ACTINOBACILLUS ACTINOMYCETEMCOMITANS AND PERIODONTAL DISEASE (LABORATORY AND CLINICAL STUDIES)

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Thesis Submitted for the Degree of Doctor of Philosophy
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May, 1990

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. T.W MacFarlane for his invaluable advice, guidance, criticism and encouragement throughout this study.

Special thanks are due to Mr. W.M.M Jenkins (Periodontology unit, Glasgow Dental Hospital and School) for his help in the clinical study in carrying out the examination of patients and in supplying specimens.

Many thanks are due also to Mr. W Marshall and Mr. D Mackenzie for their help.

Ibrahim

DECLARATION

None of the work presented in this thesis has been submitted in support of an application for another degree of this or another university or institution of learning.

The author,

I A A Taher

SUMMARY

Actinobacillus actinomycetemcomitans has been regarded as one of the major microbiological aetiological agents in certain types of human periodontal disease especially Localized Juvenile Periodontitis. However, relatively few reports are available concerning its prevalence in chronic periodontitis, and there is some doubt concerning the laboratory methods used to isolate and identify *A.actinomycetemcomitans* from subgingival plaque.

In the present investigation the ability of two semi-selective media (TSBV and MGB) to isolate *A.actinomycetemcomitans* from dental plaque samples was examined. TSBV medium was found to be less inhibitory with pure cultures and more selective in mixed cultures than MGB. In the laboratory, the presence of star-shaped colonies on primary culture plates is commonly used to differentiate *A.actinomycetemcomitans* from other bacteria, even although non-star-shaped variants are known to occur. Since there is no information concerning the presence of star-shaped and non-star-shaped colonies on primary isolation by *A.actinomycetemcomitans* in the literature, the prevalence of both colonial forms of *A.actinomycetemcomitans* on primary culture on TSBV medium was investigated in a subgroup of samples collected from patients with chronic periodontitis (see below). Surprisingly more than half of the isolates (52%) produced non-star-shaped colonies, the remainder having the star-shaped format. As a result if only star-shaped colonies had been selected for identification in this thesis, the prevalence

data for the chronic periodontitis patients would have been reduced from 38 to 19 patients. Since no standard method for identifying *A.actinomycetemcomitans* was available, the API 20 A system was used in this thesis. Four different biochemical profiles were obtained, and the system was found to be simple to use and gave reproducible results.

In a clinical study of the prevalence of *A.actinomycetemcomitans* in 98 patients with chronic periodontitis (302 subgingival samples), the organism was isolated from 39% of patients and from 27% of the total samples collected. However, in a healthy control group of 55 volunteers only one (2%) of the subjects possessed *A.actinomycetemcomitans*. In the chronic periodontitis group there were 55 females and 43 males, and although *A.actinomycetemcomitans* was isolated overall from more sites in male patients (60%) compared to female patients (40%), the distribution of *A.actinomycetemcomitans* was found to be similar in both groups. In general there were no clear differences between the prevalence of *A.actinomycetemcomitans* in the six different segments sampled in the 98 patients.

Although adherence and hydrophobicity are believed to play an important role in the pathogenicity of a number of bacteria, little is known about these properties with regard to *A.actinomycetemcomitans*. In addition, previously published studies have used small numbers of strains. In the present investigation the ability of 33 strains of *A.actinomycetemcomitans* to adhere to human buccal epithelial cells in the presence of Saliva Ions Buffer (SIB), clarified mixed saliva

and serum, using an epifluorescence technique was assessed *in vitro*. The hydrophobicity of the same 33 strains was assessed using adsorption to xylene. The adherence results clearly showed that overall *A.actinomycetemcomitans* can adhere well to buccal epithelial cells *in vitro* and in comparable numbers when experiments were performed in mixed saliva and in SIB. The adherence values were 12.7 and 12.0 adherent bacteria per buccal epithelial cell respectively. Similarly there was no significant difference between the adherence of fresh isolates (mean 13.3 bacteria) compared to the Type strains (10.7). The addition of human serum to the adherence assay system significantly inhibited the adherence of *A.actinomycetemcomitans* strains to buccal cells ($p \leq 0.0005$). All isolates of *A.actinomycetemcomitans* were hydrophobic and produced a reduction in absorbance which ranged from 39% to 82% (mean 67%). No significant difference between the hydrophobicity of fresh and Type strains of *A.actinomycetemcomitans* was found. When the adherence to buccal epithelial cells and hydrophobicity results for each strain were compared a significant overall correlation ($p \leq 0.05$) was found. These results tend to confirm the view that hydrophobicity and adherence to buccal cells are related.

The pathogenicity of *A.actinomycetemcomitans* has been related to its ability to produce a number of toxic factors, especially leucotoxin. The leucotoxic activity of twelve fresh isolates and six Type strains of *A.actinomycetemcomitans* against human polymorphonuclear leucocytes (PMNLs) or a

promyelocyte cell line (HL-60) was investigated using the trypan blue exclusion or lactate dehydrogenase release (LDH) methods, *in vitro*. Only one of the fresh isolates of *A.actinomycetemcomitans* (Aa 40) was found to be leucotoxic for both HL-60 cells and PMNLs as monitored by both detection methods. The other fresh isolates failed to show any definite toxic activity. The Type strains were leucotoxic negative for both PMNLs and HL-60 cells. In view of the previous literature these results were unexpected and the possible reasons for the discrepancies between this study and that of previous workers are discussed in full. There is no agreed method of testing strains of *A.actinomycetemcomitans* for leucotoxicity, and no criteria for categorizing strains as leucotoxic positive or negative. In view of the apparent importance of this organism in some types of periodontal disease, appropriate methods and criteria should be developed in the near future.

In conclusion, although it appears that much is known about the ecology and pathogenicity of *A.actinomycetemcomitans* in the human mouth, the results of this study suggest that the current state of knowledge is far from complete, and that a substantial effort is needed, initially in relation to methodology, and later in linked clinical-laboratory studies, before this area will become clear.

PREFACE

Some parts of the work reported in this thesis have been presented at scientific meetings and published as abstracts as follows:

SCIENTIFIC MEETINGS

I.A.A TAHER and T.W MACFARLANE: Problems in the use of colonial morphology for the identification of *Actinobacillus actinomycetemcomitans*. *The British Society for Dental Research,* Liverpool, 1989.

T.W MACFARLANE, I.A.A TAHER and W.M.M JENKINS: The recovery of *Actinobacillus actinomycetemcomitans* from chronic periodontitis patients. *The British Society for Dental Research,* Liverpool, 1989.

PRINTED ABSTRACTS

TAHER, I.A.A. & MACFARLANE, T.W. (1989) Problems in the use of colonial morphology for the identification of *Actinobacillus actinomycetemcomitans*. *Journal of Dental Research,* **68**, 602, abstract number 287.

MACFARLANE, T.W., TAHER , I.A.A & JENKINS, W.M.M. (1989) The recovery of *Actinobacillus actinomycetemcomitans* from chronic periodontitis patients. *Journal of Dental Research,* **68**, 594, abstract number 347.

ABBREVIATIONS

Aa: *Actinobacillus actinomycetemcomitans*

ABB: Anaerobic Blood Broth Supplemented

ATCC: American Type Culture Collection

BECs: Buccal Epithelial Cells

CBA: Columbia Blood Agar

cfu/ml: Colony forming units per millilitre

DST: Diagnostic Sensitivity Agar

HBSS: Hanks Balanced Salt Solutions

LDH: Lactate dehydrogenase

LJP: Localized Juvenile Periodontitis

MGB: Malchite Green Bacitracin Agar

NCTC: National Collection of Type Cultures

O.D: Optical Density

p: Probability

±SEM: Standard error of mean

PBS: Phosphate Buffered Saline

PMNLs: Polymorphonuclear leucocytes

SIB: Saliva Ions Buffer

TGB: Thioglycollate Broth

TSBV: Tryptic Soy-Serum-Bacitracin-Vancomycin Agar

CHAPTER 1

LITERATURE REVIEW

1.1 Historical Review

Actinobacillus actinomycetemcomitans was first described in 1912 by the German microbiologist Klinger who isolated the organism from a case of cervicofacial actinomycosis. *A.actinomycetemcomitans* has been closely associated and frequently present with *Actinomyces israelii* in actinomycotic lesions (Klinger, 1912; Colebrook, 1920) and Holm (1951) proposed that actinomycosis should be regarded as a mixed infection of these two microorganisms. The original name given to the *A.actinomycetemcomitans* was *Bacterium actinomycetum comitans* (Klinger, 1912). This was changed to *Bacterium comitans* (Lieske, 1921) and later to *Actinobacillus actinomycetemcomitans* (Topley and Wilson, 1929). However, in view of genetic and serological relationships and previous inclusion of X and V factor independent bacterial species in the genus *Haemophilus, A.actinomycetemcomitans* was reassigned to the genus *Haemophilus actinomycetemcomitans* by Potts, Zambon and Genco (1985). However, this has not been widely accepted and both terms are in use at present.

Until recently little was known about the pathogenicity of *A.actinomycetemcomitans*, although it was described as non-pathogenic for laboratory animals (Buchanan and Gibbons, 1974). Pathogenicity for man was thought to be doubtful, since it occurs usually in combination with *A.israelii* (Heinrich and Pulverer, 1959 b; Buchanan and Gibbons, 1974).

Thus *A.actinomycetemcomitans* was regarded as a harmless member of the oral microflora which could occasionally cause pyogenic infections either alone (Thjotta and Sydnes, 1951) or in mixed culture with *A.israelii* (Heinrich and Pulverer, 1959b). The organism has also been recovered from severe non-oral infections (see section 1.6.1).

Kilian and Schiott (1975) reported that *A.actinomycetemcomitans* was indigenous to dental plaque, whereas, studies by Slots, Reynolds and Genco (1980a) indicated that subgingival dental plaque (especially from periodontal pockets) and the buccal mucosa were the organism's main oral ecological niches. During the past ten years studies have demonstrated a close association between juvenile periodontitis and the presence of *A.actinomycetemcomitans* (see review by Zambon, 1985, and section 1.7.3).

1.2 Morphological Characteristics

1.2.1 Colonial morphology

Fresh isolates cultured on solid media form small colonies about 0.5-1.0mm in diameter. Colonies are usually convex, circular, translucent and glistening with a slightly irregular edge (Slots *et al.*, 1980a; Slots, 1982a; Zambon, 1985). In addition, the internal shape of colonies is often described as "star-shaped" (Colebrook, 1920) or as "crossed-cigars" (Heinrich and Pulverer, 1959a). Other studies have also reported similar findings (Mandell and Socransky, 1981; Slots, 1982b). However, not all isolates of *A.actinomycetemcomitans* possess this cultural morphology, since non-

star-shaped variants have also been observed (Mandell and Socransky, 1981; Slots, 1982b). On primary isolation colonies tend to adhere to the agar surface and are difficult to emulsify, but become mucoid and non-adherent after repeated subculturing (Buchanan and Gibbons, 1974; Slots, 1982a). Rosan *et al.*, (1988) reported similar observations where some freshly isolated *A.actinomycetemcomitans* strains showed a "cross" or "star-shaped" interior structure which was lost after repeated subculturing *in vitro*. In addition these non-star-shaped strains, adhered weakly to the agar surface, grew rapidly in broth cultures and formed uniform suspensions (Rosan *et al.*, 1988).

1.2.2 Light microscopy

A.actinomycetemcomitans cells are Gram-negative, non motile, spheres or rods. They measure about 1.0-1.5 by 0.4-0.5μm in size (Slots, 1982a). Cells may occur singly, in pairs or in small clumps (Zambon, 1985). In older cultures or after repeated subculturing longer cells may be produced by some strains (Slots, 1982a).

1.2.3 Electron microscopy

The ultrastructure of *A.actinomycetemcomitans* using transmission electron microscopy is similar to that of other Gram-negative bacteria consisting of an outer cytoplasmic membrane, a periplasmic space and an inner cytoplasmic membrane (Holt, Tanner and Socransky, 1980). The outer membrane is covered by a ruthenium staining polymeric material (Holt *et al.*, 1980) which is said to be a possible carbohydrate

microcapsule (Zambon, Slots and Genco, 1983a). In addition membrane vesicles, ("blebs") are present in large numbers which are probably derived from the outer cytoplasmic membrane. Morphologically these structures are identical to lipopolysaccharide vesicles being released into the external environment during growth (Holt *et al.*, 1980; Lai, Listgarten and Hammond, 1981; Nowotny *et al.*, 1982). Scanning electron microscopy has also revealed the presence of surface projections and amorphous surface material which cover and connect adjacent cells (Holt *et al.*, 1980). Further studies have revealed the presence of fimbriae on freshly isolated *A.actinomycetemcomitans* strains (Scannapieco, Kornman and Coykendall, 1983; Scannapieco *et al.*, 1987; Preus, Namork and Olsen, 1988; Rosan *et al.*, 1988).

1.3 Biochemical Characteristics

The biochemical characteristics of *A.actinomycetemcomitans* are summarized in (Table 1.1). Members of the species are fermentative, non-haemolytic, indole negative and produce catalase. Although many sugars and carbohydrates have been tested, *A.actinomycetemcomitans* is limited in its saccharolytic activity. However, the results in Table 1.1 highlight the sugar fermentation reactions which are useful in identification. *A.actinomycetemcomitans* can be distinguished from other bacteria such as, *Haemophilus* species by its ability to grow in the absence of X (haemin) or V (nicotinamide adenine dinucleotide). Using biochemical tests it can also be differentiated from other X and V factor independent organisms like *Haemophilus aphrophilus* (see Table 1.1). Most wor-

Table 1.1 Biochemical characteristics of *A.actinomycetemcomitans* and *H.aphrophilus*

Test	*A.actinomycetemcomitans*	*H.aphrophilus*
Catalase	+	-
Nitrate	+	+
Glucose	+	+
Galactose	v	+
Lactose	-	+
Maltose	v	+
Mannitol	v	-
Xylose	v	-
Sucrose	-	+
Trehalose	-	+
Raffinose	-	+
Glycerol	-	+
Starch	v	+
β-glucosidase	-	+
β-galactosidase	-	+
Sodium fluoride	-	+

+: Positive reaction

-: Negative reaction

V: Variable reaction

kers have reported similar biochemical profiles for *A.actinomycetemcomitans* (King and Tatum, 1962; Sneath and Johnson, 1973; Buchanan and Gibbons, 1974; Kilian and Schiott, 1975; Kilian and Frederisken, 1981; Slots, 1982a). However, Tanner *et al.*, (1982) reported that three (16%) of their *A.actinomycetemcomitans* strains did not decompose hydrogen peroxide.

1.4 Serological Characteristics

Most studies have found 3 different serogroups using a variety of techniques including tube precipitation (Slots *et al.*, 1980a), immunodiffusion and indirect immunofluoresence (Zambon *et al.*, 1983a) and monoclonal antibodies (McArthur, Stroup and Wilson, 1986) (see Table 1.2). Some of these groups were said to be similar eg. the serogroups a, b and c of Zambon *et al.*, (1983a) and those of King and Tatum (1962). *A.actinomycetemcomitans* serogroups a and b are most commonly found in the human oral cavity, while serotype c accounts for only 10% of oral isolates. However, serotype a is mainly present in samples from patients with localized juvenile periodontitis suggesting that this serotype may be important in the pathogensis of this disease (Zambon *et al.*, 1983a). Zambon and Jones (1987) indicated that all 3 serotypes described above (a, b and c) are predominantly mannose containing polysaccharide antigens.

A.actinomycetemcomitans possess at least one common antigen (King and Tatum, 1962; McArthur *et al.*, 1982), which is shared with other members of the *Actinobacillus* genus eg. *A.*

Table 1.2 Summary of the different serogroups and biotypes of *A.actinomycetemcomitans* described in the literature.

Author	Sero-groups	Bio-types	Anti-gens	Agglut. Patterns	Techniques
King & Tatum (1962)	3	7	1		Capillary tube precipitation and fermentation of sugars
Pulverer & Ko (1970/72)	3	8	6	24	Tube agglutination assay
Slots *et al.*, (1980a)	3	10			Fermentation of dextrin, maltose mannitol and xylose
Slots *et al.*, (1982)	3				Heat stable polysaccharide component
Zambon *et al.*, (1983a)	3	10	12	3	Immunodiffusion and immunofluorescence
McArthur *et al.*, (1982)	4				Immunodiffusion and enzyme linked immunosorbence
Taichman *et al.*, (1982)	4				Surface antigens and proteinaceous leucotoxin
McArthur *et al.*, (1986)	3				Monoclonal antibodies

Agglut.: Agglutination

suis and *A.equuli* (Zambon *et al.*, 1983a) and one other antigen shared with *H.aphrophilus* (McArthur *et al.*, 1982; Zambon *et al.*, 1983a).

1.5 Isolation and Selectivity

A useful approach to help determine the role of an organism in the aetiology of a particular disease is to study its prevalence and numbers in diseased and healthy sites. It is very difficult using non selective culture media such as blood agar to isolate and enumerate *A.actinomycetemcomitans* from subgingival plaque samples. Therefore two different semi-selective media have been developed.

The medium developed by Slots *et al.*, (1980a) contained tryptic case soy agar, supplemented with 75µg/ml bacitracin, 10% heat-inactivated horse serum, 0.1% yeast extract and 0.2% glucose. This medium was later improved by Slots (1982b) by the addition of 5 mg/ml vancomycin and removal of glucose (TSBV). Slots (1982b) showed that at least as many colonies of *A.actinomycetemcomitans* were isolated from subgingival plaque samples on this semi-selective medium compared with enriched brain heart infusion blood agar. The new selective medium (TSBV) also suppressed the growth of most other bacteria present in plaque and it was possible for *A.actinomycetemcomitans* to be distinguished from other species isolated by colonial morphology and catalase activity.

In 1981 Mandell and Socransky developed a medium composed of tryptic soy agar 40g/litre, bacitracin 128µg/ml, malachite green 8 µg/ml and 5% defibrinated sheep blood (MGB). The

MGB when incubated in air plus 10% CO_2 gave a recovery rate of 83-99% of pure cultures of *A.actinomycetemcomitans* when compared to non selective media. The efficacy of both MGB and TSBV media in recovering *A.actinomycetemcomitans* from periodontal pockets and the ability of laboratory strains to grow on both media were compared by Van Steenbergen *et al.*, (1986). The results showed that the highest colony forming units/ml were obtained on Blood agar, TSBV and MGB media respectively. Low counts were detected on MGB and six strains of *A.actinomycetemcomitans* did not grow on this medium (Van Steenbergen *et al.*, 1986). In addition the colony characteristics of *A.actinomycetemcomitans* were easily recognized and colonies appeared larger on TSBV than on MGB medium. Further attempts were made to improve the selectivity of both TSBV and MGB media by adding other drugs such as spiramycin, carbenicillin and fusidic acid to TSBV (Holm *et al.*, 1987), or penicillin to both MGB and TSBV (Riches and Marsh, 1987). However, the addition of these agents to both media did not significantly improve their selectivity (Holm *et al.*, 1987; Riches and Marsh, 1987).

1.6 Oral Ecology

The mouth contains several ecological niches available for bacterial growth, these include the buccal mucosa, tongue, hard and soft palate, gingiva, gingival crevice, teeth and saliva, as well as dentures and various filling materials (Gibbons, 1984). The oral cavity contains very few organisms at birth but shortly thereafter, various bacteria colonize these oral sites (Christersson *et al.*, 1985). After

tooth eruption, additional bacterial species are acquired from the mother and other family members and colonize the tooth surface and newly formed gingival sulcus (Socransky and Manganiello, 1971; Berkwoitz and Jordan, 1975). However, for a microorganism to colonize the human mouth, certain requirements have to be met, such as variation in temperature, oxidation-reduction potential, pH and nutrition (Slots and Genco, 1984). Other factors include serum or saliva components (Gibbons, 1980), growth conditions (Peros and Gibbons, 1981) and salivary enzymes of bacterial origin (Nakamura and Slots, 1983). In addition persistent colonization in the mouth requires the bacteria to become sufficiently attached to oral surfaces to avoid removal by the mechanical washing action of saliva and the crevicular fluid together with other cleansing mechanisms (Van Houte, 1983).

Dental plaque can be defined as a complex mixed microbial community which forms on hard surfaces within the mouth consisting predominantly of extracellular polymers synthesized by bacteria and macromolecules derived from saliva, crevicular fluid and dietary sources in the mouth (Fitzgerald, 1985). Saliva is believed to play a central role in the microbial ecology of the mouth; it protects mucosal surfaces from desiccation (Tabak *et al.*, 1982), and may modulate bacterial adhesion and aggregation to oral surfaces (Stinson *et al.*, 1982). It can also be an important source of nutrients for bacterial growth (Van der Hoeven *et al.*, 1984). The mouth is also supplied with crevicular fluid via the gingival crevice, which has a protective function due to its

flushing action and to the presence of immune system components such as, immunoglobulins (IgG, IgA & IgM), complement, neutrophils, lymphocytes and monocytes (Cimasoni, 1983).

1.7 Pathogenicity of *A.actinomycetemcomitans*

The role of specific Gram-negative bacteria in the aetiology of human periodontal disease has been increasingly appreciated in recent years (Slots, 1984). However, periodontal disease activity has not been fully investigated and it remains difficult to associate a specific bacterial species with active periodontal destruction (Slots and Dahlen, 1985). *A.actinomycetemcomitans* for a number of years has been considered a pathogen mainly in LJP and to a lesser extent in adult periodontitis (Slots and Dahlen, 1985). In general for an organism to cause periodontal disease, it must possess virulence factors to (a) allow it to initially colonize and grow in the periodontal environment (b) resist the host defence mechanism and (c) to produce tissue damage (Smith, 1975). *A.actinomycetemcomitans* has been shown to produce various factors which may play an important role in the pathogenicity of this organism (see Table 1.3).

1.7.1 Leucotoxic activity of *A.actinomycetemcomitans*

Polymorphonuclear leucocytes (PMNLs) are commonly present in periodontal pockets constituting >90% of the leucocyte population (Slots, 1986). They probably play a central role in the host defence against periodontal infection due to their ability to phagocytose and destroy microorganisms (Van Dyke,

Levine and Genco, 1982a). During the last 10 years investigators have studied the interaction between PMNLs and bacteria present in human dental plaque (Taichman and McArthur, 1976; Taichman *et al.*, 1977; Tsai *et al.*, 1978; Baehni *et al.*, 1978). Although the majority of certain Gram-negative bacteria (eg. *Capnocytophaga, Bacteroides* and *Leptotrichia* species) as well as whole plaque, triggered the release of certain lysosomal constituents present in PMNLs (eg. lysozyme, β-glucuroindase or myeloperoxidase), there was no evidence of PMNL death in these experiments (Taichman *et al.*, 1977; Baehni *et al.*, 1978; Tsai *et al.*, 1978). More interestingly Tsai *et al.*, (1978) reported that a Bacteroides like Gram-negative microorganism designated Y4 failed to stimulate PMNLs lysosomal release and showed no cytotoxic activity. In 1979, Baehni *et al.*, presented the first study which demonstrated the cytotoxic activity of *A.actinomycetemcomitans* Y4 for human PMNLs. The reason for the lack of toxicity of Y4 in the previous study (Tsai *et al.*, 1978) is not understood (Baehni *et al.*, 1979), although it may be related to the fact that different growth conditions and a fresh stock culture of Y4 were used in later experiments. In addition, it was suggested that reculturing of *A.actinomycetemcomitans* Y4 *in vitro* may result in the loss of leucotoxic activity (Baehni *et al.*, 1979). Similar experiments were carried out later by a number of investigators and all agreed that *A.actinomycetemcomitans* Y4 possessed cytotoxic activity (Tsai *et al.*, 1979; McArthur *et al.*, 1981; Baehni *et al.*, 1981).

The toxin produced by *A.actinomycetemcomitans* is widely described as a leucotoxin which kills human PMNLs and monocytes (Tsai *et al.*, 1979; Taichman, Dean and Sanderson, 1980; Taichman *et al.*, 1982; Simpson, Berthold and Taichman, 1988). The toxin is known to be heat-labile, active at 37°C but inactivated at 56°C for 30 minutes and trypsin sensitive (Taichman *et al.*, 1980; McArthur *et al.*, 1982; Ohta *et al.*, 1987). The leucotoxin is found in both viable as well as in soluble sonic extracts or in the membrane vesicles of *A.actinomycetemcomitans* cells (Tsai.*et al.*, 1979; Nowotny *et al.*, 1982). The cytotoxic activity of *A.actinomycetemcomitans* leucotoxin is described to be dose, time and temperature dependent. As the number of bacteria per PMNL is increased more PMNLs were killed, reaching a maximum after 45 to 60 minutes exposure, as monitored by either trypan blue staining or lactate dehydrogenase release (Baehni *et al.*, 1979; Tsai *et al.*, 1979; McArthur *et al.*, 1981; Zambon *et al.*, 1983c).

There is some evidence that the addition of fresh human sera (Baehni *et al.*, 1979; McArthur *et al.*, 1982; Zambon *et al.*, 1983c) as well as sera from patients with other types of periodontal or systemic diseases (Tsai *et al.*, 1981; Taichman and Wilton, 1981) enhances the cytotoxicity of *A.actinomycetemcomitans in vitro*. However, sera from juvenile periodontitis patients or pooled sera from rabbits immunized with *A.actinomycetemcomitans*, neutralized the leucotoxin and no killing of PMNLs occurred (Tsai *et al.*, 1979; McArthur *et al.*, 1981). It appears that this protective activity was

due to the presence of antibodies to the leucotoxin, since Ebersole *et al.*, (1982) and Tsai *et al.*, (1981) found that LJP patients had high serum IgG levels to *A.actinomycetemcomitans* Y4. Microbiological and immunological studies have demonstrated that specific IgG was later identified as the inhibitor of *A.actinomycetemcomitans* leucotoxin activity (McArthur *et al.*, 1981). While the leucotoxin produced by *A.actinomycetemcomitans* Y4 has been most extensively studied, Baehni *et al.*, (1981) examined a number of *A.actinomycetemcomitans* strains including some dental plaque isolates, and found that not all strains tested exhibited leucotoxic activity for PMNLs. The leucotoxic strains were found to possess specific antigens which were absent from the non-leucotoxic strains (Baehni *et al.*, 1981).

A.actinomycetemcomitans leucotoxin can kill gingival crevice PMNLs (Taichman and Wilton, 1981; Taichman *et al.*, 1982), promyelocytic tissue cell lines HL-60, U937 and KG-I as well as T and B-cell lines (Zambon *et al.*, 1983c; Simpson *et al.*, 1988). Furthermore, *A.actinomycetemcomitans* leucotoxin was found to be cytotoxic for other non human PMNLs and monocytes including those of monkeys and other primates which varied significantly in their sensitivity to the *A.actinomycetemcomitans* leucotoxin (Taichman *et al.*, 1984; Taichman *et al.*, 1987a). The ability of *A.actinomycetemcomitans* to produce leucotoxin appears to be correlated with virulence (Slots and Genco, 1984; Slots, 1986). This is evident from a study by Zambon *et al.*, (1983c) who showed that 55% of *A.actinomycetemcomitans* isolates from LJP patients were leu-

cotoxic, whereas, only 16% of *A.actinomycetemcomitans* isolates from healthy subjects possessed cytotoxic activity. However, Taichman *et al.*, (1982) hypothesized that infection by *A.actinomycetemcomitans* may occur during the early stages in the development of juvenile periodontitis and that leucotoxin production in the gingival crevice may deplete the area of an essential host defence mechanism. In addition, Tsai and Taichman (1986) suggested that the ability of *A.actinomycetemcomitans* to destroy human PMNLs could be altered during the course of infection since leucotoxic producing *A.actinomycetemcomitans* were only found in isolates obtained from young patients (6-12 years) but not in older patients (13-25) years old.

Although some *A.actinomycetemcomitans* strains possess plasmids (Hammond, Peindl and Socransky, 1975) and are infected with bacteriophage (Stevens, Hammond and Lai, 1982; Preus, Olsen and Namork, 1987), there seems to be no correlation between these factors and leucotoxin production (Stevens *et al.*, 1982). Moreover, leucotoxin-producing *A.actinomycetemcomitans* strains may be more resistant to the hydrolytic enzymes of PMNLs than non-producing strains, since enzymatic extracts from human PMNLs cause greater damage to the cell wall and cytoplasmic membrane of non-leucotoxic *A.actinomycetemcomitans* strains than to leucotoxic *A.actinomycetemcomitans* Y4 (Sela, 1983; Sela and Romano, 1983).

Table 1.3 Potentially important pathogenic factors produced by *A.actinomycetemcomitans* strains.

FACTORS PRODUCED	EFFECT on HOST
Leucotoxin	kill PMNLs and monocytes
Endotoxin	platelet aggregation, macrophage killing
Lipopolysaccharide (LPS)	Lymphocyte activation
LPS	Bone resorption
Epitheliotoxin	Periodontal tissues damage
Collagenase	~ ~
Catalase	~ ~
Fibroblast growth inhibitor	~ ~
Acid phosphatase	~ ~
Alkaline phosphatase	~ ~

1.7.2 Chemotaxis suppression

With regard to the immune system, defective neutrophil chemotaxis has been reported in patients with juvenile or **rapidly** Progressive periodontitis(Ciancola *et al.*, 1977; Lavine *et al.*, 1979; Clark, Page and Wilde, 1977; Van Dyke *et al.*, 1980) as well as defective phagocytosis (Van Dyke *et al.*, 1986). However, Kinane *et al.*, (1989a, b) could not confirm the presence of depressed PMNL chemotaxis in juvenile or rapidly progressive periodontitis patients using the Boyden chamber method. Differences in techniques were thought to account for much of the variation with respect to PMNL chemotaxis in periodontal disease (Kinane *et al.*, 1989b).

A reduced level of complement-derived chemotactic activity has been demonstrated in serum from patients with LJP (Clark *et al.*, 1977). This may play a role in periodontal infection in that defects in PMNLs may predispose individuals to infection (Van Dyke *et al.*, 1980). For instance, Van Dyke *et al.*, (1982b) showed that *A.actinomycetemcomitans* can inhibit human neutrophil chemotaxis and Sundqvist and Johansson (1982) demonstrated that *A.actinomycetemcomitans* was not killed by a pooled human serum with known active complement. Other workers have shown that *A.actinomycetemcomitans* strains were resistant to killing by complement alone or by complement and serum containing IgG and IgM antibodies to *A.actinomycetemcomitans* (Evans and Genco, 1983). However, in the presence of neutrophils (PMNLs), complement and antibodies, killing did occur indicating the importance of these cells in the immune system (Genco and Slots, 1984).

1.7.3 Lymphocyte suppression

Despite the fact that sonic extracts of *A.actinomycetemcomitans* do not appear to be cytotoxic for human lymphocytes (Tsai *et al.*, 1979), it is known that such extracts can suppress the responsiveness of human lymphocytes to mitogens and antigens affecting DNA, RNA, and protein synthesis (Shenker, McArthur and Tsai, 1982a). Sonic extracts also suppress both B and T-cell functions (Shenker, Tsai and Taichman, 1982b). In addition a lipopolysaccharide from *A.actinomycetemcomitans* was shown to enhance human lymphocyte killing of the natural killer cell-sensitive human erythroleukaemia cell line K562 (Lindemann, Miyasaki and Wolinsky, 1988; Lindemann, 1988). Moreover, using animal models Bick *et al.*, (1981) and Yoshie *et al.*, (1985) found that *A.actinomycetemcomitans* possessed a potent B-cell activator which may contribute to *A.actinomycetemcomitans* pathogenicity by inducing B lymphocytes to produce antibodies and possibly other mediators of inflammation (Carpenter *et al.*, 1984). More recent studies have also shown that *A.actinomycetemcomitans* can strongly induce interleukin-1 (IL-1) production by mouse macrophages or human monocytes which may be important in the development of periodontal disease, since IL-1 may express a range of biological activities eg. alteration of gingival connective tissues during the development of periodontal disease (Ohmori *et al.*, 1988; Lindemann and Economou, 1988).

Finally *A.actinomycetemcomitans* is reported to be resistant to killing by physiological concentrations of (85 - >1000μM)

hydrogen peroxide (H_2O_2). This property could be sufficient to protect it from direct H_2O_2-mediated killing by host phagocytes (Miyasaki *et al.*, 1984). However, the addition of sodium azide, which inhibits cell-associated catalase activity did not affect the resistance of *A.actinomycetemcomitans* to H_2O_2. Thus catalase activity may not be important in the resistance of *A.actinomycetemcomitans* to H_2O_2. Nonetheless, *A.actinomycetemcomitans* was found to be sensitive to killing by myeloperoxidase-hydrogen peroxide-chloride system *in vitro* under aerobic and anaerobic conditions (Miyasaki, Wilson and Genco, 1986b). In addition most oxygen-dependent killing requires myeloperoxidase activity which is essential but not alone sufficient to kill *A.actinomycetemcomitans* (Miyasaki *et al.*, 1986a; Miyasaki *et al.*, 1987). This may suggest a possible role for oxidative inactivation of *A.actinomycetemcomitans* leucotoxin by secretory products of neutrophils (Clark, Leidal and Taichman, 1986). Another possibility is that lactoferrin contributes to the killing of *A.actinomycetemcomitans* by human neutrophils and may thus play a significant role in the innate secretory defenses against *A.actinomycetemcomitans* (Kalmar and Arnold, 1988).

1.7.4 Tissue destruction by *A.actinomycetemcomitans*

Endotoxin:

Bacteria and their products may lead to the destruction of the periodontal tissues by direct action or indirectly by inducing reactions that are partly unfavorable to the host

(Slots and Genco, 1984). *A.actinomycetemcomitans* produces endotoxic lipopolysaccharide (LPS) which can cause tissue destruction in animals and appears to be similar in structure to the LPS from other Gram-negative bacteria, ie. a polysaccharide chain with a hydrophobic lipid-A which is covalently joined by an inner core and an outer carbohydrate layer (Kiley and Holt, 1980; Hammond and Stevens, 1982). The LPS of *A.actinomycetemcomitans* appears to possess a range of biological activities which include the Schwartzman reaction, macrophage activation, platelet aggregation and limulus amoebocyte activity (Kiley and Holt, 1980; Nowotny *et al.*, 1982; Nishihara *et al.*, 1986; Koga *et al.*, 1987; Nishihara, Koga and Hamada, 1987; Hamada *et al.*, 1988). The LPS material from *A.actinomycetemcomitans* can exert potent bone resorption activity as demonstrated by the *in vitro* release of calcium from mouse calvaria similar to that obtained with 'capsular' material under the same conditions (Kiley and Holt, 1980; Iino and Hopps, 1984; Wilson, Kamin and Harvey, 1985). Also significant increase in bone resorption has been seen in rats who received T-cells sensitized to *A.actinomycetemcomitans* LPS compared with a non-sensitized control group (Yoshie *et al.*, 1987). These results tend to suggest that the LPS activity of *A.actinomycetemcomitans* may be important in mediating bone loss in chronic periodontitis. *A.actinomycetemcomitans* also produces acid and alkaline phosphatases (Slots, 1981), and the former has been implicated in bone destruction which may enable *A.actinomycetemcomitans* to cause tissue destruction (Slots, 1986).

Collagenase:

Collagen is an important component of the periodonitum which is mainly composed of Type I and III. It is arranged in the tissues as precisely ordered aggregates of many similar molecules (Gross, 1970). The amino acid composition of collagen is made up of glycine, proline and hydroxyproline (Fietzek and Kuhn, 1976) and fibres are generally resistant to enzymatic degradation. However, it is sensitive to certain bacterial enzymes such as collagenase which has been defined as an enzyme capable of degrading native collagen at physiological pH and temperature (Gross, 1970). Collagenase is produced by most *A.actinomycetemcomitans* strains (Slots, 1982a; Robertson *et al.*, 1982; Rozanis *et al.*, 1983) and this enzyme may play an important role in collagen loss in destructive disease of the periodontium (Kamin *et al.*, 1986). However, not all *A.actinomycetemcomitans* strains produce collagenase and while Rozanis and Slots (1982) found 11 of 140 (8%) strains possessed collagenolytic activity, Uitto *et al.*, (1988) found that 5 out 5 *A.actinomycetemcomitans* strains did not produce collagenase. It has been suggested that if collagenase derived from *A.actinomycetemcomitans* was combined with collagenase released from leucotoxin-damaged PMNLs then the mixture may produce a significant level of tissue destruction (Zambon, 1985). From these results it is not clear if collagenase produced by *A.actinomycetemcomitans* is important in pathogenicity, since only very small percentage (8%) of *A.actinomycetemcomitans* isolates produced col-

lagenase. In addition very little data is available and more studies should be carried out in this field.

Fibroblasts:

Fibroblasts are the predominant cells in connective tissues and show morphological changes at sites of inflammation (Stevens, Gatewood and Hammond, 1983). *A.actinomycetemcomitans* was found to produce a dose-dependent inhibition of both human and murine fibroblast proliferation as assessed by DNA and RNA synthesis with an overall decrease in cell growth but not viability (Shenker, Kushner and Tsai, 1982c; Stevens *et al.*, 1983; Kamin *et al.*, 1986; Stevens and Hammond, 1988).

Epitheliotoxin:

Epitheliotoxin is another pathogenic factor capable of directly causing tissue destruction (Birkedal-Hansen *et al.*, 1982; Kamen, 1983) and which may assist *A.actinomycetemcomitans* to penetrate the sulcular epithelium and invade the underlying connective tissues. There is little information known about this factor, though it has been suggested that the epitheliotoxin of *A.actinomycetemcomitans* may destroy the epithelial lining of the periodontal pocket and interfere with healing (Slots, 1986).

More recently Christersson *et al.*, (1987a) using immunofluoresence and electron microscopic studies reported the presence of *A.actinomycetemcomitans* in the connective tissue of humans. A positive correlation was found between the

presence of *A.actinomycetemcomitans* in gingival biopsies and the number of *A.actinomycetemcomitans* colonies cultured from minced tissues and from plaque collected from periodontal pockets (Christersson *et al.*, 1987a,b). It is possible that the production of epitheliotoxin played a role in the invasion of these tissues by *A.actinomycetemcomitans*.

1.7.5 Adherence

It is clear that the ability of an organism to adhere to host surfaces or commensal bacteria is important in the colonization of the mouth. The literature contains only three studies which have dealt with the adherence of *A.actinomycetemcomitans* to different oral surfaces, namely, buccal epithelial cells, tissue culture cells, tooth enamel and hydroxyapatite. Unfortunately different methods have been used to study the adherence of *A.actinomycetemcomitans* to these surfaces which makes a comparison of the results difficult. Gibbons and Etherden (1983) examined two *A.actinomycetemcomitans* strains (ATCC 29523 and Y4) which were both found to adhere in low numbers to saliva treated hydroxyapatite. For example when both strains were tested at a concentration of 10^9/ml, only 0.3% of cells adhered to hydroxyapatite (HA). Nonetheless, Kagermeier and London (1985) reported that *A.actinomycetemcomitans* strains Y4 and N27 adhered to hydroxyapatite in approximately the same numbers. The percentages of *A.actinomycetemcomitans* cells which adhered to the HA was higher than that reported earlier by Gibbons and Etherden (1983), although in Kagermeier's study the concentration of bacteria was less than that used by

Gibbons and Etherden (1983). Sweet (1986) examined the adherence of one *A.actinomycetemcomitans* strain (NCTC 9710) and showed that this isolate adhered in high numbers to buccal epithelial cells, tooth enamel and Hela tissue culture cells.

The ability of *A.actinomycetemcomitans* to adhere to oral surfaces is thought to be related to a number of bacterial cell surface structures, such as a 'capsule', membrane vesicles or fimbriae (Holt *et al.*, 1980; Lai *et al.*, 1981; Zambon *et al.*, 1983a). A preliminary study by Rosan *et al.*, (1988) showed that two fimbriated *A.actinomycetemcomitans* strains adhered to hydroxyapatite about 3 to 4-fold times higher than non-fimbriated variants which were obtained after repeated subculturing. However, results obtained with a third strain showed no difference in adherence. Other factors which may affect adherence, eg. the composition of the growth medium appear to have little or no effect on the adherence of *A.actinomycetemcomitans* to HA *in vitro* (Kagermier and London, 1985), whereas repeated subculturing over a period of one year did reduce the ability of *A.actinomycetemcomitans* to adhere to HA (Kagermeier and London, 1985). There seems to be different views on the effect of serum or saliva on the adherence of *A.actinomycetemcomitans* to oral surfaces. Kagermeier and London (1985) have shown that the ability of *A.actinomycetemcomitans* to adhere to HA was reduced when the hydroxyapatite was coated with either saliva or serum *in vitro*. However, Rosan *et al.*, (1988) reported that the addition of saliva had no effect on the adherence of two

fimbriated *A.actinomycetemcomitans* strains compared to the results obtained using hydroxyapatite alone. Finally *A.actinomycetemcomitans* adhered in high numbers to Hela cell monolayers which were pretreated with both saliva and serum as compared to the results obtained when the assays were performed in buffer alone (Sweet, 1986).

1.7.6 Hydrophobicity

Hydrophobicity is described as the free energy change associated with the process of bringing two entities from infinite separation to distances of the order of magnitude of molecular dimensions (Ben-Naim, 1977). The outcome of many *in vitro* studies suggest that hydrophobic interactions are important in mediating the adherence of most oral bacteria to various oral surfaces eg. tooth surfaces and buccal epithelial cells (Rosenberg *et al.*, 1981; Rosenberg *et al.*, 1983a). A number of laboratory methods have been developed to measure bacterial hydrophobicity including, hydrophobic interaction chromatography, salting out of bacteria by increasing the concentration of ammonium sulphate, adhesion to solid surfaces (eg. polystyrene, plastic or glass), binding of radiolabelled hydrocarbons, two phase partioning, contact angle measurements and bacterial adhesion to hydrocarbons (for review see Rosenberg and Kjelleberg, 1986).

A number of factors appear to affect hydrophobicity and therefore bacterial adhesion, which include fimbriae as well as other surface projections such as fibrils (Marshall and Cruickshank, 1973; Rosenberg *et al.*, 1982). In general, the

structure and chemical composition of the bacterial cell surface may vary due to differences in culture conditions such as the composition of the growth medium, the degree of aeration, the incubation temperature and the age of the microbial cells, all of which may affect cell surface hydrophobicity (review Rosenberg and Kjelleberg, 1986). The presence of plasmids may similarly affect hydrophobicity by allowing the expression of fimbriae or other surface components (Martinez, 1983). Moreover, hydrophobicity varies not only from species to species but also from strain to strain (Rosenberg, 1982; Olsson and Westergren, 1982). While there is some information about the effect of saliva and serum on the adherence of bacteria to oral surfaces, there is very little data concerning the effect of either saliva or serum on bacterial hydrophobicity *in vitro*.

Relatively little is known about the hydrophobicity of *A.actinomycetemcomitans in vitro* or about the role of this property in the colonization of the dentogingival area (Kozlovsky, Metzger and Eli, 1987). Nonetheless, Gibbons and Etherden (1983) reported that two *A.actinomycetemcomitans* strains (ATCC 29523 and Y4) were hydrophilic as assessed by their lack of ability to adsorb to hexadecane and because they adhered in only low numbers to saliva coated hydroxyapatite. However, these results were not supported by the finding of Kozlovsky *et al.*, (1987) who reported that *A.actinomycetemcomitans* strain Y4 was hydrophobic when measured using adherence to the hydrocarbon, octane. They also noted that hydrophobicity could be varied from 60 to 90%,

depending on the medium in which the organism was grown, age of culture and the buffer in which the assay was carried out. One possible explanation for the discrepancies found in these two studies is the difference in growth conditions used in the two studies (Kozlovsky *et al.*, 1987). Another reason for the differences may be due to the use of different hydrocarbons (ie hexadecane and octane). Since Rosenberg, Gutnick and Rosenberg (1980) have reported that the hydrophobicity of the same bacterial strain showed slight variations depending on the hydrocarbon used (hexadecane, octane and xylene). In another study the hydrophobicity of supragingival dental plaque was found to be higher in the presence of xylene than in octane or hexadecane respectively (Rosenberg, Judes and Weiss, 1983b). More recently Sweet (1986) investigated the hydrophobicity of one Type strain of *A.actinomycetemcomitans* (NCTC 9710) and found it hydrophobic when measured using adherence to xylene. Apparently neither saliva nor the type of buffer used (SIB or PBS) had any affect on the hydrophobicity of *A.actinomycetemcomitans* NCTC 9710.

