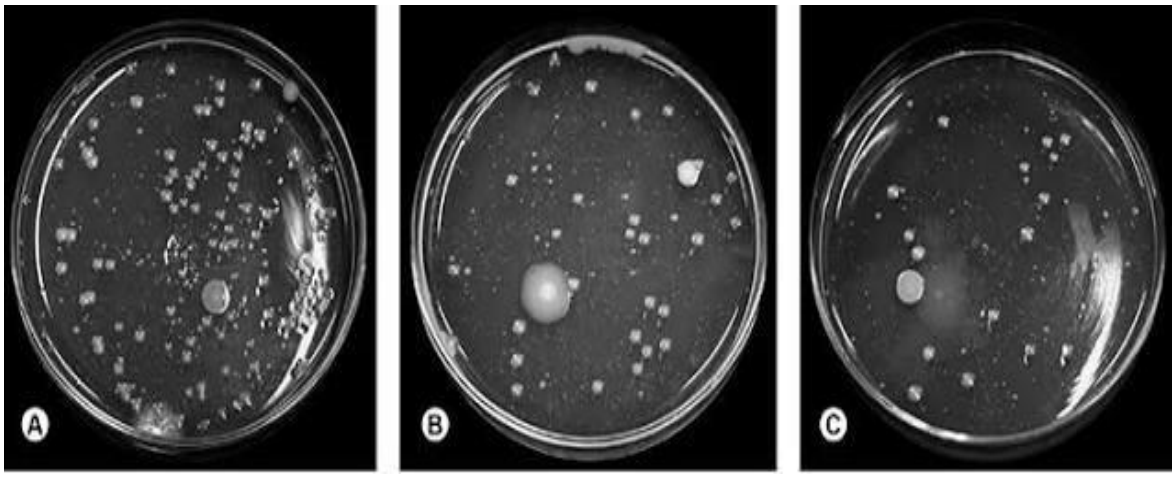

Fig 5.2: Comparison of A. actinomycetemcomitans growth in Control (A), Dye (B) and Dye/ Laser (C) groups.

Immunodiagnostic methods

These include indirect immunofluorescence, flow cytometry, Evalusite ™ test – an antibody-based sandwich enzyme linked immunosorbent assay, bacterial concentration fluorescence immunoassay.

Nucleic acid probes

Digixogenin labelled whole genomic DNA, Radiolabelled cloned DNA, Digixogenin labelled genomic DNA, DMDx ™ detection method, Radiolabelled oligonucleotide. These DNA probe methods are rapid and are efficient tools for clinical detection of periodontopathic bacteria, mainly Aa and Porphyromonas gingivalis. For Aa, the DNA from ATCC 43718, JP2, ATCC 29524, 310a, 146 HE are used for the probes.

Treatment modalities for periodontal diseases mediated by A. actinomycetemcomitans

A highly virulent microbial flora has been described, with Aggregatibacter actinomycetemcomitans considered to be a risk factor because its persistence is associated with recurrence.[99]

The primary objective of treating this kind of periodontal disease is to modify the pathogenic subgingival microbiota to non-pathogenic and to prevent the re-establishment of virulent bacteria in the subgingival biofilm. The identification of the bacterial species that colonise the pockets might be a tool for predicting the outcome or success of treatment, especially with the use of additional antiseptic and antibiotic treatment.[100, 101]

Non-Surgical Periodontal therapy

Several studies have reported the effectiveness and clinical safety of non-surgical periodontal therapy combined with systemic antibiotics for the treatment of Aggressive Periodontitis.[102-104] Hughes et al[99] conducted a study on patients with GAP following treatment with superficial root debridement and oral hygiene instructions. In the re-assessment, the authors reported a mean decrease in PD and an increase in CAL.

Adjunctive antibiotic therapy

Systemically administered antibiotics are recommended for elimination of this bacterium from the subgingival and adjacent intra oral areas. Efficacy of mechanical therapy combined with 875 mg amoxicillin and 500 mg metronidazole every 12 hours for 10 days and Clorhexidine 0.12% rinses to eliminate pathogens and thereby restore periodontal tissue, have been put forth in past studies.[99] The rationale for adding amoxicillin to the prescription of this combined drug regimen is based on a synergistic effect of amoxicillin on metronidazole and its hydroxymetabolite against Aa.[105]

Haffajee et al[106] showed that systemic administration of these antibiotics may suppress periodontal bacteria efficiently and thereby improve the therapeutic response.

Further studies are needed to discuss the balance of the risk/benefits ratio of the prescription adjunctive antimicrobial regimen, in particular when high doses of amoxicillin are used (825mg/10 days/ 2 times a day/) and Aa is identified in low frequency.

Longitudinal studies conducted to compare the clinical and microbial data in patients with severe periodontal disease, representing either subgingival suppression or recurrence of Aa found that in adult periodontitis, the periodontal treatment response is negatively affected by the persistence of subgingival Aa over a 3-year maintenance period.[9]

Study of the microbiological effects of initial periodontal therapy using DNA probes and PCR, indicates that the initial conventional therapy can eliminate Porphyromonas gingivalis and Bacteroides forsythus, but not Aggregatibacter actinomycetemcomitans. This indicates that monitoring the levels of these periodontopathic bacteria and the elimination of all these three microorganisms is a prerequisite for successful treatment. While adjunctive therapy is effective, few studies have reported whether periodontal species have developed resistance to antibiotics prescribed.[99]

Antibiotic resistance has been described amongst bacterial species colonizing the periodontal pockets. Some strains of Aa have been found to exhibit resistance to metronidazole, but a combination of amoxicillin and metronidazole has been found to be effective against subgingival aerobic and capnophilic mixed flora. Pristinamycin and ciprofloxacin are effective alternate monotherapies against Aa.[107] Threat of β lactam resistance by production of β lactamase production is currently not a problem seen with A. actinomycetemcomitans.