1.8 General Infections Related to *A.actinomycetemcomitans*

A.actinomycetemcomitans can cause severe medical infections throughout the body. It has been associated with abscess of the abdomen, brain, face, hand, thyroid gland, actinomycosis, endarteritis, endocarditis, meningitis, pneumonia, septicaemia, urinary tract infection, vertebral osteomyelitis and more recently from a case of endophthalmitis

(Garner, 1979; Page and King, 1966; Mauff *et al.*, 1983; Burgher, Loomis and Ware, 1973; Colebrook, 1920; Symbas *et al.*, 1967; AhFat, Patel and Pickens, 1983; Meyers *et.al*, 1971; Townsend and Gillenwater, 1969; Muhle, Rau and Ruskin, 1979; Ishak, Zablit and Dumas, 1986). Infective endocarditis is probably the most common type of extra-oral infection with *A.actinomycetemcomitans* and approximately two-thirds of these cases occur in subjects with a history of rheumatic heart disease or prosthetic valve replacement (Zambon, 1985).

1.9 Periodontal Infections Related to *A.actinomycetemcomitans*

Microbiological studies have strongly associated *A.actinomycetemcomitans* with localized juvenile periodontitis (Slots *et al.*, 1980a), since it tends to be found in only small numbers of healthy subjects or patients with other types of periodontal disease (Slots *et al.*, 1980a; Zambon, Chistersson and Slots, 1983b). More recently the role of *A.actinomycetemcomitans* in chronic periodontitis has been investigated and reports have yielded conflicting results (Wolff *et al.*, 1985; Dahlen *et al.*, 1989).

1.9.1 Prepubertal periodontitis

Localized prepubertal periodontitis (LPP) is described as a host-defect mediated form of bacterially induced periodontitis with an early onset and rapid progression around a few teeth in children prior to puberty (Delaney and Kornman, 1987). Although the number of studies in this field are

limited, it has been shown that subjects with LPP harbour high levels of several suspected pathogens including *A.actinomycetemcomitans*, *Bacteroides intermedius* and *Capnocytophaga* species (Delaney and Kornman, 1987; Sweeney *et al.*, 1987; Alaluusua and Asikainen, 1988; Frisken *et al.*, 1987). It has been suggested that these results indicate that localized prepubertal periodontitis may be more common than previously realized and associated with bacteria generally regarded as periodontal pathogens particularly *A.actinomycetemcomitans* (Sweeney *et al.*, 1987; Preus and Gjermo, 1987).

More recently Preus and Gjermo (1987) presented two clinical cases of Papillon-Lefevre Syndrome (PLS) in two siblings with prepubertal periodontitis. Although no microbiology was performed the authors postulated that the microorganisms responsible for the extreme tissue destruction in PLS periodontitis must be virulent. Preliminary studies have indicated elevated serum titers of antibodies against *A.actinomycetemcomitans* in both siblings, and later *A.actinomycetemcomitans* was isolated from one of the patients (Preus *et al.*, 1987).

1.9.2 Juvenile periodontitis

Juvenile periodontitis is described as a disorder of the supporting apparatus of the teeth which occurs in adolescents (Baer, 1971). It is characterized by the early loss of alveolar bone around the permanent first molar and incisor teeth (Liljenberg and Lindhe, 1980; Listgarten, Lai and

Evian, 1981). This form of disease is known as Localized Juvenile Periodontitis (LJP). However, the extent of periodontal destruction in these patients is generally disproportional to the minimal amounts of dental plaque and gingival inflammation which are found clinically (Zambon, 1983). The disease tends to occur with higher frequency in certain families (Zambon, 1983).

Although the precise aetiology of juvenile periodontitis is unknown, many theories have been suggested, including a genetic or immunological defect of the host with or without specific microbial infection (Lindemeyer, 1986). An association between juvenile periodontitis and certain bacterial species especially Gram-negative rods has been reported (Newman *et al.*, 1976; Slots, 1976; Newman and Socransky, 1977). *A.actinomycetemcomitans* is believed to be the most important Gram-negative rod present in plaque from these patients (Tanner *et al.*, 1979). *Capnoytophaga* has also been commonly isolated from LJP patients (Newman *et al.*, 1976; Newman and Socransky, 1977; Holt, Leadbetter and Socransky, 1979; Socransky *et al.*, 1979). However the relationship of *Capnocytophaga* with periodontal disease is still unclear (Genco, Christersson and Zambon, 1986).

The recent evidence implicating *A.actinomycetemcomitans* as an important organism in the aetiology of juvenile periodontitis is as follows: (a) The increased prevalence of *A.actinomycetemcomitans* in almost all juvenile periodontitis patients, while subjects with a healthy periodontium harbour very few if any *A.actinomycetemcomitans* (Slots *et al.*,

1980a); (b) the presence of high titers of antibodies against *A.actinomycetemcomitans* in the serum, saliva and gingival crevicular fluid of patients with this disease (Listgarten *et al.*, 1981); (c) the capability of *A.actinomycetemcomitans* to produce a range of virulence factors which can depress defence mechanisms; eg. inhibition of human fibroblast proliferation and leucotoxin production (Review Zambon, 1985); (d) and finally clinical studies have shown that juvenile periodontitis can be treated by eliminating *A.actinomycetemcomitans* from periodontal pockets in diseased subjects using tetracycline therapy (Lindemeyer, 1986).

Studies by Slots *et al.*, (1980a); Mandell and Socransky (1981) and Slots (1982b) reported that *A.actinomycetemcomitans* was present in high numbers in almost all localized juvenile periodontitis lesions, whereas, it was less frequently detected in healthy periodontal sites, normal juveniles, normal adults or adult periodontitis patients. In a study involving 403 healthy and diseased subjects, *A.actinomycetemcomitans* was detected in 97% of subgingival dental plaque samples from LJP patients (Zambon *et al.*, 1983b), but in very small numbers from only 15% of other individuals which included healthy adults, chronic periodontitis patients and insulin-dependent juvenile diabetics. Similar findings were reported by Slots *et al.*, (1982) and Genco *et al.*, (1986) where 95% of LJP patients samples yielded *A.actinomycetemcomitans*.

Moore (1987) summarized a number of recent studies on *A.actinomycetemcomitans* and concluded that the subgingival

plaque from about 17% of 137 affected juvenile periodontitis sites contained 10% to 99% *A.actinomycetemcomitans*, 20% contained 1% to 10% and 64% of affected sites contained less than 1% *A.actinomycetemcomitans*. He concluded that if at least 1% *A.actinomycetemcomitans* is required for tissue destruction, then *A.actinomycetemcomitans* could be the destructive agent in about one-third of the sites examined (Moore, 1987). The relationship between *A.actinomycetemcomitans* proportions and disease progression was evaluated by Bragd *et al.*, (1987) using multiple-regression analysis. A recovery rate below 0.01% for *A.actinomycetemcomitans* distinguished non-progressing from progressing periodontal sites with a sensitivity and specificity of about 85% for both variables.

The prevalence of *A.actinomycetemcomitans* was also reported in 17 Finnish LJP patients with 89% positive (Asikainen *et al.*, 1986), in 12 Panamanian LJP patients 100% positive (Eisenmann *et al.*, 1983) and in 5 Korean LJP patients again 100% positive (Chang *et al.*, 1984).

Further evidence of a close association between *A.actinomycetemcomitans* and LJP comes from immunological studies. Genco *et al.*, (1980) and Tsai *et al.*, (1981) found that 69 to 94% of LJP patients had specific serum antibodies to *A.actinomycetemcomitans*. In other investigations, only 3% to 19% of gingivitis subjects were positive and 15% to 30% of adult periodontitis patients exhibited any antibody response (Genco *et al.*, 1980; Tsai *et al.*, 1981; Ebersole *et al.*, 1982; Ranney *et al.*, 1982).

Finally a number of clinical studies have also associated *A.actinomycetemcomitans* with the following groups of patients: generalized juvenile periodontitis (Tanner *et al.*, 1979), insulin-dependent juvenile diabetics with periodontitis (Mashimo *et al.*, 1983) and rapidly progressing periodontitis (Moore *et al.*, 1982; Page *et al.*, 1983).

1.9.3 Chronic periodontitis

While the aetiology of *A.actinomycetemcomitans* in juvenile periodontitis is well documented, there is much less information regarding a possible association between *A.actinomycetemcomitans* and chronic periodontitis. However, the results summarized in Table (1.4) clearly show that *A.actinomycetemcomitans* is not infrequently isolated from patients with chronic periodontitis, although the clinical significance of the data is not clear. Past reports suggest that *A.actinomycetemcomitans* is less frequently isolated from chronic periodontitis patients compared to LJP patients. For example Slots *et al.*, (1980a) found 50% of chronic periodontitis patients harboured *A.actinomycetemcomitans* compared to 90% of the LJP patients. *A.actinomycetemcomitans* was mainly isolated from subgingival plaque samples and lesions which were described as progressing compared to non-progressing lesions (Slots *et al.*, 1986). Since subgingival plaque samples exhibited higher recovery rates than supragingival samples, the presence of *A.actinomycetemcomitans* in these sites was found to correlate with high plaque index and pocket depths of 3 to 5mm (Wolff *et al.*, 1985).

Table 1.4 The occurrence of *A.actinomycetemcomitans* in subgingival plaque collected from chronic periodontitis patients.

Author's	No. patients /Sites	Age (yrs)	% recovery subgingival patients/sites	Method of investigation
Slots *et al.*, (1980a)	12/49	30-65	50%/35	Medium of Slots *et al.*, (1980a)
Zambon *et al.*, (1983b)	134/ND	21-78	21%/ND	TSBV medium
Okuda *et al.*, (1984)	8/8	37-52	14%/14%	MGB medium
Kolkotrnis, Sofinanou & Konstantini, (1985)	22/22	35-50	14%/14%	TSBV medium
Wolff *et al.*, (1985)	284/533	20-40	13%/9%	Medium of Slots *et al.*, (1980a)
Bonta *et al.*, (1985)	10/60	28-69	30%/10%	TSBV medium and immunofluoresence
Slots *et al.*, (1985)	40/81	24-59	ND/21%	TSBV medium and immunofluoresence
Slots *et al.*, (1986)	146/235	18-79	ND/50%	TSBV medium
Dahlen *et al.*, (1989)	20/40	30-65	40%/28%	TSBV medium

TSBV and MGB medium (see section 1.5)

ND: No data available

1.10 Aims of this study

As cited in Section 1.5 that there are different views on the suitability of the two semi-selective media TSBV and MGB in the isolation of *A.actinomycetemcomitans* from subgingival plaque samples. Therefore the first series of experiments in this thesis were designed to compare these two media for their inhibitory action against pure isolates of *A.actinomycetemcomitans* and their ability to differentiate this organism from other bacterial species present in mixed plaque samples.

Due to the variation in prevalence data associated with the recovery of *A.actinomycetemcomitans* from healthy volunteers and chronic periodontitis patients, a group of Scottish patients were investigated. Since there is no information concerning the prevalence of both star-shaped and non-star-shaped colonies of *A.actinomycetemcomitans* on primary isolation, it was decided to investigate this phenomena in the subgingival plaque cultures on semi-selective media. While previous workers have employed different identification methods, the commercially provided kit (API 20 A) was used throughout this study for the identification of suspected *A.actinomycetemcomitans* isolates.

It is clear from the earlier review of the literature that little information is available concerning the adherence of *A.actinomycetemcomitans* to oral surfaces (especially buccal epithelial cells). In addition the small amount of information concerning hydrophobicity which is available tends

to be conflicting. Thus it was decided to study the adherence of 33 strains of *A.actinomycetemcomitans* to buccal cells, and to examine the effect of both saliva and serum on adherence. The hydrophobicity of *A.actinomycetemcomitans* to xylene was also investigated with the same strains.

Finally the pathogenicity of *A.actinomycetemcomitans* has been related to the ability of this organism to produce leucotoxin capable of killing human PMNLs and other target cells such as HL-60 *in vitro*. Freshly isolated strains of *A.actinomycetemcomitans* were included in leucotoxicity experiments in this thesis to provide further information concerning toxin production by *A.actinomycetemcomitans*.

CHAPTER 2

THE PREVALENCE OF *A.ACTINOMYCETEMCOMITANS* IN CHRONIC PERIODONTITIS (MICROBIOLOGICAL AND CLINICAL STUDIES)

2.1 Introduction

This chapter is divided into two parts, the first part deals with a preliminary study in which a comparison of semi-selective media for the isolation of *A.actinomycetemcomitans* was carried out. The other part deals with a clinical study in which the prevalence of *A.actinomycetemcomitans* in subgingival plaque samples from 98 patients and 55 control subjects was investigated.

2.2 Comparison of selective media

2.2.1 Introduction

The isolation and identification of *A.actinomycetemcomitans* has become increasingly important because of its suspected role in certain types of human periodontal disease, eg. patients with Localized Juvenile Periodontitis (LJP), (Slots *et al.*, 1980a; Slots, 1982b). Development of a fully selective medium for the specific recovery of *A.actinomycetemcomitans* from subgingival plaque would of particular value, as the organism forms small translucent colonies on primary cultures and may be easily overlooked due to the presence of other microorganisms.

Two semi-selective media have been developed for the isolation of *A.actinomycetemcomitans* from subgingival plaque (Mandell and Socransky, 1981; Slots 1982b). However, there

seems to be some confusion about the suitability of using these two media (Riches and Marsh, 1987). In this study the two semi-selective media were compared with respect to (I) their ability to inhibit the growth of pure strains of *A.actinomycetemcomitans*, and (II) their effectiveness in the isolation of *A.actinomycetemcomitans* from mixed subgingival plaque samples. The medium which performed best in these tests was subsequently used for the isolation of *A.actinomycetemcomitans* from both patients and control subjects in the follow-up clinical study.

2.2.2 Materials and methods

2.2.3 Bacteria used to compare the selective media

A total of 17 *A.actinomycetemcomitans* strains were used in this study, 4 Type strains, (NCTC 9709, NCTC 9710, NCTC 10979 and NCTC 10982) obtained from the National Collection of Type Cultures, London, UK, and 13 freshly isolated *A.actinomycetemcomitans* strains from subgingival sites in chronic periodontitis patients who attended Glasgow Dental Hospital and School (see Table 2.1). All the freshly *A.actinomycetemcomitans* strains were isolated as described in section 2.3.3. Both the Type strains and the fresh isolates were identified according to the procedures described below.

2.2.4 Identification of *A.actinomycetemcomitans* strains

Microscopic examination (Gram-stain)

All bacterial isolates were stained by Gram's method using a similar technique to that described by Preston and Morrell

(1962). A bacterial smear was prepared by removing 2-3 colonies using a sterile loop and emulsifying them in a loopful of sterile distilled water on a glass microscope slide. The smear was dried by passing it over a Bunsen flame and stained with a 0.5% w/v crystal violet solution for 60 seconds. The crystal violet was poured off and the slide was flooded with Gram's iodine solution which was left to act for a further 60 seconds. The smear was then decolourized using acetone (M & A, Pharmchem Ltd., Bolton, England) which was left to act for about 5 seconds. Next the smear was washed with running tap water and counterstained with 5% v/v carbolfuchsin solution for two minutes. Finally the counterstain was washed off, and the smear dried and examined at 1000X magnification using an Olympus microscope (Tokyo, Japan). With the exception of acetone all the chemicals used in the Gram-stain were supplied by Clin-Tech Ltd., London, England.

CO_2 requirement

The requirement of each strain for CO_2 was examined by comparing the growth of *A.actinomycetemcomitans* on Columbia Blood Agar plates (CBA) (Gibco-Europe, Paisley, Scotland), (Appendix I) after incubation for 48 hours at 37°C in air (LEEC Ltd., Nottingham, England) with that obtained from growth in 5% CO_2 in air using a Qualitemp 80 MI incubator (Laboratory Terminal Equipment Ltd., Oldham, UK). Results were recorded on the basis of either a light, medium or heavy growth obtained under the two different atmospheric conditions.

Table 2.1 ***A.actinomycetemcomitans*** **strains used in the selective media study.**

Strain Number	Source	Site of Isolation	API 20 A Profile
Aa 01	NCTC 9709	Abscess (site not given)	4110404
Aa 02	NCTC 9710	Abscess (site not given)	4110405
Aa 03	NCTC 10979	Mandibular abscess	4150404
Aa 06	NCTC 10982	Chest aspirate	4050505
Aa 07	GDH 310	Juvenile periodontitis	4110404
Aa 08	GDH 312	Chronic periodontitis	4110404
Aa 09	GDH 143	~ ~	4110404
Aa 10	GDH 216	~ ~	4110404
Aa 11	GDH R8529	~ ~	4150404
Aa 12	GDH 156	~ ~	4110404
Aa 13	GDH 471	~ ~	4110404
Aa 14	GDH 1954	~ ~	4050404
Aa 34	GDH 1116	~ ~	4050404
Aa 35	GDH 1123	~ ~	4050404
Aa 36	GDH 28	~ ~	4110404
Aa 37	GDH 33	~ ~	4110404
Aa 38	GDH 39	Juvenile periodontitis	4110404

NCTC: National Collection of Type Cultures

ATCC: American Type Culture Collection

GDH: Glasgow Dental Hospital

Catalase test

Catalase production by *A.actinomycetemcomitans* was tested on a glass slide. One drop of 3% H_2O_2 from a pasteur pipette was dispensed onto a clean glass slide. Three to four colonies were selected from a Tryptic Soy-Serum-Bacitracin-Vancomycin Agar (TSBV) (Appendix I) using the edge of a clean glass coverslip and smeared near the drop of H_2O_2. A coverslip was then carefully placed over the slide and the formation of bubbles within 10 seconds was recorded as a positive result.

X and V requirements

The requirement of *A.actinomycetemcomitans* for either X or V growth factors was examined by emulsifying the growth from one CBA plate in 3.0 ml sterile distilled water. This was then inoculated onto Peptone Agar (Mast Lab., Merseyside, England), (Appendix I) with a sterile cotton swab (Medical Wire & Equipment Co., Wiltshire, England). Using a sterile needle X, V and XV discs (Mast Lab., Merseyside, England) were applied to the agar surface and plates were incubated for 48 hours at 37°C in a Qualitemp 80 MI incubator with an atmosphere of 5% CO_2 in air. The presence or absence of growth around the discs was then recorded.

API 20 A System

The API 20 A system (API Laboratories, La Balme, Les Grottes, France) which consists of 20 biochemical tests in pre-prepared plastic microtubes, was used as the standard method

for the identification of *A.actinomycetemcomitans* (Figure 2.1). The inoculum for the test was collected by removing the pure heavy growth from a CBA plate using a sterile cotton swab. This was then emulsified in the broth medium supplied with the API kit. The microtubes were inoculated with the bacterial suspension to the recommended level using a pasteur pipette and then incubated for 48 hours at 37°C in 5% CO_2 in air. At the same time a drop from the test suspe-nsion was inoculated onto a fresh CBA to check for purity and incubated under the same conditions used for the API strips.

The results were recorded as indicated by colour changes of the inoculated API strips after 48 hours of incubation (see Figure 2.1) The results were recorded on the report sheet supplied and the biochemical reactions obtained for each test organism were then compared to the four different API 20 A profiles for *A.actinomycetemcomitans.* As the API comm-ercial system has no database for *A.actinomycetemcomitans,* these profiles were produced by testing five different NCTC strains of *A.actinomycetemcomitans* on three different occas-ions. The profiles were 4110, 4150, 4050 and 4010.

2.2.5 Inoculation procedure

Due to the difficulty in growing the majority of the freshly isolated strains of *A.actinomycetemcomitans* in broth culture without homotypic aggregation occurring (Figure 2.2), each of the test strains was grown on two CBA plates for 48 hours in 5% CO_2 plus air in a Qualitemp 80MI incubator (Laboratory

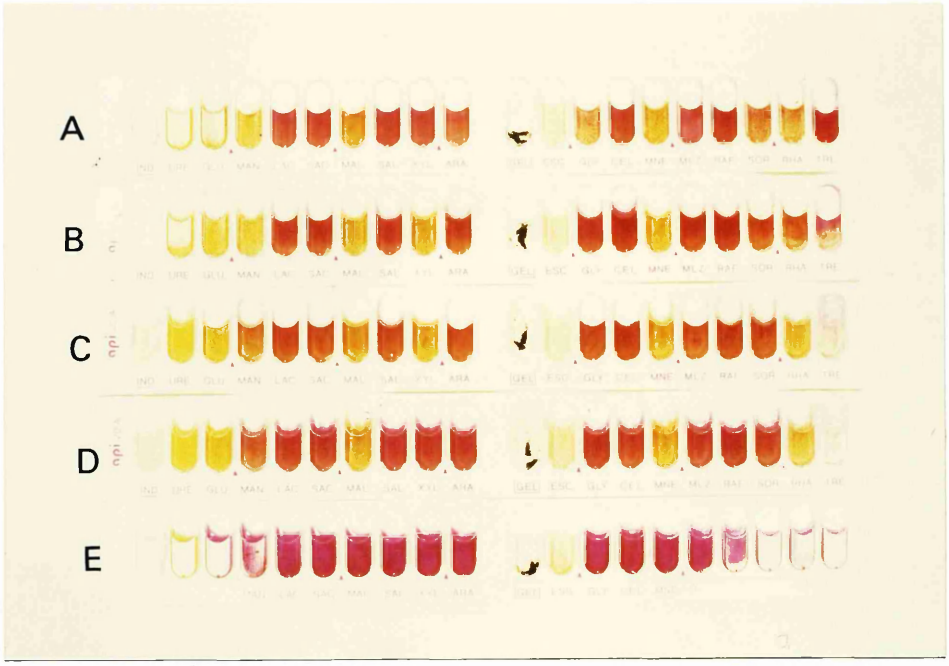

Figure 2.1 API 20 A (System) used for the biochemical identification of *A.actinomycetemcomitans*. The control test (E) shows no change in colour, while the four test strips (A, B, C & D) show different biochemical profiles.

Figure 2.2 Strains of *A.actinomycetemcomitans* cultured in thioglycollate broth; the type strain Y4 shown here produces uniform turbidity while the the fresh isolates GDH 87034 & GDH 114 show marked aggregation.

Terminal Equipment Ltd., Oldham, UK). At the end of this time, growth was harvested using a sterile cotton swab and transferred into 5 ml Anaerobic Blood Broth (ABB, Appendix I) (Gibco-Europe Ltd., Paisley, Scotland), vortex mixed for 60 seconds (Fisons Scientific Apparatus, Leicestershire, England) and 10-fold serial dilutions then made in Phosphate Buffered Saline, pH 7.2 (PBS, Appendix II) (Flow Laboratories, Rickmansworth, England). A dilution of 10^{-4} to 10^{-5} was used to inoculate the following media; TSBV, Malachite Green Bacitracin Agar (MGB), and CBA (Appendix I). Using a spiral plater (Model D, Spiral Systems, Clough Pike, Cincinnati, USA), 50 µl aliquots were inoculated onto each agar medium in duplicate and incubated in an atmosphere of 5% CO_2 in air for 5 days at 37°C. The spiral plater operates by dispensing 50 µl onto the surface of the agar plate at a constant rate in a spiral pattern from the centre outwards (Figure 2.3). Therefore, the concentration of the sample per surface area becomes progressively less on moving from the centre to the periphery of the culture plate.

2.2.6 Colony counting

The colony forming units (cfu/ml) on each plate were counted by using a 10 cm, Spiral Systems, manual counting grid. The grid divides the surface of the agar plate into zones which represent known volumes of the inoculum (Figure 2.4). Areas containing 30-50 colonies were chosen and the number of colonies present on sectors on contralateral sides of the plate were counted to obtain more accurate results. The mean number of colonies on duplicate plates was then calculated.

Figure 2.3 Spiral plater model D used for inoculating agar plates.

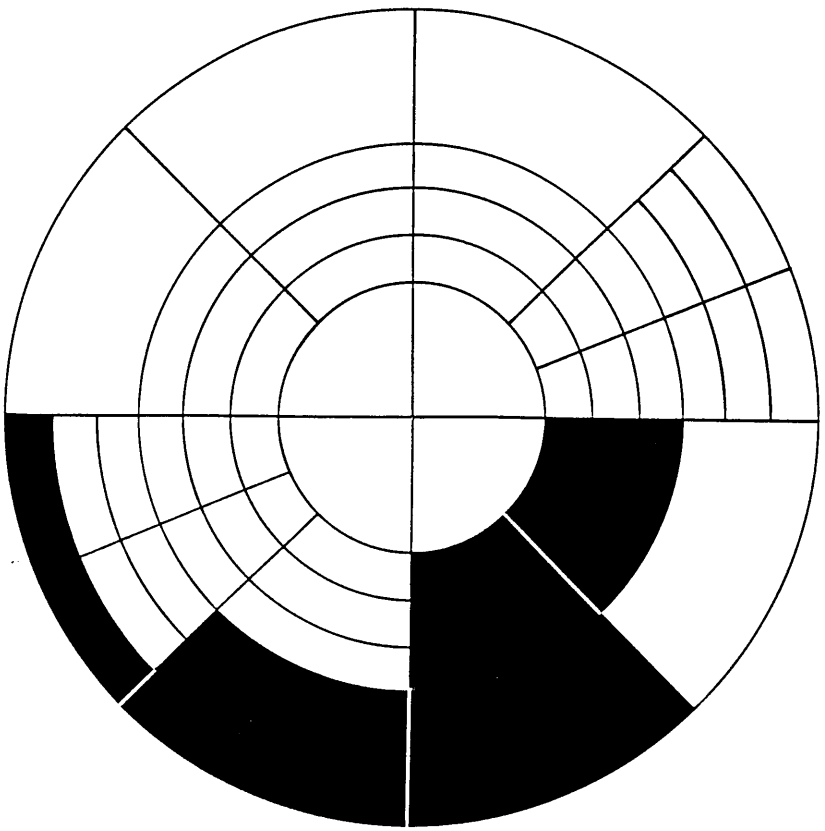

Figure 2.4 A manual grid used in conjunction with the spiral plater for counting bacterial colonies in different zones on agar plates.

2.2.7 Statistical analysis

All assays were performed on three different occasions for each bacterial strain under study. Results were analysed using STAT-WORK software (Apple Macintosh computer), and Significant differences were determined using the paired t-Test.

2.2.8 Results:

Comparison of selective media

The $\log_{10}$ counts of the colony forming units (cfu/ml) of pure cultures of *A.actinomycetemcomitans* recovered on each of the three tested media (CBA, TSBV and MGB) are shown in Tables 2.2, 2.3 & 2.4 and a summary of these results is presented in Table 2.5. There was a clear evidence that both of the semi-selective media TSBV and MGB suppressed the growth of *A.actinomycetemcomitans* to a different degree compared to growth on CBA. As shown in Table 2.5, the over-all mean number of *A.actinomycetemcomitans* was highest on CBA plates followed by TSBV while MGB gave the lowest number of *A.actinomycetemcomitans* recovered. The growth of *A.actinomycetemcomitans* was significantly inhibited on MGB and TSBV agar plates as compared to CBA plates ($p \leq 0.005$). In addition, MGB was significantly more inhibitory to the growth of *A.actinomycetemcomitans* compared to TSBV medium ($p \leq 0.01$). Furthermore, the colony characteristics of *A.actinomycetemcomitans* strains were more easily recognized on the TSBV than on the MGB agar plates, while colony size was maximal on CBA followed by TSBV and MGB respectively.

Table 2.2 $\log_{10}$ cfu/ml of 17 *A.actinomycetemcomitans* strains recovered on CBA, TSBV and MGB agar plates (figures represent the mean of two observations). Experiment No. 1

Strain number	$\log_{10}$ cfu/ml of *A.actinomycetemcomitans* CBA	TSBV	MGB
Aa 01	10.04	9.18	10.08
Aa 02	10.0	9.36	9.93
Aa 03	10.11	9.54	10.18
Aa 06	9.83	9.20	9.81
Aa 07	9.65	9.90	9.70
Aa 08	9.86	9.71	9.52
Aa 09	9.23	8.40	4.0
Aa 10	8.59	8.20	3.86
Aa 11	9.85	9.59	5.65
Aa 12	8.94	8.61	4.55
Aa 13	9.11	8.23	4.73
Aa 14	9.70	9.43	5.32
Aa 34	10.15	10.08	9.63
Aa 35	9.63	10.08	9.57
Aa 36	9.76	9.84	9.50
Aa 37	9.94	9.65	9.72
Aa 38	9.77	9.77	9.59

Table 2.3 $\log_{10}$ cfu/ml of 17 *A.actinomycetemcomitans* strains recovered on CBA, TSBV and MGB agar plates (figures represent the mean of two observations). Experiment No. 2

Strain number	$\log_{10}$ cfu/ml of *A.actinomycetemcomitans* CBA	TSBV	MGB
Aa 01	9.82	9.77	10.04
Aa 02	9.84	9.60	9.81
Aa 03	10.15	9.96	10.20
Aa 06	9.81	9.86	9.98
Aa 07	9.84	9.86	9.67
Aa 08	9.72	9.54	9.62
Aa 09	9.46	9.34	6.20
Aa 10	9.18	8.81	3.80
Aa 11	10.04	10.0	5.65
Aa 12	8.0	9.0	7.04
Aa 13	9.28	8.81	3.85
Aa 14	9.23	9.0	5.0
Aa 34	9.89	9.71	9.79
Aa 35	9.18	9.84	9.74
Aa 36	9.72	9.83	9.45
Aa 37	9.99	9.71	9.78
Aa 38	9.94	9.75	9.62

Table 2.4 $\log_{10}$ cfu/ml of 17 *A.actinomycetemcomitans* strains recovered on CBA, TSBV and MGB agar plates (figures represent the mean of two observations). Experiment No. 3

Strain number	$\log_{10}$ cfu/ml of *A.actinomycetemcomitans* CBA	TSBV	MGB
Aa 01	10.32	10.04	10.08
Aa 02	9.87	9.57	9.94
Aa 03	10.25	9.38	9.90
Aa 06	9.96	9.54	9.97
Aa 07	9.81	9.89	9.84
Aa 08	9.68	9.81	9.71
Aa 09	9.48	9.45	4.30
Aa 10	9.38	8.98	3.85
Aa 11	10.15	9.96	5.50
Aa 12	9.25	9.08	3.90
Aa 13	9.15	8.82	3.82
Aa 14	NT	NT	NT
Aa 34	10.20	9.86	9.96
Aa 35	10.25	9.92	9.77
Aa 36	9.83	9.93	9.65
Aa 37	10.20	10.20	10.20
Aa 38	9.86	9.87	9.54

NT: Not tested

Table 2.5 Summary of the mean $\log_{10}$ cfu/ml of 17 ***A.actinomycetemcomitans*** strains recovered on CBA, TSBV and MGB agar plates (figures represent the mean of three different experiments).

Strain number	Mean $\log_{10}$ cfu/ml of *A.actinomycetemcomitans* CBA (±SEM)	TSBV (±SEM)	MGB (±SEM)
Aa 01	10.06 (0.14)	9.66 (0.25)	10.07 (0.01)
Aa 02	9.90 (0.05)	9.51 (0.07)	9.89 (0.04)
Aa 03	10.17 (0.04)	9.63 (0.17)	10.09 (0.1)
Aa 06	9.87 (0.05)	9.53 (0.2)	9.92 (0.1)
Aa 07	9.77 (0.06)	9.88 (0.01)	9.74 (0.05)
Aa 08	9.75 (0.05)	9.69 (0.08)	9.62 (0.05)
Aa 09	9.39 (0.08)	9.06 (0.33)	4.83 (0.69)
Aa 10	9.05 (0.24)	8.66 (0.24)	3.84 (0.02)
Aa 11	10.0 (0.09)	9.85 (0.13)	5.60 (0.05)
Aa 12	8.73 (0.38)	8.89 (0.14)	5.16 (0.96)
Aa 13	9.18 (0.05)	8.62 (0.19)	4.13 (0.3)
Aa 14	9.46 (0.23)	9.21 (0.21)	5.16 (0.16)
Aa 34	10.08 (0.1)	9.88 (0.11)	9.79 (0.09)
Aa 35	9.69 (0.31)	9.95 (0.07)	9.69 (0.06)
Aa 36	9.77 (0.03)	9.87 (0.03)	9.53 (0.06)
Aa 37	10.04 (0.08)	9.85 (0.17)	9.90 (0.15)
Aa 38	9.86 (0.05)	9.80 (0.04)	9.58 (0.04)
Over all	9.69 (0.09)	9.50 (0.11)	8.03 (0.61)

±SEM: Standard error of mean

Comparisons: determined by paired t-Test

CBA v TSBV= ≤ 0.005

CBA v MGB= ≤ 0.005

TSBV v MGB= ≤ 0.01

2.2.9 Discussion:

The selectivity and sensitivity of the two semi-selective media TSBV and MGB for the isolation of *A.actinomycetemcomitans* have been examined by few researchers. In 1981 Mandell and Socransky investigated the ability of MGB medium to grow 9 strains of *A.actinomycetemcomitans* (4 Type strains and 5 fresh isolates), and reported that using MGB, a range of 83% to 99% (mean 90%) of *A.actinomycetemcomitans* cells were recovered compared to the number which grew on a trypticase soy agar plates. In the present investigation, 17 strains of *A.actinomycetemcomitans* were examined including 4 Type strains, and a range of 42% to 100% (mean 83%) of *A.actinomycetemcomitans* cells were recovered on MGB compared to the number obtained on CBA plates. Only one of the Type strains of *A.actinomycetemcomitans* (ATCC 29522) examined by Mandell and Socransky (1981) was included in the present investigation. While Mandell and Socransky (1981) showed that 94% of ATCC 29522 cells were recovered on MGB, a figure of 99% was obtained in this study. However, a marked difference was found between the two studies in relation to the inhibitory activity of MGB. The lowest recovery rate reported by Mandell and Socransky (1981) was 83%, whereas a figure of 42% was obtained in this study. The importance of this difference is doubtful since it could be related to the fact that different *A.actinomycetemcomitans* strains and blood agar plates were used in both studies. VAN Steenbergen *et al.*, (1986) who examined 21 isolates of *A.actinomycetemcomitans* including three Type strains reported low

recovery rates on MGB which ranged from approximately 10% to 100% giving an overall mean of 55%, which agrees with the results of the present study, and confirms that MGB medium significantly inhibits the growth of pure cultures of *A.actinomycetemcomitans* compared to growth on blood agar plates. Riches and Marsh (1987) reported that the growth of three Type strains of *A.actinomycetemcomitans* ranged between 61% to 73% on MGB medium. Although two of the strains examined by Riches and Marsh (1987) were included in the present study, comparison of results is not possible because there was no data reported for these strains by these workers.

The other semi-selective medium TSBV has been used by a number of workers. Slots (1982b) has reported a mean recovery rate of 96% for 12 pure cultures on TSBV compared to the number on blood agar plates, but no information was given about the individual strains. However, VAN Steenbergen *et al.*, (1986) reported that using TSBV, a range of 50% to >100% (mean 85%) pure cultures of 21 strains of *A.actinomycetemcomitans* were recovered as compared to blood agar plates. In the present investigation recovery rates of 94% to >100% (mean 98%) of pure cultures of *A.actinomycetemcomitans were obtained* which agrees with the results of both Slots (1982b) and VAN Steenbergen *et al.*, (1986). Nevertheless, Riches and Marsh (1987) reported the lowest recovery rate of 3% to 19% for three Type strains of *A.actinomycetemcomitans* on TSBV. These results which disagree with the previously published data and that reported in this study

may be explained by the fact that Riches and Marsh (1987) used only Type strains of *A.actinomycetemcomitans*.

In this study both TSBV and MGB significantly suppressed the growth of *A.actinomycetemcomitans* compared to CBA agar plates ($p \leq 0.005$), but MGB was significantly more inhibitory than the TSBV ($p \leq 0.01$). The suppression of growth of *A.actinomycetemcomitans* strains on the these semi-selective media may be related to the presence of either antimicrobials or other agents such as malachite green. Riches and Marsh (1987) reported that TSBV was more inhibitory to three Type strains of *A.actinomycetemcomitans* (Y4, NCTC 10979, NCTC 9710) than MGB. Viable counts of *A.actinomycetemcomitans* obtained ranged from 3% to 19% on TSBV, whereas MGB recovered a range of 61% to 73% (Riches and Marsh, 1987). These results disagree with the findings of the present investigation and those by VAN Steenbergen *et al.*, (1986). In the present study, MGB mainly suppressed the growth of 6 (46%) fresh isolates of *A.actinomycetemcomitans*, while this was not observed with the Type strains of *A.actinomycetemcomitans* (see Table 2.5). On the contrary, the NCTC Type strains examined, grew equally well on both TSBV and MGB plates. In support of this findings, VAN Steenbergen *et al.*, (1986) used three Type strains of *A.actinomycetemcomitans* (Y4, ATCC 29523 and ATCC 29524) in their study (none of which were examined in the present investigation), and reported that all grew in high numbers on TSBV, MGB and blood agar plates. This discrepancy between Type and fresh isolates may possibly be explained by the fact that the Type

strains have adapted to growing under laboratory conditions over a period of time (VAN Steenbergen *et al.*, 1986). The results of the present investigation and those by (VAN Steenbergen *et al.*, 1986) highlight the importance of using fresh isolates as well as Type strains to examine the selectivity of culture media to recover pure cultures of *A.actinomycetemcomitans*.

2.2.10 Isolation of *A.actinomycetemcomitans* from mixed cultures

This part of study was carried out to examine the effectiveness of both semi-selective media (TSBV and MGB) in recovering *A.actinomycetemcomitans* from subgingival plaque samples. Nine patients with 31 sites were examined in this study. One subgingival plaque sample was taken from the deepest pocket in each segment in which pocket depths of 6 mm or more were found. Thus depending on the distribution of disease, one to 6 samples were taken from each patient. Samples were collected after isolating the relevant tooth with cotton wool rolls and drying with an air syringe. Supragingival plaque was collected with a sterile curette and discarded. One to three sterile paper points (depending on the space available) were inserted to the base of the pocket and left for 10 seconds. The paper points were then transferred to one ml ABB and immediately transported to the laboratory. All plaque samples were dispersed using a vortex mixer (Fisons Scientific Apparatus, Leicestershire, England) for 60 seconds and 10-fold dilutions were made in PBS. Using a spiral plater (Spiral Systems, Clough Pike, Cincin-

nati, USA), 50 µl aliquots from the neat, 10^{-1} and 10^{-2} dilutions were inoculated onto the TSBV and CBA plates. Incubation was carried out for 5 days at 37°C in 5% CO_2 in air, and plates were examined daily for up to 5 days for the presence of colonies resembling *A.actinomycetemcomitans*. Colonies were selected using the criteria described in section 2.3.5, and all isolates were identified using the same method described earlier in section 2.2.4.

2.2.11 Results:

A total number of 31 sites from nine patients were examined for the presence of *A.actinomycetemcomitans* using the two semi-selective media TSBV and MGB. Although only nine patients were included in this study, four patients (44%) and 8 sites (26%) were found to harbour *A.actinomycetemcomitans*. However, as shown in Table 2.6 all positive cultures were detected only on TSBV medium since the MGB medium failed to recover *A.actinomycetemcomitans* from any of the mixed samples.

Table 2.6 Isolation of *A.actinomycetemcomitans* from 31 mixed samples using both TSBV and MGB media.

Patient Number	No. sites examined	No. +ve sites	Medium +ve for *A.actinomycetemcomitans*
01	1	1	TSBV
02	1	0	None
03	2	2	TSBV
04	2	0	None
05	3	3	TSBV
06	5	0	None
07	5	0	None
08	6	2	TSBV
09	6	0	None

Number of patients examined = 9

Total sites examined = 31

Number of patients with *A.actinomycetemcomitans* = 4 (44%)

Number of sites with *A.actinomycetemcomitans* = 8 (26%)

2.2.12 Discussion:

There are only few reported studies which have investigated the isolation of *A.actinomycetemcomitans* from mixed samples using both TSBV and MGB media. Although only a small number of samples (31 sites) were included in this study, TSBV medium was found to be far superior to MGB medium in the recovery of *A.actinomycetemcomitans* from mixed samples obtained from patients with periodontal disease. A similar study was carried out by VAN Steenbergen *et al.*, (1986) where the selectivity of both TSBV and MGB media in the recovery of *A.actinomycetemcomitans* from 123 patients was investigated. Thirty patients (24%) were found to harbour *A.actinomycetemcomitans* as detected on TSBV medium incubated in air-5% CO_2. In comparison only 16 patients (13%) were positive for *A.actinomycetemcomitans* using MGB medium. Both the present investigation and the study by VAN Steenbergen *et al.*, (1986) demonstrate the effectiveness of the TSBV medium in the recovery of *A.actinomycetemcomitans* from mixed samples. However, the fact remains that *A.actinomycetemcomitans* was not detected on MGB in this investigation, which disagree with the findings of VAN Steenbergen *et al.*, (1986). This could be due to the small number of samples examined, 31 compared to the 123 samples investigated by VAN Steenbergen *et al.*, (1986). Certainly colonies of *A.actinomycetemcomitans* were larger, more characteristics and more easily recognized on TSBV compared with MGB medium, facts which were also noted by VAN Steenbergen *et al.*, (1986). Furthermore, TSBV medium was suitable for detecting catalase

production unlike MGB which contains blood and can thus produce false positive results (Slots, 1982b; VAN Steenbergen *et al.*, 1986).

In conclusion, the results of the present investigation and those by other researchers are in agreement that TSBV medium is preferable to MGB for the isolation of *A.actinomycetemcomitans* from subgingival plaque samples from periodontitis patients.

2.3 Clinical Study

2.3.1 Introduction

A.actinomycetemcomitans has been implicated in the aetiology of different forms of periodontitis, especially Localized Juvenile Periodontitis (LJP) (Zambon *et al.*, 1983b; Mandell, 1984). However, it has not been frequently found in high proportions in patients with chronic periodontitis or in healthy subjects (Moore, 1987). Wolff *et al.*, (1985) found *A.actinomycetemcomitans* in 13% of of 284 people with healthy gingiva or moderate periodontitis, and the presence of *A.actinomycetemcomitans* was found to correlate with high plaque index and intermediate pocket depths of 3-5mm. Since there is no information about the the prevalence of *A.actinomycetemcomitans* in a Scottish population, a study was undertaken to quantify the prevalence of *A.actinomycetemcomitans* in subgingival plaque in 98 patients with chronic periodontitis and 55 healthy volunteers.

The identification and enumeration of *A.actinomycetemcomitans* is important as there are close morphological and some biochemical similarities between *A.actinomycetemcomitans* and the other facultative Gram-negative rods present in dental plaque especially *H.aphrophilus*. *A.actinomycetemcomitans* has often been described as producing star-shaped colonies, and this characteristic has been heavily used in the isolation, identification and enumeration on primary selective culture plates. However, the literature lacks information on the prevalence of both star-shaped and non-star-shaped (SS/NSS) variants of this organism on primary culture. Therefore, using a number of the samples collected from the chronic periodontitis and the healthy control group, the prevalence of the different types of colonies (SS and NSS) was investigated. Since systemic antimicrobial agents are sometimes used in the management of LJP and chronic periodontitis (Slots and Rosling, 1983), it was decided to perform antimicrobial sensitivity tests on all isolates from diseased patients.

2.3.2 Material and methods

Patients & controls

The population studied comprised 98 patients, (43 males and 55 females), with advanced periodontitis who were referred to Glasgow Dental Hospital and School for specialist periodontal assessment and treatment. Their ages ranged from 14-63 years with a mean age of 38.5 years.

A healthy group of 55 young adults (27 males and 28 females) between the ages of 18-40 years with a mean age of 22.5 were selected to serve as a control group.

2.3.3 Clinical Assessment

Chronic periodontitis patients

A full periodontal examination was carried out by a single consultant periodontologist using a WHO 621 probe (Ainamo *et al.*, 1982). Each jaw was divided into 3 segments with the incisor and canine teeth forming one segment and the premolar and molar teeth on either side the other two segments. Subgingival plaque samples were removed as described in section 2.2.9 and were later examined for the presence of *A.actinomycetemcomitans* as described in Figure 2.5.

Healthy volunteers

The majority of the 55 healthy volunteers were dental students at the Glasgow Dental Hospital and School. The mouths of these volunteers were examined by the same consultant periodontologist who assessed the periodontitis group using WHO 621 probes (this was done gently to avoid any bleeding). All subjects were inspected to confirm that they had a generally healthy mouth without evidence of inflammatory periodontal diseases. Samples were collected by first removing any supragingival plaque which was discarded. Using a sterile curette subgingival plaque was removed from one gingival sulcus/pocket in each quadrant. Due to the small amounts of plaque collected from each site, plaque samples were then immediately pooled in one ml of ABB, before transfer to the laboratory.

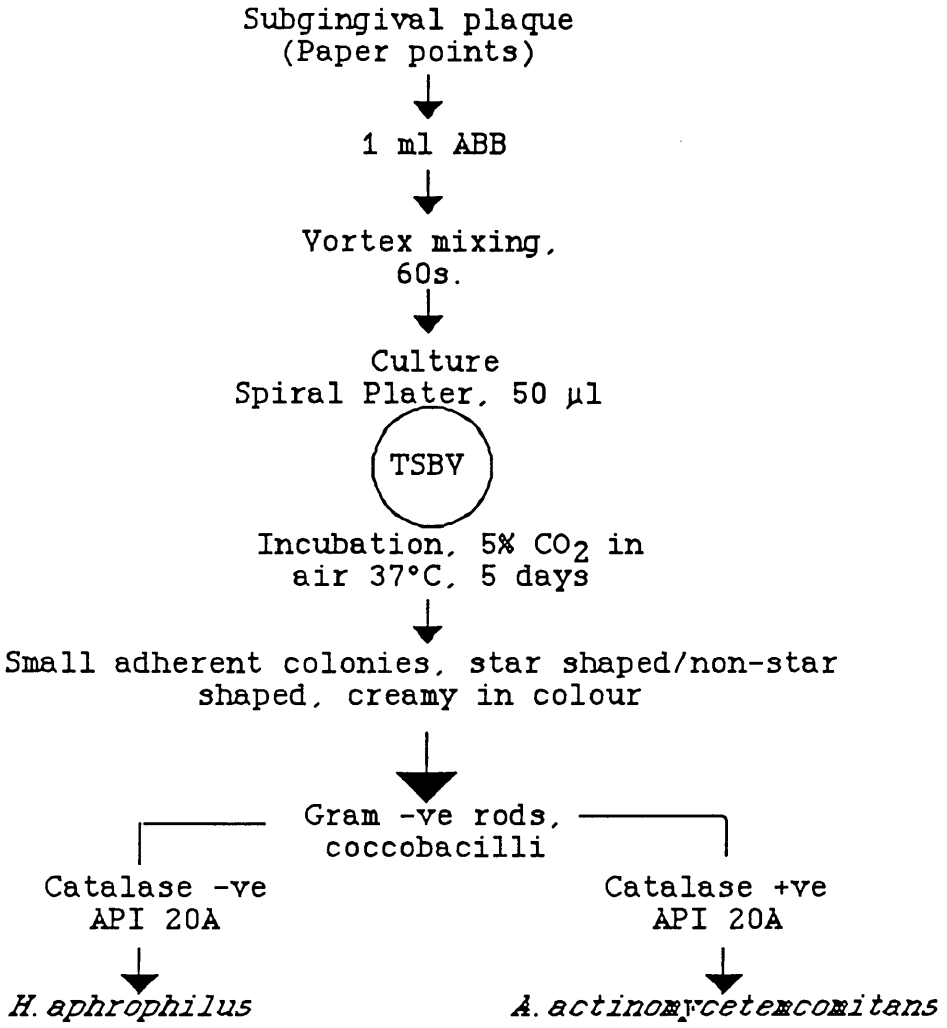

Figure 2.5 Chart for the isolation and identification of *A. actinomycetemcomitans*.

2.3.4 Culture of samples

All plaque samples were dispersed using a vortex mixer (Fisons Scientific Apparatus, Leicestershire, England) for 60 seconds, then 10-fold dilutions made in PBS. Using a spiral plater (Spiral Systems Clough Pike, Cincinnati, USA), 50 µl aliquots of neat, 10^{-1} and 10^{-2} dilutions were inoculated onto the TSBV plates. Incubation was carried out for 5 days at 37°C in 5% CO_2 in air using a Qualitemp 80 MI incubator (Laboratory Terminal Equipment Ltd., Oldham, UK) and using a manual magnifying glass the plates were examined daily for up to 5 days for the presence of colonies resembling *A.actinomycetemcomitans*. Colonies were selected using the criteria described in section (2.3.5).

2.3.5 Identification of the freshly isolated *A.actinomycetemcomitans* strains

Colonial morphology

The colonies selected for isolation and identification were small, about 0.5-1.0mm diameter in size, circular, glistening with a slightly irregular edge and creamy in colour. Since both star-shaped and non-star-shaped colonies have been described previously, it was decided to examine plates for both variants in this study. Examples of the types of colonies selected are shown in Figure 2.6. A minimum of 3 to 4 colonies from each TSBV agar plate were subcultured onto a fresh blood agar plate (CBA) and incubated for purity in 5% CO_2 in air for 48 hours. Pure isolates were then

identified using the morphological and biochemical tests described earlier in section 2.2.4.

API-ZYM test

The API-ZYM (API Laboratories, La Balme, Les Grottes, France) is a semi-quantitative micro-method system which can be used to investigate the enzyme patterns produced by microorganisms. The API-ZYM colorimetric kit system was used to test the ability of 26 *A.actinomycetemcomitans* strains to produce different patterns of enzymes some of which may be involved in the pathogenicity of this organism. The assay was carried out by emulsifying a pure growth of *A.actinomycetemcomitans* from one CBA plate in 2 ml of sterile distilled water. The strips were inoculated using a pasteur pipette as recommended by the manufacturer and incubated in an atmosphere of 5% CO_2, in air at 37°C for 4 hours. After incubation, the API-ZYM reagents A and B (API Laboratories, La Balme, Les Grottes, France) were added to the strips as recommended and the colour change (Figure 2.7) was recorded on a 0 to 5 scale according to the colour chart supplied with the test kit. Grade 0 was regarded as negative enzyme activity, grade 1 or 2 as a weak reaction and grade 3, 4 or 5 as strong activity.

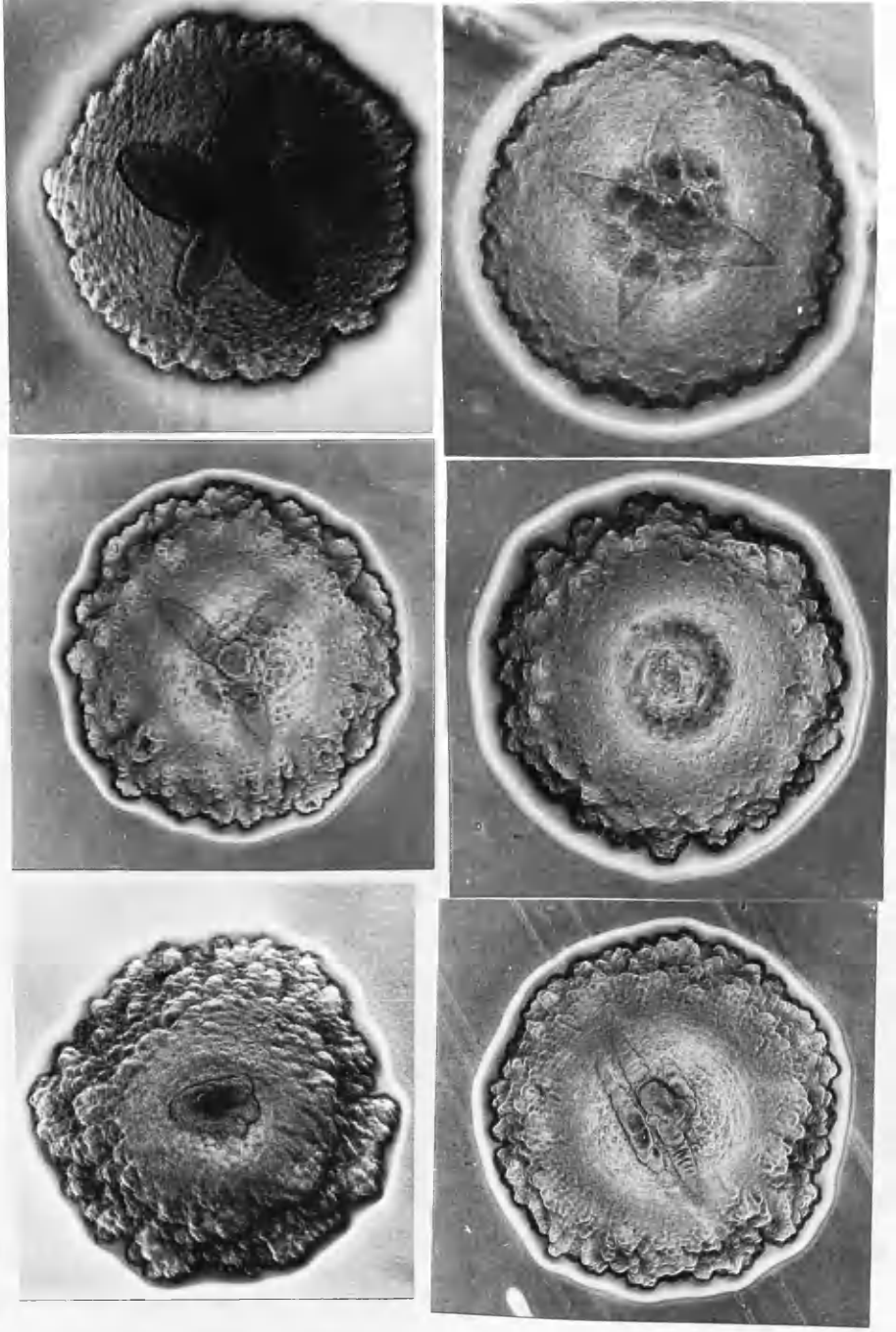

Figure 2.6 Variation in the colonial morphology of *A.actinomycetemcomitans strains* selected for isolation and identification.

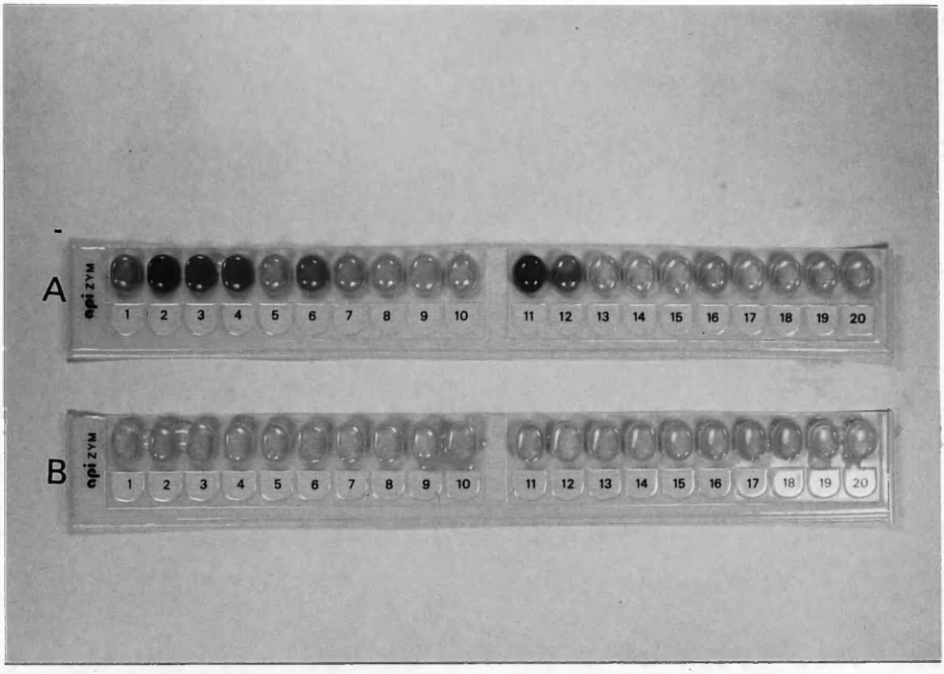

Figure 2.7 API-ZYM strip inoculated with *A.actinomycetemcomitans* (the test (A) shows change in colour, while the control (B) remains unchanged).

2.3.6 Antimicrobial sensitivity testing

A total of 46 *A.actinomycetemcomitans* isolates including 3 reference strains were subjected to antimicrobial sensitivity testing using Stokes method (Stokes and Waterworth, 1972). The bacterial inoculum was prepared by growing each test organism on a CBA plate for 48 hours in an atmosphere of 5% CO_2 in air at 37°C. Five to seven colonies were removed with a sterile loop and transferred to 2 ml of peptone water (Oxoid, Ltd., Basingstoke, Hampshire, England) then vortex mixed for 60 seconds. However, in the case of the control organism (*Staphylococcus aureus* Oxford NCTC 6571), only one colony was removed and emulsified in 2 ml peptone water. For both control and test organisms a sterile cotton wool swab was dipped into the bacterial suspension and the excess fluid removed by turning the swab against the side of the tube. The swab containing the test organism was then spread evenly in a broad band across the centre of the Diagnostic Sensitivity Test Agar plate (DST, Appendix I) (Oxoid, Ltd., Basingstoke, Hampshire, England). The control organism was seeded evenly in two bands on either side of the test inoculum (see Figure 2.8). All tests were run in duplicate on a single occasion. Antimicrobial discs (see Table 2.7) (Mast Lab., Merseyside, England) were applied with a sterile needle on the line between the test and the control organisms and pressed gently onto the surface to ensure even contact with the medium. Due to the presence of a metronidazole antibiotic disc, all plates were incubated in an anaerobic chamber (Don Whithley

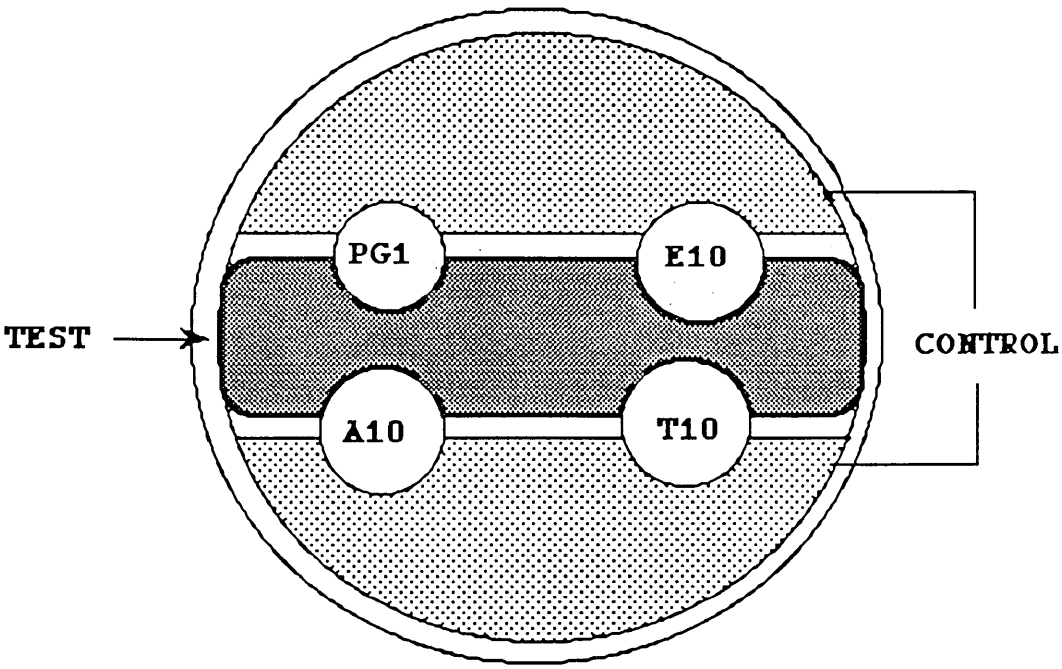

Figure 2.8 Diagrammatic representation of an antimicrobial sensitivity test plate using Stokes method before incubation.

Table 2.7 Antimicrobial agents used for testing the sensitivity patterns of *A.actinomycetemcomitans*.

Antimicrobial Agent	Abbreviation	Disc Potency
Amoxycillin	A	10 µg
Erythromycin	E	10 µg
Tetracycline	T	10 µg
Penicillin	PG	1.0 unit
Metronidazole	MZ	2.5 µg

Scientific Ltd., West Yorkshire, England) at 37°C in an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 for 48 hours.

Zones of inhibition were measured from the centre of the disc to the edge of the zone (Figure 2.9) and the difference between the test organism and control was calculated. Results were interpreted as follows:

Sensitive: Zone size equal to, wider than, or not more than 3 mm smaller than the control.
Intermediate : Zone size greater than 3 mm, but smaller than the control by more than 3 mm.
Resistant: Zone size 3 mm or less.

2.3.7 Maintenance of cultures

Due to the possibility of phenotypic variation occurring in strains of *A.actinomycetemcomitans* as a result of continuous subculture *in vitro*, freshly isolated bacteria were freeze dried within two to three subcultures after isolation and identification. Type strains supplied by NCTC or ATCC were also freeze dried.

Freeze dried ampoules (R and J Woods, Paisley, Scotland) were prepared for sterilization by placing a thin piece of card containing information to identify the isolate inside the glass tube. A small piece of cotton wool was inserted loosely into the top of the tube and the ampoule sterilized by dry heat at 160°C for 90 minutes. Using a cotton wool swab, the growth from two 48 hour CBA cultures was harvested and added to 3 ml of ABB. Approximately 0.5 ml of this emu-

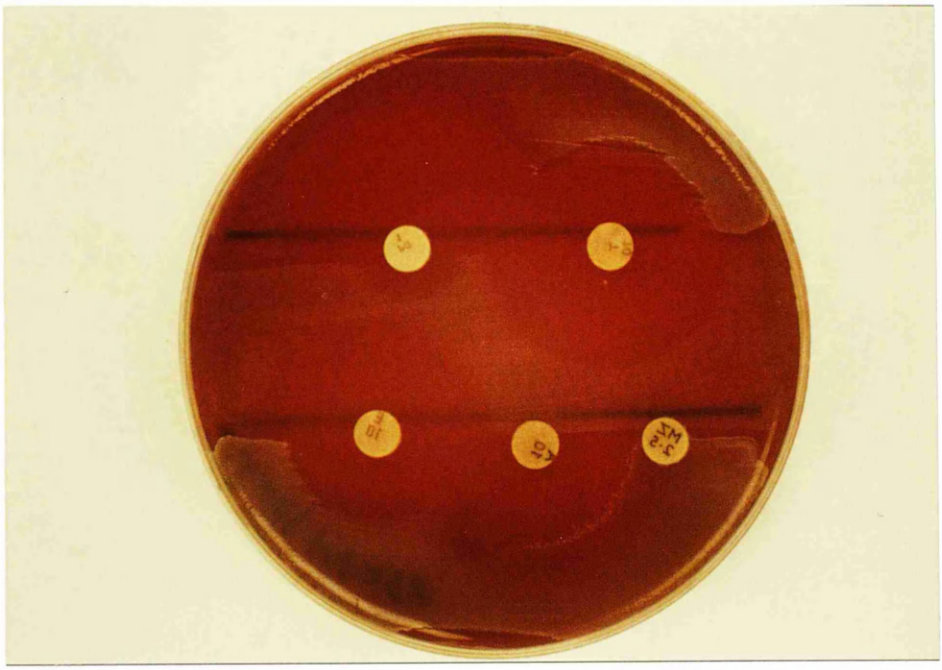

Figure 2.9 A DST agar plate inoculated with the test organism *A.actinomycetemcomitans* and *S.aureus* NCTC 6571 as control, for sensitivity testing of amoxycillin, erythromycin, tetracycline, penicillin and metronidazole using Stokes method.

lsion was added to prelabelled sterile ampoules. Freeze drying was subsequently performed using an Edwards EF4 Modulyo freeze drier (Edwards High Vacuum, Crawley, England). Freezing was carried out on two stages, firstly during centrifugation and secondly when the vials had stopped spinning. This secondary freezing was continued until a pressure of 1.3×10^{-1}m bar or below was achieved. The vials were then constricted using an Edwards Ampoule constricter and subjected to the final stage of freeze drying under a vacuum for 60 to 90 minutes. After this procedure the ampoules were sealed using an Edwards Flamemaster hand torch and stored at room temperature until required. Fresh ampoules were opened after the strains in use had been subcultured on more than five occasions. During experimental work, cultures were maintained on blood agar plates at 4°C.

When required, ampoules were opened by making a mark around the top half of the glass vial with a diamond marker. A clean paper napkin was placed around the ampoule and the top of the ampoule was broken off. Two to three drops of ABB were added to the culture using a pasteur pipette. The bacterial suspension was then inoculated onto a fresh CBA plate and incubated at 37°C in an atmosphere of 5% CO_2 in air for 48-72 hours, each culture was then checked for purity as described in section 2.2.4 before use.

2.4 Results:

2.4.1 Isolation of *A.actinomycetemcomitans* from 98 patients with chronic periodontitis

Ninety eight patients with chronic periodontitis were examined in this study. The male group consisted of 43 patients aged from 20 to 63 years (mean 41); and the female group of 55 patients aged between 14 to 59 (mean 36.4). The results for the isolation of *A.actinomycetemcomitans* from the 98 patients with chronic periodontitis are shown in Table 2.8. *A.actinomycetemcomitans* was isolated from 38 out of 98 patients (39%), aged between 14 to 63 years (mean 38.5). As shown in Table 2.8, the percentage carriage of *A.actinomycetemcomitans* among the different age groups, varied from 28% to 63% excluding age groups 11-20 and 61-70 because only very few patients were examined. The highest percentage carriage of 63% was evident in the age group of 20 to 30 years, with a figure of 45% being recorded for the 51-60 group (Table 2.8). Of the 43 males and 55 females examined; 39.5% of male and 38.2% of female patients were found to be positive for *A.actinomycetemcomitans*.

2.4.2 Isolation of *A.actinomycetemcomitans* from healthy control subjects

There were 55 control subjects examined in the present investigation (Table 2.9). The male group consisted of 27 subjects aged from 20 to 30 years old with a mean age of 23 years; and the female group of 28 subjects aged between 18 and 40 years of age (mean 22 years). As shown in Table 2.9

almost all of the subjects tested were aged from 20 to 30 years. *A.actinomycetemcomitans* was isolated from only one (4%) male control subject and from none of the female control subjects giving an overall mean prevalence of 2% for the 55 control subjects.

2.4.3 Isolation frequency of *A.actinomycetemcomitans* from 302 sites sampled in 98 patients

The isolation frequency of *A.actinomycetemcomitans* from the different diseased sites in the 98 periodontitis patients are shown in Tables 2.10, 2.11, 2.12 and summarized in Table 2.13. The sites were divided into 6 different segments, namely, (I) upper right, (II) upper left posterior, (III) upper anterior, (IV) lower anterior, (V) lower right posterior, and (VI) upper left posterior. Of the 302 subgingival plaque samples examined, 82 (27%) harboured *A.actinomycetemcomitans;* 49 (60%) of these sites were detected in males and 31 (40%) sites from female patients . As shown in Tables 2.10, 2.11 and 2.12, there were approximately similar numbers of sites examined and sampled in both male and female patients.

In general the isolation of *A.actinomycetemcomitans* from these sites tended to be higher in male patients compared to females. The only exception to this was in the upper right posterior segments where the number of sites positive for *A.actinomycetemcomitans* in females was twice of that in male patients (32% v 16%). When the results for males and females are combined (Table 2.13), the isolation frequency

of *A.actinomycetemcomitans* in the six different sites examined, ranged from 19% to 34% with no large differences noted between any of the sites examined.

2.4.4 Viable counts of *A.actinomycetemcomitans*

In order to reduce the bulk of data, all the colony forming units per millilitre (cfu/ml) obtained for *A.actinomycetemcomitans* on TSBV agar plates were converted into a logarithmic format ($\log_{10}$ cfu/ml). The $\log_{10}$ cfu/ml of *A.actinomycetemcomitans* for both male and female patients are shown in Tables 2.14 and 2.15. *A.actinomycetemcomitans* was detected in variable concentrations among different patients as well as in different sites in the same individual. As shown in Table 2.14 the concentration of *A.actinomycetemcomitans* in samples from male patients ranged from $\log_{10}$ 1.15 to 6.52 (mean 5.2), while a range of 1.3 to 6.34 (mean 4.34) was recorded for samples obtained from female patients (Table 2.15). The overall mean $\log_{10}$ cfu/ml of *A.actinomycetemcomitans* was found to be significantly higher in males $\log_{10}$ 5.2 than in females $\log_{10}$ 4.34 ($p \leq 0.03$) using paired t-Test. Although 63 and 62 samples were examined in both males and females respectively, more samples were positive for *A.actinomycetemcomitans* from male patients 49 (78%) compared with female patients 33 (53%) (Tables 2.14 and 2.15).

Table 2.8 Age range and prevalence of *A.actinomycetemcomitans* isolated from 98 patients with chronic periodontitis.

Age distribution (Years)	No. tested M	No. tested F	No. +ve M	No. +ve F	Overall % +ve
0-10	0	0	0	0	0%
11-20	1	2	1	1	67%
21-30	6	13	2	10	63%
31-40	13	26	4	7	28%
41-50	15	10	6	2	32%
51-60	7	4	4	1	45%
61-70	1	0	0	0	0%
Total	43	55	17	21	39%

Age range: 14-63 yrs

Mean age: 38.5 yrs

M: Male patients

F: Female patients

Table 2.9 Age range and prevalence of *A.actinomycetemcomitans* isolated from male and female control subjects.

Age distribution (Years)	No. tested		No. +ve		Overall % +ve
	M	F	M	F	
0-10	0	0	0	0	0%
11-20	3	7	0	0	0%
21-30	24	19	1	0	2.3%
31-40	0	2	0	0	0%
Total	27	28	1	0	2%

Age range: 20-40 yrs

Mean age: 22.5 yrs

M: Male subjects

F: Female subjects

Table 2.10 Isolation frequency of *A.actinomycetem-comitans* in the upper posterior segments of male and female patients.

	Upper Right Posterior Segments			Upper Left Posterior Segments		
	No.S.T	No.+ve	% ve	No.S.T	No.+ve	% ve
Males	25	4	16	24	6	25
Females	25	8	32	29	4	14
Total	50	12	24	53	10	19

No.S.T: Number of sites tested

Table 2.11 Isolation frequency of *A.actinomycetem-comitans* in the lower posterior segments of male and female patients.

	Lower Right Posterior Segments			Lower Left Posterior Segments		
	No.S.T	No.+ve	% ve	No.S.T	No.+ve	% ve
Males	21	10	48	16	9	56
Females	22	2	9	25	5	20
Total	43	12	24	41	14	34

No.S.T: Number of sites tested

Table 2.12 Isolation frequency of *A.actinomycetem-comitans* in the anterior segments of male and female patients.

	Upper Anterior Segments			Lower Anterior Segments		
	No.S.T	No.+ve	% ve	No.S.T	No.+ve	% ve
Males	30	12	40	22	8	36
Females	43	9	21	20	5	25
Total	73	21	29	42	13	31

No.S.T: Number of sites tested

Table 2.13 Summary of the isolation frequency of *A.actinomycetemcomitans* in the different oral segments of male and female patients.

Segment	No.sites tested	No.+ve sites	% +ve
Upper Right Post. Seg.	50	12	24%
Upper Left Post. Seg.	53	10	19%
Lower Right Post. Seg.	43	12	28%
Lower Left Post. Seg.	41	14	34%
Upper Anter. Segment	73	21	29%
Lower Anter. Segment	42	13	31%
Total	802	82	27%

Post.: Posterior

Anter: Anterior

Seg.: Segment

Table 2.14 Viable counts of *A.actinomycetemcomitans* (log_{10} cfu/ml) on TSBV from subgingival plaque samples of male patients.

Patient Number	Age (Yrs)	No. Sit. tested	No.+ve Sites	Range of log_{10} cfu/ml	Mean +ve sites
01	20	6	2	3.0-3.9	3.65
02	23	6	3	5.08-5.11	5.08
03	30	3	3	5.62-6.52	6.30
04	33	5	5	1.15-6.11	5.76
05	34	4	4	3.0-4.83	4.36
06	35	2	2	4.08-5.36	5.08
07	38	3	3	2.78-4.95	4.54
08	42	2	2	5.18-5.59	5.43
09	42	5	5	2.9-5.65	5.23
10	42	5	3	3.56-5.0	4.67
11	43	4	3	5.0-5.62	5.43
12	43	1	1	5.23	5.23
13	44	3	3	3.68-6.11	5.9
14	52	3	1	5.38	5.38
15	55	4	4	2.5-5.54	5.04
16	55	3	3	2.3-5.95	5.58
17	58	4	2	4.34-6.0	5.7

No. Sit: Number of sites

Table 2.15 Viable counts of *A.actinomycetemcomitans* (log_{10} cfu/ml) on TSBV from subgingival plaque samples of female patients.

Patient Number	Age (Yrs)	No. Sit. tested	No.+ve Sites	Range of log_{10} cfu/ml	Mean +ve sites
01	20	4	1	NT	NT
02	21	3	3	5.0-5.25	5.0
03	24	3	3	NT	NT
04	27	2	1	2.5	2.5
05	28	1	1	4.89	4.89
06	29	5	1	4.99	4.99
07	29	4	1	5.04	5.04
08	30	3	1	5.78	5.78
09	30	6	6	1.6-5.25	4.5
10	30	1	1	5.2	5.2
11	30	3	3	1.3-3.48	3.15
12	33	5	1	2.3	2.3
13	33	1	1	4.25	4.25
14	34	1	1	2.82	2.82
15	35	4	1	3.68	3.68
16	36	5	1	4.0	4.0
17	37	1	1	NT	NT
18	38	3	1	4.99	4.99
19	41	2	1	6.34	6.34
20	48	2	1	2.48	2.48
21	53	3	2	1.78-5.53	5.23

NT: Not tested

No. Sit: Number of site

2.4.5 Biochemical results of the freshly isolated strains of *A.actinomycetemcomitans*

The biochemical profiles of *A.actinomycetemcomitans* using the API 20 A system are summarized in Table 2.16. Results obtained for 38 fresh isolates, showed that all *A.actinomycetemcomitans* strains (100%) fermented glucose, maltose and mannose. However, 68% of isolates fermented mannitol; 42% fermented xylose and only 8% of all isolates fermented glycerol or rhamanose. In addition all isolates produced catalase and did not require either X or V growth factors. Using the API 20 A system, the majority of isolates 21 (55%) were found to produce the profile 4110404 followed by 4050404 (29%), 4150404 (13%), and 4010404 (3%) (see Table 2.16).

2.4.6 The enzymatic characterization of *A.actinomycetemcomitans*

The enzyme patterns of 26 different strains of *A.actinomycetemcomitans* were examined using the API ZYM test. These *A.actinomycetemcomitans* strains included both Type strains and fresh isolates from the present investigation. Results summarized in Table 2.17 clearly shows that all 26 *A.actinomycetemcomitans* strains (100%) were strongly positive for alkaline and acid phosphatases, leucine arylamidase, but weakly positive for esterase and phosphohydrolase (see Table 2.17).

Table 2.16 Summary of the biochemical results for the 38 freshly isolated *A.actinomycetemcomitans* strains as determined by the API 20 A system.

	A.actinomycetemcomitans (38 strains)	
Test	RESULT	% Positive
Indole	-	0
Urea	-	0
Glucose	+	100
Mannitol	v	68
Lactose	-	0
Saccharose	-	0
Maltose	+	100
Salicin	-	0
Xylose	v	42
Arabinose	-	0
Gelatin	-	0
Esculin	-	0
Glycerol	v	8
Cellobiose	-	0
Mannose	+	100
Melezitose	-	0
Raffinose	-	0
Sorbitol	-	0
Rhamnose	v	8
Trehalose	-	0
Catalase	+	100

Sign: +, positive reaction; -, negative reaction; v, variable reaction.

Number of strains which produced the profile 4110404 = 21 (55%)

Number of strains which produced the profile 4050404 = 11 (29%)

Number of strains which produced the profile 4150404 = 5 (13%)

Number of strains which produced the profile 4010404 = 1 (3%)

Table 2.17 Summary of the enzymatic characteristics of *A.actinomycetemcomitans* (26 strains) as determined by the API-ZYM system.

	A.actinomycetemcomitans (26 strains)	
Enzyme tested	RESULT	% positive
Alkaline phoshatase	+ (S)	100
Esterase (butyrate)	+ (W)	100
Esterase-Lipase (caprylate)	+ (W)	100
Lipase (myristate)	-	0
Leucine arylamidase	+ (S)	100
Valine arylamidase	-	0
Cystine arylamidase	-	0
Trypsin	-	0
Chymotrypsin	-	0
Acid phoshatase	+ (S)	100
Naphtol-AS-BI-phosphohydrolase	+ (W)	100
α galctosidase	-	0
β galctosidase	-	0
β glucuronidase	-	0
α glucosidase	-	0
β glucosidase	-	0
N-acetyl-β glucosamindase	-	0
α mannosidase	-	0
α fucosidase	-	0

Sign: + (S), strong positive reaction

Sign: + (W), weak positive reaction

Sign: -, negative reaction

2.4.7 The prevalence of star-shaped and non-star-shaped colonies in primary cultures of *A.actinomycetemcomitans* isolated from subgingival plaque samples

The prevalence of star-shaped and non-star shaped colonies of *A.actinomycetemcomitans* was examined in the primary cultures of samples from patients employed in this study. Although, 38 patients were found to be positive for *A.actinomycetemcomitans,* full information concerning star-shaped and non-star-shaped colonies was only obtained for 31 of these patients. As shown in Table 2.18, *A.actinomycetemcomitans* isolates formed star-shaped colonies in only 16 (52%) of patients, whereas in 20 (64%) patients, *A.actinomycetemcomitans* produced only non-star shaped colonies on primary isolation on TSBV medium. In 5 (16%) of patients, both colonial types of *A.actinomycetemcomitans* were present on primary isolation.

While there were 31 patients examined in this study, a total of 157 colonies (star-shaped and non-star-shaped) were selected from the subgingival plaque samples of these patients for identification. As shown in Table 2.19, 120 colonies were identified as *A.actinomycetemcomitans;* 50 (68%) of which appeared star-shaped, whereas the other 70 (83%) colonies were non-star shaped on primary isolation. The other 37 colonies were identified as *H.aphrophilus* which also produced star-shaped and non-star-shaped colonies on primary isolation. Furthermore, in a number of cases all of the star-shaped colonies selected were identified as *H.aphrophilus* (eg. patients number 7 and 30).

Table 2.18 The prevalence of star-shaped and non-star-shaped strains of *A.actinomycetemcomitans* in subgingival plaque samples on primary isolation on TSBV medium from 31 patients.

Patient Number	A.a Source	*A.actinomycetemcomitans* SSC	NSSC
07	GDH 310	-ve	+ve
08	GDH 312	-ve	+ve
18	GDH 1014	+ve	-ve
19	GDH 1212	-ve	+ve
20	GDH 1214	-ve	+ve
27	GDH 110	-ve	+ve
28	GDH 114	+ve	-ve
29	GDH 127	+ve	-ve
30	GDH 1114	-ve	+ve
32	GDH 125	-ve	+ve
33	GDH 129	-ve	+ve
34	GDH 1116	-ve	+ve
35	GDH 1123	+ve	+ve
36	GDH 28/78	-ve	+ve
37	GDH 33	+ve	-ve

Table 2.18 Continued.

Patient Number	A.a Source	*A.actinomycetemcomitans* SSC	NSSC
38	GDH 39	-ve	+ve
39	GDH 1115	-ve	+ve
41	GDH 1125	-ve	+ve
42	GDH 227916	+ve	-ve
43	GDH 117	+ve	+ve
44	GDH 119	+ve	-ve
45	GDH 130376	+ve	-ve
46	GDH 226824	+ve	+ve
47	GDH 229075	+ve	-ve
48	GDH 231393	+ve	-ve
50	GDH 121	+ve	+ve
51	GDH 228	-ve	+ve
52	GDH 213705	-ve	+ve
53	GDH 225843	+ve	+ve
54	GDH 229473	+ve	+ve
55	GDH 239911	+ve	-ve
Total	31 strains	16	20

A.a: *A.actinomycetemcomitans*

SSC: Star-shaped colonies

NSSC: Non-star-shaped colonies

Number of subjects with *A.actinomycetemcomitans* = 31

Number of subjects with star-shaped colonies = 16 (52%)

Number of subjects with non-star-shaped colonies = 20 (64%)

Number of subjects with both SSC and NSSC = 5 (16%)

Table 2.19 The prevalence of star-shaped and non-star-shaped colonies of *A.actinomycetemcomitans* and *H.aphrophilus* in subgingival plaque samples on primary isolation on TSBV medium from 31 patients.

Patient Number	*A.actinomycetemcomitans*		*H.aphrophilus*	
	No.SSC	No.NSSC	No.SSC	No.NSSC
07	0	4	3	0
08	0	4	0	0
18	2	0	0	0
19	0	2	0	1
20	0	3	2	0
27	0	1	1	1
28	1	0	1	1
29	8	0	0	3
30	0	5	3	0
32	0	1	1	0
33	0	3	0	1
34	0	6	0	6
35	2	1	0	0
36	0	1	1	1
37	2	0	0	0

Table 2.19 Continued.

Patient Number	*A.actinomycetemcomitans*		*H.aphrophilus*	
	No.SSC	No.NSSC	No.SSC	No.NSSC
38	0	8	6	0
39	0	4	2	0
41	0	1	1	0
42	5	0	0	0
43	2	7	0	0
44	4	0	1	0
45	6	0	0	0
46	1	5	0	0
47	5	0	0	0
48	2	0	0	0
50	2	0	0	0
51	0	1	1	0
52	0	4	0	0
53	2	6	0	0
54	3	3	0	0
55	3	0	0	0
Total	50	70	23	14

Total number of colonies counted = 157

Number of colonies identified as *A.actinomycetemcomitans* = 120

Number of SSC identified as *A.actinomycetemcomitans* = 50 (42%)

Number of NSSC identified as *A.actinomycetemcomitans* = 70 (52%)

Number of colonies identified as *H.aphrophilus* = 37

2.4.8 The antimicrobial sensitivity of *A.actinomycetemcomitans*

The antimicrobial sensitivity of 46 strains of *A.actinomycetemcomitans* was investigated. The agents used were, tetracycline, erythromycin, penicillin, amoxycillin and metronidazole. The results obtained from duplicate readings of zones of inhibition showed that, all 46 *A.actinomycetemcomitans* strains (100%) were sensitive to tetracycline and amoxycillin. Overall, 44 strains (96%) were sensitive to erythromycin. The sensitivity to penicillin was variable with only 11 strains (24%) giving sensitive results. All isolates (100%) were resistant to metronidazole (see Table 2.20).

Table 2.20 The effect of five antimicrobial agents on *A.actinomycetemcomitans* (results represent duplicate readings of inhibition zones using *S. aureus* NCTC 6571 as control.

Antimicrobial agent	Disc Sensitivity Results		
	No. Strains Sensitive (%)	No. Strains Moderately Sensitive (%)	No. Strains Resistant (%)
Amoxycillin	46 (100%)	0	0
Tetracycline	46 (100%)	0	0
Erythromycin	44 (96%)	2 (4%)	0
Penicillin	11 (24%)	29 (63%)	6 (13%)
Metronidazole	0	0	46 (100%)

Sensitive: Zone size equal to, greater than, or not more than 3 mm smaller than the control zone.

Moderate: Zone size greater than 3 mm, but smaller than the control by more than 3 mm.

Resistant: Zone size 3 mm or less and criteria as described by Stokes and Waterworth (1972).

2.5 Discussion:

2.5.1 The prevalence of *A.actinomycetemcomitans* in healthy subjects and chronic periodontitis patients

(I) Health

There are only a few studies which have investigated the prevalence of *A.actinomycetemcomitans* in chronic periodontitis patients and healthy individuals using culture techniques involving selective media. In the present investigation *A.actinomycetemcomitans* was isolated from only one sample (2%) from 55 healthy subjects aged 18 to 40 years (mean 22.5). However, other researchers have reported higher prevalence rates of *A.actinomycetemcomitans* in their healthy control groups. For example, Slots *et al.*, (1980a) reported that 4 (36%) of 11 adults and 17% of the 66 samples analysed were positive for *A.actinomycetemcomitans.* In a large group of 142 healthy individuals aged 9 months to 54 years, 24/142 (17%) of patients had *A.actinomycetemcomitans* (Zambon *et al.*, 1983b). In 1984, Okuda, *et al.*, reported that the prevalence of *A.actinomycetemcomitans* in an healthy adult group was higher than that in periodontitis patients. *A.actinomycetemcomitans* was recovered from 5/6 samples collected from healthy subjects. Furthermore, Mombelli *et al.*, (1990) examined 29 periodontally healthy adults with partially erupted lower third molars aged 19-38 years (mean 24) and found that 34% of subjects possessed *A.actinomycetemcomitans.* The reason for the low prevalence figures obtained in the present study is uncertain but may be related to the fact that the majority of volunteers were

dental students with a higher level of oral hygiene compared to other groups in the population.

(II) Chronic Periodontitis

The prevalence of *A.actinomycetemcomitans* in patients with chronic periodontitis have differed among researchers. Studies which have used methods similar to those employed in this investigation have produced a range of prevalence figures for *A.actinomycetemcomitans* (see Table 1.4). Slots *et al.*, (1980a) reported that *A.actinomycetemcomitans* was isolated from 6 (50%) adult patients with chronic periodontitis aged 30 to 65 years, and this is the highest prevalence level to be reported by any research group. Other workers have found *A.actinomycetemcomitans* at levels ranging between 13% to 40% in adult chronic periodontitis patients. For instance, Bonta *et al.*, (1985) reported that 30% of 10 chronic periodontitis patients harboured *A.actinomycetemcomitans,* while Dahlen *et al.*, (1989) detected *A.actinomycetemcomitans* in 40% of 20 patients aged 30 to 65 years with the same condition. The results of the present investigation which used culture techniques similar to those described by Dahlen *et al.*, (1989) produced a prevalence carriage of 39%. In a large group of 284 subjects with either healthy gingiva or with moderate chronic periodontitis, Wolff *et al.*, (1985) reported an overall recovery rate of 13% for *A.actinomycetemcomitans.* However, no details were given concerning the percentage carriage in the different patients examined by Wolff *et al.*, (1985).

The prevalence data for sites will be discussed next. In the present investigation 302 samples were collected from patients with periodontal pockets of ≥ 6mm. *A.actinomycetemcomitans* was detected in 82 (27%) of samples. This is comparable with figures reported by Slots *et al.*, (1980a) and Dahlen *et al.*, (1989) who found that 35% and 28% of sites examined in patients with chronic periodontitis yielded *A.actinomycetemcomitans.* However, other researchers have found smaller values for positive sites which varied between 9 and 21% (Okuda *et al.*, 1984; Wolff *et al.*, 1985; Slots *et al.*, 1985). The prevalence of *A.actinomycetemcomitans* in 6 different oral segments in the mouths of diseased individuals was examined in the present investigation, and although the prevalence in positive sites ranged from 19% to 31% there were no large differences noted. Although there have been a number of studies which have investigated the prevalence of *A.actinomycetemcomitans* in chronic periodontitis patients, no details have been given about the isolation of *A.actinomycetemcomitans* in the different sites examined.

In conclusion the study by Slots *et al.*, (1980a) presented some evidence in which the prevalence of *A.actinomycetemcomitans* in chronic periodontitis was only slightly higher (50% v 36%) compared to the prevalence in healthy subjects. Zambon *et al.*, (1983b) reported similar observations, showing that while 21% of 134 patients with chronic periodontitis harboured *A.actinomycetemcomitans*, 17% of 142 healthy individuals in the same study were positive. In one paper by Okuda *et al.*, (1984) the opposite appeared to occur, ie.

more healthy subjects (5/6) harboured *A.actinomycetemcomitans* compared to patients (3/8). In the present investigation there was clear evidence that the number of patients with *A.actinomycetemcomitans* is more than that of healthy adults (39% v 2%). Although, not all studies agree, there is a trend which suggests that more patients with chronic periodontitis harbour *A.actinomycetemcomitans* compared to healthy individuals.

(III) Progressive and Non-progressive Chronic Periodontitis

Although there are only a few studies performed in this field, it has been suggested that *A.actinomycetemcomitans* could mainly be found in progressive rather than non-progressive sites in chronic periodontitis patients (Slots and Genco, 1984). Tanner *et al.*, (1979) found that 4 out of 16 progressive sites in patients with chronic periodontitis harboured *A.actinomycetemcomitans*, but none of the five non-progressive sites examined yielded positive cultures. Further evidence was reported by Slots *et al.*, (1986), who found that 50% of 130 sites from 71 patients with active periodontal pockets (6-11mm depth) yielded *A.actinomycetemcomitans*, but only 5% of 105 non-progressive sites from 75 patients proved to be positive. In addition Mandell, Ebersole and Socransky (1987) reported that while 90% of the progressing sites (18/20) harboured *A.actinomycetemcomitans*, only 44% of the inactive sites were positive.

Thus, the little information available suggests that *A.actinomycetemcomitans* is more likely be isolated from 'active' compared to 'inactive' sites (Slots *et al.*, 1986; Mandell *et al.*, 1987). However, further studies are needed to confirm these observations, and to establish the clinical importance of isolating *A.actinomycetemcomitans* from chronic periodontitis patients and healthy subjects. Finally, it would also be important to establish if the leucotoxic activity and other pathogenic properties of the different isolates from these groups are different. Only one study appears to have investigated this matter and suggested that there were more *A.actinomycetemcomitans* isolates from chronic periodontitis possessing leucotoxic activity (43%) compared to those from healthy subjects (11%) (Zambon *et al.*, 1983c).

2.5.2 Percentage proportion of *A.actinomycetemcomitans* in total microbial counts of subgingival plaque in healthy subjects and chronic periodontitis

The numbers of *A.actinomycetemcomitans* isolated on semi-selective media expressed as a percentage of the total micrbial counts on blood agar plates from healthy or infected areas in chronic periodontitis patients have been reported by a number of researchers.