Host Modulation therapy

Host modulation therapy with low dose doxycycline – 20 mg doxycycline hyclate is also followed contemporarily. 250 mg tetracycline over a period of 2 – 7 years is a regimen that has been used over the past several years. For tetracycline resistant strains, combination of amoxicillin and metronidazole is the drug of choice.

Mechanical instrumentation significantly changes the composition of the subgingival microflora by decreasing virulent microorganisms and increasing beneficial cocci and bacilli, with this bacterial pattern leading to a healthy bacteriological profile.[99]

However, few reports have shown limited effect of SRP, because their complete elimination might be difficult as a result of re-infection of successfully treated sites. Johnson et al[108] suggest that after 6 months, these bacteria recolonise the plaque when they remain in the epithelial cells of the oral mucosa and are unaltered by treatment.[99]

Mechanical debridement (scaling and root planning- SRP) in combination with amoxicillin – metronidazole therapy is effective in subgingival suppression of Aa in patients with severe periodontitis.

Chlorhexidine 0.2% mouthwash has shown significant antibacterial effects on A. actinomycetemcomitans species isolated from subgingival plaque of periimplantitis patients. In a study by Zeinab Kadkhoda et al[109] in 2015, the results of disc diffusion test demonstrated the mean diameter of Aa growth inhibition zone around discs impregnated with CHX to be significantly larger in standard group and biofilm samples of Aa compared to the negative control group and was zero after 48 hours of incubation.

Laser therapy

The clinical benefits of SRP are derived from the disruption of the subgingival biofilm, which reduces the bacterial load and results in a delay in repopulation by pathogenic microbes. The effects of SRP on selected bacterial species have been evaluated for both the short and the long term. However, mechanical therapy alone has failed time and again to completely eradicate pathogenic bacteria because of their ability to invade within periodontal tissues.[110] These bacteria include Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Campylobacter rectus, and some species of spirochetes.

Joanna J. Kamma et al in demonstrated the effects of Diode Laser (980 nm) therapy on Aggressive Periodontitis patients.[111] According to the findings of this study, diode laser-assisted periodontal treatment with SRP had a superior effect over that of SRP or laser alone in reducing probing depth and increasing clinical attachment level over the 6-mo study period.

Similarly, Moritz et al. earlier reported considerable bacterial elimination from periodontal pockets using irradiation with an 810-nm diode laser at 2.5 W power used in pulse mode (50 Hz, pulse duration 10 msec) following scaling as compared to scaling alone.[112]

Laser has shown a positive effect on elimination of pathogenic bacteria, when used as adjunct to SRP.

Summary

A. Scaling and root planing alone cannot remove A.actinomycetemcomitans from lesions of localized aggressive periodontitis. Non-surgical therapy has the least effect on Aa counts in heavily infected periodontal lesions. This is because of the ability of the organism to invade gingival tissue and evade the effect of mechanical debridement and periodontal healing. The cells of the bacterium in gingival may constitute a reservoir for repopulating periodontal pockets.

B. Periodontal therapy often fails to effectively control subgingival Aa Modified Widman flap surgery is shown to have about 50 % effect.

C. An apically displaced flap with osseous recontouring is more effective than an apically displaced without osseous recontouring in reducing subgingival levels of Aa.

D. Superior performance of resective periodontal surgery may be due to the excision of Aa – infected gingival tissue and reduction of pocket depth.

E. Systemic metronidazole has good anti Aa activity in localized aggressive periodontitis patients, but not in cases of adult periodontitis.

F. Systemic amoxicillin-metronidazole combination shows striking clinical results in treatment of localized aggressive periodontitis, adult periodontitis and refractory periodontitis, even in the absence of other periodontal therapy. The prescribed regimen is 250 mg amoxicillin and 250 mg metronidazole- thrice daily for 8 days.

In summary, for complete treatment of Aa mediated periodontal infections, the treatment plan should include scaling and root planning with a surgical procedure with/ without osseous recontouring along with systemic and local antibiotic therapy.[9]

Other discoveries

1. Taichman et al. divided A. actinomycetemcomitans into 4 serogroups based on surface antigens and proteinaceous leukotoxin.[93]

2. Fives-Taylor et al in 1999 study demonstrated an effective migration of Aggregatibacter actinomycetemcomitans through the gingival epithelium.[113]

3. Baker & Wilson, in their study conducted in 1989 observed that patients with localized aggressive periodontitis had circulating opsonic IgG antibodies, which were secreted by mature B cells (plasma cells) against Aa.[114]

4. A. actinomycetemcomitans stimulates interferon-c (IFN-c) and interleukin-12 (IL-12) production that polarize Th1 cells and IL-4 that polarizes Th2 cells as observed by Garlet et al in 2006.[115]

5. Aa has the ability to adhere to mucosal and gingival epithelial cells, which is believed to be essential for its initial colonization in the oral cavity (Meyer & Fives-Taylor, 1994.[116]

6. Guentsch et al in 2009 isolated PMNs from patients with aggressive periodontal disease with IgG titers against Aa, greater PMN phagocytosis and killing compared with PMNs isolated from periodontally healthy control subjects. Relative to healthy control subjects, PMNs from patients with aggressive periodontal disease were characterized by a significant increase in basal activity of human neutrophil elastase.[117]

7. Permpanich et al in 2006 suggested that self-aggregating Aa, possessing a rough fimbriated surface, impaired PMN phagocytosis, which perhaps is simply secondary to PMN limitations in engulfing large foreign bodies.[118]

8. The LPS from Aa has been shown to promote osteoclastic differentiation and activation, resulting in alveolar bone resorption (Rogers et al., 2007).[119]

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