(I) Health

There are only a few studies that have reported the proportion of *A.actinomycetemcomitans* present in the total microbial counts of subgingival plaque collected from healthy subjects. Slots *et al.*, (1980a) have shown that in 11 posi-

tive sites from 12 healthy subjects, the concentration of *A.actinomycetemcomitans* ranged from 0.9% to 5% of the total count, with 85% of patients possessing levels of less than 1%. Mombelli *et al.*, (1990) have reported that *A.actinomycetemcomitans* constituted <0.01% to 0.07% (mean 0.03%) of the total microbial count in subgingival plaque samples from 29 healthy individuals. Finally the proportion of *A.actinomycetemcomitans* in 5 subgingival plaque samples collected from 5 subjects was reported to range from 0.05% to 1.5% (mean 0.72%) by Okuda *et al.*, (1984). Although only one healthy individual in the present study was positive for *A.actinomycetemcomitans,* no information is available concerning the concentration of *A.actinomycetemcomitans* in this subject.

(II) Chronic Periodontitis

The concentration of *A.actinomycetemcomitans* in samples from chronic periodontitis patients have varied among different workers. Slots *et al.*, (1980a) have reported that the concentration of *A.actinomycetemcomitans* in 17 positive sites ranged from ≤0.9% to 40%. However, more than 75% of these sites had *A.actinomycetemcomitans* at a concentration of ≤0.9%, while only one sample exhibited a range of 20 to 40% (Slots *et al.*, 1980a). Similar observations were reported by Wolff *et al.*, (1985) who found *A.actinomycetemcomitans* levels to be equal to or less than 1% of the total microbial flora in 87% of 47 positive sites from 37 subjects. In the other 13% of sites, *A.actinomycetemcomitans* was found at levels of >1% to 12% of the total flora. Okuda *et al.*,

(1984) examined a small number of patients and showed that 3/8 patients harboured *A.actinomycetemcomitans* at a concentration ranging from 0.7% to 1.9% (mean 1.5%) of the total microbial count. In 1989, Dahlen *et al.*, reported that *A.actinomycetemcomitans* accounted for 0.03% to 0.5% (mean (0.2%) of the total microbial flora in 11 positive sites from 8 patients with chronic periodontitis.

In the present study, a total microbial count as obtained from anaerobic blood agar culture was not available , and therefore a comparison with the results of other workers is not possible. However, the overall mean count of *A.actinomycetemcomitans* on TSBV was $\log_{10}$ 4.8 (range 1.15-6.52) cfu/ml in 82 positive sites. Using the same semi-selective medium used in this study incubated under anaerobic conditions, Christersson *et al.*, (1985) reported that *A.actinomycetemcomitans* was present in an overall mean $\log_{10}$ 5.9 in 24 samples from two patients. In addition, a mean $\log_{10}$ 3.5 has been reported by Mandell (1984) using the MGB semi-selective medium for isolation in either aerobic or anaerobic conditions.

(III) Progressive and Non-Progressive Chronic Periodontitis

There are only three papers which have reported the proportions of *A.actinomycetemcomitans* in plaque from active and non-progressive sites in patients with chronic periodontitis. Tanner *et al.*, (1979) reported a mean plaque concentration of zero percent and 14% for *A.actinomycetem-*

comitans in non-progressive and active sites respectively. In comparison, Slots *et al.*, (1986) showed that *A.actinomycetemcomitans* accounted for 4.3% (range 0.0002-50%) of the total microbial count of plaque collected from active periodontal sites compared to 0.6% (range 0.002-2.1%) in non-progressive sites. In addition, Mandell *et al.*, (1987) reported that 20 progressive sites in 8 patients harboured *A.actinomycetemcomitans* at a concentration ranging from 0%-73% (mean 20%), this compared with a range of 0%-19% (mean 3%) in subgingival samples from non-progressive sites in the same patients. In contrast to the prevalence of *A.actinomycetemcomitans*, these results all agree that overall diseased sites harboured higher concentrations of *A.actinomycetemcomitans* compared to either healthy or inactive sites. However, considerable variations between the diseased and non-diseased sites also occurred.

Slots and Genco (1984) have suggested that even if the percentage proportion of *A.actinomycetemcomitans* in subgingival plaque is less than 2%, this low level may still cause disease due to the high virulence of *A.actinomycetemcomitans*. If this is true then the mere presence of *A.actinomycetemcomitans* in any subject could lead to periodontal disease. Since this is not clearly supported by clinical data, it has been argued that the isolation of leucotoxic strains from an individual must be demonstrated before a significant relationship between *A.actinomycetemcomitans* and disease can be considered. The question of leucotoxicity and pathogenicity will be discussed in Chapter 5.

2.5.3 Biochemical characteristics of *A.actinomycetemcomitans*

In the present investigation, the API 20 A system used in identification, gave reproducible results with all isolates tested. The freshly isolated *A.actinomycetemcomitans* strains (38) fermented glucose, maltose, mannose and produced catalase, results which agree with those of King and Tatum, 1962; Pulverer and Ko, 1970; Sneath and Johnson, 1973; Slots, 1982a. However, 68% of isolates fermented mannitol, 42% fermented xylose and only 8% fermented both glycerol and rhamnose. The variable fermentation activity of *A.actinomycetemcomitans* strains towards mannitol and xylose have been previously reported by King and Tatum (1962); Pulverer and Ko (1970); Slots (1982a) and Tanner *et al.*, (1982). However, two papers only have reported positive results for the two carbohydrates xylose and mannitol (Mannheim, Pohl and Hollander, 1980; Sneath and Johnson, 1973). In addition, three (8%) of the freshly isolated *A.actinomycetemcomitans* strains in the present study fermented glycerol and rhamnose, a finding which disagrees with the results reported by previous researchers.

Urease has been reported to be positive by Sneath and Johnson, (1973) and variable by Mannheim *et al.*, (1980) both of which disagree with the uniform negative results found in this investigation and in those of other researchers (King and Tatum, 1962; Pulverer and Ko, 1970; Slots, 1982a; Tanner *et al.*, 1982). Differences in the composition of the basal media and the methods used to investigate the biochemical

activity of *A.actinomycetemcomitans* may explain some of the conflicting results reported by different investigators. Thus, it would help if all researchers used one standard identification system such as the the API 20 A (used in this investigation), which although expensive is readily available and easy to use.

Slots (1982a) examined the enzyme patterns of 136 strains of *A.actinomycetemcomitans* and found that all strains (100%) produced strong alkaline and acid phosphatases but produced weak leucine aminopeptidase (86%) using the API-ZYM system. Results obtained using the same detection system in the present study with 26 *A.actinomycetemcomitans* strains, agree with these findings. However, only weak activity was evident with esterase (butyrate), esterase lipase (carpylate), and phsophohydrolase similar to those reported by Slots (1982a).

2.5.4 Colonial morphology of *A.actinomycetemcomitans* on primary isolation

A.actinomycetemcomitans has been described as producing star-shaped colonies (Slots, 1982a; Zambon, 1985), a feature which has been commonly used to isolate fresh strains of *A.actinomycetemcomitans* on primary isolation. Surprisingly no information is available about the prevalence of either star-shaped or non-star-shaped variants of *A.actinomycetemcomitans* on primary culture. In this study the prevalence of non-star-shaped colonies on primary isolation was greater than that of star-shaped variants; of the 120 colonies

subsequently identified as *A.actinomycetemcomitans*, 70 (58%) appeared as non-star-shaped colonies, compared to 50 (42%) which had a star-shaped colonial morphology on TSBV plates. Although, only a relatively small number of isolates and colonies were included in this study, the results highlight the danger of using star-shaped morphology as a key criterium when isolating and identifying *A.actinomycetemcomitans* from subgingival plaque samples. For instance, if only catalase positive star-shaped colonies had been counted, only about half (52%) of the total number of positive *A.actinomycetemcomitans* patients would have been recorded.

H.aphrophilus was also identified in a number of samples and was found to produce both star-shaped and non-star-shaped colonies on primary isolation similar to those of *A.actinomycetemcomitans*. While a catalase test will distinguish between the two species, failure to perform the test for every isolate could well lead to false positive results in prevalence data.

2.5.5 Antimicrobial susceptibility of *A.actinomycetemcomitans*

Since it has been claimed that the successful treatment of periodontitis associated with *A.actinomycetemcomitans* requires the use of antibiotics (Slots and Rosling, 1983), the *in vitro* antimicrobial activity of 5 antimicrobial agents against 46 strains of *A.actinomycetemcomitans* was monitored using a disc sensitivity method. All strains of *A.actinomycetemcomitans* examined in this study were sensitive to tetr-

acycline and amoxycillin which is in agreement with the findings of Slots *et al.*, (1980b) and Walker *et al.*, (1985). In the present investigation almost all strains (96%) were found to be sensitive to erythromycin (10 µg) which agrees with the results of Baker *et al.*, (1985) where 11 strains of *A.actinomycetemcomitans* were inhibited at an MIC concentration of 25µg/ml. However, Slots *et al.*, (1980b) (59 strains) and Walker *et al.*, (1985) (39 strains) reported that only 13% and 50% respectively of their *A.actinomycetemcomitans* strains were susceptible to erythromycin at MIC concentrations of 2µg and 4µg respectively. The low activity of erythromycin as reported by both Slots *et al.*, (1980b) and Walker *et al.*, (1985) appears to be related to the low concentration (2µg and 4µg) used by these workers, compared to a high concentration of 10µg used in the present investigation. Similarly, reports concerning the sensitivity of *A.actinomycetemcomitans* to penicillin has been varied. For instance, Slots *et al.*, (1980b) found that only about 50% of isolates were susceptible to penicillin at 4µg/ml, but 41/42 strains examined by Walker *et al.*, (1985) were inhibited at a smaller concentration of 2µg/ml. In the present study penicillin was used at a concentration of 1.0 unit per disc and only 11 strains (24%) were sensitive to penicillin at this concentration. Baker *et al.*, (1985) reported that penicillin was only effective at high concentration ie. 6.3 µg/ml or more. Metronidazole (2.5µg/ml) was ineffective against the 46 strains examined in this study. However, using a higher concentration of ≥ 16µg/ml, more

than 80% of isolates examined by both Slots *et al.*, (1980b) and Walker *et al.*, (1985) were killed.

Some of the differences in these results may be due to a number of factors which include differences in the methods and concentrations of the antimicrobial agents used by researchers, variation in the media used as well as natural variation to antimicrobial agents found in the strains of *A.actinomycetemcomitans* investigated. However, there is overall agreement that *A.actinomycetemcomitans* is sensitive to tetracycline, which has been recommended for the treatment of patients with periodontal disease associated with *A.actinomycetemcomitans*.

2.6 Conclusions:

TSBV medium was less inhibitory to pure cultures of *A.actinomycetemcomitans* compared to MGB medium.

A.actinomycetemcomitans was isolated from 8 of 31 (26%) samples of mixed plaque on TSBV, but not on MGB medium.

A.actinomycetemcomitans was recovered from 39% of patients with chronic periodontitis and from 27% of the total number of sites examined.

Only one (2%) of 55 healthy subjects harboured *A.actinomycetemcomitans*.

A.actinomycetemcomitans can produce both star-shaped and non-star-shaped colonies on primary isolation and the preva-

lence of non-star-shaped colonies (58%) was greater than that of star-shaped colonies (42%).

If star-shaped colonies alone were selected for identification, 48% of the positive *A.actinomycetemcomitans* patients would have been missed.

A.actinomycetemcomitans was found in mean concentration of 6.5×10^4 cfu/ml in subgingival plaque samples on TSBV.

All strains of *A.actinomycetemcomitans* (46) were sensitive to both tetracycline and amoxycillin, and 44 strains were sensitive to erythromycin. However, only 11 strains were sensitive to penicillin and none was sensitive to metronidazole.

CHAPTER 3

ADHERENCE OF *A.ACTINOMYCETEMCOMITANS* TO BUCCAL EPITHELIAL CELLS

3.1 Introduction

The ability to adhere to oral surfaces is believed to be important for bacteria to colonize the human mouth, and enables bacteria to become attached on surfaces exposed to the mechanical washing action of saliva (Van Houte, 1983). Human buccal epithelial cells have been widely used as a test surface to which bacteria can adhere. However, as cited in section 1.7.5 there have been very few studies regarding the adherence of *A.actinomycetemcomitans* to oral surfaces. There is information that *A.actinomycetemcomitans* can adhere to hydroxyapatite (Kagermeier and London, 1985; Rosan *et al.*, 1988), and to buccal epithelial cells (Sweet, 1986). However, there is no satisfactory data on the effect of either saliva, or serum (both of which are important intraoral environmental factors), on the subsequent adherence of *A.actinomycetemcomitans* to the hard and soft tissue surfaces of the mouth.

Since there is evidence implicating *A.actinomycetemcomitans* in the aetiology of human periodontal disease (see section 1.9), it is perhaps surprising that relatively little is known about the mechanisms by which this organism colonizes the mouth. Therefore, this study was performed to assess the ability of 33 strains of *A.actinomycetemcomitans* to adhere to buccal epithelial cells using an *in vitro* assay.

3.2 Materials and methods

3.2.1 Determination of bacterial concentration

Thirty three *A.actinomycetemcomitans* strains (Aa 01-33) were included in this study. The source and identification profile of these strains are listed in Table 3.1. Although many bacteria can be enumerated microscopically using a counting chamber, it was impossible to use this technique to obtain accurate counts of *A.actinomycetemcomitans* due to their small size. Therefore, an alternative method described by Sweet, MacFarlane and Samaranayake (1988) was adopted to determine the bacterial concentration of different strains of *A.actinomycetemcomitans*. Bacterial suspensions were prepared by growing the test organism on two CBA plates for 48 hours at 37°C, in an atmosphere of 5% CO_2 in air and harvested in 6 ml of PBS (Appendix II) using sterile cotton wool swabs. This emulsion was designated the neat suspension. Ten-fold dilutions were then made in PBS from 10^{-1} to 10^{-3}, the latter being taken as the working suspension. The optical density of this suspension was measured at 520 nm in a Pye Unicam SP 8-100 spectrophotometer (Pye Unicam Cambridge, England). From the working suspension, a series of dilutions were made in PBS as shown in Table 3.2. The bacterial concentration of the *A.actinomycetemcomitans* strain under test was then determined by diluting the contents of tube number 3 to 10^{-3} in PBS. One millilitre of this dilution was then added to one ml of 0.025% acridine orange in distilled water (Hopkin and Williams Ltd., Essex, England). Previously the acridine orange was filter sterilized through a

0.45 µm pore Sterifil D-HA filtration unit (Nihon Millipore, Kogyo K.K., Yonezawa, Japan) to remove any debris which could be mistaken for bacteria. The mixture of bacteria and acridine orange was then added to a DEFT manifold (Direct Epifluorescent Filter Technique) (Micromeasurements Ltd., Saffron Walden, Essex, U.K), as shown in (Figure 3.1) which contained a 0.6 µm, 25 mm diameter polycarbonate filter (Nucleopore, Corporation, USA). After two minutes the acridine orange was removed from the filter unit by switching on the negative pressure valve attached to the DEFT unit. The stained bacteria were washed once with 2.5 ml of PBS for two minutes and the buffer was removed as before. The filter with attached bacteria was then removed from the filtration unit and mounted on a clean glass microscope slide with Uvinert immersion oil (BDH Chemicals Ltd., Poole, England). The mounted filters were then examined under ultra violet light at a magnification of 1000 times using a Nikon Optiphot microscope (Nippon Kogaku K.K., Yokyo, Japan). A minimum of fifty fields were randomly selected and the number of bacteria present in each field was counted. This procedure was repeated on three different occasions for each bacterial strain used in the adherence studies.

Table 3.1 List of *A.actinomycetemcomitans* strains used in adherence and hydrophobicity studies.

Strain number	Source	Site of Isolation	API 20 A Profile
Aa 01	NCTC 9709	Abscess (site not given)	4110404
Aa 02	NCTC 9710	Abscess (site not given)	4110405
Aa 03	NCTC 10979	Mandibular abscess	4150404
Aa 04	NCTC 10980	Blood	4110405
Aa 05	NCTC 10981	Neck abscess	4010505
Aa 06	NCTC 10982	Chest aspirate	4050505
Aa 07	GDH 310	Juvenile periodontitis	4110404
Aa 08	GDH 312	Chronic periodontitis	4110404
Aa 09	GDH 143	~ ~	4110404
Aa 10	GDH 216	~ ~	4110404
Aa 11	GDH R8529	~ ~	4150404
Aa 12	GDH 156	~ ~	4110404
Aa 13	GDH 471	~ ~	4110404
Aa 14	GDH 1954	~ ~	4050404
Aa 15	GDH 107	~ ~	4050404
Aa 16	GDH 87034	Prepubertal periodontitis	4050404

Table 3.1 Continued.

Strain number	Source	Site of Isolation	API 20 A Profile
Aa 17	GDH 510/87	Chronic periodontitis	4050404
Aa 18	GDH 1014	Juvenile periodontitis	4110404
Aa 19	GDH 1212	~ ~	4110404
Aa 20	GDH 1214	Chronic periodontitis	4110404
Aa 21	AA Y4	Juvenile periodontitis	4150404
Aa 22	ATCC 29522	Mandibular abscess	4110404
Aa 23	ATCC 29523	Blood	4050505
Aa 24	ATCC 29524	Chest aspirate	4050505
Aa 25	GDH 105	Juvenile periodontitis	4050505
Aa 26	GDH 1028	Chronic periodontitis	4050505
Aa 27	GDH 110	~ ~	4050505
Aa 28	GDH 114	~ ~	4110404
Aa 29	GDH 127	~ ~	4110404
Aa 30	GDH 1114	~ ~	4150404
Aa 31	GDH 2351	Juvenile periodontitis	4150404
Aa 32	GDH 125	Chronic periodontitis	4050404
Aa 33	GDH 129	~ ~	4050404

NCTC: National Collection of Type Cultures

ATCC: American Type Culture Collection

GDH: Glasgow Dental Hospital

Table 3.2 Dilutions made to determine the total counts of bacteria.

Tube number	Volume of PBS (ml)	Volume of suspension (ml)	Final volume (ml)	Dilution
1	4.5	3.0	7.5 ml	1 in 2.5
2	6.0	3.0	9.0 ml	1 in 3.0
3	6.0	2.0	8.0 ml	1 in 4.0
4	8.0	2.0	10.0 ml	1 in 5.0
5	7.0	1.0	8.0 ml	1 in 8.0
6	9.0	1.0	10.0 ml	1 in 10.0

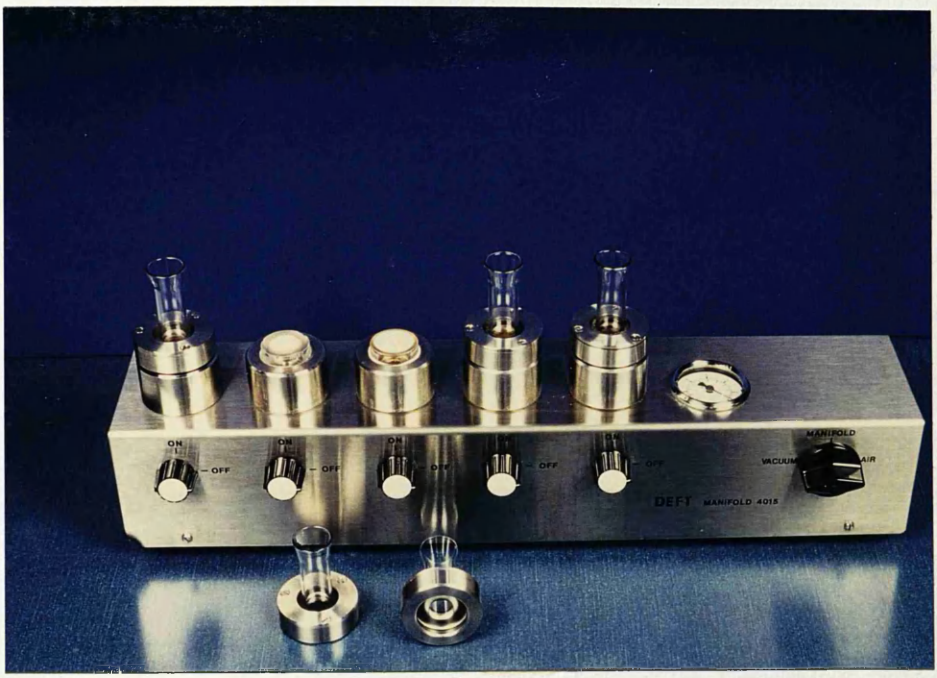

Figure 3.1 DEFT-Filter unit which was used in the determination of bacterial concentration and in adherence studies.

3.2.2 Calculation of the total number of bacteria in each suspension

From the known dilution factors, ie. the area of the filter over which the bacteria are deposited, the area of the microscopic field at a magnification of 1000 times and the mean number of bacteria per field, the bacterial concentration was calculated using the following equation:

$$\text{Concentration/ml} = \frac{\text{Mean number bacteria x filter area x dilution}}{\text{Microscope field area}}$$

Once the number of bacteria present in a given tube was calculated, the number of bacteria in the other tubes could be easily obtained. The optical density of all tubes was then determined at 520 nm in a Pye Unicam SP 8-100 spectrophotometer (Pye Unicam Cambridge, England), and a series of pairs of data of optical density versus bacterial concentration was obtained. A graph of these two parameters was then constructed (Figure 3.2) so that the optical density of the bacterial suspension at any given concentration could be determined.

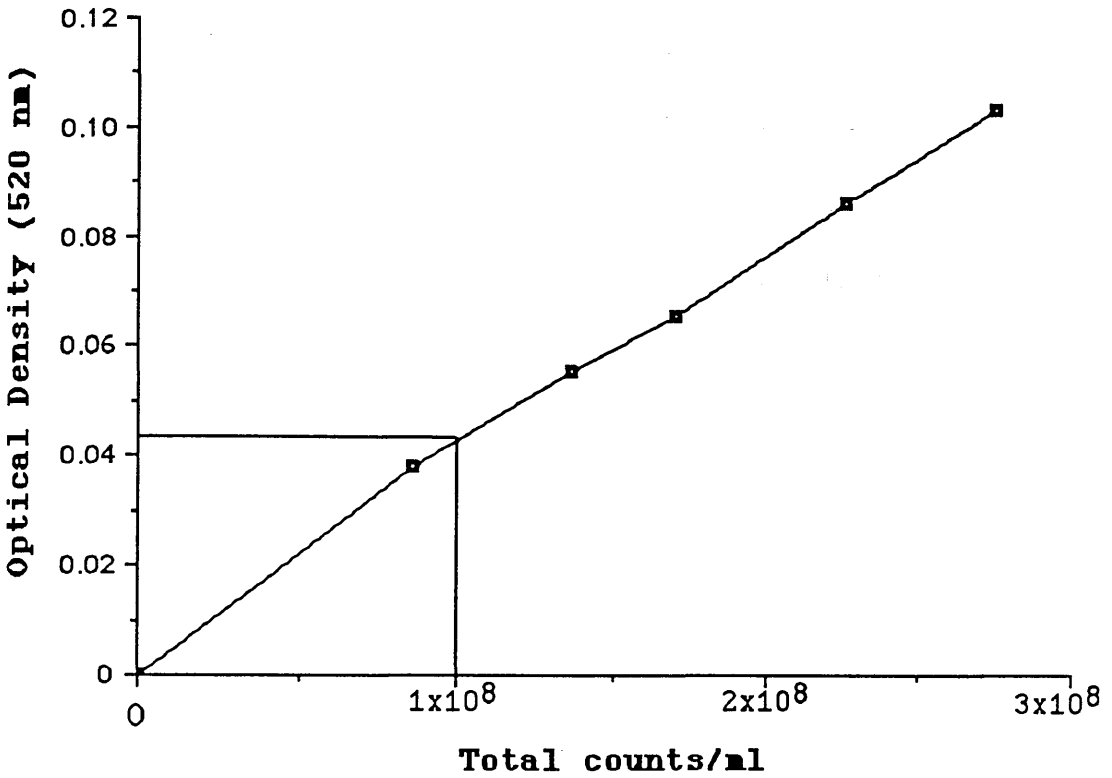

Figure 3.2 An example of a typical plot used to determine the optical density of Aa 02. A suspension containing 1 x 10^8 bacteria/ml is equivalent to an optical density of 0.042 at 520 nm.

3.2.3 Donor

In this study the buccal epithelial cells, saliva, blood and serum were all obtained from the same healthy 30 year old male donor, who had no previous history of periodontal disease. The donor was free from *A.actinomycetemcomitans* as determined by the sampling and microbiological techniques described in section 2.3.4.

3.2.4 Preparation and clarification of saliva

Whole unstimulated saliva was collected by expectoration into a sterile universal bottle held in ice. Saliva was diluted with an equal volume of Saliva Ions Buffer, pH 7.2 (SIB) as recommended by Sweet, MacFarlane and Samaranayake (1987), (Appendix II). The diluted salivary sample was next clarified by centrifugation at 20,000 g for 60 minutes at 4°C (Ericson, Pruitt and Wedel, 1975) using an MSE High Speed 18 Centrifuge (Crawley, England). This method was used to prevent the significant loss of glycoprotein which can occur when undiluted saliva is centrifuged (Ericson, 1966). Finally the supernatant was collected and dispensed in 2 ml volumes into sterile bijou bottles and stored at -20°C until required.

3.2.5 Separation of serum

Ten millilitres of venous blood were collected and allowed to clot at room temperature in a sterile glass bottle. Serum was separated by centrifugation at 3000 g for 15 minutes in an MSE centrifuge (MSE Scientific Instruments, Craw-

ley, England) at room temperature. Serum was then removed and aliquoted into 2 ml volumes in sterile bijou bottles and stored at -20°C until required.

3.2.6 Collection of buccal epithelial cells (BECs)

Buccal epithelial cells (BECs) were collected from a single donor (section 3.2.3) by gently scraping the inside of the cheeks with a sterile cotton wool swab and suspending the cells in 5 ml of SIB. The cells were then washed twice in SIB by centrifugation at 200g for 5 minutes (MSE Scientific Instruments, Crawley, England) and resuspended in one ml of the same buffer.

3.2.7 Enumeration of BECs

The BECs were counted using a haemocytometer (Hawksley and Sons Ltd., Lancing, England). This was done by touching the edge of the coverslip with the pipette tip and allowing the well to fill by capillary action. After leaving the cells to settle for 60 seconds, the BECs were counted in the centre square and the four large corner squares of the haemocytometer under 250 times magnification using an Olympus microscope. The final concentration of BECs was then adjusted to 2 or 4 x 10^5 cells per ml in SIB.

3.2.8 Buccal cell adherence assay

The same 33 *A.actinomycetemcomitans* isolates listed in Table 3.1 were used in this investigation. The adherence assay was performed in 5 ml plastic disposable bijou bottles (Sterilin Ltd., Feltham, England). Aliquots (0.2 ml) of the

buccal cell suspension which contained 2.0 x 10^5 cells/ml were added to 0.2 ml of the bacterial suspension (2.0 x 10^8 bacterial cells/ml) in SIB buffer. In addition, controls were set up which consisted of 0.2 ml buccal cells plus 0.2 ml of SIB to obtain background counts of indigenous bacteria. Both test and control bottles were then incubated in an orbital shaker (A. Gallenkamp and Co. Ltd., London, England) at a setting of 60 to 80 revolutions per minute for 60 minutes, at 37°C.

Immediately after incubation, 5 ml of SIB was added to all bottles to minimize any further attachment. The buccal cells were then collected on 25 mm diameter polycarbonate filters with a pore size of 12 µm (Nucleopore Corp., Pleasanton, CA., USA) mounted on a DEFT manifold unit. The retained buccal cells were washed 3 to 4 times with 2.5 ml of SIB to remove any unattached bacteria. While such bacteria can pass easily through the 12 µm pore filters the buccal cells with attached bacteria were retained.

3.2.9 Adherence in the presence of saliva

The affect of saliva on the adherence of *A.actinomycetemcomitans* to BECs was examined using the same method described in section 3.2.8 with the following minor modification. In these experiments saliva was added to the bacterial/BECs mixture during the incubation period. The test consisted of 0.2 ml saliva, 0.1 ml bacteria at a concentration of 4.0 x 10^8/ml in SIB and 0.1 ml buccal cells containing 4.0 x 10^5/ml. The saliva control consisted of 0.2 ml of saliva

plus 0.1 ml SIB and 0.1 ml BECs. These dilutions were made to allow comparison of the results with the standard adherence assay described earlier in section 3.2.8.

3.2.10 Adherence in the presence of serum

The affect of serum on the adherence of *A.actinomycetemcomitans* to BECs was also examined using the method described in section 3.2.8 with the following minor modification. The assay was carried out by adding 0.24 ml of bacteria at a concentration of 2.0×10^8/ml to 0.24 ml of buccal cells containing 2.0×10^5 cells/ml and 0.03 ml of sterile serum giving a final concentration of 5%. The serum control consisted of 0.24 ml SIB plus 0.24 ml buccal cells and 0.03 ml serum. In addition serum was added to the control bottle to find out if serum had any effect on the adherence of indigenous bacteria.

3.2.11 Adherence of repeated subcultured strains

Ten freshly isolated *A.actinomycetemcomitans* were subcultured for 50 times on blood agar plates (CBA). Subculturing was repeated every 48 hours by sweeping a sterile loop a cross the plate and inoculating the material onto a fresh CBA plate which was then incubated at 37°C in 5% CO_2 in air. After 50 subcultures, only four of the *A.actinomycetemcomitans* isolates (Aa 09, 16, 17 & 33) were found to produce smooth colonies which did not adhere to the agar surface and produced smooth turbid growth in broth cultures. The four strains were subsequently tested for their ability to adhere

to buccal epithelial cells using the same procedure described in 3.2.8.

3.2.12 Staining procedure

While the filters still remained on the DEFT unit the buccal cells and bacteria were stained using 2.5 ml of a 0.025% solution of acridine orange (Hopkin and Williams Ltd., Essex, England) in pH 3.3, 0.1 M citrate/sodium hydroxide buffer (BDH, Chemicals Ltd., Poole, England), (Appendix II) for two minutes. The filter preparation was then washed twice with 2.5 ml of SIB, and the excess background fluorescence was quenched with 2.5 ml of 0.01% potassium permanganate solution (BDH, Chemicals Ltd., Poole, England) for two minutes. Finally the cells were given two more rinses with SIB. Both the acridine orange and the potassium permanganate were filter sterilized before use through a 0.45 µm Sterifil D-HA filtration unit (Nihon Millipore, Kogyo K.K., Yonezawa, Japan) to ensure that no particulate matter was present which could confuse counting of attached bacteria.

3.2.13 Enumeration of attached bacteria

Air dried filters were mounted on clean glass slides (1.0-1.2mm thick) using Uvinert immersion oil (BDH Chemicals Ltd., Poole, England). The mounted filters were then examined under ultra-violet light at a magnification of 1000 times using a Nikon Optiphot Microscope (Nippon Kogaku K.K., Tokyo, Japan). A minimum of fifty BECs were selected at random and the number of adherent bacteria per BEC was counted. Buccal cells were only included for counting if they

appeared morphologically normal, possessed a single nucleus, were separated from each other, appeared flat and not folded and were free from debris that could interfere with counting. The same criteria were applied for buccal cells used in the control preparations and the number of indigenous adherent bacteria was counted. Finally after both control and test adherent bacteria were counted, the number of indigenous adherent bacteria was subtracted from the total number of adherent bacteria in the tests. The assay procedure was repeated on three different occasions for each strain tested and the mean number of bacteria attached per buccal cell was then calculated.

3.2.14 Statistical analysis

All assays were performed on three different occasions for each bacterial strain under study. Results were analysed using STAT-WORK software (Apple Macintosh computer), and significant differences were determined using the paired t-Test.

3.3 Results:

3.3.1 Determination of *A.actinomycetemcomitans* concentration

The optical densities (O.D) of the *A.actinomycetemcomitans* strains used in the different experiments are summarized in Table 3.3. The O.D of the different *A.actinomycetemcomitans* isolates tested at a concentration of eg. 10^8 bacteria/ml varied from 0.023 to 0.049 with an average of 0.034 at a wave length of 520 nm. These differences are probably due to variations in the cell size of the different strains used. In particular some of the Type strains (eg. NCTC 9709 and NCTC 10979) appeared to be larger than the freshly isolated strains of *A.actinomycetemcomitans* when examined microscopically at 1000 magnification. Nonetheless, these variation were noted not only between Type and fresh isolates, but also when the freshly isolated strains of *A.actinomycetemcomitans* were compared.

The stained bacteria were deposited evenly over the polycarbonate filter surface and the *A.actinomycetemcomitans* cells fluoresced bright orange when examined under ultra-violet light and contrasted well against the non-staining polycarbonate filter thus making counting of bacteria easy.

Table 3.3 Summary of the optical densities at a concentration of 10^8 bacteria/ml obtained for *A.actinomycetemcomitans* strains used in adhesion experiments.

A.actinomycetemcomitans strain No.	O.D equivalent to 10^8 bacteria/ml	±SEM
Aa 01	0.026	0.0009
Aa 02	0.042	0.001
Aa 03	0.029	0.001
Aa 04	0.037	0.004
Aa 05	0.023	0.001
Aa 06	0.025	0.002
Aa 07	0.030	0.008
Aa 08	0.024	0.002
Aa 09	0.034	0.003
Aa 10	0.033	0.002
Aa 11	0.033	0.002
Aa 12	0.039	0.003
Aa 13	0.036	0.003
Aa 14	0.034	0.003
Aa 15	0.037	0.001
Aa 16	0.040	0.0009

Table 3.3 Continued.

A. actinomycetemcomitans strain No.	O.D equivalent to 10^8 bacteria/ml	±SEM
Aa 17	0.037	0.002
Aa 18	0.035	0.001
Aa 19	0.041	0.003
Aa 20	0.041	0.009
Aa 21	0.026	0.0006
Aa 22	0.028	0.001
Aa 23	0.027	0.0006
Aa 24	0.028	0.002
Aa 25	0.045	0.002
Aa 26	0.034	0.001
Aa 27	0.042	0.001
Aa 28	0.030	0.0006
Aa 29	0.035	0.006
Aa 30	0.026	0.003
Aa 31	0.032	0.001
Aa 32	0.049	0.001
Aa 33	0.037	0.001

The results represent the mean of three experiments performed on three different occasions.

±SEM: Standard error of mean

3.3.2 Adherence of *A.actinomycetemcomitans* to BECs

Once the bacteria had been stained with acridine orange, they could be easily visualized and counted. The bacterial cells appeared bright orange and the buccal epithelial cells as pale green with brighter green nuclei (see Figure 3.3). The number of adherent bacteria on fifty test and fifty control buccal epithelial cells were counted in each assay. Examples of the raw data obtained for two *A.actinomycetemcomitans* isolates (Aa 03 and Aa 18) are presented in Tables 3.4a,b,c and 3.5a,b,c. In these examples the number of bacteria adhering to the individual buccal cells varied between one to 84 in the case of Aa 03 and between 0 and 90 for Aa 18 (Tables 3.4 and 3.5). The frequency distribution of the number of adherent bacteria per buccal epithelial cell was calculated for both of the two *A.actinomycetemcomitans* strains (see Figures 3.4 and 3.5). These histograms showed clearly that more than 80% of the buccal cells counted had ≥ 10 adhered bacteria. Although the results of only two strains were presented in Tables 3.4 and 3.5, they are representative of the results obtained with the other *A.actinomycetemcomitans* isolates.

The results of all the *A.actinomycetemcomitans* strains which were tested are summarized in Table 3.6. These results show clearly that for the majority of the strains tested a mean adherence score of ≥ 10 bacteria per buccal epithelial cell was recorded. However, overall adhesion values varied among the different strains tested. The number of adherent bacteria ranged from 1.7 to 27.7 bacteria/BEC with a mean of

12.7 bacteria and a mean standard error of 1.6. The number of indigenous adherent bacteria per buccal cell was found to be low with a range of 0.2 to 2.0 bacteria/BEC. More than 80% of the freshly isolated *A.actinomycetemcomitans* strains (GDH isolates) adhered very well to the buccal cells with a minimum mean adherence value of 10 adherent bacteria per buccal cell. However, the ATCC Type strains (Aa 22, Aa 23 & Aa 24) were found to adhere in smaller numbers/BEC, compared to both the fresh isolates and to the NCTC Type strains (Aa 01 to Aa 06).

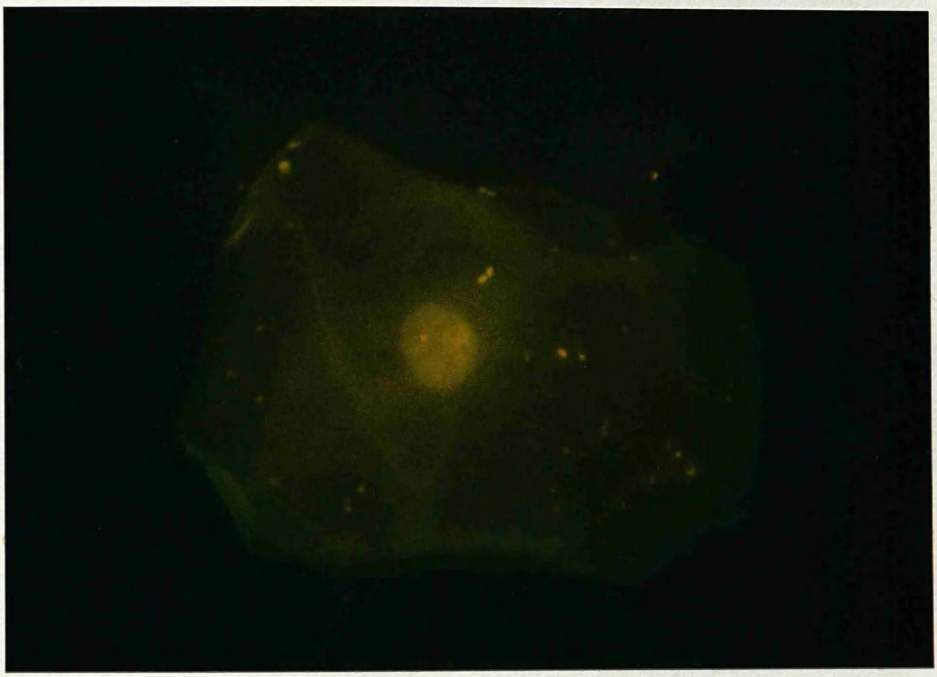

Figure 3.3 *A.actinomycetemcomitans* adhering to a buccal epithelial cell *in vitro* stained by acridine orange, examined at 1000 magnification.

Table 3.4a An example of the raw adherence data obtained for Aa 03 (data represent the number of bacteria/BEC for test and control in SIB). Experiment No. 1

BEC No.	Test	Control	Control	Test	BEC No.
01	55	0	0	7	26
02	13	0	0	40	27
03	23	0	0	11	28
04	29	0	0	49	29
05	17	0	0	33	30
06	20	0	1	21	31
07	17	0	1	1	32
08	27	0	2	5	33
09	52	1	0	16	34
10	9	0	1	63	35
11	28	0	1	23	36
12	25	0	1	33	37
13	62	1	0	5	38
14	25	0	0	34	39
15	36	0	1	44	40
16	14	0	0	80	41
17	65	0	0	33	42
18	23	0	1	60	43
19	47	2	0	66	44
20	21	0	1	28	45
21	4	3	0	13	46
22	57	0	0	13	47
23	8	6	0	33	48
24	11	1	0	8	49
25	15	1	2	18	50

Number of bacteria/BEC = 27/50 = 0.54 (Control)

Number of bacteria/BEC = 1440/50 = 28.8 (Test)

Mean No. of test bacteria adhering/BEC = 28.3

Range of adhering test bacteria = 1 to 80

Table 3.4b An example of the raw adherence data obtained for Aa 03 (data represent the number of bacteria/BEC for test and control in SIB). Experiment No. 2

BEC No.	Test	Control	Control	Test	BEC No.
01	64	2	9	16	26
02	58	1	0	49	27
03	16	1	0	28	28
04	23	4	1	36	29
05	65	2	1	28	30
06	52	0	2	31	31
07	41	3	0	6	2
08	34	3	0	46	33
09	76	0	0	80	34
10	6	3	0	19	35
11	12	6	0	32	36
12	47	0	1	31	37
13	22	0	0	39	38
14	15	0	0	21	39
15	25	0	28	21	40
16	55	0	1	42	41
17	20	4	0	20	42
18	34	0	0	13	43
19	18	0	0	11	44
20	48	2	0	70	45
21	51	0	10	24	46
22	50	1	0	14	47
23	23	0	5	48	48
24	32	9	2	11	49
25	36	0	0	20	50

Number of bacteria/BEC = 100/50 = 2.0 (Control)

Number of bacteria/BEC = 1679/50 = 33.6 (Test)

Mean No. of test bacteria adhering/BEC = 31.6

Range of adhering test bacteria = 6 to 80

Table 3.4c An example of the raw adherence data obtained for Aa 03 (data represent the number of bacteria/BEC for test and control in SIB). Experiment No. 3

BEC No.	Test	Control	Control	Test	BEC No.
01	15	0	0	21	26
02	20	0	0	3	27
03	44	0	0	1	28
04	77	0	0	7	29
05	8	0	0	17	30
06	7	1	5	9	31
07	36	1	3	54	32
08	24	0	3	8	33
09	73	0	0	22	34
10	20	0	0	18	35
11	43	0	5	19	36
12	14	1	0	19	37
13	57	0	2	20	38
14	5	0	3	15	39
15	36	2	0	37	40
16	59	0	0	2	41
17	10	1	0	38	42
18	18	10	2	20	43
19	46	0	0	17	44
20	6	0	0	84	45
21	21	0	1	18	46
22	24	6	0	16	47
23	8	0	3	25	48
24	18	0	4	14	49
25	13	2	2	16	50

Number of bacteria/BEC = 57/50 = 1.14 (Control)

Number of bacteria/BEC = 1222/50 = 24.4 (Test)

Mean No. of test bacteria adhering/BEC = 23.3

Range of adhering test bacteria = 1 to 84

Table 3.5a An example of the raw adherence data obtained for Aa 18 (data represent the number of bacteria/BEC for test and control in SIB). Experiment No. 1

BEC No.	Test	Control	Control	Test	BEC No.
01	9	2	5	49	26
02	15	0	0	13	27
03	2	1	2	3	28
04	22	2	0	9	29
05	1	1	0	12	30
06	16	3	0	24	31
07	5	0	1	45	32
08	34	6	0	13	33
09	14	2	0	29	34
10	6	5	2	9	35
11	10	1	0	22	36
12	31	1	4	8	37
13	13	0	0	15	38
14	10	1	0	30	39
15	4	0	0	18	40
16	11	1	0	42	41
17	6	2	0	26	42
18	11	0	0	1	43
19	26	1	0	9	44
20	5	1	1	26	45
21	18	2	0	21	46
22	26	2	1	16	47
23	8	3	0	14	48
24	19	1	0	5	49
25	19	1	0	24	50

Number of bacteria/BEC = 55/50 = 1.1 (Control)

Number of bacteria/BEC = 824/50 = 16.5 (Test)

Mean No. of test bacteria adhering/BEC = 13.4

Range of adhering test bacteria = 1 to 49

Table 3.5b An example of the raw adherence data obtained for Aa 18 (data represent the number of bacteria/BEC for test and control in SIB). Experiment No. 2

BEC No.	Test	Control	Control	Test	BEC No.
01	7	0	0	1	26
02	22	0	0	42	27
03	90	2	0	5	28
04	20	2	0	5	29
05	23	0	10	18	30
06	0	0	0	34	31
07	7	0	20	9	32
08	36	3	0	12	33
09	33	0	0	26	34
10	10	1	2	17	35
11	8	3	0	17	36
12	4	5	0	7	37
13	10	0	0	5	38
14	22	0	27	20	39
15	35	0	0	22	40
16	24	1	0	6	41
17	56	1	0	10	42
18	16	0	0	23	43
19	16	2	1	27	44
20	54	0	1	2	45
21	38	0	1	15	46
22	20	0	0	13	47
23	5	14	0	2	48
24	8	2	0	6	49
25	7	10	2	23	50

Number of bacteria/BEC = 100/50 = 2.0 (Control)

Number of bacteria/BEC = 938/50 = 18.8 (Test)

Mean No. of test bacteria adhering/BEC = 16.8

Range of adhering test bacteria = 0 to 90

Table 3.5c An example of the raw adherence data obtained for Aa 18 (data represent the number of bacteria/BEC for test and control in SIB). Experiment No. 3

BEC No.	Test	Control	Control	Test	BEC No.
01	8	0	0	3	26
02	17	0	0	14	27
03	16	2	0	7	28
04	40	0	0	14	29
05	9	0	0	19	30
06	20	0	2	18	31
07	90	0	0	8	32
08	14	0	0	8	33
09	15	0	0	13	34
10	11	0	0	13	35
11	11	0	1	13	36
12	33	0	0	5	37
13	8	0	0	15	38
14	11	0	0	0	39
15	19	0	0	7	40
16	8	0	1	13	41
17	8	0	0	7	42
18	19	0	0	28	43
19	4	0	0	2	44
20	0	0	0	18	45
21	15	0	0	2	46
22	3	0	0	20	47
23	2	0	1	9	48
24	11	0	1	40	49
25	80	0	0	12	50

Number of bacteria/BEC = 8/50 = 0.16 (Control)

Number of bacteria/BEC = 663/50 = 13.3 (Test)

Mean No. of test bacteria adhering/BEC = 13.0

Range of adhering test bacteria = 0 to 80

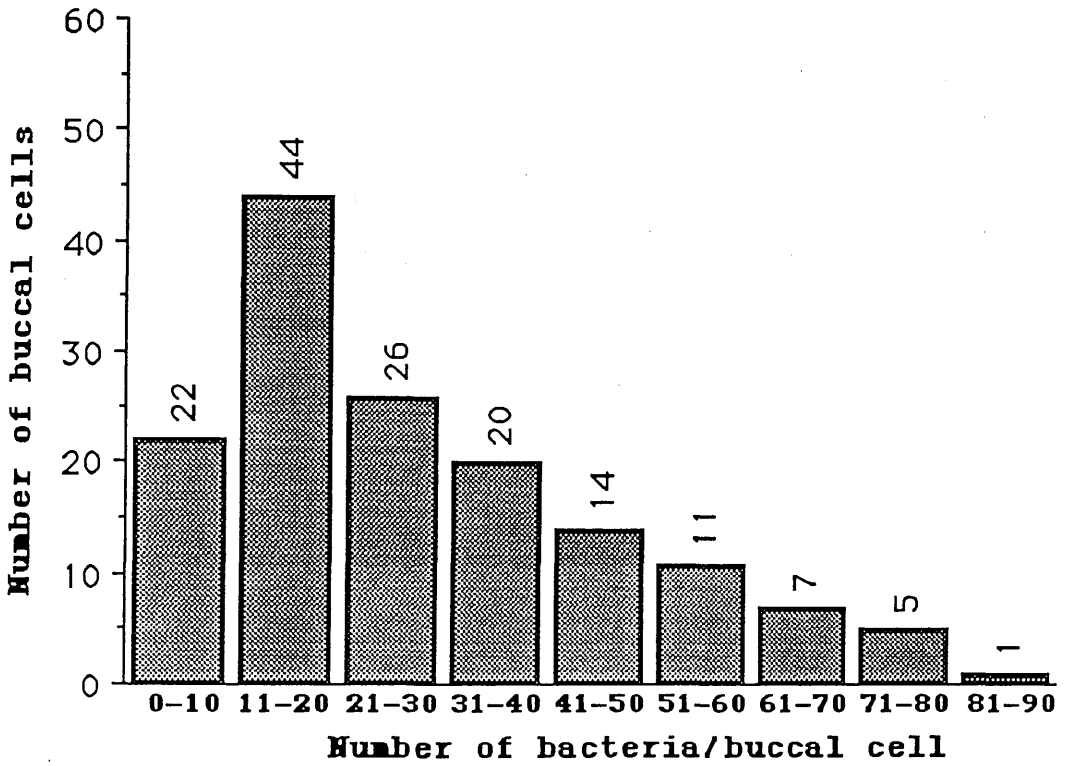

Figure 3.4 Distribution of the number of Aa 03 (NCTC 10979) cells adhering to 150 buccal epithelial cells in SIB, (the data represents the combined results from three different experiments).

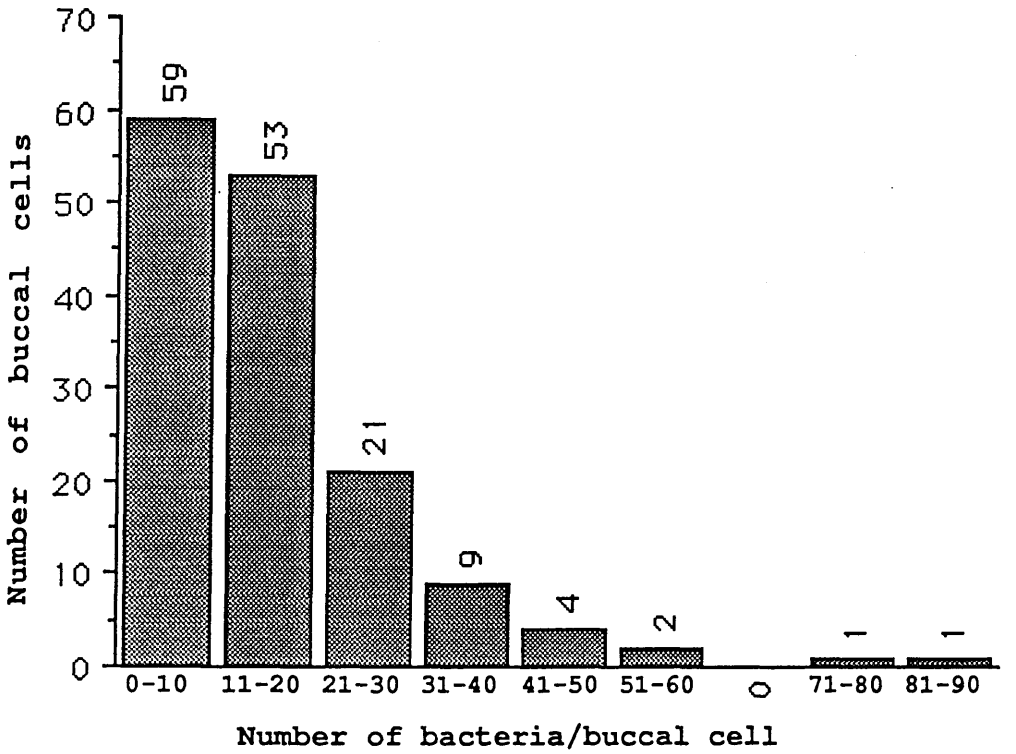

Figure 3.5 Distribution of the number of Aa 18 (GDH 1014) cells adhering to 150 buccal epithelial cells in SIB, (the data represents the combined results from three different experiments).

Table 3.6 Summary of the adherence results for *A.actinomycetemcomitans* strains to buccal epithelial cells in SIB buffer.

Bacterial strain No.	Mean No. of adhering bacteria/BEC Experiment Number 1	2	3	Mean (±SEM)
Aa 01	12.5	7.5	3.0	7.7 (2.7)
Aa 02	14.7	11.8	13.6	13.4 (0.8)
Aa 03	28.3	31.6	23.3	27.7 (2.4)
Aa 04	15.0	9.3	13.0	12.4 (1.7)
Aa 05	16.0	6.0	5.0	9.0 (3.5)
Aa 06	21.4	25.7	11.4	19.5 (4.2)
Aa 07	6.0	6.0	9.8	7.3 (1.3)
Aa 08	19.8	11.6	11.0	14.0 (2.8)
Aa 09	9.6	12.2	8.7	10.2 (1.0)
Aa 10	14.3	12.0	14.8	13.7 (0.8)
Aa 11	19.6	18.9	14.8	17.8 (1.5)
Aa 12	22.1	16.6	18.9	19.2 (1.6)
Aa 13	17.5	17.5	15.0	16.7 (0.8)
Aa 14	17.4	14.7	17.0	16.4 (0.9)
Aa 15	25.7	20.8	18.0	21.5 (2.2)
Aa 16	12.0	12.8	8.2	11.0 (1.4)

Table 3.6 Continued.

Bacterial strain No.	Mean No. of adhering bacteria/BEC Experiment Number 1	2	3	Mean (±SEM)
Aa 17	7.0	7.6	3.0	5.9 (1.4)
Aa 18	15.5	16.8	13.0	15.0 (1.1)
Aa 19	12.4	16.0	17.0	15.0 (1.4)
Aa 20	10.3	14.5	15.0	13.3 (1.5)
Aa 21	4.0	9.0	4.0	5.7 (1.6)
Aa 22	5.4	4.7	3.4	4.5 (0.6)
Aa 23	2.2	2.2	0.6	1.7 (0.5)
Aa 24	6.5	5.0	3.3	5.0 (0.9)
Aa 25	16.0	13.7	19.0	16.3 (1.6)
Aa 26	6.9	11.4	18.8	12.4 (3.5)
Aa 27	11.0	10.7	8.7	10.0 (0.7)
Aa 28	12.6	12.4	16.0	13.7 (1.2)
Aa 29	9.7	15.0	8.6	11.0 (2.0)
Aa 30	4.0	8.4	7.0	6.4 (1.3)
Aa 31	15.0	17.6	10.3	14.3 (2.1)
Aa 32	12.0	16.0	13.6	13.9 (1.1)
Aa 33	13.0	10.3	12.0	11.8 (0.8)

±SEM: Standard error of mean

3.3.3 Effect of saliva on the adherence of *A.actinomycetemcomitans* to BECs

These experiments were performed to examine the effect of saliva on the adherence of *A.actinomycetemcomitans* to BEC *in vitro*. Saliva free assays for comparison purposes were not performed at the same time. An example of the raw data obtained with Aa 18 (GDH 1014) is shown in Table 3.7a, b & c and represents that obtained for the other strains tested. As can be seen the number of bacteria adhering to the individual buccal cells varied between 0 to 83. The frequency distribution histogram (Figure 3.6) shows that more than 80% of buccal cells counted, had greater than 10 adherent bacteria. The saliva treated control buccal cells had very few indigenous adherent bacteria with an overall mean adherence value of 1.3 bacteria per buccal cell.

The results of all the *A.actinomycetemcomitans* strains which were tested are summarized in Table 3.8. These results show clearly that for the majority of the strains tested a mean adherence score of $\geq$ 10 bacteria per buccal epithelial cell was recorded. However, overall adhesion values varied among the different strains tested. The number of adherent bacteria ranged from 1.7 to 26.8 bacteria/BEC with a mean of 12 bacteria. The freshly isolated *A.actinomycetemcomitans* strains (GDH isolates) adhered very well to the buccal cells with a mean adherence value of 16 adherent bacteria/BEC. However, the ATCC Type strains (Aa 22, Aa 23 & Aa 24) and NCTC Type strains (Aa 01, Aa 02 & Aa 05) were found to adhere in smaller numbers/BEC compared with the fresh isolates.

Table 3.7a An example of the raw adherence data obtained for Aa 18 (data represent the number of bacteria/BEC in the presence of saliva for test and control assays). Experiment No. 1

BEC No.	Test	Control	Control	Test	BEC No.
01	11	0	0	32	26
02	13	4	0	38	27
03	18	0	0	33	28
04	18	0	1	15	29
05	12	0	0	16	30
06	15	0	0	13	31
07	2	5	0	9	32
08	17	0	1	6	33
09	19	0	0	31	34
10	42	0	4	57	35
11	22	0	2	28	36
12	42	3	0	32	37
13	17	2	0	15	38
14	27	4	0	10	39
15	20	2	1	13	40
16	4	0	0	9	41
17	16	0	0	27	42
18	15	0	1	3	43
19	43	0	0	42	44
20	19	0	0	18	45
21	11	0	0	52	46
22	8	0	0	30	47
23	32	0	6	4	48
24	4	0	6	4	49
25	10	1	0	14	50

Number of bacteria/BEC = 40/50 = 0.8 (Control)

Number of bacteria/BEC = 1011/50 = 20.2 (Test)

Mean No. of test bacteria adhering/BEC = 19.4

Range of adhering test bacteria = 4 to 52

Table 3.7b An example of the raw adherence data obtained for Aa 18 (data represent the number of bacteria/BEC in the presence of saliva for test and control assays). Experiment No. 2

BEC No.	Test	Control	Control	Test	BEC No.
01	0	12	2	19	26
02	0	5	0	83	27
03	6	3	5	10	28
04	49	0	16	0	29
05	1	26	0	0	30
06	0	30	7	9	31
07	6	5	0	20	32
08	0	6	0	10	33
09	4	26	2	2	34
10	0	4	0	4	35
11	3	3	0	4	36
12	2	9	0	15	37
13	1	12	0	5	38
14	0	0	1	30	39
15	0	4	1	8	40
16	6	7	8	19	41
17	2	7	0	9	42
18	11	42	1	8	43
19	0	18	0	0	44
20	0	18	0	7	45
21	0	18	4	7	46
22	0	16	1	22	47
23	0	2	0	14	48
24	0	25	0	5	49
25	0	18	0	3	50

Number of bacteria/BEC = 141/50 = 2.8 (Control)

Number of bacteria/BEC = 629/50 = 12.6 (Test)

Mean No. of test bacteria adhering/BEC = 9.6

Range of adhering test bacteria = 0 to 83

Table 3.7c An example of the raw adherence data obtained for Aa 18 (data represent the number of bacteria/BEC in the presence of saliva for test and control assays). Experiment No. 3

BEC No.	Test	Control	Control	Test	BEC No.
01	11	0	0	6	26
02	2	0	0	3	27
03	30	0	0	9	28
04	15	0	0	21	29
05	39	0	0	33	30
06	23	1	3	0	31
07	15	0	0	3	32
08	3	0	0	13	33
09	17	0	0	11	34
10	15	0	1	4	35
11	16	0	1	1	36
12	20	1	2	25	37
13	11	0	0	27	38
14	23	0	1	10	39
15	29	0	0	7	40
16	26	2	0	1	41
17	6	1	0	21	42
18	42	0	0	6	43
19	7	0	1	10	44
20	1	0	0	21	45
21	17	0	0	18	46
22	17	0	0	7	47
23	9	0	0	16	48
24	9	8	2	22	49
25	19	1	7	29	50

Number of bacteria/BEC = 32/50 = 0.64 (Control)

Number of bacteria/BEC = 746/50 = 14.9 (Test)

Mean No. of test bacteria adhering/BEC = 14.3

Range of adhering test bacteria = 0 to 42

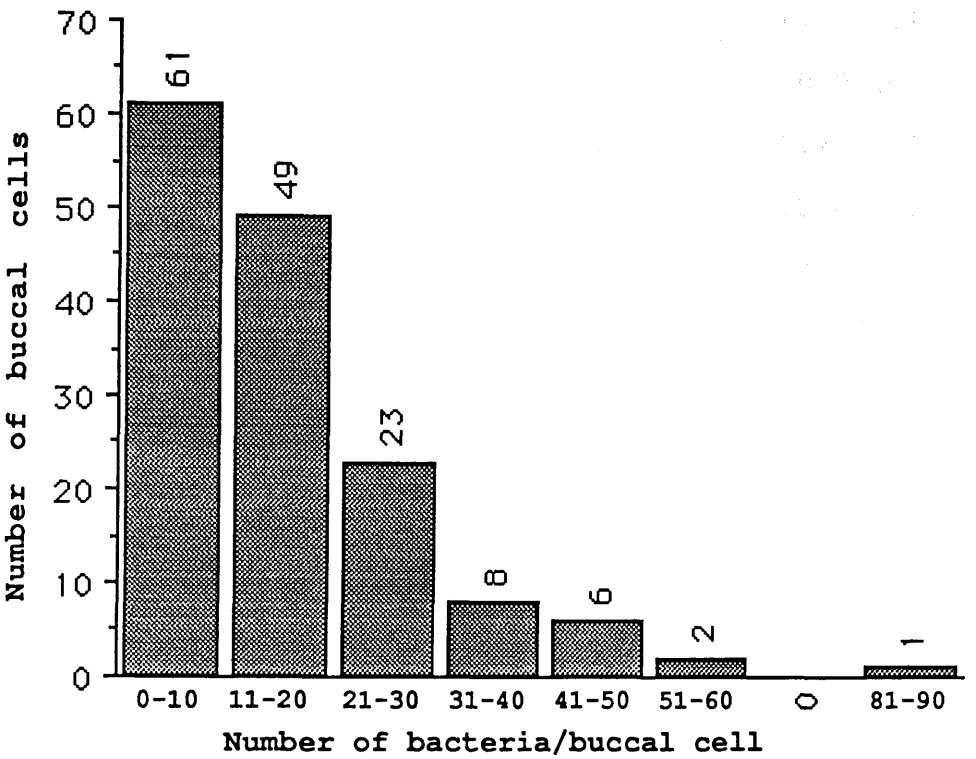

Figure 3.6 Distribution of the number of Aa 18 (GDH 1014) cells adhering to 150 buccal epithelial cells in the presence of saliva, (the data represents the combined results from three different experiments).

Table 3.8 Summary of the adherence results for *A.actinomycetemcomitans* strains to buccal epithelial cells in the presence of saliva.

Bacterial strain No.	Mean No. of adhering bacteria/BEC Experiment Number 1	2	3	Mean (±SEM)
Aa 01	6.9	6.0	5.4	6.0 (0.4)
Aa 02	11.7	9.4	8.7	10.0 (0.9)
Aa 05	8.6	5.0	5.0	6.2 (1.2)
Aa 09	11.3	13.4	10.6	11.8 (0.8)
Aa 10	14.4	7.4	17.0	13.0 (2.9)
Aa 13	14.6	14.0	13.0	14.0 (0.4)
Aa 14	20.3	12.6	16.2	16.4 (2.2)
Aa 15	37.3	23.4	19.8	26.8 (5.3)
Aa 16	15.6	19.0	9.0	14.5 (2.9)
Aa 17	15.0	8.4	9.2	11.0 (2.1)
Aa 18	19.4	9.6	14.3	14.4 (2.8)
Aa 21	7.7	11.0	3.6	7.4 (2.1)
Aa 22	2.7	8.5	3.6	7.0 (2.0)
Aa 23	4.9	11.0	5.3	1.7 (0.5)
Aa 24	4.6	9.0	4.7	6.0 (1.4)
Aa 25	26.0	22.4	23.2	23.8 (1.1)
Aa 26	19.2	12.8	16.8	16.3 (1.8)
Aa 28	15.4	7.2	13.3	12.0 (2.5)

±SEM: Standard error of mean

3.3.4 Effect of serum on the adherence of *A.actinomycetemcomitans* to BECs

Fourteen freshly isolated *A.actinomycetemcomitans* strains were examined for their ability to adhere to buccal epithelial cells in the presence of human serum or SIB. Two sets of experiments (ie. adherence of *A.actinomycetemcomitans* strains in the presence of serum and SIB alone) were carried out simultaneously on three different occasions. An example of the raw data obtained with Aa 09 (GDH 143) when serum was present is shown in Table 3.9a, b & c and is representative of the results obtained for the other strains tested. The frequency distribution histogram (Figure 3.7) shows that more than 85% of buccal cells had only 0 to 4 adherent bacteria when serum was present in the assay.

A summary of the adherence results obtained in SIB alone and in serum are given in Tables 3.10 and 3.11 respectively. The addition of serum to the bacteria/buccal cells mixture generally reduced the number of adherent bacteria per buccal epithelial cell, and in a number of experiments complete inhibition of bacterial adherence occurred (see Aa 20 experiment 3 and Aa 39 experiment 2, Table 3.11). Using a paired t-Test the adherence of *A.actinomycetemcomitans* in the presence of serum was overall and individually highly significantly reduced ($p < 0.0005$) as compared to that obtained in SIB buffer alone (Table 3.12).

Table 3.9a An example of the raw adherence data obtained for Aa 09 (data represent the number of bacteria/BEC in the presence of serum for test and control assays). Experiment No. 1

BEC No.	Test	Control	Control	Test	BEC No.
01	1	7	0	0	26
02	2	6	0	0	27
03	7	0	0	7	28
04	0	0	0	2	29
05	12	0	0	8	30
06	1	0	0	0	31
07	2	0	0	0	32
08	2	0	0	0	33
09	0	0	0	0	34
10	0	2	0	0	35
11	0	0	0	0	36
12	0	0	0	0	37
13	0	0	0	0	38
14	8	0	0	10	39
15	8	0	0	10	40
16	0	0	0	9	41
17	4	0	0	3	42
18	0	0	2	0	43
19	0	0	4	0	44
20	10	0	0	0	45
21	3	0	0	2	46
22	0	0	0	0	47
23	0	0	0	2	48
24	0	0	0	0	49
25	0	0	0	0	50

Number of bacteria/BEC = 21/50 = 0.42 (Control)

Number of bacteria/BEC = 113/50 = 2.26 (Test)

Mean No. of test bacteria adhering/BEC = 1.84

Range of adhering test bacteria = 0 to 12

Table 3.9b An example of the raw adherence data obtained for Aa 09 (data represent the number of bacteria/BEC in the presence of serum for test and control assays). Experiment No. 2

BEC No.	Test	Control	Control	Test	BEC No.
01	0	4	0	4	26
02	0	1	0	20	27
03	0	4	0	0	28
04	0	2	0	0	29
05	3	0	0	0	30
06	4	0	0	16	31
07	0	0	0	0	32
08	0	0	0	0	33
09	0	0	0	0	34
10	0	0	2	2	35
11	0	0	5	0	36
12	4	0	0	0	37
13	3	0	0	0	38
14	0	0	0	0	39
15	0	0	0	0	40
16	0	0	0	0	41
17	0	3	0	0	42
18	0	2	0	3	43
19	0	3	0	5	44
20	0	0	0	0	45
21	4	0	0	2	46
22	4	0	0	3	47
23	3	0	0	0	48
24	3	0	0	0	49
25	4	0	3	1	50

Number of bacteria/BEC = 29/50 = 0.6 (Control)
Number of bacteria/BEC = 88/50 = 1.76 (Test)
Mean No. of test bacteria adhering/BEC = 1.16
Range of adhering test bacteria = 0 to 20

Table 3.9c An example of the raw data obtained for Aa 09 (data represent the number of bacteria/BEC in the presence of serum for test and control assays). Experiment No. 3.

BEC No.	Test	Control	Control	Test	BEC No.
01	15	0	3	3	26
02	2	0	0	0	27
03	4	0	0	0	28
04	4	0	0	0	29
05	3	0	0	0	30
06	0	0	2	0	31
07	0	0	0	0	32
08	1	0	0	0	33
09	0	0	0	0	34
10	0	2	0	0	35
11	0	0	0	0	36
12	0	0	0	0	37
13	0	0	0	0	38
14	22	0	0	2	39
15	0	0	0	1	40
16	0	2	0	0	41
17	1	6	0	0	42
18	5	0	0	0	43
19	0	0	0	0	44
20	0	0	0	0	45
21	0	0	2	0	46
22	3	0	8	0	47
23	0	0	0	8	48
24	5	0	0	0	49
25	12	3	0	7	50

Number of bacteria/BEC = 28/50 = 0.56 (Control)

Number of bacteria/BEC = 98/50 = 1.96 (Test)

Mean No. of test bacteria adhering/BEC = 1.4

Range of adhering test bacteria = 0 to 22

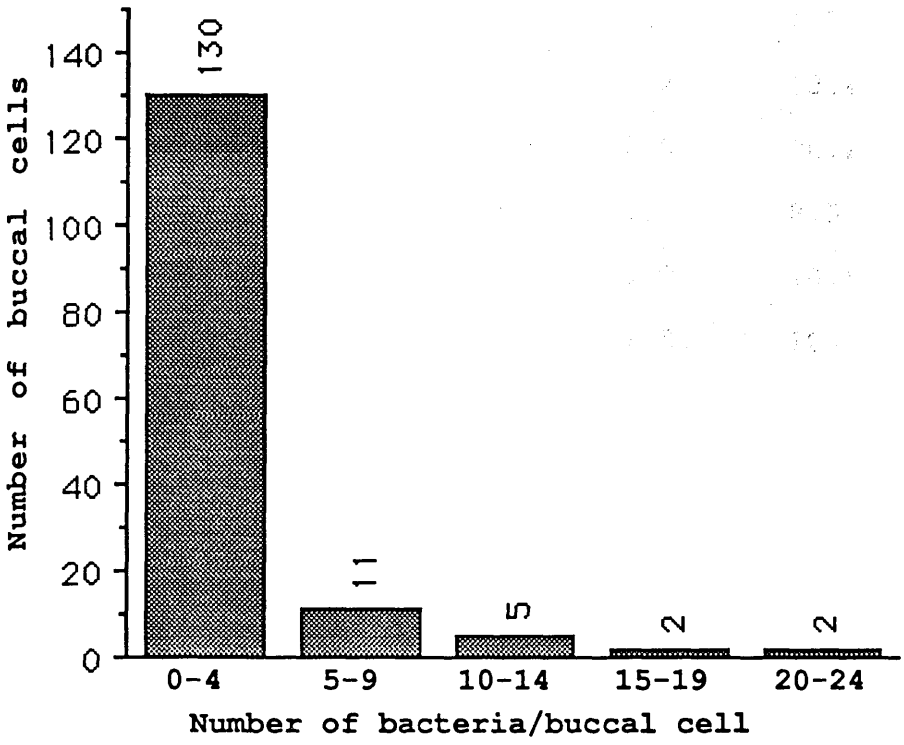

Figure 3.7 Distribution of the number of of Aa 09 (GDH 143) cells adhering to 150 buccal epithelial cells in the presence of serum, (the data represents the combined results from three different experiments).

Table 3.10 Adherence of *A.actinomycetemcomitans* to buccal epithelial cells in SIB.

Bacterial strain number	Mean No. of adhering bacteria/BEC Experiment number 1	2	3	Mean (±SEM)
Aa 07	4.5	6.0	5.0	5.2 (0.4)
Aa 08	6.0	6.0	8.0	6.7 (0.7)
Aa 09	12.7	9.4	15.2	12.4 (1.7)
Aa 10	12.9	7.7	10.0	10.2 (1.5)
Aa 15	11.0	7.0	7.0	8.3 (1.3)
Aa 16	10.5	10.0	11.0	10.5 (0.3)
Aa 17	12.0	7.0	11.3	10.0 (1.6)
Aa 18	5.0	5.0	4.0	4.5 (0.3)
Aa 19	5.0	6.0	8.0	6.3 (0.9)
Aa 20	4.0	5.0	4.0	4.3 (0.3)
Aa 25	9.0	15.0	12.0	12.0 (1.7)
Aa 27	11.0	14.0	10.0	12.0 (1.2)
Aa 32	10.0	4.0	5.0	6.3 (1.8)
Aa 39	6.0	9.0	5.0	6.7 (1.2)

±SEM: Standard error of mean

Table 3.11 Adherence of *A.actinomycetemcomitans* to buccal epithelial cells in the presence of serum.

Bacterial strain number	Mean No. of adhering bacteria/BEC Experiment number 1	2	3	Mean (±SEM)
Aa 07	0.4	0.5	0.3	0.4 (0.06)
Aa 08	1.0	0.7	0.2	0.6 (0.2)
Aa 09	1.8	1.2	1.4	1.5 (0.2)
Aa 10	2.0	1.5	1.6	1.7 (0.1)
Aa 15	1.4	1.2	0.3	1.0 (0.3)
Aa 16	3.6	4.0	2.6	3.4 (0.4)
Aa 17	3.5	1.4	0.6	1.8 (0.9)
Aa 18	1.5	2.0	0.1	1.2 (0.6)
Aa 19	0.3	0.1	1.8	0.7 (0.5)
Aa 20	1.7	0.7	0.0	0.8 (0.5)
Aa 25	0.2	0.7	0.4	0.4 (0.1)
Aa 27	4.0	1.4	2.0	2.5 (0.8)
Aa 32	4.5	0.5	0.4	1.8 (1.3)
Aa 39	0.7	0.0	1.4	0.7 (1.4)

±SEM: Standard error of mean

Table 3.12 Comparison of adherence of *A.actinomycetemcomitans* to buccal epithelial cells in SIB and in the presence of serum.

Bacterial strain number	Mean No. of adhering bacteria/BEC SIB	SERUM	p
Aa 07	5.2	0.4	0.005
Aa 08	6.7	0.6	0.01
Aa 09	12.4	1.5	0.01
Aa 10	10.2	1.7	0.02
Aa 15	8.3	1.0	0.02
Aa 16	10.5	3.4	0.005
Aa 17	10.0	1.8	0.02
Aa 18	4.5	1.2	0.005
Aa 19	6.3	0.7	0.005
Aa 20	4.3	0.8	0.02
Aa 25	12.0	0.4	0.01
Aa 27	11.0	2.5	0.02
Aa 32	12.0	1.8	0.01
Aa 39	6.7	0.7	0.05

p: Probability as determined by paired t-Test.

3.3.5 Effect of repeated subculturing on adherence

Although there were ten freshly isolated *A.actinomycetemcomitans* subcultured on 50 occasions, only four strains changed in morphology and produced colonies that did not adhere to the agar surface and when grown in broth culture did not aggregate. The adherence results for the four strains of *A.actinomycetemcomitans* (Aa 9, 10, 16 & 17) as freshly isolated (rough form) and after subculture (smooth form) are shown in Tables 3.13 and 3.14 respectively. The combined results are presented in Table 3.15. No significance difference in adherence values between the smooth and rough forms of the same isolate were noted.

Table 3.13 Adherence of rough (fresh) strains of *A.actinomycetemcomitans* to buccal cells in SIB.

Bacterial strain No.	Mean No. of adhering bacteria/BEC Experiment number 1	2	3	Mean (±SEM)
Aa 09	12.7	9.4	15.2	12.4 (1.7)
Aa 10	12.9	7.7	10.0	10.2 (1.5)
Aa 16	10.5	10.0	11.0	10.5 (0.3)
Aa 17	12.0	7.0	11.3	10.1 (1.6)

±SEM: Standard error of mean

Table 3.14 Adherence of smooth (subcultured) variants of *A.actinomycetemcomitans* to buccal cells in SIB.

Bacterial strain No.	Mean No. of adhering bacteria/BEC Experiment number 1	2	3	Mean (±SEM)
Aa 09	13.0	11.0	10.8	11.6 (0.7)
Aa 10	10.0	10.0	10.2	10.0 (0.07)
Aa 16	19.0	11.3	11.7	14.0 (2.5)
Aa 17	10.7	13.0	11.6	11.8 (0.7)

±SEM: Standard error of mean

Table 3.15 Comparison of adherence between rough and smooth variants of *A.actinomycetemcomitans*.

Bacterial strain No.	Mean No. of adhering bacteria/BEC		
	Rough	Smooth	P
Aa 09	12.4	11.6	NS
Aa 10	10.2	10.1	NS
Aa 16	10.5	14.0	NS
Aa 17	10.1	11.8	NS
Overall			NS

P: probability as determined by paired t-Test

NS: Not significant

3.4 Discussion:

3.4.1 Experimental methods

The methods commonly used to standardize bacterial concentrations, are microscopical enumeration using a counting chamber or viable counts by culture techniques. Other methods which have been used for counting bacteria include, spectrophotometric, coulter counters, filtration and dry-weight methods. Preliminary studies suggested that the use of counting chambers to obtain accurate results with *A.actinomycetemcomitans* was not possible due to the difficulty of accurately visualizing the small *A.actinomycetemcomitans* cells. Staining of these cells with simple stains such as carbolfuchsin or crystal violet did not markedly improve the results using a counting chamber. The viable plate counting method is only appropriate to determine viable counts but not total counts which were preferred in this study. Moreover, it is a retrospective method which could not be used to standardize bacterial suspensions before performing either the adherence or hydrophobicity assays. The present method used to determine the total counts of *A.actinomycetemcomitans* in the bacterial suspension for the adherence assay employed acridine orange staining, and was based on a technique developed to count bacteria in milk (Pettipher *et al.*, 1980). The method was subsequently modified by Sweet (1986) to determine counts of a variety of Gram-negative rods including *A.actinomycetemcomitans* prior to adherence assays. Once the optical density of a particular strain has been determined, bacterial suspensions of a wide range of concentrations can

then be prepared and adjusted accurately. The main disadvantage of this technique is that it is time consuming.

Fresh isolates of *A.actinomycetemcomitans* were found to aggregate in broth cultures which presented some difficulties in performing adherence assays. However, suspensions of fresh strains grown on CBA did not readily aggregate and were therefore used to prepare the bacterial suspensions in this study. Although the problem of aggregation by *A.actinomycetemcomitans* has been previously reported (Slots, 1982b), no information has been given about how previous workers have solved this problem in relation to adherence assays (Gibbons and Etherden, 1983; Kagermeier and London, 1985; Rosan *et al.*, 1988). Almost all papers give the impression that fresh isolates of *A.actinomycetemcomitans* can be easily grown in broth cultures and enumerated using counting chambers both of which have been found to be unsatisfactory in the present study.

The adherence method used here was developed by Sweet *et al.*, (1988) to investigate the adherence of oral bacteria (especially small Gram-negative rods) to buccal epithelial cells. Since the technique overcomes some of the problems encountered when using other adherence techniques, such as inadequate visualization of attached bacteria and poor standardization of bacterial suspensions it was the method of choice. This epifluorescence technique overcomes the problems mentioned above since staining with acridine orange permits clear visualization of attached bacteria to buccal cells. In addition, the saliva ions buffer (SIB) used thr-

oughout this study is believed to contain the main ionic constituents of saliva (Sweet *et al.*, 1988). A similar buffer has been used by a number of other researchers (Clark, Bammann and Gibbons, 1978; Applebaum *et al.*, 1979) to study the adherence of bacteria to oral surfaces. In general the adherence of bacteria to buccal cells *in vitro* may be affected by different factors such as, the type of buffer used (Yamazaki, Ebisu and Okada, 1981) and the source of epithelial cells. It has been shown that the same bacterial strains may vary in their ability to adhere to buccal cells obtained from different donors (Gibbons and Dankers, 1983). Moreover, it has been suggested that buccal cells that harbour small numbers of indigenous bacteria may have low affinity for bacterial adherence and vice versa (Sweet *et al.*, 1988). Therefore, in order to minimize the effect of these different parameters in the present adherence assay, both saliva and buccal cells were collected from the same donor.

3.4.2 Adherence of *A.actinomycetemcomitans* to BECs

This study was performed to examine the ability of a wide range of *A.actinomycetemcomitans* strains to adhere to human buccal epithelial cells since little is known about the ecology of this organism. As stated previously in section 3.1 there is little literature concerning the adherence of *A.actinomycetemcomitans* to buccal cells. In fact there is only one report which has investigated the adherence of a single Type strain of *A.actinomycetemcomitans* (NCTC 9710) to buccal epithelial cells in the presence of SIB (Sweet *et al.*, 1988). Since this strain of *A.actinomycetemcomitans*

was also investigated in the present study using SIB, a direct comparison of results is possible. Sweet *et al.*, (1988) reported a mean adherence value of 7.5 bacteria/buccal epithelial cell, while the value obtained in this study was 13.4 bacteria per buccal cell; an increase of almost 50 percent. This difference may be due to a number of factors such as the source of epithelial cells or growth conditions used in the two studies. Sweet *et al.*, (1988) used Anaerobic Blood Broth supplemented medium for growing *A.actinomycetemcomitans*, whereas Columbia Blood Agar medium was used in the present investigation. However, results from both studies clearly demonstrate the ability of *A.actinomycetemcomitans* to adhere to buccal epithelial cells *in vitro*.

There was a wide variation of adherence levels obtained with the different strain of *A.actinomycetemcomitans* examined in the present study. A mean range of 1.7 to 27.7 bacteria/buccal epithelial cell was obtained with the 33 *A.actinomycetemcomitans* isolates giving an overall mean of 12.7 bacteria/BEC. These results highlight the importance of testing a large number of bacterial strains to investigate the adherence of a particular species to buccal epithelial cells. In addition, it would be interesting to carry out similar adherence experiments using pooled buccal epithelial cells from different donors, since the present study only used buccal cells from the same donor.

3.4.3 Effect of saliva on the adherence of *A.actinomycetemcomitans* to BECs

The effect of clarified whole saliva on the adherence of *A.actinomycetemcomitans* to buccal cells *in vitro* was investigated using 18 strains of *A.actinomycetemcomitans*. The same adherence assay described in Section 3.2.8 was empolyed, but with the addition of saliva. Although the *in vitro* adherence system used in this study is not the same as the *in vivo* situation, the results of these experiments may help to identify some of the factors which are involved in the colonization of human oral surfaces by *A.actinomycetemcomitans*.

Since the adherence experiments in SIB alone and in the presence of saliva alone were performed at different times, any direct comparison of the results must take into account the possible effect of variations in bacterial suspensions and buccal cells. However, *A.actinomycetemcomitans* adhered to buccal epithelial cells in the presence of saliva in similar numbers to those found when the same isolates were tested in SIB buffer alone. In general the presence of saliva had little or no effect on the number of adherent bacteria per buccal cell, although noticeable differences were recorded for a few strains eg. Aa 02, 09, 13 and 25. Saliva was found to increase the number of adherent bacteria of strains Aa 09, and Aa 25 compared with assays using SIB. On the other hand it reduced the number of adherent bacteria of strains Aa 02 and Aa 13 when compared with SIB assays. The reason for these differences is not known.

Unfortunately there are no reported studies on the effect of saliva on the adherence of *A.actinomycetemcomitans* to buccal cells, with which to compare the findings of this study. Sweet (1986) has reported that the number of *A.actinomycetemcomitans* NCTC 9710 which adhered to saliva treated Hela cells was 93 bacteria/0.018 mm^2. A value of 85 adherent bacteria/0.018 mm^2 to buccal cells was obtained in this study using the conversion formula described by Sweet (1986). However, it is difficult to compare these results since Hela cells are of non-oral origin and may possess different receptors for adherence compared with oral tissues. Saliva from only one healthy individual was studied here, and it would be interesting to carry out further work to find out if saliva from patients with *A.actinomycetemcomitans* and healthy subjects have the same or different effects on the adherence of this organism to buccal epithelial cells.

3.4.4 Effect of serum on the adherence of *A.actinomycetemcomitans* to BECs

Serum was added to the adherence system used in this study to mimic the *in vivo* environment where crevicular epithelial cells are exposed to crevicular fluid, which has a similar composition to serum (Cimasoni, 1983). Results obtained in this study showed that human serum almost completely inhibited *A.actinomycetemcomitans* from adhering to buccal epithelial cells. An average mean number of 0.4 to 3.4 bacteria per buccal epithelial cell was obtained for the 14 freshly isolated *A.actinomycetemcomitans* strains. These

results are compared with an average mean of 4.3 to 12.5 bacteria/BEC obtained with the same *A.actinomycetemcomitans* strains tested in SIB buffer alone (Table 3.15). This significant difference ($P \leq 0.0005$) can not be attributed to experimental error, as both sets of experiments were carried out simultaneously using the same preparation of buccal cells and bacteria. Moreover, the same pattern of results was found with all tested strains. There appears to be no previous publication concerning the adherence of *A.actinomycetemcomitans* to buccal cells in the presence of serum.

3.4.5 Effect of repeated subculturing on adherence

In a number of recent studies some freshly isolated strains of *A.actinomycetemcomitans* have been shown to possess fimbriae (Scannapieco *et al.*, 1987; Rosan *et al.*, 1988), which appeared to be irreversibly lost after 30 to 40 subcultures onto TSBV agar plates (Rosan *et al.*, 1988). The fimbriated strains were found to produce star-shaped colonies and tended to aggregate readily in fluid media, whereas the non-fimbriated cells formed uniform suspensions and did not produce star-shaped colonies. Two of the fimbriated strains of *A.actinomycetemcomitans* tested by (Rosan *et al.*, 1988) adhered 3 to 4-times better than non-fimbriated variants to both hydroxyapatite and saliva treated hydroxyapatite. However, one strain showed no difference in adherence to the tested surfaces when the fimbriated and non-fimbriated variant were compared. The presence of fimbriae on *A.actinomycetemcomitans* was detected using electron microscopy which showed that in any positive culture many of the cells had

only a single fimbria, whereas others possessed several fimbriae (Rosan *et al.*, 1988). These authors suggested that the presence of fimbriae is an important mediator for adhesion to hydroxyapatite by *A.actinomycetemcomitans.* However, it must be remembered that one of the 3 strains tested by (Rosan *et al.*, 1988) failed to exhibit any difference in adherence whether fimbriated or not. Moreover, it is not known if the number of fimbria present on each bacterial cell plays any role in adherence. Kagermeier and London (1985) reported that *A.actinomycetemcomitans* Y4 lost some of its capacity to adhere to hydroxyapatite after it had been subcultured over a period of one year. In the present study four freshly isolated *A.actinomycetemcomitans* strains (Aa 09, 10, 16 & 17) were subcultured 50 times on blood agar plates and examined for their ability to adhere to buccal epithelial cells *in vitro.* Interestingly the four strains tested gave almost the same numbers of adhered bacteria per buccal cell after repeated subculture when compared to results obtained with fresh strains subcultured on only 5 or 6 occasions. These results suggest that repeated subculturing has no effect on the adherence of *A.actinomycetemcomitans* to buccal cells. It was not possible to confirm the presence or absence of fimbriae in the *A.actinomycetemcomitans* strains used in the present investigation. In conclusion, further investigations are necessary before the role of fimbriae in the adherence of *A.actinomycetemcomitans* to oral surfaces can be clearly defined.

3.4.6 Adherence of fresh isolates compared to Type strains

There is no published information with which to compare the adherence of fresh and Type strains of *A.actinomycetemcomitans* to buccal epithelial cells. In the present study more than 80% of the fresh isolates of *A.actinomycetemcomitans* adhered well to buccal cells at levels of ≥ 10 bacteria/BEC with an overall mean of 13.3 bacteria. Results obtained with the Type strains also demonstrated similar findings to that obtained with the fresh isolates giving an overall mean of 10.7 bacteria/BEC and no significant difference was found. However, there was a wide variation between the ability of different strains to adhere to buccal cells. These results emphasize the inaccuracies which may occur if only one or two strains of a bacterial species are examined and conclusions are then drawn on the adherence of that microorganism. For example, if by chance Aa 23 (adherence value of 1.7 bacteria/BEC) had been the only strain tested in this investigation, then the conclusion would have been that *A.actinomycetemcomitans* adheres poorly to buccal epithelial cells. However, the opposite would have been the case if another strain which adhered in higher numbers was chosen eg. Aa 03 with a value of 27.7/BEC. It is interesting to note that although strains NCTC 10979 and ATCC 29522 are said to be same isolate (American Type Culture Catalogue, 1985), different adherence values to BECs were obtained. While *A.actinomycetemcomitans* NCTC 10979 showed the highest adherence value of 27.7 bacteria/BEC; ATCC 295222 only

adhered at a level of 4.5 bacteria/BEC. The reason for this 5-fold difference in adherence between what is believed to be the same Type culture is unknown. Therefore as a general rule it is important to study a wide selection of isolates including fresh and Type strains when investigating the adherence of a bacterial species.

3.4.7 Adherence of *A.actinomycetemcomitans* to other surfaces

There are a few papers which deal with the adherence of *A.actinomycetemcomitans* to hydroxyapatite and other oral surfaces, and although this is not directly related to the present investigation, it is interesting to consider the results. Two strains of *A.actinomycetemcomitans* (ATCC 29523 and Y4) were reported to adhere poorly to hydroxyapatite beads (Gibbons and Etherden, 1983). Surprisingly due to the differences in the surfaces tested and the nature of adherence assay used, the results obtained for both strains in the present study were also low. For instance, *A.actinomycetemcomitans* ATCC 29523 showed the lowest level of all strains tested at 1.7/BEC, while Y4 adhered at 5.7 bacteria per buccal cell.

The effect of saliva on the adherence of *A.actinomycetemcomitans* to hydroxyapatite has also been reported by Kagermeier and London (1985). They found that the adherence of *A.actinomycetemcomitans* strains Y4 and N27 to hydroxyapatite was reduced after coating with saliva. This reduction was said to be around 50% for the N27 strain and was

only slightly reduced for Y4. However, Rosan *et al.*, (1988) showed that three strains of *A.actinomycetemcomitans* adhered to both hydroxyapatite and saliva treated hydroxyapatite to a similar extent. In addition, one Type strain (NCTC 9710) was found to adhere in high numbers (324/0.018 mm^2) to saliva treated tooth enamel (Sweet, MacFarlane and Samaranayake, 1990).

There is little information about the affect of serum on the adherence of *A.actinomycetemcomitans* to oral surfaces. However, it has been shown that serum reduced the adherence of two *A.actinomycetemcomitans* strains (N27 and Y4) to hydroxyapatite by 20 and 66% respectively (Kagermeier and London, 1985). Although the test surfaces and adherence assays used in the Kagermeier and London (1985) study and the present investigation are very different, they do both report a marked inhibitory effect of serum on the adherence of *A.actinomycetemcomitans.* Finally it should be emphasized that until more investigations are carried out using serum, saliva and buccal epithelial cells from different individuals as well as more strains of *A.actinomycetemcomitans*, the factors which affect the adherence of *A.actinomycetemcomitans* will remain uncertain. However, the assay method described here and the results obtained, should provide a start for subsequent studies in this field.

3.5 Conclusions:

The results of this study showed clearly that all 33 strains of *A.actinomycetemcomitans* had the ability to adhere to hu-

man buccal epithelial cells *in vitro* with an overall mean of 12.5 bacteria/BEC.

In general the freshly isolated *A.actinomycetemcomitans* isolates adhered better than the Type strains, but the difference was not statistically different.

Adherence of *A.actinomycetemcomitans* in the presence of saliva was similar to that observed in SIB buffer alone.

Human serum significantly reduced the number of adhered bacteria per buccal epithelial cells compared to that obtained in either SIB or in the presence of saliva.

Continuously subcultured strains of *A.actinomycetemcomitans* were found to adhere to buccal epithelial cells to a similar extent compared with the fresh isolates.

CHAPTER 4

HYDROPHOBICITY OF *A.ACTINOMYCETEMCOMITANS*

4.1 Introduction

Hydrophobic interactions have been considered primarily responsible for mediating bacterial adherence to most oral surfaces (Rosenberg *et al.*, 1983a). Thus if the adherence of microorganisms to oral surfaces is mediated by hydrophobicity, there should be a clear association between hydrophobicity and adherence. There is little information concerning the hydrophobic activity of *A.actinomycetemcomitans* and what has been reported is conflicting (see section 1.7.6). For example, Gibbons and Etherden (1983) reported that two strains of *A.actinomycetemcomitans* were hydrophilic as assessed by their inability to adsorb to hexadecane, and adhered in only low numbers to hydroxyapatite. However, Kozlovsky *et al.*, (1987) found that one *A.actinomycetemcomitans* strain possessed hydrophobic activity, which was found to vary depending on the medium used for growth, the age of culture and the suspending medium employed.

Therefore, the aim of this study was (I), to evaluate the hydrophobic activity of the same 33 isolates of *A.actinomycetemcomitans* which had been used in Chapter 3 to study adherence to BECs and (II), to determine if there is any correlation between hydrophobicity and the adherence of *A.actinomycetemcomitans* to BECs.

4.2 Materials and methods

4.2.1 Preparation of *A.actinomycetemcomitans* suspensions

Thirty three *A.actinomycetemcomitans* isolates were tested for their hydrophobic activities. The isolates included 10 reference strains and 23 GDH fresh isolates (see Table 3.1). Suspensions of all *A.actinomycetemcomitans* strains were prepared by growing the test bacteria on two CBA plates for 48 hours at 37°C in an atmosphere of 5% CO_2 in air. Growth was harvested using a sterile cotton swab and then added to 5 ml SIB. After centrifugation at 8000 g for five minutes using an MSE centrifuge (MSE Scientific Instruments, Crawley, England), the resultant pellet was resuspended in 10 ml SIB, and diluted to give an optical density of 1.0 at 520 nm (SP 8-100 Spectrophotometer, Pye Unicam, England) representing a concentration of approximately 3 x 10^9/ml.

4.2.2 Hydrophobicity assay

The assay procedure used was that described by Sweet *et al.*, (1987) with the minor modification that aeration of the *A.actinomycetemcomitans* suspensions was extended from 1 to 4 minutes since the recommended time of one minute failed to remove all xylene from the bacterial test suspensions. For each *A.actinomycetemcomitans* strain tested, 5 ml of the bacterial suspension (3 x 10^9/ml) was added to each of two test tubes (15mm diameter and 150mm long), one tube labelled as test and the other as control. A second set of test and control tubes containing the same suspending medium were

included in each assay to provide a background reading (see Figure 4.1). To all tubes labelled 'test', one millilitre of xylene (Raymed, Leeds, England) was carefully added to ensure that the xylene formed a layer on top of the suspension medium, without mixing occurring. All tubes (both test and control) were then incubated in a water bath (HETO, Denmark) at 37°C for 10 minutes. Following incubation, all the tubes were then vortex mixed for 30 seconds and immediately returned to the water bath for a further 30 minutes. During this period, xylene formed a separate layer at the top. The aqueous lower layer was next carefully removed using a clean glass pasteur pipette to a new test tube, the first few drops being discarded. Since it is impossible to carry this out without transferring some xylene, the latter was removed by bubbling compressed air through the fluid for 4 minutes using a clean pasteur pipette.

The tubes were once more vortex mixed and the optical densities were measured at 520 nm using a Pye Unicam SP 8-100 Spectrophotometer. The tube containing the suspending medium without xylene was used to adjust the spectrophotometer to a zero reading for measuring the optical densities of the xylene-free control bacterial suspensions. The suspending medium containing xylene was used to zero the spectrophotometer before measuring the optical densities of the bacterial suspensions to which xylene was added.

4.2.3 Hydrophobicity of *A.actinomycetemcomitans* after repeated subculturing

Four freshly isolated *A.actinomycetemcomitans* strains (Aa 9, 16, 17 & 33) were subcultured on 50 occasions on blood agar plates (CBA). Subculturing was repeated every 48 hours by sweeping some of the growth using a sterile loop and inoculating it onto a fresh CBA plate which was then incubated under 5% CO_2 in air at 37°C. These isolates were found to produce smooth colonies and grew in broth culture without aggregation occurring. The four strains were subsequently tested for their hydrophobic properties as opposed to the rough variants (only subcultured for 5 to 6 times) using the same method described earlier in section 4.2.2. The results obtained with the rough and smooth subcultured variants were then compared.

4.2.4 Effect of suspending media on hydrophobicity

The effect of suspending media (ie. SIB and PBS) on the hydrophobicity of *A.actinomycetemcomitans* (Aa 09, 10 and 16) was examined using the same procedure described in section 4.2.2. The bacteria were prepared as described in section 4.2.1 and then suspended in either SIB or PBS.

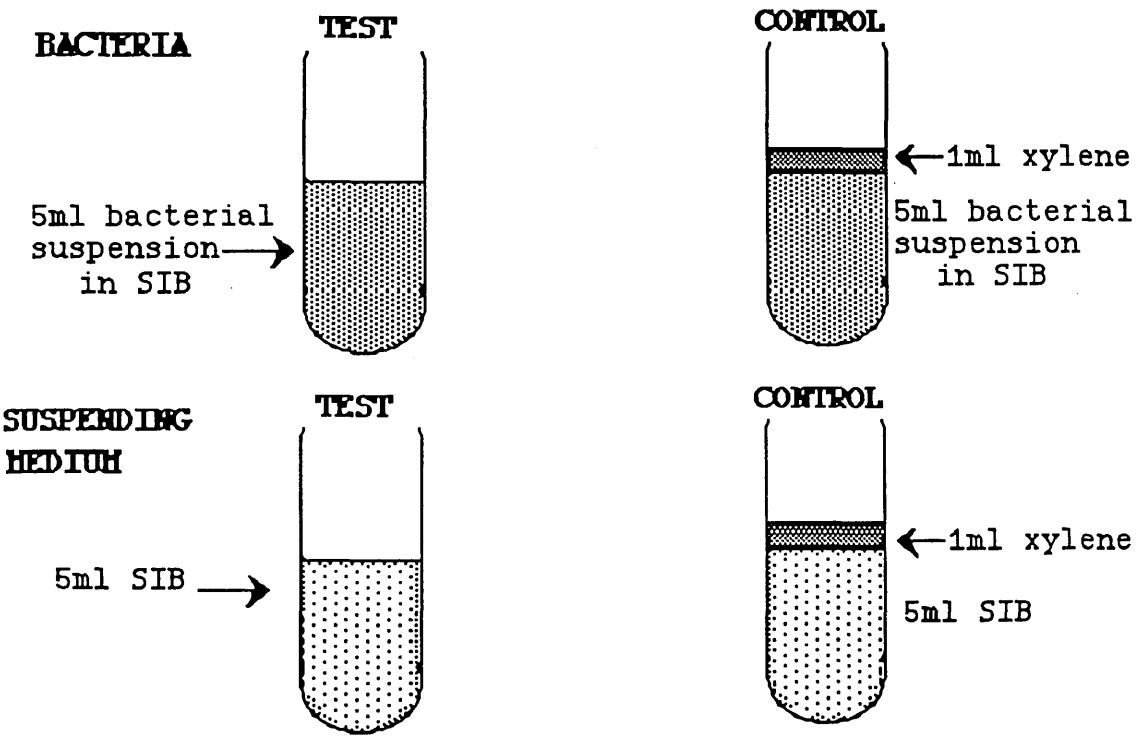

Figure 4.1 Diagrammatic representation of the hydrophobicity assay used in this study.

4.2.5 Effect of growth conditions on the hydrophobicity of the subcultured strains

Five of the freshly isolated *A.actinomycetemcomitans* strains (Aa 9, 10, 16, 17 & 18) were grown on CBA for 24 and 48 hours in 5% CO_2 in air. Bacterial suspensions of these isolates were then prepared in SIB as described in section 4.2.1. The hydrophobic activities of these strains was then determined using the same procedure described in section 4.2.2.

4.2.6 Calculation of hydrophobicity values

To determine the hydrophobicity of the test strains, the percentage reduction in optical density of the test suspension which contained xylene as compared with the control suspension which was xylene free, were calculated. These percentage values are proportional to the hydrophobicity of the test bacteria in any given suspending medium (Rosenberg *et al.*, 1980). The assay was repeated once on three different occasions for each *A.actinomycetemcomitans* strain tested.

4.2.7 Statistical analysis

All assays were performed on three different occasions for each bacterial strain under study. Results were tested to discover if they were normally distributed or not using STAT WORK software (APPLE Macintosh computer). Significant differences were determined using the non-parametric Mann-whitney U test. In addition, correlation coefficients were det-

ermined for comparisons of the hydrophobicity results with the adherence results reported in Chapter 3.

4.3 Results:

4.3.1 Hydrophobicity of *A.actinomycetemcomitans* in SIB

The hydrophobicity results for the same 33 strains of *A.actinomycetemcomitans* (Aa 01 to Aa 33) used in the adherence assay are shown in Table 4.1. In general all *A.actinomycetemcomitans* isolates were shown to be hydrophobic as assessed by their ability to adsorb to xylene. The percentage reduction in absorbance at a wave length of 520 nm for these isolates ranged from 39 to 82% with an overall mean of 67%. All the fresh GDH strains of *A.actinomycetemcomitans* exhibited over a 50% reduction in absorbance with an overall mean of 70% reduction in SIB. The Type strains showed an overall mean reduction of 58%. Moreover, the ATCC strain (Aa 22) gave the lowest hydrophobic value (39%), and the highest recorded value of 82% was obtained with the fresh GDH strain (Aa 18). The Mann-Whitney U test was used to compare these results which showed no significant difference between fresh and Type strains ($p > 0.05$).

Table 4.1 Hydrophobicity results of 33 strains of *A.actinomycetemcomitans* in SIB buffer.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 01	61	58	62	60 (1.2)
Aa 02	75	79	83	79 (2.3)
Aa 03	61	63	60	61 (0.9)
Aa 04	83	75	82	80 (2.5)
Aa 05	65	76	48	63 (8.1)
Aa 06	48	53	46	49 (2.0)
Aa 07	82	74	64	73 (5.2)
Aa 08	86	62	73	74 (6.9)
Aa 09	74	77	76	76 (0.9)
Aa 10	73	73	72	73 (0.3)
Aa 11	62	59	71	64 (3.6)
Aa 12	81	73	76	77 (2.3)
Aa 13	71	80	77	76 (4.6)
Aa 14	81	76	80	79 (2.6)
Aa 15	66	62	62	63 (1.3)
Aa 16	54	59	70	61 (4.7)
Aa 17	68	76	62	69 (4.0)

±SEM: Standard error of mean

Table 4.1 Continued.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 18	72	84	90	82 (5.3)
Aa 19	81	85	67	78 (5.4)
Aa 20	88	70	78	79 (5.2)
Aa 21	44	47	46	46 (0.9)
Aa 22	35	40	42	39 (2.1)
Aa 23	51	66	51	56 (5.0)
Aa 24	52	54	45	50 (2.7)
Aa 25	77	79	73	76 (1.8)
Aa 26	64	73	63	67 (3.2)
Aa 27	51	63	50	55 (4.2)
Aa 28	68	78	70	72 (3.0)
Aa 29	82	77	69	76 (3.8)
Aa 30	66	61	73	67 (3.5)
Aa 31	58	67	76	67 (5.2)
Aa 32	58	61	58	59 (1.0)
Aa 33	66	57	62	62 (2.6)

±SEM: Standard error of mean

4.3.2 Effect of repeated subculturing on hydrophobicity

Repeated subculturing on 50 occasions on CBA plates, resulted in the loss of homotypic aggregation in broth cultures and in adherence to agar surfaces by the strains of *A.actinomycetemcomitans* tested. However, as shown in Tables 4.2 and 4.3, repeated subculturing did not affect the hydrophobicity of these strains. Although only four strains were tested, results showed clearly that both variants were hydrophobic. *A.actinomycetemcomitans* (Aa 31) lost some of its hydrophobic activity after it had been subcultured for 50 times (Table 4.4). However, there was no significant difference between any of the results presented in Table 4.4 as determined by the Mann Whitney-U test.

4.3.3 Effect of suspending medium on hydrophobicity

Three strains (Aa 9, 10 & 16) were tested for their hydrophobic activity in different suspending media (ie. SIB, Table 4.5, and PBS, Table 4.6). None of the strains showed a significant difference in hydrophobicity when suspended in either of the two buffers used, and the mean % drop in adsorption was 70.3 for SIB and 71% for PBS (see Table 4.7).

Table 4.2 Hydrophobicity of the 4 rough (fresh) isolates of *A.actinomycetemcomitans* strains before subculture.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 09	74	77	76	76 (0.9)
Aa 16	54	59	70	61 (4.7)
Aa 17	68	76	62	69 (4.0)
Aa 31	58	67	76	67 (5.2)

±SEM: Standard error of mean

Table 4.3 Hydrophobicity of 4 *A.actinomycetemcomitans* strains after 50 repeated subcultures over a period of 16 weeks (smooth forms).

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 09	77	80	73	77 (2.0)
Aa 16	68	55	61	61 (3.7)
Aa 17	71	71	78	73 (2.3)
Aa 31	45	53	51	50 (2.4)

±SEM: Standard error of mean

Table 4.4 A comparison of the hydrophobicity of rough and smooth colonial forms of *A.actinomycetemcomitans*.

Bacterial strain number	% Drop in Absorbance at 520 nm		
	Rough	Smooth	p
Aa 09	76	78	NS
Aa 16	61	61	NS
Aa 17	69	73	NS
Aa 31	67	50	NS

p: Probability determined by Mann-Whitney U-test

NS: Not significant

Table 4.5 Hydrophobicity of three strains of *A.actinomycetemcomitans* in SIB buffer.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 09	77	80	73	77 (2.0)
Aa 10	73	73	72	73 (0.3)
Aa 16	68	55	61	61 (3.7)

±SEM: Standard error of mean

Table 4.6 Hydrophobicity of three strains of *A.actinomycetemcomitans* in PBS buffer.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 09	75	78	79	77 (1.2)
Aa 10	73	65	70	69 (2.3)
Aa 16	65	66	69	67 (1.2)

±SEM: Standard error of mean

Table 4.7 Comparison of hydrophobicity of three strains of *A.actinomycetemcomitans* in SIB and PBS.

Bacterial strain number	% Drop in Absorbance at 520 nm SIB	PBS	P
Aa 09	77	77	NS
Aa 10	73	69	NS
Aa 16	61	67	NS
Mean	70.3	71	NS

p: Probability determined by Mann-Whitney U-test

NS: Not significant

4.3.4 Effect of incubation time on hydrophobicity

These results were obtained from five different strains (Aa 9-10 and 16-18) after they had been incubated on CBA plates for 24 and 48 hours. As shown in Tables 4.8 and 4.9 there were variations between the percentage reduction in absorbance under these conditions. A range of 57 to 93% reduction in absorbance was obtained for the test strains after incubation for 24 hours on CBA compared with a range of 50 to 80% reduction, after 48 hours incubation. As expected these slight variations shown in Table 4.10 were not significant.

Table 4.8 Hydrophobicity of *A.actinomycetemcomitans* after incubation for 24 hours on CBA.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 09	96	92	90	93 (1.8)
Aa 10	80	86	78	81 (1.4)
Aa 16	47	49	44	47 (2.4)
Aa 17	58	56	58	57 (0.7)
Aa 18	88	90	88	89 (0.7)

± SEM: Standard error of mean

Table 4.9 Hydrophobicity of *A.actinomycetemcomitans* after incubation for 48 hours on CBA.

Bacterial strain number	% Drop in Absorbance at 520 nm Experiment number 1	2	3	Mean (±SEM)
Aa 09	82	80	77	80 (1.4)
Aa 10	70	74	84	76 (4.2)
Aa 16	46	46	57	50 (3.7)
Aa 17	57	63	76	65 (5.6)
Aa 18	77	74	79	77 (1.4)

± SEM: Standard error of mean

Table 4.10 Comparison of hydrophobicity of *A.actinomycetemcomitans* after incubation for 24 and 48 hours on CBA.

Bacterial strain number	% Drop in Absorbance at 520 nm 24 hour	48 hour	p
Aa 09	93	80	NS
Aa 10	81	76	NS
Aa 16	47	50	NS
Aa 17	57	65	NS
Aa 18	89	77	NS

p: Probability determined by Mann-Whitney U-test

NS: Not significant

4.4 Discussion:

4.4.1 Experimental methods

The original method for determining bacterial hydrophobicity was described by Rosenberg *et al.*, (1980) in which three different hydrocarbons (hexadecane, octane and xylene) were used. Results obtained from testing different bacteria showed different affinities towards the three hydrocarbons used (Rosenberg *et al.*, 1980). This suggested that the affinity for hydrocarbons may vary among different bacteria. The methods used to study the hydrophobicity of *A.actinomycetemcomitans* include the adherence to hexadecane (Gibbons and Etherden, 1983); octane (Kozlovsky *et al.*, 1987) and xylene (Sweet, 1986). In the present study hydrophobicity of *A.actinomycetemcomitans* was determined using the method described by Sweet *et al.*, (1987). This technique was performed on all isolates and showed reproducible results with xylene as the test hydrocarbon. A standard error range of 0.3 to 8.1 was recorded for the 33 tested *A.actinomycetemcomitans* strains with an overall mean of 3.3. The main problem encountered in the present study in using this technique was the removal of xylene from the aqueous bacterial suspensions. Although Sweet *et al.*, (1987) found that aeration of bacterial suspensions for one minute resulted in the removal of xylene, this time proved to be inadequate in this study as xylene droplets were still noted in the bacterial suspensions containing *A.actinomycetemcomitans*. Therefore, suspensions were aerated for four minutes which resulted in the removal of visible xylene. It has been sugges-

ted that although hexadecane is widely used by researchers to study bacterial hydrophobicity, failure to aerate hexadecane may account for the poor reproducibility of experiments using this hydrocarbon (Rosenberg *et al.*, 1980; Olsson and Westergren, 1982; Sweet *et al.*, 1987). However, at present there is insufficient evidence to confirm or refute the finding that a period of aeration is important in hydrophobicity assays and more studies are needed in this field.

4.4.2 Hydrophobicity results

This is the first report when a large number of *A.actinomycetemcomitans* isolates (33 strains) were examined for their hydrophobic activity. In general all the strains tested were hydrophobic as assessed by their ability to adsorb to xylene. These isolates exhibited an overall range of 39% to 82% reduction in the bacterial suspensions with an overall mean of 67%. Previous studies have only tested one or two Type strains (Gibbons and Etherden, 1983; Sweet *et al.*, 1986; Kozlovsky *et al.*, 1987). The first report was that of Gibbons and Etherden (1983) who studied *A.actinomycetemcomitans* ATCC 29523 and Y4. They found both of these strains to be hydrophilic as assessed by their inability to adsorb to hexadecane. Later Kozlovsky *et al.*, (1987) tested Y4 and concluded that it was hydrophobic as determined by its ability to adsorb to octane. The adherence of Y4 to octane varied between 60 and 90% depending on the medium used for cultivation, age of culture and the suspending medium (these factors are discussed in sections 4.4.3-4.4.5). In this study using xylene however, *A.actinomycetemcomitans* Y4 and

ATCC 29523 both exhibited moderately hydrophobic activities with a 46% and 56% reduction in optical density respectively. Sweet (1986) examined a single strain of *A.actinomycetemcomitans* (NCTC 9710) and found it to be highly hydrophobic (89% reduction in absorbance). This is in agreement with the findings of the present study where a level of 79% reduction was obtained for the same strain. Since both investigations used almost the same methodology, there is thus a good evidence that the method chosen to evaluated hydrophobicity was reproducible. In conclusion the findings of this study strongly confirm the earlier suggestions that *A.actinomycetemcomitans* is hydrophobic.

4.4.3 Effect of repeated subculturing on hydrophobicity

Continuous repeated subculturing for 50 times was found not to affect the hydrophobicity of *A.actinomycetemcomitans* (Table 4.4). Although only four strains were studied, there was little difference between the hydrophobicity of the two different colonial forms of *A.actinomycetemcomitans*. Nevertheless, one strain (Aa 31) was found to lose some of its hydrophobic activities after it had been repeatedly subcultured. A decrease of 17% in the absorbance of the bacterial suspension was obtained with the smooth variant compared with the value obtained with the rough colonial form. However, this change was not significant when compared to that obtained with the rough variant of the same isolate (Table 4.4).

Surprisingly there is no information in the literature concerning the effect of repeated subculture on hydrophobicity although there are some reports dealing with other oral microorganisms. Sweet *et al.*, (1986) failed to show any difference in hydrophobicity when 8 fresh and 8 Type oral bacterial species were compared. However, *A.actinomycetemcomitans* was not included in Sweet's comparison. In another study *Streptococcus mutans* was found to be less hydrophobic after repeated subculturing (Olsson and Westergren, 1982). However, this was subsequently shown to occur with serotype c strains only (Westergren and Olsson, 1983). In conclusion the hydrophobicity of *A.actinomycetemcomitans* appears to be generally unaffected by laboratory maintenance.

4.4.4 Suspending medium

In a previous report the hydrophobicity of *A.actinomycetemcomitans* was said to be affected by the type of buffer in which the bacteria was suspended (Kozlovsky *et al.*, 1987). In their investigation potassium/urea/magnesium (PUM) buffer gave the highest hydrophobicity results for *A.actinomycetemcomitans* Y4 compared with phosphate and PBS buffers. Kozlovsky *et al.*, (1987) did not statistically analyse their results, but using the published results, this was carried out as part of the present investigation. Analysis showed that their results were not significantly different as determined by t-Test. The three *A.actinomycetemcomitans* isolates examined in the present study appeared to be unaffected by the type of buffer used, ie. SIB and PBS (see Table 4.7). However, other researchers have demonstrated that suspending

medium may affect hydrophobicity of oral bacteria other than *A.actinomycetemcomitans* (Rogers, Pilowsky and Zilm, 1984; Sweet, 1986). Rogers *et al.*, (1984) found oral ***Streptococci*** to be more hydrophobic in PUM buffer compared to hydrophobicity in phosphate or Hepes buffer. Sweet (1986) on the other hand, showed that oral bacteria gave higher hydrophobic values when suspended in SIB than in PBS buffer. Rogers *et al.*, (1984) suggested that the main factor affecting hydrophobicity may be related to the ionic concentration of the buffers used, which can enhance the partitioning of the bacteria between phases. Increased salt concentration is known to cause the precipitation of proteins or bacteria according to their degree of hydrophobicity (Lindhal *et al.*, 1981). This would suggest that buffers with a higher ionic strength may yield higher bacterial hydrophobicity values. According to this assumption it would be expected that PBS with a higher ionic strength (0.284) than SIB (0.055) would produce higher bacterial hydrophobicity values (Sweet, 1986). However, this was not demonstrated in the case of *A.actinomycetemcomitans* when tested in the two buffers (PBS and SIB). Nevertheless, it may be of interest to examine the hydrophobicity of *A.actinomycetemcomitans* in other types of buffers.

4.4.5 Age of culture

The overall hydrophobicity results of *A.actinomycetemcomitans* after cultivation for 24 or 48 hours on CBA were not significantly different as compared by either Mann-Whitney U test or t-Test. Nonetheless, it has been suggested that the

age of bacteria may affect hydrophobicity. Kozlovsky *et al.*, (1987) found that *A.actinomycetemcomitans* Y4 grown for 24 hours in fluid thioglycollate broth or brain heart infusion agar gave the highest hydrophobic activities compared to the results obtained from cells cultured for 48 hours on the same media. These authors did not present any evidence that these differences were statistically significant. However, when Kozlovsky's data were analysed by the present author using a t-Test, the differences were found not to be significant. Thus on statistical grounds the results of this study with five fresh isolates of *A.actinomycetemcomitans* agree with the findings of Kozlovsky *et al.*, (1987).

4.4.6 Correlation between hydrophobicity and adherence

Recent studies have tended to directly relate bacterial adherence with hydrophobicity (Rosenberg *et al.*, 1981; Rosenberg *et al.*, 1983a). However, not all findings agree with the previous statement (Rosan, Eifert and Golub, 1985) and therefore, the relationship between the two phenomena is not entirely clear. Gibbons and Etherden (1983) have suggested that *A.actinomycetemcomitans* was hydrophilic since it failed to adsorb to hexadecane and only adhered in low numbers to hydroxyapatite. The adherence of other oral bacteria such as *Actinomyces viscosus, S.sanguis* and *Bacteroides gingivalis* to hydroxyapatite and the adherence of *Acinetobacter calcoaceticus* to buccal epithelial cells, correlated with their hydrophobic activities (Gibbons and Etherden, 1983; Rosenberg *et al.*, 1983a). However, there was no statistical

analysis carried out in these papers to confirm these observations.

The combined adherence results (Table 3.6) and hydrophobicity values (Table 4.1) obtained for the same 33 *A.actinomycetemcomitans* isolates investigated in this study are shown in Table 4.11. There was a clear significant overall correlation ($p \leq 0.05$) between adherence to BECs and hydrophobicity in xylene of *A.actinomycetemcomitans* (Spearman correlation coefficient 0.371). These results are similar with the findings of Sweet (1986) where a significant level of 0.536 ($p < 0.05$) was obtained for 18 pairs of different oral bacteria which did not include *A.actinomycetemcomitans.* As two distinct groups, the freshly isolated *A.actinomycetemcomitans* strains were found to adhere in higher numbers to buccal epithelial cells and showed higher hydrophobic activities compared to the Type cultures. However, these differences were found to be not significant.

Table 4.11 Comparison between *A.actinomycetemcomitans* adherence to buccal epithelial cells and hydrophobicity.

Bacterial strain number	Hydrophobicity % drop in abs. at 520 nm (±SEM)	Adherence No. bacteria/BEC (±SEM)
Aa 01	60 (1.2)	7.7 (2.7)
Aa 02	79 (2.3)	13.4 (0.8)
Aa 03	61 (0.9)	27.7 (2.4)
Aa 04	80 (2.5)	12.4 (1.7)
Aa 05	63 (8.1)	9.0 (3.5)
Aa 06	49 (2.0)	19.5 (4.2)
Aa 07	73 (5.2)	7.3 (1.3)
Aa 08	74 (6.9)	14.0 (2.8)
Aa 09	76 (0.9)	10.2 (1.0)
Aa 10	73 (0.3)	13.7 (0.8)
Aa 11	64 (3.6)	17.8 (1.5)
Aa 12	77 (2.3)	19.2 (1.6)
Aa 13	76 (4.6)	16.7 (0.8)
Aa 14	79 (2.6)	16.4 (0.9)
Aa 15	63 (1.3)	21.5 (2.2)
Aa 16	61 (4.7)	11.0 (1.4)

Table 4.11 Continued.

Bacterial strain number	Hydrophobicity % drop in abs. at 520 nm (±SEM)	Adherence No. bacteria/BEC (±SEM)
Aa 17	69 (4.0)	5.9 (1.4)
Aa 18	82 (5.3)	15.0 (1.1)
Aa 19	78 (5.4)	15.0 (1.4)
Aa 20	79 (5.2)	13.3 (1.5)
Aa 21	46 (0.9)	5.7 (1.6)
Aa 22	39 (2.1)	4.5 (o.6)
Aa 23	56 (5.0)	1.7 (0.5)
Aa 24	50 (2.7)	5.0 (0.9)
Aa 25	76 (1.8)	16.3 (1.6)
Aa 26	67 (3.2)	12.4 (3.5)
Aa 27	55 (4.2)	10.0 (0.7)
Aa 28	72 (3.0)	13.7 (1.2)
Aa 29	76 (3.8)	11.0 (2.0)
Aa 30	67 (3.5)	6.4 (1.3)
Aa 31	67 (5.2)	14.3 (2.1)
Aa 32	59 (1.0)	13.9 (1.1)
Aa 33	62 (2.6)	11.8 (0.8)

Comparison of hydrophobicity v adherence, $p = 0.03$

abs: absorbance

4.5 Conclusions:

The method used to determine the hydrophobicity of *A.actinomycetemcomitans* was simple, reproducible and rapid to perform. In addition, the use of xylene was of advantage since it was easily removed by aeration for four minutes.

All of the freshly isolated *A.actinomycetemcomitans* strains were hydrophobic when tested in SIB buffer. However, some of the Type strains appeared to be only moderately hydrophobic (especially the ATCC strains).

There were no overall significant differences between the hydrophobicity of fresh and Type strains of *A.actinomycetemcomitans*.

Factors such as repeated subculturing (50 times), type of buffer used and incubation time were found not to affect the hydrophobicity of *A.actinomycetemcomitans*.

A significant overall correlation was found between hydrophobicity and adherence to buccal epithelial cells by *A.actinomycetemcomitans* which may suggest that the adherence of this organism to oral surfaces *in vivo* can be influenced by hydrophobic forces.

CHAPTER 5

LEUCOTOXIC ACTIVITY OF *A.ACTINOMYCETEMCOMITANS*

5.1 Introduction

A.actinomycetemcomitans is a suspected pathogen in Localized Juvenile Periodontitis (LJP) (Reviewed by Zambon, 1985). This organism has been shown to produce several biologically active factors including a leucotoxin which kills human polymorphonuclear leucocytes (PMNLs), monocytes (Taichman *et al.*, 1984) and promyelocytic HL-60 cell line, *in vitro*. It has been suggested that PMNLs may play a defensive role in the normal and diseased gingival crevice (Miyasaki *et al.*, 1987). The mechanisms by which *A.actinomycetemcomitans* leucotoxin causes destruction of target cells is not completely understood, but recently the leucotoxin has been cloned (Kolodrubetz *et al.*, 1989; Lally *et al.*, 1989). Both leucotoxic and non-leucotoxic *A.actinomycetemcomitans* strains have been isolated from LJP or patients with other types of periodontal disease, eg. adult periodontitis, generalized juvenile periodontitis as well as healthy individuals (Zambon *et al.*, 1983c). This variation in toxicity has not been fully explained at the molecular gene level, nor is there any information to suggest that bacteriophages acts as mediators for leucotoxicity (Ohta *et al.*, 1987). Tsai and Taichman (1986) found that leucotoxic *A.actinomycetemcomitans* strains were isolated from younger patients (6-12 years of age) but not from older patients (13-25 years old).

This investigation was designed to examine the leucotoxicity of a number of freshly isolated and Type strains of *A.acti-*

nomycetemcomitans for human PMNLs obtained from a single donor with no history of periodontal disease and for a promyelocyte tissue cell line (HL-60) *in vitro*.

5.2 Materials and methods

5.2.1 Bacterial strains

Test strains of *A.actinomycetemcomitans* included 6 Type strains (NCTC 9709, 9710, 10979, 10980, and 10981), *A.actinomycetemcomitans* Y4 and twelve fresh isolates from patients attending Glasgow Dental Hospital and School (see Table 5.1). The bacterial suspension for the leucotoxic assay was prepared by growing the test bacteria in 20 ml NIH-Thioglycollate Broth (Difco, Detroit, Michigan, USA), (Appendix I) in an anaerobic chamber (Don Whitley, Scientific Ltd., West Yorkshire, England) with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 at 37°C for 48 hours. This medium and growth conditions were used so that results could be compared with previous reports (Zambon *et al.*, 1983c). Bacteria were harvested by centrifugation in a MSE High Speed 18 Centrifuge (MSE Ltd., Crawley, England) at 16000 g for 10 minutes and washed twice in PBS at 8000 g for 5 minutes. The collected pellet was then resuspended in Hanks Balanced Salt Solutions (HBSS) (Gibco-Europe, Paisley, Scotland), (Appendix II) and the optical densities were adjusted to 0.6-0.7 at 520 nm using a Pye Unicam SP 8-100 Spectrophotometer. The resultant suspension contained 2.0×10^9 bacteria/ml (see section 3.2.1).

Table 5.1 ***A.actinomycetemcomitans*** **strains used in the leucotoxicity studies.**

Strain number	Source	Site of Isolation	API 20 A Profile
Aa 01	NCTC 9709	Abscess (site not given)	4110404
Aa 02	NCTC 9710	Abscess (site not given)	4110405
Aa 03	NCTC 10979	Mandibular abscess	4150404
Aa 04	NCTC 10980	Blood	4110405
Aa 05	NCTC 10981	Neck abscess	4010505
Aa 07	GDH 310	Juvenile periodontitis	4110404
Aa 08	GDH 312	Chronic periodontitis	4110404
Aa 09	GDH 143	~ ~	4110404
Aa 10	GDH 216	~ ~	4110404
Aa 15	GDH 107	~ ~	4050404
Aa 16	GDH 87034	Prepubertal periodontitis	4050404
Aa 17	GDH 510/87	Chronic periodontitis	4050404
Aa 18	GDH 1014	Juvenile periodontitis	4110404
Aa 19	GDH 1212	~ ~	4110404
Aa 20	GDH 1214	Chronic periodontitis	4110404
Aa 21	GDH 1115	Juvenile periodontitis	4150404
Aa 39	GDH 1115	Chronic periodontitis	4110404
Aa 40	GDH 1224	Juvenile periodontitis	4110404

NCTC: National Collection of Type Cultures

ATCC: American Type Culture Collection

GDH: Glasgow Dental Hospital

5.2.2 Isolation of Polymorphonuclear leucocytes (PMNLs)

Human peripheral blood PMNLs were isolated from a healthy 30 year old male donor by collecting 10 ml venous blood in a sterile universal bottle containing one millilitre heparin at 100 units/ml (Leo Laboratories Ltd., Bucks., UK). This sample was then added to 30 ml of Hank's Balanced Salt Solution (HBSS) giving a dilution of one in 4. Twenty millilitres of this diluted blood sample was then carefully layered onto 4 ml of histopaque solution (Histopaque-1077, Sigma, St., Louis, USA) in a universal bottle and centrifuged at 600 g for 30 minutes at room temperature (MSE Centaur Centrifuge, Crawley, England). The supernatant was discarded and the pelleted cells were collected and suspended in 20 ml lysis buffer (Boyum, 1968, see Appendix II for constituents) and left at room temperature to lyse any blood cells other than PMNLs. Treatment with lysis buffer was repeated once more to make sure that very few contaminant cells remained in the PMNL suspension. The PMNLs in the lysis buffer were centrifuged at 180 g for 10 minutes, suspended and washed three times in PBS. Finally the cells were collected and resuspended in 2 ml HBSS after which they were counted using a haemocytometer and adjusted to give a concentration of 10^7 cells/ml. Before use, cells were stained with trypan blue to determine the percentage of viable and dead cells (see Section 5.2.5 for details). The PMNLs were used in the assay experiments when $\geq$ 90% of the cells were

designated viable as monitored by the trypan blue exclusion test.

5.2.3 Promyelocytic Leukaemia Cell Line (HL-60)

The HL-60 cell line was originally established from the peripheral blood of a patient with acute promyelocytic leukaemia (Collins, Gallo and Gallagher, 1977). These cells develop along the myelocytic lineage and in culture maintain many enzymatic and cell surface characteristics of mature granulocytes (Gallagher *et al.*, 1979). The HL-60 cell line was obtained from the European Collection of Animal Cell Cultures (PHLS Centre, Salisbury, England). These cells were cultured in 50 ml Nunc tissue culture cell flasks (Inter Med, Denmark) using RPMI 1640 medium with glutamine (Gibco-Europe, Paisley, Scotland), (Appendix I) supplemented with 200 µg/ml gentamicin, 2.5 µg/ml fungizone (both supplied by Gibco, Europe, Paisley, Scotland) and 10% sterile foetal calf serum (Globepharm Ltd., Surrey, UK). Cultures were maintained at 37°C in a humified CO_2 incubator (LEEC, Ltd., Nottingham, England) containing an atmosphere of 5% CO_2 and 95% air. The cells do not attach to surfaces and so grow in the fluid phase of the medium. Subcultures were performed twice weekly as described below. Since most of the HL-60 cells remained at the bottom of the tissue culture flask, it was possible to discard the old medium by tilting the flask and leaving 2 to 3 ml of cells. Thirty to forty millilitres of the freshly prepared RPMI 1640 medium was then added to the cells and incubated as before. In preparation for the leucotoxic assay, the cells were collected in universal bot-

tles and centrifuged at 180 g for 10 minutes. The supernatant was discarded and the pelleted cells were collected and suspended in 5 ml PBS and washed twice by centrifugation at 180 g (MSE Centaur 2, Crawley England) for 5 minutes. Finally the cells were resuspended in 2 ml HBSS and cell viability was determined by trypan blue staining (see method below). The concentration of the HL-60 cells was adjusted to give 10^7 cells/ml using HBSS. The HL-60 cells were only used when more than 75% of cells were viable.

5.2.4 Leucotoxicity assay procedure

The assay was performed by adding 0.5 ml of the test bacteria containing 2.0 x 10^9/ml to 0.5 ml of human PMNLs or HL-60 (both at a concentration of 10^7/ml) in bijou bottles, thus producing a ratio of approximately 200 bacteria per target cell. A number of controls were included in each leucotoxicity assay as described in Table 5.2. All tubes were incubated in a shaking water bath (HETO, Denmark) at 37°C for 1, 2 or 7 hours depending on the experiment being performed. Target cells (ie. PMNLs or HL-60 cells) were incubated with bacteria for up to 7 hours because a number of *A.actinomycetemcomitans* strains failed to kill the target cells when incubated for one hour in preliminary experiments. Death of target cells was measured using two different methods namely the trypan blue exclusion and lactate dehydrogenase release (LDH) methods.

Table 5.2 The constituents of the test and control tubes in the leucotoxic assays.

Test: 0.5 ml PMNLs or HL-60 cells + 0.5 ml test bacteria
Control (a): 0.5 ml PMNLs or HL-60 cells + 0.5 ml HBSS
Control (b): 0.5 ml PMNLs or HL-60 cells + 0.5 ml of a 0.2% solution of Triton-X 100 (TX-100)
Control (c): 0.5 ml bacteria + 0.5 ml HBSS
Control (d): 0.5 ml of a 0.2% solution of Triton-X 100 + 0.5 ml HBSS

HBSS = Hanks Balanced Salt Solutions (see appendix II)

(TX-100): Triton-X 100

Controls (a, b, c and d) were employed in the lactate dehydrogenase release assays.

Control (a) only was employed in the trypan blue exclusion method.

5.2.5 Measurement of cell death by the trypan blue exclusion method

The viability of both PMNLs and HL-60 cells was assessed by the trypan blue (BDH, Laboratory Chemicals, Poole, England) exclusion test (Baehni *et al.*, 1979). At the end of the incubation period the tubes containing the assay mixture were removed from the water bath and placed in a vessel containing ice to limit any further leucotoxic activity of *A.actinomycetemcomitans*. Filter sterilized trypan blue (0.1 ml of a 0.4% solution) in HBSS was added to 0.1 ml of the test or control mixtures in bijou bottles and stained for 5 minutes at room temperature. The number of cells which were stained by trypan blue (dead cells) or remained dye free (viable cells) (Figures 5.1a, 5.1b) were counted in a haemocytometer using microscopical examination at 250 times magnification (Olympus Optical CO LTD., Japan). A minimum total of 100 target cells were counted and the percentages of dead cells was calculated using the formula below. Any bacterial strain causing greater than 25% kill of target cells within 60 minutes exposure was regarded as leucotoxic.

$$\% \text{ Dead Cells} = \frac{\text{Total No. of cells counted - No. of cells stained}}{\text{Total No. of cells counted}} \times 100$$

5.2.6 Measurement of cell death by the Lactate dehydrogenase method (LDH)

Lactate dehydrogenase (LDH) release was also used to measure the damage which occurred to both PMNLs and HL-60 cells after they have been exposed to *A.actinomycetemcomitans*. The technique described by Zambon *et al.*, (1983c) was used

in this investigation. The release of LDH is used to monitor cytotoxicity, and is considered to be directly proportional to the percentage of total cellular LDH released (Zambon *et al.*, 1983c). After the end of the incubation period the test and control bottles were removed from the water bath and placed on ice for 5 minutes to inhibit further toxic activity. All test and control samples were centrifuged at 4°C using an MSE High Speed 18 centrifuge at 1200g for 10 minutes. Supernatants from the test and control were removed and a series of two-fold dilution was made in HBSS from neat to 1/128. Aliquots (50 µl) were removed from each dilution and added to a fresh bijou bottle. The LDH detection system was prepared by adding 1 ml of pyruvate substrate (Sigma Kit No. 500 L-1) (Sigma, Diagnostics, St., Louis, USA) to a vial containing 1 mg ß-Nicotinamide adenine dinucleotide (Sigma) and preheated in a water bath at 37°C for 5 minutes. Aliquots (0.1 ml) of this solution were added to each bottle containing the supernatant of both test and control samples which were then incubated in a shaking water bath at 37°C for 15 minutes. Bottles were then removed and 100 µl of a Sigma colour reagent containing 2,4-dinitrophenyl-hydrazine in 1 N-hydrochloric acid were added and the bottles held at room temperature for 20 minutes. A reference blank was included with every set of experiments by adding 0.1 ml of the buffer substrate to 0.1 ml of the coloured reagent and treated as other samples. Finally a one ml volume of 0.4 M NaOH was added to all bottles and after rapid mixing the optical densities were read against a

water blank at 450 nm using a Pye Unicam SP 8-100 spectrophotometer (Pye Unicam Cambridge, England).

5.2.7 Quantitation of LDH release

The optical density readings were plotted against dilutions (for example see Figure 5.2) and the percentage LDH release was determined using the data obtained from the plotted graphs. The optical density reading of the 'reference blank' containing pyruvate and the Sigma colour reagent given above, was taken as the maximum amount of substrate present in each assay. Leucotoxic assays were employed to determine the amount necessary to reduce about 50% of the pyruvate used in the standard assay (ie. reference blank). In this region, activity is proportional to dilution (Zambon *et al.*, 1983c). Lysis of cells by Triton-X100 (Sigma, Diagnostics, St., Louis, USA) is used to indicate the total cellular release of LDH from target cells. The dilution of Triton-X100 required to produce a 50% reduction of the pyruvate used in the standard assay was then compared to the dilution of the test bacteria at the 50% point on the graph. Experiments were repeated on three different occasion and the mean values were calculated. A minimum level of 15% LDH release after one hour was considered as a positive leucotoxic effect by *A.actinomycetemcomitans*.

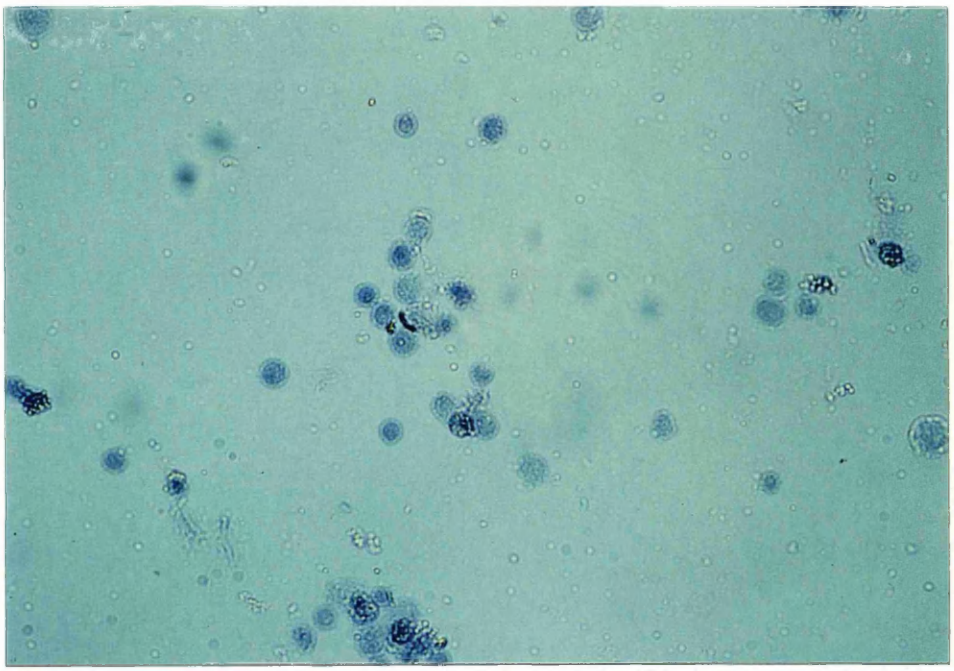

Figure 5.1a, HL-60 cells stained by trypan blue after 60 minutes exposure to whole cells of *A.actinomycetemcomitans* (Aa 40).

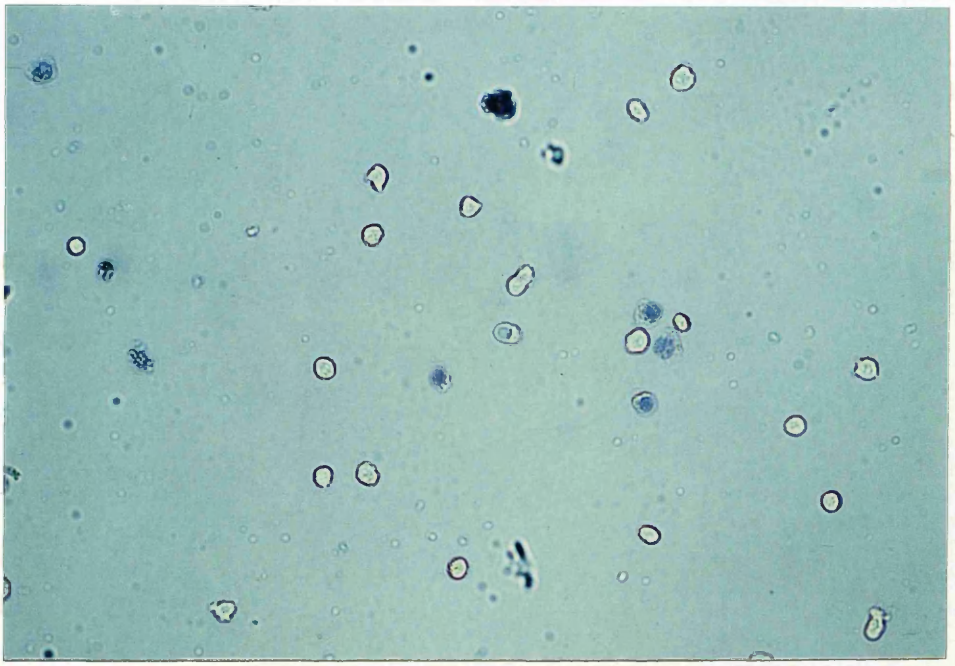

Figure 5.1b, HL-60 cells stained by trypan blue after 60 minutes incubation in Hanks Balanced Salt Solutions buffer.

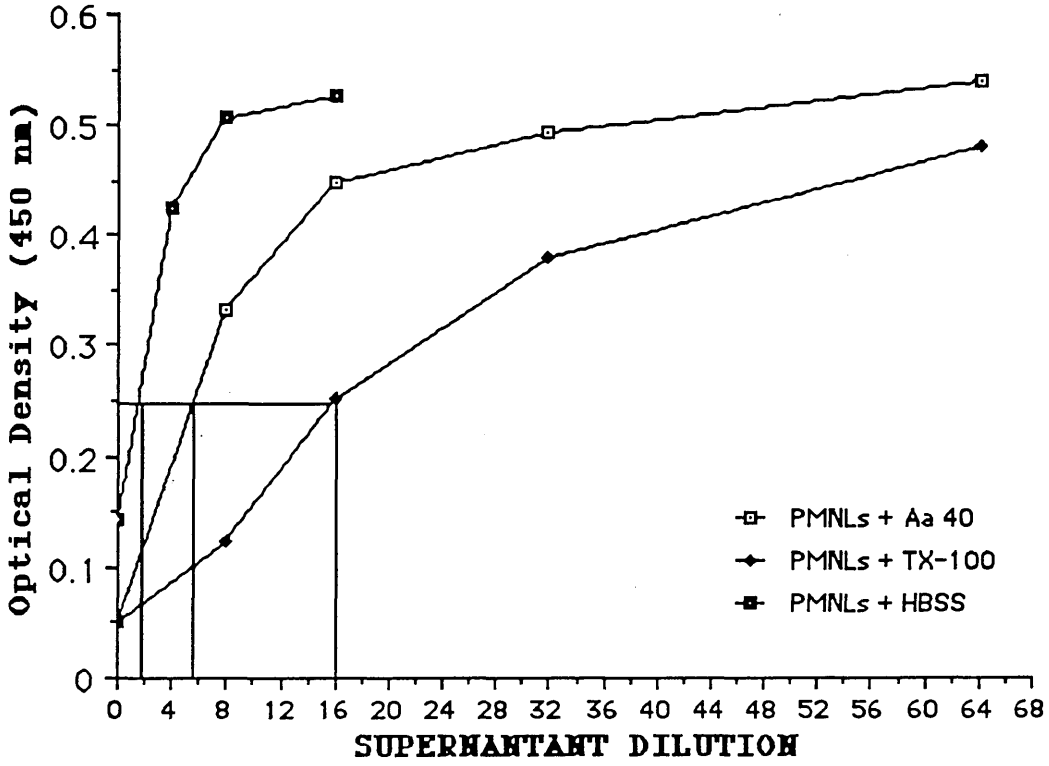

Figure 5.2 An example of a graph used for measuring lactate dehydrogenase release from PMNLs using whole cells of Aa 40 (GDH 1224). The optical density reading of the pyruvate substrate (reference blank not shown) was 0.6 at 450 nm.

In Figure 5.2 shown above, the dilutions required to reduce 50% of the pyruvate used in the leucotoxicity assay were as follows:

100% LDH release is equivalent to a dilution of 1:16 (ie 50% reduction of pyruvate) in the presence of Triton-X100

Aa 40 + PMNLs (Test) at dilution of 1:6 = 37.5% LDH release

PMNLs + HBSS (Control) at dilution of 1:2 = 12.5% LDH release

Therefore, 37.5 - 12.5 = 25% LDH release for Aa 40

5.3 Results:

5.3.1 Leucotoxic activity as measured by trypan blue exclusion of five Type strains of *A.actinomycetemcomitans* for HL-60 cells after 7 hours exposure

The leucotoxic activity of five Type strains of *A.actinomycetemcomitans* for HL-60 cells was determined using the trypan blue exclusion test (Tables 5.3 and 5.4). In these experiments the HL-60 cells were exposed to 200 bacteria per target cell in HBSS for up to 7 hours. The percentage kill of target cells was calculated as described in section 5.2.5 and the percentage killed in the presence of HBSS alone (negative control) was then subtracted from all experiments were the test bacteria were used. None of the *A.actinomycetemcomitans* strains showed any detectable toxic activity against the HL-60 cells after 2 and 3 hours incubation. However, as the incubation time was extended up to 5 then 7 hours, a modest increase in cell death compared to controls occurred with all five *A.actinomycetemcomitans* strains. The highest percentage kill of 22% of HL-60 cells was obtained with Aa 05, while the other tested strains gave similar results and produced percentage kill levels of 13% to 19% (Figure 5.3). Thus, despite the long incubation time of HL-60 cells with *A.actinomycetemcomitans*, only a small number of tissue culture cells were killed and according to the criteria used in this study all of the tested strains are designated non-leucotoxic.

Table 5.3 Leucotoxic activity of five Type strains of *A.actinomycetemcomitans* for HL-60 cells at zero time (the data represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live HL-60 cells No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		13 (4.4)	109 (9.8)	11 %	89 %
Aa 01	NCTC 9709	14 (4.4)	122 (6.7)	10 %	90 %
Aa 02	NCTC 9710	16 (2.0)	105 (12.3)	13 %	87 %
Aa 03	NCTC 10979	11 (2.3)	114 (11.0)	9 %	91 %
Aa 04	NCTC 10980	11 (4.3)	108 (6.1)	9 %	91 %
Aa 05	NCTC 10981	10 (3.4)	108 (12.0)	9 %	91 %

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean

Table 5.4 Leucotoxic activity of five Type strains of *A.actinomycetemcomitans* for HL-60 cells after 7 hours exposure (the data represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live HL-60 cells No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		41 (7.9)	95 (7.5)	30 %	70 %
Aa 01	NCTC 9709	49 (4.6)	59 (3.5)	45 %	55 %
Aa 02	NCTC 9710	57 (8.4)	60 (9.9)	49 %	51 %
Aa 03	NCTC 10979	61 (8.5)	63 (12.0)	49 %	51 %
Aa 04	NCTC 10980	52 (4.5)	68 (3.7)	43 %	57 %
Aa 05	NCTC 10981	66 (5.2)	61 (1.0)	52 %	48 %

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean
No.L: Number of Live; No.D: Number of dead

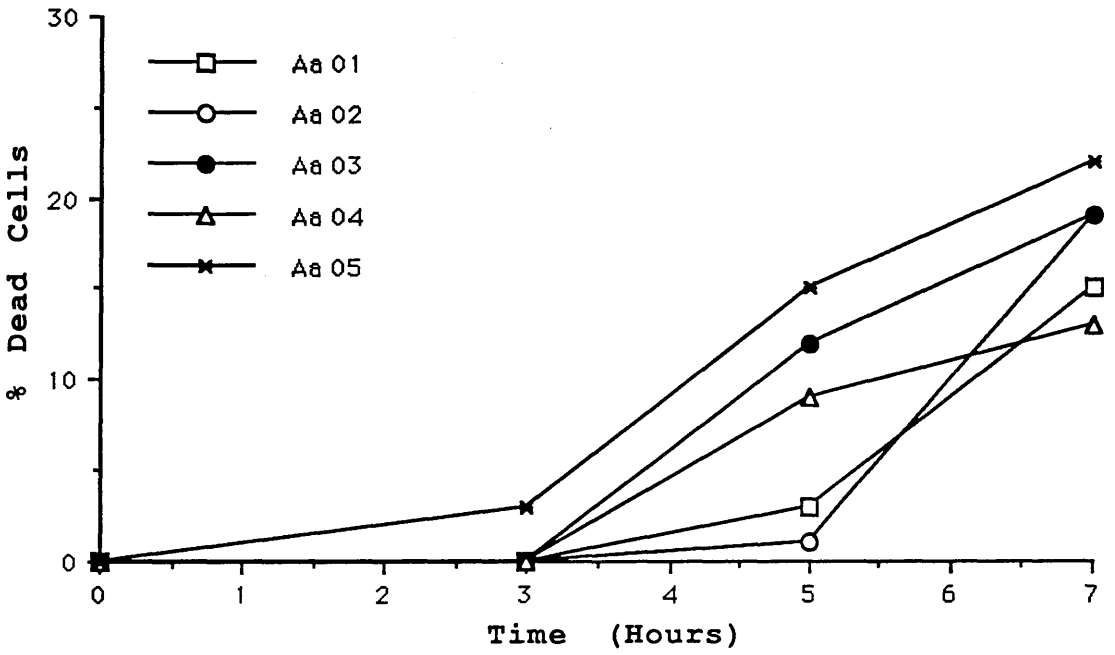

Figure 5.3 Leucotoxic activity of five Type strains of *A.actinomycetemcomitans* for HL-60 cells over a period of 7 hours using the trypan blue exclusion method (figures represent the mean of three different experiments after subtracting the % dead cells in control assays).

5.3.2 Leucotoxic activity as measured by trypan blue exclusion of five Type strains of *A.actinomycetemcomitans* for PMNLs after 7 hours exposure

The results of these experiments are shown in Tables 5.5 and 5.6. When the *A.actinomycetemcomitans* strains were examined for their leucotoxic activity using PMNLs over a period of seven hours, *A.actinomycetemcomitans* killed fewer PMNLs compared to the percentage kill of HL-60 cells. As shown in Tables 5.6 and 5.7 the highest percentage kill recorded was only 8% after 7 hours incubation and the lowest being 3%. In conclusion, as shown in Table 5.7, none of the five Type strains of *A.actinomycetemcomitans* was toxic for either HL-60 cells or PMNLs *in vitro.*

5.3.3 Leucotoxic activity as measured by trypan blue exclusion of 12 GDH-fresh isolates of *A.actinomycetemcomitans* against HL-60 cells

In this investigation 12 freshly isolated *A.actinomycetemcomitans* strains were examined for their leucotoxic activity against HL-60 cells *in vitro.* All experiments were repeated on three different occasions for a maximum of two hours using a bacterial/HL-60 cell ratio of 200:1. Leucotoxicity was assessed by trypan blue exclusion method as described in Section 5.2.5. Despite the two hour incubation period employed only one (Aa 40) of the 12 *A.actinomycetemcomitans* strains tested showed any toxic activity (Tables 5.8-5.11) as compared to control HL-60 cells incubated in HBSS alone. The leucotoxic effect of Aa 40 increased only slightly with the time of incubation; 33% of cells were killed at 60 minutes and 42% at 120 minutes.

Table 5.5 Leucotoxic activity of five Type strains of *A.actinomycetemcomitans* for PMNLs at zero time (the data represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live PMNLs No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		7 (0.7)	121 (2.3)	6 %	94 %
Aa 01	NCTC 9709	7 (1.3)	118 (3.7)	6 %	94 %
Aa 02	NCTC 9710	8 (1.8)	108 (4.3)	7 %	93 %
Aa 03	NCTC 10979	8 (2.3)	106 (5.2)	7 %	93 %
Aa 04	NCTC 10980	10 (1.2)	100 (2.9)	9 %	91 %
Aa 05	NCTC 10981	7 (2.2)	101 (4.0)	7 %	93 %

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean

Table 5.6 Leucotoxic activity of five Type strains of *A.actinomycetemcomitans* for PMNLs after 7 hours exposure (the data represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live PMNLs No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		5 (2.6)	100 (4.0)	5 %	95 %
Aa 01	NCTC 9709	13 (4.3)	105 (8.1)	11 %	89 %
Aa 02	NCTC 9710	13 (4.9)	118 (7.7)	10 %	90 %
Aa 03	NCTC 10979	9 (1.8)	105 (3.7)	8 %	92 %
Aa 04	NCTC 10980	17 (2.5)	112 (7.9)	13 %	87 %
Aa 05	NCTC 10981	14 (4.4)	102 (8.2)	12 %	88 %

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean

Table 5.7 Summary of the leucotoxic activity on both HL-60 cells and PMNLs after 7 hours exposure to five Type strains of *A.actinomycetemcomitans*.

Strain number	Source	PERCENTAGE of DEAD CELLS* HL-60 cells 0 time	HL-60 cells 7 hours	PMNLs 0 time	PMNLs 7 hours
Aa 01	NCTC 9709	0 %	15 %	0 %	6 %
Aa 02	NCTC 9710	2 %	19 %	1 %	5 %
Aa 03	NCTC 10979	0 %	19 %	1 %	3 %
Aa 04	NCTC 10980	0 %	13 %	3 %	8 %
Aa 05	NCTC 10981	0 %	22 %	1 %	7 %

* : All figures were obtained after subtracting the % death of control HL-60 cells incubated in HBSS alone.

Table 5.8 Leucotoxic activity of 12 GDH strains of *A.actinomycetemcomitans* for HL-60 cells at Zero time (figures represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live HL-60 cells No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		26 (4.4)	96 (4.3)	21	79
Aa 07	GDH 310	33 (5.3)	93 (6.7)	26	74
Aa 08	GDH 312	28 (4.9)	101 (4.5)	22	78
Aa 09	GDH 143	28 (0.3)	100 (4.8)	21	79
Aa 10	GDH 216	28 (5.3)	98 (3.2)	22	78
Aa 15	GDH 107	28 (1.5)	106 (8.5)	21	79
Aa 16	GDH 87034	30 (2.0)	97 (6.6)	24	76
Aa 17	GDH 510/87	28 (5.2)	97 (3.3)	22	78
Aa 18	GDH 1014	27 (1.3)	97 (3.0)	22	78
Aa 19	GDH 1212	25 (0.9)	94 (3.8)	21	79
Aa 20	GDH 1214	34 (3.2)	105 (2.3)	24	76
Aa 39	GDH 1115	28 (3.5)	90 (9.0)	24	76
Aa 40	GDH 1224	25 (1.7)	97 (4.7)	21	79

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean

No.D: Number of dead

No.L: Number of Live

Table 5.9 Leucotoxic activity of 12 GDH strains of *A.actinomycetemcomitans* for HL-60 cells after 60 minutes exposure (figures represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live HL-60 cells No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		26 (4.0)	103 (4.5)	20	80
Aa 07	GDH 310	34 (6.4)	96 (5.8)	26	74
Aa 08	GDH 312	34 (5.8)	95 (3.2)	26	74
Aa 09	GDH 143	27 (1.5)	100 (9.0)	21	79
Aa 10	GDH 216	29 (2.3)	100 (11.0)	22	78
Aa 15	GDH 107	24 (5.0)	105 (2.0)	19	81
Aa 16	GDH 87034	30 (8.2)	99 (12.0)	23	77
Aa 17	GDH 510/87	33 (6.5)	95 (9.9)	26	74
Aa 18	GDH 1014	30 (5.6)	98 (5.7)	23	77
Aa 19	GDH 1212	26 (2.2)	97 (7.6)	21	79
Aa 20	GDH 1214	31 (5.9)	97 (1.2)	24	76
Aa 39	GDH 1115	23 (5.0)	94 (13.0)	20	80
Aa 40	**GDH 1224**	**69 (4.9)**	**61 (9.7)**	**53**	**47**

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean

No.D: Number of dead

No.L: Number of Live

Table 5.10 Leucotoxic activity of 12 GDH strains of *A.actinomycetemcomitans* for HL-60 cells after 120 minutes exposure (figures represent the mean of three different experiments).

Strain number	Source	Number and % of Dead/Live HL-60 cells No.D. (±SEM)	No.L. (±SEM)	% Dead	% Live
Bacteria free control		23 (2.3)	102 (3.8)	20	80
Aa 07	GDH 310	27 (1.0)	99 (2.7)	21	79
Aa 08	GDH 312	26 (2.5)	95 (7.4)	22	78
Aa 09	GDH 143	30 (1.7)	95 (7.0)	24	76
Aa 10	GDH 216	27 (2.3)	103 (5.3)	21	79
Aa 15	GDH 107	27 (4.0)	107 (4.5)	20	80
Aa 16	GDH 87034	23 (5.5)	106 (12.0)	18	82
Aa 17	GDH 510/87	26 (4.8)	98 (4.9)	21	79
Aa 18	GDH 1014	20 (3.6)	101 (8.5)	17	83
Aa 19	GDH 1212	28 (7.6)	101 (3.8)	22	78
Aa 20	GDH 1214	29 (4.9)	97 (5.1)	23	77
Aa 39	GDH 1115	26 (8.5)	98 (13.0)	21	79
Aa 40	**GDH 1224**	**76 (5.9)**	**47 (2.6)**	**62**	**38**

Bacteria free control: HL-60 cells incubated in HBSS buffer alone

±SEM: Standard error of mean

No.D: Number of dead

No.L: Number of Live

Table 5.11 Summary of the leucotoxic activity of 12 GDH *A.actinomycetemcomitans* strains for HL-60 cells.

Strain number	Source	Mean Percentage Death of HL-60 cells* Zero time	60 minutes	120 minutes
Aa 07	GDH 310	5	6	1
Aa 08	GDH 312	1	6	2
Aa 09	GDH 143	0	1	4
Aa 10	GDH 216	1	2	1
Aa 15	GDH 107	0	0	0
Aa 16	GDH 87034	3	3	0
Aa 17	GDH 510/87	1	6	1
Aa 18	GDH 1014	1	3	0
Aa 19	GDH 1212	0	1	2
Aa 20	GDH 1214	3	4	3
Aa 39	GDH 1115	3	0	1
Aa 40	**GDH 1224**	**0**	**33**	**42**

* : All figures were obtained after subtracting the % death of control HL-60 cells incubated in HBSS alone.

5.3.4 Leucotoxic activity of five strains of *A.actinomycetemcomitans* for HL-60 cells as determined by both trypan blue and LDH release

In this investigation five *A.actinomycetemcomitans* strains (Aa 03, 09, 16, 21 and Aa 40) were tested *in vitro* for their leucotoxic activity for HL-60 cells. Target cells were exposed to 200 *A.actinomycetemcomitans* per cell for 60 minutes and leucotoxicity was assessed by trypan blue and LDH release. As shown in Table 5.12 an average mean of 0.5 to 3.2% kill was obtained with *A.actinomycetemcomitans* strains 3, 9, 16 and 21, and all were designated as non-leucotoxic. However, Aa 40 killed 73% of HL-60 cells after 60 minutes exposure and was accepted as leucotoxic (Table 5.12).

The percentage of LDH released from HL-60 in the presence of the test bacteria is shown in Table 5.13. An average mean of 2.2% to 4.6% LDH release were obtained for Aa 03, 9, 16 and 21, while Aa 40 produced a 50% LDH release, compared to that obtained with Triton X-100 (total LDH release). Thus using the criteria used in this study for designating leucotoxicity, only Aa 40 possess leucotoxic activity using either trypan blue or LDH release methods.

Table 5.12 Leucotoxic activity of five strains of *A.actinomycetemcomitans* for HL-60 cells after 60 minutes exposure as determined by trypan blue exclusion method.

Bacterial Strain	Source	Mean % Death of HL-60* Experiment Number 1	2	3	Mean	(±SEM)
Aa 03	NCTC 10979	6	0	3.5	3.2	(1.7)
Aa 09	GDH 143	2	2	0.0	1.3	(0.7)
Aa 16	GDH 87034	0	1.5	0.0	0.5	(0.5)
Aa 21	AA Y4	2.5	2.0	0.0	1.5	(0.8)
Aa 40	**GDH 1224**	**78**	**69**	**71**	**73**	**(2.7)**

±SEM: Standard error of mean

* : All figures were obtained after subtracting the % death of control HL-60 cells incubated in HBSS alone.

Table 5.13 Leucotoxic activity of five strains of *A.actinomycetemcomitans* for HL-60 cells after 60 minutes exposure as determined by lactate dehydrogenase release method.

Bacterial Strain	Source	Mean % LDH Release from HL-60 cells* Experiment Number 1	2	3	Mean	(±SEM)
Aa 03	NCTC 10979	2.5	2	2	2.2	(0.2)
Aa 09	GDH 143	5	3	6	4.6	(0.9)
Aa 16	GDH 87034	5	6	2.8	4.6	(0.9)
Aa 21	AA Y4	5	4.7	2.8	4.2	(0.7)
Aa 40	**GDH 1224**	**51**	**49**	**50**	**50**	**(0.6)**

±SEM: Standard error of mean

* : All figures were obtained after subtracting the % death of control HL-60 cells incubated in HBSS alone.

5.3.5 Leucotoxic activity of five strains of *A.actinomycetemcomitans* for PMNLs as determined by both trypan blue and LDH release

The experiments which were carried out using HL-60 as the target cells were repeated using PMNLs and leucotoxicity was determined using both the trypan blue exclusion method and LDH release. Four of the *A.actinomycetemcomitans* strains (Aa 03, 9, 16 and 21) failed to kill target cells (PMNLs), as shown in Table 5.14. An average mean of 0.3% to 5.3% kill was recorded for the four *A.actinomycetemcomitans* strains tested. However, Aa 40 killed 29.3% of PMNLs used in the assay, being the only fresh isolate showing leucotoxic activity.

Further confirmation of these early negative results were obtained from the LDH release experiments where only a small percentage LDH release was obtained with the four strains (Aa 03, 09, 16, and Aa 21). An average mean of 0% to 4.2% LDH release was obtained for these strains (Table 5.15). However, Aa 40 caused 25% LDH release from PMNLs *in vitro*.

In conclusion, Aa 40 was the only *A.actinomycetemcomitans* strain which possessed leucotoxic activity against both HL-60 cells and human PMNLs as determined by both the trypan blue exclusion method and LDH release (Table 5.16). There was no LDH release from any of the bacteria incubated in HBSS or in the Triton X-100 incubated in HBSS alone.

Table 5.14 Leucotoxic activity of five strains of *A.actinomycetemcomitans* for PMNLs after 60 minutes exposure as determined by trypan blue exclusion method.

Bacterial Strain	Source	Mean % Death of PMNLs* Experiment Number 1	2	3	Mean (±SEM)
Aa 03	NCTC 10979	0	0	2	0.7 (0.7)
Aa 09	GDH 143	0	0	1	0.3 (0.3)
Aa 16	GDH 87034	5	4	7	5.3 (0.9)
Aa 21	AA Y4	0	0	4	1.3 (1.3)
Aa 40	**GDH 1224**	**31**	**29**	**28**	**29.3 (0.9)**

±SEM: Standard error of mean

* : All figures were obtained after subtracting the % death of control PMNLs incubated in HBSS alone.

Table 5.15 Leucotoxic activity of five strains of *A.actinomycetemcomitans* for PMNLs after 60 minutes exposure as determined by lactate dehydrogenase release.

Bacterial Strain	Source	Mean % LDH Release from PMNLs* Experiment Number 1	2	3	Mean (±SEM)
Aa 03	NCTC 10979	3.2	1	7.7	4.0 (2.0)
Aa 09	GDH 143	7.6	3	2	4.2 (1.7)
Aa 16	GDH 87034	0	0	0	0 (0)
Aa 21	AA Y4	7	2	2.5	3.8 (1.6)
Aa 40	**GDH 1224**	**24**	**26**	**25**	**25 (0.6)**

±SEM: Standard error of mean

* : All figures were obtained after subtracting the % death of control PMNLs incubated in HBSS alone.

Table 5.16 Summary of leucotoxic activity of five strains of *A.actinomycetemcomitans* for PMNLs and HL-60 cells after 60 minutes exposure as determined by both lactate dehydrogenase release and trypan blue exclusion.

Bacterial Strain	Source	Trypan Blue (% Death) HL-60	Trypan Blue (% Death) PMNLs	% LDH Release HL-60	% LDH Release PMNLs
Aa 03	NCTC 10979	3.2	0.7	2.2	4.0
Aa 09	GDH 143	1.3	0.3	4.6	4.2
Aa 16	GDH 87034	0.5	5.3	4.6	0
Aa 21	AA Y4	1.5	1.3	4.2	3.8
Aa 40	**GDH 1224**	**73**	**29**	**50**	**25**

5.4 Discussion:

5.4.1 Experimental methods

The common methods which have been used to determine the leucotoxicity of *A.actinomycetemcomitans* are the exclusion of trypan blue from viable cells, lactate dehydrogenase release and ^{51}Cr release from prelabelled cells (Tsai *et al.*, 1979; Taichman and Wilton, 1981; Taichman *et al.*, 1987a; Simpson *et al.*, 1988). Other techniques less widely used include measurement of lysosomal constituents release, and electron microscopic examination of target cells (Baehni *et al.*, 1979; Taichman *et al.*, 1980).

The trypan blue method is simple and rapid to perform, and thus can be used to monitor leucotoxicity of a wide range of bacterial strains at any given time. The sensitivity of the method under the conditions used in this thesis is unknown and although the technique has been used by other workers no information about the sensitivity of PMNLs or HL-60 cells to trypan blue after exposure to *A.actinomycetemcomitans* has been reported. It has been suggested that if host cells are suspended in a protein rich medium with high affinity for trypan blue, this may give rise to false positive results, because of protein-dye complexes attached to the surface of viable cells. In particular serum at a concentration of 5% may cause errors with trypan blue (Paul, 1975). Although HL-60 cells were cultured in a medium containing 10% serum, the cells were washed twice in PBS, before use. Furthermore, all experiments used in this investigation were serum

free, and therefore errors due to serum rich conditions are very unlikely.

The lactate dehydrogenase method used in this investigation to determine the leucotoxicity of *A.actinomycetemcomitans* for HL-60 cells and human PMNLs was first described by Zambon *et al.*, (1983c). The method depends on the LDH released from injured target cells to catalyse the reduction of pyruvate to lactate, and results are expressed as "percentage release" of LDH. As in the case of trypan blue there is no information about the sensitivity of this technique and its suitability in determining the toxicity of bacteria such as *A.actinomycetemcomitans*. Nonetheless, the technique proved to be reproducible and was in agreement with the trypan blue exclusion method. However, it was more difficult to perform and more time consuming compared to the dye exclusion method.

5.4.2 Factors affecting the leucotoxicity of *A.actinomycetemcomitans*

Although there were 18 strains of *A.actinomycetemcomitans* (6 Type strains plus 12 fresh isolates) included in this study, only one of the freshly isolated *A.actinomycetemcomitans* was found to be leucotoxic according to the criteria used in this thesis. These results were not expected because a number of the Type strains tested (ie. Y4 & ATCC 29522) have been shown to posses leucotoxic activity by previous researchers (Baehni *et al.*, 1981; Zambon *et al.*, 1983c). The fac-

tors which may be responsible for the negative results obtained, will be discussed separately.

Effect of media

The effect of media on the leucotoxic activity of *A.actinomycetemcomitans* has not been previously investigated. However, thioglycollate broth medium (Difco) under anaerobic conditions has been the most widely used medium for culturing *A.actinomycetemcomitans* for leucotoxic studies (see Table 5.17 for details). Since the same medium and culture conditions as described by previous workers, were used in the present investigation, it was hard to argue that failure to demonstrate leucotoxicity in certain Type strains of *A.actinomycetemcomitans* was due to the use of different growth conditions. However, it has been suggested that media may affect the leucotoxic activity of *A.actinomycetemcomitans*. Baehni *et al.*, (1979) reported that *A.actinomycetemcomitans* Y4 was non-leucotoxic when grown on trypticase soy agar, but was later found to be toxic when grown in thioglycollate broth medium (TGB). However, there was no strong scientific evidence to support this observation and it should be regarded as a personal view. Other media such as Peptone Yeast Extract broth (Taichman *et al.*, 1987a) or Lombard-Dowell broth (Kalmar, Arnold and Van Dyke, 1987) have been used to culture Y4 prior to leucotoxin assays which proved to be positive.

In preliminary experiments carried out in the present investigation, *A.actinomycetemcomitans* Y4 and NCTC 10979 which

have been reported previously as leucotoxin positive, were grown in this study on Columbia blood agar, Anaerobic Blood Broth (ABB) and thioglycollate broth (TGB-Difco) in 5% CO_2 in air. Bacterial suspensions made from these different media failed to show any strong evidence of leucotoxic activity for HL-60 cells after 2 hours exposure. These experiments were only carried out once, and therefore, it is impossible to reach any definite conclusions. Since there is little or no scientific data on the effect of media on leucotoxin production by *A.actinomycetemcomitans,* specific studies are required to clarify this area.

Effect of bacterial concentration

A.actinomycetemcomitans has been used in leucotoxic assays as either whole cells (Baehni *et al.*, 1979; Baehni *et al.*, 1981; Zambon *et al.*, 1983c; Miyasaki *et al.*, 1986a; Tsai and Taichman, 1986; Chung *et al.*, 1989), sonic extracts of whole cells (Tsai *et al.*, 1979; Taichman and Wilton, 1981; Ohta *et al.*, 1987) or as purified toxin (Simpson *et al.*, 1988).

Whole cells

Although, HL-60 cells have been used as target cells in leucotoxicity studies, there is no information on the effect of different concentrations of whole cells of *A.actinomycetemcomitans* on the viability of these cells using the trypan blue exclusion method. However, using LDH release, Zambon *et al.*, (1983c) have reported that increasing the concentration of whole cells of Y4 using optical density at 540 nm from 0.3, 0.6 to 1.0, resulted in an increase in the rel-

ease of LDH from approximately 12% to 17% and 26% respectively.

In the case of human PMNLs, the lowest number of *A.actinomycetemcomitans* cells used by researchers was 5 bacteria, the maximum number being 200 bacteria per target cell (see Table 5.17). Baehni *et al.*, (1979) showed that more PMNLs were killed as the number of bacteria was increased from 50 to 200. The percentage kill of PMNLs increased from 85% to 95% and 96% in the presence of 50, 100 and 200 bacteria respectively, using trypan blue staining. However, it is questionable if the results reported by Baehni *et al.*, (1979) were significant. In a study in 1981 Baehni *et al.*, reported results using both the trypan blue exclusion and LDH release methods, only the LDH results were published. An investigation of the killing of *A.actinomycetemcomitans* by human neutrophils (PMNLs) at a ratio of ≤10 bacteria per cell using six strains of *A.actinomycetemcomitans* was carried out by Miyasaki *et al.*, (1986a). They reported that two of the strains (Y4 and 650) which have previously been reported to be leucotoxic for PMNLs by Baehni *et al.*, (1981) and another four isolates, were non-leucotoxic, and their assays in fact the bacteria were killed by the PMNLs. In addition, Miyasaki *et al.*, (1986a) reported that 88% and 99% of the *A.actinomycetemcomitans* cells were killed within 30 minutes of exposure to the PMNLs under anaerobic and aerobic conditions respectively. Furthermore, the viability of PMNLs remained above 90% as assessed by trypan blue exclusion over the 30 minute incubation period. Although the previously

characterized leucotoxic and non-leucotoxic strains were killed rapidly by the PMNLs, Miyasaki *et al.*, (1986a) suggested that their results did not contradict an earlier report by Baehni *et al.*, (1981) which recorded positive results for leucotoxin activity. It is difficult to understand how Miyasaki *et al.*, (1986a) reached this conclusion especially in the case of Y4 and 650 both of which had been leucotoxic at a level of 5 bacteria per PMNL (Baehni *et al.*, 1981) with no evidence of *A.actinomycetemcomitans* cells being killed by PMNLs.

In one of the preliminary experiments carried out in the present investigation, the concentration of bacteria used was 1400 per target cell. However, none of the six strains examined at this high concentration caused any detectable killing of target cells over a two hours period. Thus it seems unlikely that the failure to demonstrate leucotoxic activity in these strains could be related to insufficient bacteria in assay experiments. Similarly Baehni *et al.*, (1981) reported that *A.actinomycetemcomitans* strains which had been designated, non-leucotoxic failed to kill target cells even when the concentration of bacterial cells was increased to 200 per PMNL.

The effect of different concentrations of whole cells of *A.actinomycetemcomitans* on the release of LDH from PMNLs was investigated by a number of workers. Baehni *et al.*, (1979) have shown that the LDH release was increased from 12% to approximately 20% in the presence of 50 and 100 Y4 bacterial cell respectively. Similarly, McArthur *et al.*, (1982) have

reported that as the concentration of *A.actinomycetemcomitans* whole cells was increased from 25 to 100 per PMNL, there was a direct relationship with PMNL damage as measured by the release of lactate dehydrogenase (LDH). For example, LDH release in the presence of Y4 increased from 55% to 62% at a concentration of 25 to 100 bacterial cells per PMNL respectively. However, with two other strains of *A.actinomycetemcomitans* (ATCC 29522 & ATCC 29524) only a small increase in LDH release was obtained as the concentration of bacterial cells was increased from 25 to 50 per target cell (McArthur *et al.*, 1982). Similar observations were reported by Taichman *et al.*, (1982) who used a number of fresh and Type strains of *A.actinomycetemcomitans.* Although the concentration of whole cells was increased from 25 to 100, the increase of LDH did not exceed more than 20% with six of the strains tested by Taichman *et al.*, (1982). No statistics were performed in these investigations, and it seems unlikely that the difference in LDH release would be highly significant.

Sonic extracts

Sonic extracts of *A.actinomycetemcomitans* have also been used at different concentrations ranging from 0.001 to 3.6mg protein/ml. However, there is no information on the activity of different concentrations of sonic extracts of *A.actinomycetemcomitans* on the viability of HL-60 cells using either the trypan blue exclusion method or LDH release. In contrast, the effect of sonic extracts of *A.actinomycetemcomitans* on the viability of PMNLs *in vitro* has been inves-

Table 5.17 Leucotoxic activity of *A.actinomycetemcomitans* as determined by different research groups.

Author's	Aa strains tested	Cult. Medium	Conc.	Target Cells	% Dead T. blue	% LDH release	No. +ve Strains
Baehni *et al.*, (1979)	Y4	TGB	5-200	PMNLs	85%-96%	23%	
Tsai *et al.*, (1979)	Y4	TGB	0.01-1.6 mg/ml	PMNLs	> 95%	63%	
Baehni *et al.*, (1981)	(ATCC 29522, 23, 24), Y4	TGB	5-200	PMNLs	ND	2-84%	(3/4)
" "	10 fresh Aa	TGB	5-200	PMNLs	ND	<1-89%	(7/10)
McArthur *et al.*, (1981)	Y4	TGB	1.0 mg/ml	PMNLs	ND	46%	
Taichman and Wilton (1981)	Y4	TGB	0.01-1.0 µg/ml	PMNLs	56%	ND	

Table 5.17 Continued

Author's	Aa strains tested	Cult. Medium	Conc.	Target Cells	% Dead T. blue	% LDH release	No. +ve Strains
Zambon *et al.*, (1983c)	(ATCC 29522, 23, 24), Y4, Aa 67	TGB	O.D 0.6 at 540 nm	PMNLs	ND	3.4-25%	3/5
" "	" "	"		HL-60	60%	4.5-32%	3/5
Miyasaki *et al.*, (1986a)	ATCC 29523, Y4 & 4 fresh Aa	Choc. Agar	≥10	PMNLs	0%	ND	0
Ohta *et al.*, (1987)	ATCC 29522, 23 24 & 32 fresh Aa	TGB	1.7-3.6 mg/ml	PMNLs	30%	7.5%	10/35
Chung *et al.*, (1989)	46 fresh Aa	TGB	50	PMNLs	ND	2.5-10.7%	10/46

TGB: Thioglycolate broth (Difco)

T.blue: Trypan blue

ND: No data

Aa: *Actinobacillus actinomycetemcomitans*

ATCC: American Type Culture Collection

Conc: Concentration of *A.actinomycetemcomitans* (number per target cell or weight of sonic extract-protein/ml)

tigated. Taichman and Wilton (1981) reported that increasing Y4 sonic extracts concentration from zero to 1.0 µg/ml resulted in more PMNLs being killed by Y4 as monitored by trypan blue exclusion method. The percentages of dead PMNLs were increased from 22% to 50% in the presence of 0.001 and 1.0µg/ml sonic extract respectively.

There appear to be no other reports to either confirm or dispute these findings. Most of the published data has tended to highlight the effect of *A.actinomycetemcomitans* sonicates in respect to LDH release from PMNLs. Tsai *et al.*, (1979) reported that LDH release was increased from 28% to 63% in the presence of 0.25 µg and 1.5 µg sonic extract from *A.actinomycetemcomitans* Y4 respectively. Baehni *et al.*, (1981) have examined the effect of sonic extracts prepared from 13 strains of *A.actinomycetemcomitans* on PMNLs. While nine of these strains were found to be leucotoxic, there was clear evidence that increasing the sonic extract concentration from 0.1 to 0.8mg protein/10^7 PMNLs resulted in an increase of LDH release reaching a maximum at the highest concentration of extracts used. Other studies which have shown a direct relationship between extract concentration or the amount of purified leucotoxin and LDH release, have been reported by Zambon *et al.*, (1983c) and Simpson *et al.*, (1988).

A.actinomycetemcomitans ATCC 10979 and five of the freshly isolated strains in this thesis (Aa 07, 16, 17, 19 and Aa 40) have been examined using whole cells and their sonic extracts (Solanki, 1989). The results showed that whole

cells of strains ATCC 29522 and Aa 16 were non-leucotoxic when tested with both HL-60 cells and PMNLs. However, sonic extracts from both strains were found to be toxic as assessed by LDH release or by the trypan blue exclusion method. *A.actinomycetemcomitans* strains 17 and 19 were found to be non-leucotoxic whether used as whole cells or sonic extracts, and finally Aa 40 produced detectable levels of LDH when used as a sonic extract or as whole cells, but activity was higher with sonic extracts. All six strains were examined in the present investigation as whole cells at a concentration of 200 bacteria per target cells but sonicates were not used. All strains with the exception of Aa 40 were found to be non-leucotoxic which agrees with the results reported by Solanki (1989).

In conclusion, surprisingly, there are no reported direct comparisons of leucotoxic activity as produced by whole cells and sonic extracts of *A.actinomycetemcomitans*. However, the information available suggests that while sonic extracts have a dose dependent action, whole cells only poorly exhibit this relationship. Furthermore, some reports give the impression that more positive results could be achieved if sonic extracts are used instead of whole cells. However, more specific scientific studies are needed before definite conclusions can be made.

Effect of exposure time

The contact time for *A.actinomycetemcomitans* and target cells (PMNLs or HL-60 cells) has varied among research gro-

ups with the time ranging from 30 to 120 minutes (Miyasaki *et al.*, 1986a; Zambon *et al.*, 1983c). Tsai *et al.*, (1979) reported that as the exposure time was increased from 10 to 60 minutes, more PMNLs were killed by *A.actinomycetemcomitans* which resulted in an increase in LDH release from 15 to >40% respectively. Similarly, Zambon *et al.*, (1983c) have shown that more target cells (both PMNLs and HL-60) were killed by the same concentration of whole cells of leucotoxic strains of *A.actinomycetemcomitans* in a time dependent fashion. The maximum kill of target cells as indicated by LDH release was obtained within 45 minutes of incubation. However, when sonic extracts of *A.actinomycetemcomitans* were used, the maximum kill of PMNLs (% LDH release) was achieved after 60 minutes (Tsai *et al.*, 1979) or 120 minutes when human monocytes were used as target cells (Taichman *et al.*, 1980). The time used in this investigation was usually 60 minutes but was increased in some experiments to 2 and 7 hours. Since the majority of investigators used 60 to 120 minutes, the time employed in the present investigation was within the limits used by other researchers. In the experiments when incubation of *A.actinomycetemcomitans* with target cells was increased to 7 hours, both HL-60 cells and PMNLs appeared to be modestly killed by the Type strains of *A.actinomycetemcomitans* (NCTC 9709, 9710, 10979, 10980 and 10981). However, it is not possible to relate this level of toxic activity over such a long period of time directly to leucotoxin production due to the real possibility of mild injury to host cells with time, which may predispose them to more severe damage from *A.actinomycetemcomitans* cells. The

viability of HL-60 cells was reduced by approximately 20% after 7 hours incubation in the HBSS control, whereas the viability of the negative PMNLs control showed little change over the same period.

Effect of temperature

Temperature appears to have a definite effect on the leucotoxic activity of of *A.actinomycetemcomitans*. In the early studies by Tsai *et al.*, (1979), it was shown that *A.actinomycetemcomitans* leucotoxin was inactive at temperatures of 4°C but killed target cells at 37°C. In the same study the activity of the toxin was found also to be temperature dependent, with increasing numbers of PMNLs being killed as the temperature was raised from 15 to 30 then 37°C (Tsai *et al.*, 1979). The release of LDH, as a measure of cell death, was increased from 5% to 25% then 32% respectively. Similarly, Zambon *et al.*, (1983c) reported that there was no measurable LDH release at 4°C, but as the temperature was raised to 21 and 37°C, LDH release was increased to 11% and 22% respectively. In the present study all experiments were carried out at 37°C and the temperature was monitored throughout the incubation period.

Type of target cells

Almost all researchers who have studied the leucotoxic activity of *A.actinomycetemcomitans* have used human PMNLs (see Table 5.17) or human leukaemic cell lines such as, HL-60, U937 or KG-1 (Zambon *et al.*, 1983c; Taichman *et al.*, 1987b; Simpson *et al.*, 1988; Iwase *et al.*, 1989). Other types of

human cells have also been used, including monocytes, lymphocytes, fibroblasts, platelets or erythrocytes (Taichman *et al.*, 1980; Tsai *et al.*, 1979). However, only human PMNLs, monocytes and HL-60, U937 or KG-1 cell lines are killed by *A.actinomycetemcomitans* leucotoxin (Baehni *et al.*, 1981; Zambon *et al.*, 1983c; Simpson *et al.*, 1988). Similarly PMNLs and monocytes from the great apes and Old World monkeys are susceptible to the leucotoxin of *A.actinomycetemcomitans* (Taichman *et al.*, 1984; Taichman *et al.*, 1987a). In the present investigation two types of cells were used; human PMNLs and HL-60 cells, and *A.actinomycetemcomitans* strains designated non-leucotoxic in this study showed little if any activity towards either of the target cells used. However, the single strain of *A.actinomycetemcomitans* which was leucotoxin positive (Aa 40) killed both types of cells which agrees with previous published findings (Zambon *et al.*, 1983c; Simpson *et al.*, 1988).

There seems to be some disagreement in the literature about which cells are most sensitive to *A.actinomycetemcomitans* leucotoxin. Zambon *et al.*, (1983c) found the HL-60 cells are slightly more sensitive than the PMNLs to leucotoxic strains, eg. 1.5 to 2 times more LDH was released from HL-60 in the presence of whole cells of Y4 than from PMNLs. However, Simpson *et al.*, (1988) using purified leucotoxin, found PMNLs were the most susceptible cells to toxin activity compared to both HL-60 and U937 cell lines. The results obtained in the present investigation with a single positive *A.actinomycetemcomitans* strain are in agreement with those

reported by Zambon *et al.*, (1983c), ie. HL-60 cells (73% kill) were more susceptible than PMNLs (29% kill). Finally, in view of the little information available and the disagreement about the susceptibility of target cells to *A.actinomycetemcomitans,* it is clear that more scientific studies are needed to establish the sensitivity and the suitability of both HL-60 cells and PMNLs in leucotoxic studies.

Criteria used to determine leucotoxicity

Trypan Blue Exclusion

Despite the information available concerning the leucotoxicity of *A.actinomycetemcomitans,* there are no clear criteria to assist with categorizing a particular strain of *A.actinomycetemcomitans* as either leucotoxic or non-leucotoxic using either LDH release or the trypan blue exclusion method. Although a number of investigators have used trypan blue, they failed to present their results in full (Baehni *et al.*, 1981; Zambon *et al.*, 1983c; Taichman *et al.*, 1987a). There is no detailed information regarding the toxic activity of *A.actinomycetemcomitans* for HL-60 cells using trypan blue exclusion test. In the case of human PMNLs, the lowest level of dead PMNLs stained by trypan blue in the literature appears to be 30% (Ohta *et al.*, 1987), while the highest levels of dead cells range from 85% to 95% (Tsai *et al.*, 1979). However, generally figures of 50% to 60% have been most commonly reported (Taichman and Wilton, 1981; Zambon *et al.*, 1983c). In the present investigation a minimum level of 25% dead cells was taken as the cut off point to distinguish leucotoxic from non-leucotoxic strains. However, the

appropriatenes of this level of activity could be questioned, since it is less than the lowest figure previously reported for PMNLs. Since both HL-60 cells and PMNLs appear to possess different sensitivity to leucotoxin (see section 5.4.2), it would seem reasonable that different killing values should be employed to designate positive and negative strains when using these different cells. The position of borderline strains eg. those giving leucotoxic values of 20-24% needs to be clarified. Until more studies using large number of isolates and possibly an animal model are performed, this issue remains unsolved.

LDH release

The problem is rather more complicated with the LDH release method. As shown in Table 5.17 different values of LDH release have been accepted for reporting *A.actinomycetemcomitans* isolates as leucotoxin positive. Chung *et al.*, (1989) presented the lowest levels of LDH with a range of 2.5% to 10.7% for 12 *A.actinomycetemcomitans* isolates. A mean level of 7.5% was used by Ohta *et al.*, (1987) for designating positive strains. On the contrary, Zambon *et al.*, (1983c) reported a figure of 5.6% LDH release for one *A.actinomycetemcomitans* strain, which they categorized as non-leucotoxic. Nonetheless, higher LDH levels have been reported for leucotoxic strains using different methods. For instance, Baehni *et al.*, (1981) reported a range of 17% to 89% LDH with part of the variation being related to the concentration of bacterial cells used, as well as to the different strain used. Other researchers who have reported high levels of LDH rele-

ase (46% to 63%) include Tsai *et al.*, (1979) and McArthur *et al.*, (1981) (see Table 5.17). There is also evidence of marked differences in the percentage LDH release by the same *A.actinomycetemcomitans* Type strain as reported by different workers. For example, Baehni *et al.*, (1979) reported a level of 23% LDH release from PMNLs in the presence of Y4 (200/PMNL) compared with a figure of 62% obtained by Baehni *et al.*, (1981)using the same bacterial strain but at half the concentration of bacterial cells used in the earlier investigation.

Taking these different results into account a minimum level of 15% LDH release within 60 minutes of exposure to *A.actinomycetemcomitans* (200 bacteria per target cell) was taken to designate a particular strain as leucotoxic in the present study. *A.actinomycetemcomitans* strains which have produced only small LDH release values (2.5% to 7.5%) have been accepted as leucotoxic by a number of workers (Ohta *et al.*, 1987; Chung *et al.*, 1989). If this range of LDH values is applied to the strains examined in the present investigation, three strains Aa 03, 09 and Aa 21 which produced LDH release ranging from 3.8% to 4.2% would be recorded as positive for leucotoxicity. It is possibly necessary to set different values for both HL-60 cells and PMNLs since the susceptibility of these cells to *A.actinomycetemcomitans* may vary even when the same bacterial strain is used. Furthermore, other confirmatory tests are needed to be developed to clarify the position of borderline strains (eg. those between 10-14%).

In conclusion it is surprising that no clear, agreed cut-off values for leucotoxicity have been proposed for any of the tests used to assess this property of *A.actinomycetemcomitans* and that such a wide range of values has been accepted. It is possible that the results from two different tests (eg. trypan blue and LDH release) are needed to be certain about the presence or absence of leucotoxicity. In addition, there is a need for standard tests with clear-cut criteria to ensure comparability of results obtained from different laboratories.

5.4.3 Leucotoxic activity of fresh isolates of *A.actinomycetemcomitans*

There are only a few papers that have studied the leucotoxic activity of fresh isolates of *A.actinomycetemcomitans* using either PMNLs or HL-60 cells *in vitro* (Baehni *et al.*, 1981; Zambon *et al.*, 1983c; Tsai and Taichman, 1986; Ohta *et al.*, 1987; Chung *et al.*, 1989). Furthermore, little details were given by these research groups concerning the activity of the strains which they studied. Baehni *et al.*, (1981) examined 10 *A.actinomycetemcomitans* plaque isolates and reported that 7 (70%) of these strains produced leucotoxic activity as determined by LDH release. A range of 17% to 89% LDH release was obtained for the 7 isolates depending on the concentration of bacterial cells used (Baehni *et al.*, 1981). A large number of fresh isolates were studied by Zambon *et al.*, (1983c), but since 100 were isolated from 55 subjects, a number of strains were clearly isolated from the same subject. Zambon *et al.*, (1983c) reported that 16% from 11 hea-

lthy subjects; 43% from 13 adult periodontitis patients; 75% from 4 insulin dependent diabetics; 66% from 2 generalized juvenile periodontitis patients and 55% from Localized Juvenile Periodontitis, produced leucotoxin. These result highlight the fact that leucotoxic strains are present in healthy as well as diseased subjects, though diseased subjects harboured higher percentages of leucotoxic strains. Moreover, some individuals harboured both leucotoxic and non-leucotoxic strains, eg. 5 out of 10 isolates from both a LJP and a rapidly progressive periodontitis patient were leucotoxic positive (Zambon *et al.*, 1983c; Ohta *et al.*, 1987). A total number of 32 strains isolated from 3 patients were examined by Ohta *et al.*, (1987) and only 8 (25%) of these isolates were found to produce toxic activity as assessed by LDH release (mean 7.5%). Chung *et al.*, (1989) investigated the leucotoxic activity of 46 fresh isolates of *A.actinomycetemcomitans* from LJP patients, and reported that 10 of the strains (22%) from 13 diseased sites in 8 patients possessed leucotoxic activity as measured by LDH release. These strains released LDH from target cells within the range of 2.5 to 10.7 percent. Despite the low activity of these strains, they were considered leucotoxic.

In the present investigation 12 fresh isolates of *A.actinomycetemcomitans* were examined for their leucotoxic activity for HL-60 cells using trypan blue staining. As shown in the results section only Aa 40 was found to be leucotoxic for HL-60 cells and PMNLs. The other isolates failed to show any toxic activity towards the HL-60 cells despite a two

hour incubation period. Two of these strains (Aa 09 and Aa 16) were also found to be non-leucotoxic for PMNLs.

The reason for failure to produce leucotoxin by some strains of *A.actinomycetemcomitans* is not clear. Tsai and Taichman (1986) have reported that leucotoxic strains are found mainly in younger patients (6-12 years of age) with fewer isolated from older patients (13-25 years old). In addition, leucotoxic activity was detected in 100% of the isolates from 15 patients aged 6-12 years (no information was given about the number of isolates tested), whereas only 23% of isolates from 23 older subjects were found to be leucotoxic (Tsai and Taichman, 1986). However, these findings disagree with the results of Taichman *et al.*, (1987b) who reported that leucotoxic strains were isolated from adult periodontitis patients aged 30 to 65 years (no full details were given). Furthermore, Chung *et al.*, (1989) isolated leucotoxic strains from 8 (50%) LJP patients aged 18 to 33 years who are older than those described by Tsai and Taichman (1986). All of the *A.actinomycetemcomitans* strains examined in the present study were isolated from different individuals, but it would have been interesting if multiple strains from the same site or subject were examined for their leucotoxic activity since there is some evidence that different isolates can give different results. In fact, a number of multiple strains were isolated in the clinical investigation described in Chapter 2, but because of shortage of time none have been tested. It is possible

that if more isolates had been tested in this study the low leucotoxic activity found would have been much higher.

In conclusion detailed scientific studies especially on the molecular basis of toxin production are needed to explain this variation in leucotoxicity among *A.actinomycetemcomitans* strains.

5.4.4 Leucotoxic activity of Type strains of *A.actinomycetemcomitans*

In the present investigation six Type strains of *A.actinomycetemcomitans* were examined for their leucotoxic activity, these include Y4, NCTC 9709, 9710, 10980, 10981, and 10979, the latter being the same strain as ATCC 29522. There is no information in the literature about the leucotoxicity of most NCTC Type strains of *A.actinomycetemcomitans,* although ATCC 29522 (NCTC 10979) has been reported as leucotoxic positive by Baehni *et al.*, (1981); McArthur *et al.*, (1982); Zambon *et al.*, (1983c) and Ohta *et al.*, (1987). However, results obtained in the present study for NCTC 10979 (ATCC 29522) failed to show any activity under similar conditions used by these researchers. Similarly Ohta *et al.*, (1986) reported ATCC 29522 as leucotoxic negative which agrees with the findings of this study. The only evidence of very low levels of leucotoxic activity produced by the NCTC Type strains examined here appeared after an excessively prolonged incubation period of 7 hours with both target cell types (PMNLs or HL-60 cells).

The reason why NCTC strains of *A.actinomycetemcomitans* failed to produce leucotoxin in the experiments reported in this thesis is not clear. Moreover, in preliminary experiments using Type strains of *A.actinomycetemcomitans* (ATCC 29522, 29523 & 29524) and NCTC 10979, where the culture medium was Columbia Blood Agar (CBA), no evidence of leucotoxin activity for HL-60 or PMNLs over a period of 2 hours period was observed, despite the fact that, the suspensions of these strains contained 200 to 1000 bacteria per target cell. However, since these experiments were only carried out once, it is not possible to draw any firm conclusion or to compare the results with other leucotoxic studies.

A.actinomycetemcomitans strain Y4 have been extensively studied by researchers and found to be leucotoxic by many of them (Tsai *et al.*, 1979; McArthur *et al.*, 1981; Baehni *et al.*, 1981; Taichman *et al.*, 1982; Zambon *et al.*, 1983c; Chung *et al.*, 1989). Nevertheless, the activity of this Type strain has been found to vary among the different researchers. For example, Baehni *et al.*, (1979) reported a 23% LDH release from PMNLs in the presence of 200 Y4 bacterial cells, which is similar to the 25% level reported by Zambon *et al.*, (1983c) in the presence of the same bacterial strain. In comparison, 62% release was achieved by Baehni *et al.*, (1981) using Y4 and employing the same method but in the presence of only 100 bacterial cell. However, results obtained in the present study failed to demonstrate any leucotoxic activity for the Y4 strain using either PMNLs or HL-60 cells. There are two reports one by Tsai *et al.*,

(1978) and the other by Miyasaki *et al.*, (1986a) where *A.actinomycetemcomitans* Y4 failed to kill human PMNLs. The lack of leucotoxicity was not understood, but later the same organism from a different stock culture was found to be leucotoxic (Baehni *et al.*, 1979). Miyasaki *et al.*, (1986a) reported that the reason for Y4 failing to kill PMNLs was due to the low concentration of bacteria ≤ 10:1 used in the assay.

In conclusion it appears that there is some disagreement about the leucotoxic activity of Type strains of *A.actinomycetemcomitans* among different workers. This clearly highlights the importance of not depending solely on Type strains when studying the leucotoxic activity of *A.actinomycetemcomitans*, and it is important to include fresh isolates in assay experiments.

5.4.5 Action of leucotoxin

Recently the leucotoxin produced by *A.actinomycetemcomitans* has been identified and cloned (Lally *et al.*, 1989; Kolodrubetz *et al.*, 1989) and appears to be closely related to the leucotoxin of *Pasteurella haemolytica* (Kolodrubetz *et al.*, 1989) and *Escherichia coli* haemolysin (Lally *et al.*, 1989). However, the mechanism by which leucotoxin destroys specific target cells (eg. PMNLs, HL-60 cells or U937) remains unknown (Lally *et al.*, 1989). Nonetheless, it has been suggested that for the toxin to be active it must first bind to the cell membranes of target cells (Taichman *et al.*, 1980) thus causing plasma membrane damage (Simpson *et al.*, 1988).

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Killing of target cells by *A.actinomycetemcomitans* leucotoxin is inhibited in a dose-dependent manner by polyclonal and monoclonal antibodies against the leucotoxin (Lally *et al.*, 1989). Furthermore, incubation of target cells or toxin with certain saccharides such as mannose or galactose (Simpson and Taichman, 1987a), lectins such as wheat germ agglutinin and *Phaseolus vulgaris* lectin (Simpson and Taichman, 1987b), can prevent cell destruction by *A.actinomycetemcomitans* leucotoxin. Similarly, trypsin was found to significantly inhibit leucotoxic activity. In addition, extracellular calcium (Ca^{++}) appears to modulate cell killing, since leucotoxicity was greater in Ca^{++} free medium and was inhibited by the addition of $\geq$ 2mM Ca^{++} in the medium. These data suggested that the toxin reacts with the plasma membrane components of the target cells where it initially affects cell surface determinants before becoming internalized within the target cells and thus expressing its cytotoxicity (Iwase *et al.*, 1989). Finally Simpson *et al.*, (1988) have suggested that cells which are not killed by the toxin may lack or have very few leucotoxin receptors on their cell membranes. Alternatively, resistance to the leucotoxin may be a consequence of an impairment of cytopathic events that occur subsequent to toxin binding and membrane perturbation (Simpson *et al.*, (1988).

5.5 Conclusions:

Only one (Aa 40) of the 12 *A.actinomycetemcomitans* isolates from juvenile periodontitis and chronic periodontitis patients killed both PMNLs and HL-60 cells *in vitro*.

The Type strains of *A.actinomycetemcomitans* failed to show any leucotoxic activity for both HL-60 cells and PMNLs.

The trypan blue exclusion method and lactate dehydrogenase release produced similar results with regard to leucotoxicity.

For the one positive strain human PMNLs appeared to be slightly more resistant than HL-60 cells to *A.actinomycetemcomitans* leucotoxin.

It is important to develop a standard method with a clear criteria which can be used to determine leucotoxic activity of *A.actinomycetemcomitans*, so comparisons of results between different laboratories can be possible.

Further studies are required to answer many of the unsolved questions about the leucotoxin of *A.actinomycetemcomitans*, especially the mechanism by which toxin production is activated, how it destroys target cells and why it is specific for certain cells.

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

The aim of this final chapter is to investigate the results obtained in the previous 4 Chapters and to suggest lines of future investigation.

6.2 Adherence and hydrophobicity

6.2.1 Adherence

As discussed in Chapters 3 and 4 that there is little information in the literature concerning the hydrophobic activity and adherence of *A.actinomycetemcomitans* to human buccal epithelial cells *in vitro*. Although it has been reported that the main habitat for *A.actinomycetemcomitans* is dental plaque, it can also be found in low numbers in saliva, on the buccal mucosa and on the dorsum of the tongue (Slots *et al.*, 1980a). Using *in vitro* studies, Sweet *et al.*, (1988) reported that one Type strain of *A.actinomycetemcomitans* (NCTC 9710) adhered at a mean adherence value of 7.5 bacteria/BEC in SIB buffer. In the present investigation a wide range of adherence levels in SIB were obtained for 33 isolates (1.7 to 27.7 bacteria/BEC) which consisted of both fresh and Type strains of *A.actinomycetemcomitans*. Although an overall mean adherence values of 13.3 and 10.7 bacteria were obtained for both the fresh and Type strains of *A.actinomycetemcomitans* respectively, the difference was not statistically significant. These results highlighted the importance of examining large number of bacterial strains inclu-

ding fresh and Type isolates when investigating the adherence of a particular species. They also emphasize the inaccuracies which may occur if only one or two strains of a bacterial species are examined and conclusions are then drawn from such experiments.

Some studies have reported the isolation of *A.actinomycetemcomitans* from mixed saliva of both healthy and adult periodontitis patients (Slots *et al.*, 1980a), however, the intraoral reservoir of the organism especially in the healthy carriers is unknown. The results of this study certainly suggest that *A.actinomycetemcomitans* can attach to buccal cells in the presence of saliva. However, even if the same adherence mechanisms function *in vivo* it is not certain if *A.actinomycetemcomitans* would be able to colonize and profilerate at that site.

The results obtained in this study clearly showed that the presence of human serum almost completely inhibited the adherence of *A.actinomycetemcomitans* to buccal cells *in vitro*. An overall mean range adherence value of 0.4 to 3.4 bacteria/BEC was obtained for 14 freshly strains of *A.actinomycetemcomitans,* compared with an average mean of 4.3 to 12.5 adhered bacteria obtained for the same strains tested in SIB (Table 3.15). These results showed a significant deference ($p \leq 0.0005$) which was very unlikely due to experimental error since both experiments were carried out at the same time using the same buccal epithelial cells, bacterial inoculum, and controls.

The addition of serum to the adherence assay was to mimic the *in vivo* environment at the gingival crevice where the highest concentrations of *A.actinomycetemcomitans* are usually to be found (Slots *et al.*, 1980a). Thus it would seem reasonable to theorize that serum would increase the adherence of *A.actinomycetemcomitans* to intraoral sites coated with serum eg. gingival epithelial cells. However, in the present experimental studies the opposite was found which requires some explanation. If the serum used in this study contained antibodies against *A.actinomycetemcomitans*, which could aggregate the bacteria during the assay, it might result in clumps of *A.actinomycetemcomitans* which would then fail to adhere to buccal cells. Such clumps would probably not be washed through the filter, and would be arranged in a haphazard way on the filter surface. However, no evidence of this was noted microscopically, even although the counting of adherence preparations was performed anonymously. As discussed in Chapter 3, microbiological studies of plaque have shown the donor of both BECs and serum to be negative for *A.actinomycetemcomitans*. However, no immunological examination was carried out to establish if antibodies were present. It is possible that serum blocked the bacterial receptors on buccal cells in either specific or non-specific fashion, and thus prevented *A.actinomycetemcomitans* from adhering. Kagermeier and London (1985) used pooled human serum to coat hydroxyapatite and found a reduction in the number of adherent cells of two strains of *A.actinomycetemcomitans* to this surface. Although the surfaces used in these two studies were different, it is interesting that

both found serum depressed the adherence of *A.actinomycetemcomitans*. However, in spite of these *in vitro* results the fact remains that *A.actinomycetemcomitans* is mainly found in subgingival plaque where it regularly comes into contact with low levels of serum, in the form of gingival exudate, which is a transudate of plasma. It is also possible that *in vivo A.actinomycetemcomitans* interacts with serum in a way not possible to reproduce *in vitro* experiments. In view of these results, the effect of serum on the adherence of *A.actinomycetemcomitans* to buccal cells requires more *in vitro* experiments.

In conclusion, it is difficult to correlate *in vitro* results with the *in vivo* situations, but it can be postulated that *A.actinomycetemcomitans* has the ability to adhere to buccal mucosa before finally colonizing the gingival area and possibly leading to the development of periodontal disease. It would be interesting to find out if saliva and serum collected from different donors would have a similar effect on the adherence of *A.actinomycetemcomitans* to buccal epithelial cells as discussed in this thesis. Ideally, BEC pooled from a number of donors should be used.

6.2.2 Hydrophobicity

Recent studies have suggested that hydrophobic interactions can promote bacterial adhesion to buccal epithelial cells and to the tooth surface itself (Westergren and Olsson, 1983). The hydrophobic activity of *A.actinomycetemcomitans* has been investigated previously (Gibbons and Etherden,

1983; Sweet *et al.*, 1986; Kozlovsky *et al.*, 1987), but only one or two Type strains of *A.actinomycetemcomitans* have been examined. The results of the present investigation using 33 strains (10 Type strains and 23 fresh isolates) and those by Sweet *et al.*, (1986) and Kozlovsky *et al.*, (1987) all agree in describing *A.actinomycetemcomitans* as hydrophobic although one report presents the opposite view (Gibbons and Etherden, 1983). A clear and significant correlation ($p \leq 0.05$) between the adherence of *A.actinomycetemcomitans* to buccal epithelial cells and hydrophobicity using xylene, confirms the view that hydrophobic interactions are implicated in adhesion phenomena as suggested previously for other bacteria.

Since both hydrophobicity and adherence to buccal cells appear to be related, it seems likely that both saliva and serum may in return affect the hydrophobic activity of *A.actinomycetemcomitans in vivo.* There is no information to confirm or to dispute this suggestion and it would be of interest to perform adherence and hydrophobicity studies in the presence of both saliva and serum. However, it seems likely that the use of serum in hydrophobicity experiments, would pose certain technical difficulties, and new methods need to be developed.

6.3 Morphological and Biochemical Characteristics of *A.actinomycetemcomitans*

Although the formation of star-shaped colonies by *A.actinomycetemcomitans* and catalase production on primary isolation on semi-selective media has been used widely by researchers

for identification, no information has been published about the prevalence of either star-shaped or non-star-shaped variants of *A.actinomycetemcomitans* in previous studies (Slots, 1982b; Christersson *et al.*, 1985; Slots *et al.*, 1986). Thus the results of this study which showed that the prevalence of non-star-shaped colonies was greater than those of star-shaped variants on primary isolation on TSBV medium has important implications. If catalase positive star-shaped colonies had been selected for identification in the present investigation, only about half (52%) of the total number of positive *A.actinomycetemcomitans* patients would have been recorded. Since the presence of non-star-shaped colonies appears not to have been considered by previous workers, the accuracy of much of the published prevalence data must be regarded as doubtful, and new epidemiological studies perfo-rmed.

There is also a need for a reproducible and widely available commercial, method for identifying *A.actinomycetemcomitans.* In this thesis the API 20 A system was used for identification, and gave reproducible results with all Type and fresh isolates of *A.actinomycetemcomitans.* The 38 freshly isolates of *A.actinomycetemcomitans* fermented glucose, maltose, mannose and produced catalase, and produced variable reactions with mannitol and xylose which is in agreement with the majority of other workers (Sneath and Johnson, 1973; Slots 1982a). Therefore, while different basal media have been used by researchers, it would be sensible to use one standard medium such as the API 20 A system for identification to

allow satisfactory comparison of results between different laboratories.

6.4 Detection and Distribution of *A.actinomycetemcomitans* in healthy and chronic periodontitis patients

6.4.1 Prevalence of *A.actinomycetemcomitans*

Although there were 55 healthy subjects examined in the clinical study of this thesis, *A.actinomycetemcomitans* was isolated from only one (2%) of these subjects. However, other workers have reported higher prevalence rates of *A.actinomycetemcomitans* in their healthy control subjects ranging from 17% to 36% (Slots *et al.*, 1980a; Zambon *et al.*, 1983b; Mombelli *et al.*, 1990). In the case of chronic periodontitis patients, *A.actinomycetemcomitans* has been isolated from 30% to 50% of patient (Slots *et al.*, 1980a; Bonta *et al.*, 1985; Dahlen *et al.*, 1989), although other workers have reported lower prevalence rates ranging from 13% to 21% (Zambon *et al.*, 1983b; Okuda *et al.*, 1984; Wolff *et al.*, 1985). These results show clearly that the prevalence of *A.actinomycetemcomitans* in both health and disease appears to be similar in some studies, which certainly questions the significance of isolating *A.actinomycetemcomitans* from subgingival plaque collected from chronic periodontitis patients.

It can be argued that the number of *A.actinomycetemcomitans* at a given site is an important factor in pathogenicity, and that the mere presence of *A.actinomycetemcomitans* is of less

ignificance. Although the numbers of *A.actinomycetemcomi-*
ans in subgingival plaque samples was not carried out in
his study, it has been reported in a number of other
tudies. Such levels in diseased patients have ranged from
1.0% to 40% of the total microbial flora, with the majority
f patients having *A.actinomycetemcomitans* levels of less
han or equal to 1.0% (Slots *et al.*, 1980a; Okuda *et al.*,
984; Wolff *et al.*, 1985). While these figures appear to
e higher than those reported for healthy sites which ranged
rom 0.01% to 5% (Slots *et al.*, 1980a; Okuda *et al.*, 1984;
lombelli *et al.*, 1990), the prevalence of *A.actinomycetem-*
omitans in both health and disease is not substantially
lifferent. Slots and Genco (1984) have suggested that a low
oncentration of *A.actinomycetemcomitans* (≤2%) in subgin-
ival plaque could cause disease due to the high virulence
f *A.actinomycetemcomitans*. The available data show that
oth healthy and periodontitis patients harbour *A.actinomy-*
etemcomitans at low numbers. However, it must emphasized
hat most of these data were based mainly on figures repre-
enting star-shaped colonies which were only counted by
hese researchers and the presence of non-star-shaped colo-
ies have been largely ignored. If the results of this the-
is are taken into account it seems likely that higher cou-
ts of *A.actinomycetemcomitans* might have been reported, if
on-star-shaped colonies had been investigated thoroughly.

significance. Although the numbers of *A.actinomycetemcomitans* in subgingival plaque samples was not carried out in this study, it has been reported in a number of other studies. Such levels in diseased patients have ranged from ≤1.0% to 40% of the total microbial flora, with the majority of patients having *A.actinomycetemcomitans* levels of less than or equal to 1.0% (Slots *et al.*, 1980a; Okuda *et al.*, 1984; Wolff *et al.*, 1985). While these figures appear to be higher than those reported for healthy sites which ranged from 0.01% to 5% (Slots *et al.*, 1980a; Okuda *et al.*, 1984; Mombelli *et al.*, 1990), the prevalence of *A.actinomycetemcomitans* in both health and disease is not substantially different. Slots and Genco (1984) have suggested that a low concentration of *A.actinomycetemcomitans* (≤2%) in subgingival plaque could cause disease due to the high virulence of *A.actinomycetemcomitans*. The available data show that both healthy and periodontitis patients harbour *A.actinomycetemcomitans* at low numbers. However, it must emphasized that most of these data were based mainly on figures representing star-shaped colonies which were only counted by these researchers and the presence of non-star-shaped colonies have been largely ignored. If the results of this thesis are taken into account it seems likely that higher counts of *A.actinomycetemcomitans* might have been reported, if non-star-shaped colonies had been investigated thoroughly.

6.4.2 Leucotoxicity

The pathogenic activity of *A.actinomycetemcomitans* has been related to a number of factors (see section 1.7). However, much importance has been attached to its ability to produce a leucotoxin which is toxic for human PMNLs and monocytes *in vitro* (Tsai *et al.*, 1979; Taichman *et al.*, 1980). Since the number of *A.actinomycetemcomitans* in subgingival plaque in health plus disease is similar (see section 6.4.1), it has been suggested that the ability of *A.actinomycetemcomitans* to produce leucotoxin is more important than the level of cells present. There were six Type and twelve fresh strains of *A.actinomycetemcomitans* examined in this study, but only one fresh isolate (Aa 40) was found to be leucotoxic for both HL-60 cells and PMNLs *in vitro*. These results were unexpected, especially in the case of certain Type strains which have previously been reported as leucotoxin positive by other workers. However, negative results for recognized leucotoxic strains of *A.actinomycetemcomitans* have been mentioned in a few papers. For instance, Ohta *et al.*, (1986-1987) reported that two Type strains of *A.actinomycetemcomitans* (ATCC 29522 & 29524) which had been previously reported leucotoxin positive were negative in their hands, but made no attempt to discuss reasons for these negative results. These two strains were also examined in preliminary experiments in the present study and were found to be negative for toxicity which agree with the findings of Ohta's group.

The largely negative results obtained in this study require some discussion and the factors which could affect leucotoxin activity have been covered in detail in Chapter 5.

These factors include the effect of media, bacterial concentration, exposure time, temperature, type of target cell used and the criteria employed for a positive or negative test. While some of these factors have been investigated in detail by previous workers, others have been all but ignored. As discussed in Section 5.4.2 the effect of growth media on toxin production has not been specifically investigated, although it has been suggested that media may affect the leucotoxic activity of *A.actinomycetemcomitans* (Baehni *et al.*, 1979). More recently Mukherjee, Murphy and Wawszkiewcz (1988) reported that the addition of ferric ammonium citrate enhanced the virulence of one Type strain of *A.actinomycetemcomitans* (NCTC 29522) using a guinea-pig model. In view of this findings it is possible that leucotoxin production may be triggered by the presence or absence of specific nutrients. There are examples of such mechanisms in other toxic bacterial infections, eg. *Corynebacterium diphtheriae* (Collier, 1975), which shows little or no toxin production *in vitro* until the later stages of growth when nutrients become exhausted and the intracellular iron content begins to decrease. The role of growth or other factors in switching on toxin production *in vivo* and and *in vitro* is important, and studies are required to investigate the effect of a range of substances on the toxin production by *A.actinomycetemcomitans.*

The leucotoxic activity of *A.actinomycetemcomitans* has always been described as a dose dependent phenomena (McArthur *et al.*, 1982; Taichman *et al.*, 1982). However, the avail-

able data suggest that while sonic extracts exhibit a dose dependent action, whole cells of *A.actinomycetemcomitans* do not generally show this relationship. Although these results suggest that more positive results for leucotoxin could be obtained if sonic extracts rather than whole cells were used, no detailed comparisons have been performed using a range of fresh and Type isolates.

Recent studies have reported the identification and cloning of *A.actinomycetemcomitans* leucotoxin. However, there is no information to suggest that specific toxin has ever been isolated from diseased sites in patients harbouring *A.actinomycetemcomitans*. However, the release of microvesicles which may contain leucotoxin by *A.actinomycetemcomitans* into growth media and into the crevicular environment has been suggested (Nowotny *et al.*, 1982). Since serum from juvenile periodontitis has been found to contain antibodies which neutralized *A.actinomycetemcomitans* leucotoxin, this may rapidly neutralize leucotoxin in the crevicular environment, which would make toxin detection difficult from in vivo samples. It is also interesting to speculate on the possibility eg. that star-shaped and non-star-shaped variants of *A.actinomycetemcomitans* may possess different toxic activity. Obviously only large scale investigation can answer many of these unsolved problems. In the present study it was intended to investigate the activity of a large number of *A.actinomycetemcomitans* strains; (fresh and Type, star-shaped and non-star-shaped) would be tested, but shortage of time limited the work to that presented earlier.

The effect of time and temperature on leucotoxin detection is agreed by all workers, with a maximum incubation period of two hours being accepted sufficient for all strains to show toxic activity (Zambon *et al.*, 1983c). The maximum activity of leucotoxic strains appears to take place at a temperature of 37°C (Tsai *et al.*, 1979; Zambon *et al.*, 1983c), but there is little information to suggest that the usually used target cells (ie. PMNLs or HL-60) are necessarily the most suitable for leucotoxic studies. Certainly the collection of human PMNLs is a time consuming procedure compared to the collection of HL-60 cells from laboratory cultures. However, the suitability of both types of cells must be established by carrying out specific studies using a large number of fresh and Type strains of *A.actinomycetemcomitans*.

It was surprising to find that no clear criteria for cut-off points exist to categorize a particular strain of *A.actinomycetemcomitans* as leucotoxic or non-leucotoxic using either the trypan blue exclusion or the LDH release methods. Taking into account previously published data it was suggested in the present study that a minimum level of 25% cell death and 15% LDH release should be regarded as the cut-off points to distinguish between positive and negative toxic activity using HL-60 cells and PMNLs respectively. However, there is evidence that HL-60 cells and PMNLs respond differently to leucotoxin and it may be more accurate to use different values to distinguish positive and negative strains when employing these different cells. In order to make progress

in this area, standard methods and clear criteria require to be decided for testing leucotoxin activity which can be followed by all workers and so allow results to be compared. Although both HL-60 cells and PMNLs can be killed by *A.actinomycetemcomitans,* it is possible that another cell type may be more suitable. Further work is required to solve these problems using a wide range of *A.actinomycetemcomitans* isolates.

Once these problems are solved then the next step is to establish the association of *A.actinomycetemcomitans* and periodontal disease, because although research has been proceeding for ten years or more, as yet no definite conclusions have been reached. There is evidence that *A.actinomycetemcomitans* is strongly involved in localized juvenile periodontitis (Section 1.9), but not all studies have been able to relate the presence of *A.actinomycetemcomitans* in LJP (Moore *et al.*, 1985). The role of *A.actinomycetemcomitans* in chronic and other forms of periodontal disease is less clear. It might be argued that isolates from juvenile periodontitis are more pathogenic compared to isolates from chronic or other forms of periodontal disease, but there is very little information about this. Zambon *et al.*, (1983c) reported leucotoxin production by 100 strains of *A.actinomycetemcomitans* isolated from a mixture of healthy volunteers, as well as patients with chronic and juvenile periodontitis. They reported that 16% strains of *A.actinomycetemcomitans* from 11 healthy subjects, and 43% from adult periodontitis patients produced leucotoxic activity.

Although the pathogenic role of *A.actinomycetemcomitans* in LJP and to some extent in chronic periodontitis is widely reported in textbooks, a critical examination of the data cast doubts on the accuracy of some of the techniques used, as well as the interpretation of results. A clear view of the role of *A.actinomycetemcomitans* in periodontal disease will not emerge until many of the difficulties discussed earlier have been resolved and well planned and carefully linked clinical and laboratory studies are performed.

APPENDIX I

MEDIA PREPARATION

Pouring of plates

All plates were poured in a clean air Laminar Flow Cabinet (Microflow Pathfinder, Intermed). Aliquots of 15 ml per plate were poured aseptically, and plates were left to solidify at room temperature, dried, then stored at 4°C for up to one week.

Media:

Tryptic Soy-Serum-Bacitracin-Vancomycin Agar (TSBV)

Malchite Green Bacitracin Agar (MGB)

Columbia Blood Agar (CBA)

Peptone Agar (PA)

Diagnostic Sensitivity Agar (DST)

Anaerobic Blood Broth Supplemented (ABB)

NIH-Thioglycollate Broth (TGB)

RPMI 1640 Medium

Tryptic Soy-Serum-Bacitracin-Vancomycin Agar (TSBV)

Slots, J. (1982) *Journal of Clinical Microbiology*, **15**, 606-609.

Composition:

Peptone 140 (Gibco)	15 g/l
Peptone 110 (Gibco)	5.0 g/l
Sodium chloride (Gibco)	5.0 g/l
Yeast extract (Difco)	1.0 g/l
Horse serum (Gibco)	100 ml/l
Bacitracin (Sigma)	75 mg/l
Vancomycin (Sigma)	5.0 mg/l
Agar (Gibco)	15 g/l
Distilled water	1000 ml

The medium was prepared by dissolving 40 g of tryptic soy agar (Gibco-Europe, Paisley, Scotland) and 1.0 g yeast extract in distilled water by heating in a Koch steamer. The pH was adjusted to 7.4 ± 0.2 with NaOH before sterilization using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The medium was next dispensed into 100 ml amounts and sterilized by autoclaving at 121°C for 15 minutes. The medium was then left to cool to about 50°C and sterilized serum, and antimicrobials were added aseptically in a clean air cabinet and poured into sterile Petri-dishes.

Malachite Green-Bacitracin Agar (MGB)

Mandell, R.L. & Socransky, S.S. (1981) *Journal of Periodontology*, **52**, 593-598.

Composition:

Peptone 140 (Gibco)	15 g/l
Peptone 110 (Gibco)	5.0 g/l
Sodium chloride (Gibco)	5.0 g/l
Horse blood (Gibco)	50 ml/l
Bacitracin (Sigma)	128 mg/l
Malachite green (Surrey UK)	8.0 mg/l
Agar (Gibco)	15 g/l
Distilled water	1000 ml

The medium was prepared by dissolving 40 g of tryptic soy agar (Gibco-Europe, Paisley, Scotland) and malachite green in distilled water by heating in a Koch steamer. The pH was adjusted to 7.4 ± 0.2 with NaOH before sterilization using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The medium was next dispensed into 100 ml amounts and sterilized by autoclaving at 121°C for 15 minutes. The medium was then left to cool to about 50°C and sterilized horse blood and antimicrobials were added aseptically in a clean air cabinet and poured into sterile Petri-dishes.

Columbia Blood Agar (CBA)

Source: Gibco-Europe Ltd., Paisley Scotland

Composition:

Peptone 140	13 g
Peptone 100	6.0 g
Yeast extract	3.0 g
Beef extract	3.0 g
Starch	1.0 g
Sodium chloride	5.0 g
Agar	13 g
Defibrinated horse blood (Gibco)	50 ml

To prepare; 44 grams of the powdered medium was dissolved in 1000 ml distilled water by heating in a Koch steamer. The pH was adjusted to 7.4 ± 0.2 with NaOH before sterilization using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The molten agar was dispensed in 500 ml amounts and then sterilized by autoclaving at 121°C for 15 minutes. The medium was left to cool to about 50°C and 50 ml of sterile defibrinated blood was added per 1000 ml and poured into sterile Petri-dishes.

Peptone Agar (PA)

Source: Mast Laboratories, Merseyside, England

Composition:

Bacteriological peptone (RM52)	10 g
Sodium chloride	5.0 g
Agar (RM10)	14 g

To prepare; 29 grams of the powdered medium was dissolved in 1000 ml distilled water by heating in a Koch steamer. The pH was adjusted to 7.4 ± 0.2 with NaOH before sterilization using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The medium was then sterilized by autoclaving at 121°C for 15 minutes and poured into sterile Petri-dishes.

Diagnostic Sensitivity Test Agar (DST)

Source: Oxoid Ltd., Basingstoke, Hampshire England

Composition:

Proteose peptone(Oxoid L46)	10 g/l
Veal infusion solids	10 g/l
Dextrose	2.0 g/l
Sodium chloride	3.0 g/l
Disodium phosphate	2.0 g/l
Sodium acetate	1.0 g/l
Adenine sulphate	0.01 g/l
Guanine hydrochloride	0.01 g/l
Uracil	0.01 g/l
Xanthine	0.00002 g/l
Lysed horse blood (Gibco)	100 ml/l
Agar No.1 (Oxoid L11)	12 g/l

The DST medium was prepared by dissolving 40 g of powdered medium in 1000 ml distilled water by heating in a Koch steamer. The pH was adjusted to 7.3 ± 0.2 with NaOH before sterilization using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C) and sterilized by autoclaving at 121°C for 15 minutes. The medium was cooled to about 50°C and 100 ml lysed horse blood (Gibco) was added per 1000 ml of medium which was then poured into sterile Petri-dishes.

Anaerobic Blood Broth Supplemented (ABB)

Source: Gibco-Europe Ltd., Paisley Scotland

Composition:

Tryptone	10 g
Beef extract	2.0 g
Liver extract	3.0 g
Yeast extract	5.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
Vitamin K	0.005 g
Haemin	0.005 g
Cysteine hydrochloride	1.0 g
Dithiothreitol	0.1 g
Sodium bicarbonate	0.9 g

To prepare; 32 grams of the powdered medium was dissolved in 1000 ml distilled water by heating in a Koch steamer and dispensed in 20 ml volumes in glass universal bottles. The medium was then sterilized by autoclaving at 121°C for 15 minutes and stored at room temperature.

NIH-Thioglycollate Broth (Difco)

Source: Difco-laboratories, Detroit, Michigan USA

Composition:

Bacto casitone	15 g/l
Bacto yeast extract	5.0 g/l
Bacto dextrose	5.5 g/l
Sodium chloride	2.5 g/l
L-cysteine	0.5 g/l
Sodium thioglycollate	0.5 g/l

The medium was prepared by dissolving 29 grams of the powdered medium in 1000 ml distilled water by heating in a Koch steamer. The pH was adjusted to 7.4 ± 0.2 with NaOH before sterilization using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The broth was then dispensed in 20 ml volumes and sterilized by autoclaving at 121°C for 15 minutes.

RPMI 1640 Medium

Source: Gibco-Europe Ltd., Paisley Scotland

Composition:

INORGANIC SALTS:	
$Ca(NO_3)_2.4H_2O$	100 mg/l
KCl	400 mg/l
$MgSO_4.7H_2O$	100 mg/l
NaCl	6000 mg/l
$NaHCO_3$	2000 mg/l
$Na_2HPO_4.7H2O$	1512 mg/l
OTHER COMPONENTS:	
D-Glucose	2000 mg/l
Glutathione (reduced)	1.0 mg/l
Phenol Red	5.0 mg/l
AMINO ACIDS:	
L-Arginine	200 mg/l
L-Asparagine	50 mg/l
L-Aspartic Acid	20 mg/l
L-Cystine	50 mg/l
L-Glutamic Acid	20 mg/l
L-Glutamine	300 mg/l
Glycine	10 mg/l
L-Histidine	15 mg/l
L-Hydroxproline	20 mg/l
L-Isoleucine	50 mg/l
L-Leucine	50 mg/l
L-Lysine.HCl	40 mg/l
L-Methionine	15 mg/l
L-Phenylalanine	15 mg/l
L-Proline	200 mg/l
L-Serine	30 mg/l
L-Threonine	20 mg/l
L-Tryptophan	5.0 mg/l
L-Tyrosine	20 mg/l
L-Valine	20 mg/l

VITAMINS:

Biotin	0.2 mg/l
D-Ca Pantothenate	0.25 mg/l
Choline Chloride	3.0 mg/l
Folic Acid	1.0 mg/l
i-Inositol	35 mg/l
Nicotinamide	1.0 mg/l
Para-aminobenzoic Acid	1.0 mg/l
Pyridoxine HCl	1.0 mg/l
Riboflavin	0.2 mg/l
Thiamine HCl	1.0 mg/l
Vitamin B_{12}	0.005 mg/l

The RPMI 1640 medium was supplied as a sterile solution in 500 ml volumes by the manufacturer, and stored at 4°C.

APPENDIX II

BUFFERS AND STAINS

Saliva Ions Buffer (SIB)

Sweet, *et.al.*, (1987) *FEMS Microbiology Letters*, **48**, 159-163.

Source: BDH-British Drug Houses, Laboratory Chemicals, Poole, England

Composition:

Dipotassium hydrogen orthophosphate	0.5 mM
Potassium dihydrogen orthophosphate	0.5 mM
Potassium chloride	50 mM
Magnesium chloride	0.1 mM
Calcium chloride	1.0 mM
Distilled water	1000 ml

To prepare; dissolve potassium phosphates, potassium chloride and magnesium chloride in distilled water. The pH was adjusted to 7.2 with NaOH using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The medium was then dispensed into 100 ml amounts and sterilized by autoclaving at 121°C for 15 minutes.

Calcium chloride was prepared separately because it becomes turbid and autoclaved at 121°C for 15 minutes. Before use calcium chloride was added to the rest of the buffer solution and sterilized by filtration through a 0.2 µm pore size MINISART filter (Sartorius, Ltd., Epsom, Surrey, England).

Since large quantities of this buffer were needed to carry out the different experiments, it was decided to prepare the buffer at a 50 times concentrate using the method described above. The buffer was stored at room temperature.

Hanks Balanced Salt Solutions (HBSS)

Source: Gibco-Europe Ltd., Paisley Scotland

Composition:

Calcium chloride	0.14 g/l
Potassium chloride	0.40 g/l
Potassium dihydrogen orthophosphate	0.06 g/l
Magnesium sulphate	0.098 g/l
Sodium chloride	8.0 g/l
Disodium hydrogen orthophosphate	0.048 g/l
Glucose	1.0 g/l

The HBSS was supplied as sterile solution in 500 ml volumes by the manufacturer, and stored at 4°C.

Phosphate Buffered Saline (PBS)

Dulbecco's formula (modified) without magnesium and calcium

Source: Flow Laboratories, Rickmansworth, England

Composition: (Supplied as tablets)

Disodium hydrogen orthophosphate	1.15 g/l
Potassium dihydrogen orthophosphate	0.2 g/l
Sodium chloride	8.0 g/l
Potassium chloride	0.2 g/l

One tablet was dissolved per 100 ml of distilled water at room temperature and the pH adjusted to 7.2 with NaOH using a Kent EIL 3055 pH meter (TAIWAN, N.R.O.C). The buffer was then sterilized by autoclaving at 121°C for 15 minutes, and stored at room temperature.

Lysis buffer composition

] **Boyum**, A. (1968) *Journal Clin. Lab. Invest.*, **21**, *Suppl.*, 97, 31-50.

Source: BDH-British Drug Houses, Laboratory Chemicals, Poole England

Composition:

Potassium hydrogen carbonate	1.0 g/l
Ammonium chloride	8.2 g/l
EDTA	0.037 g/l
Distilled water	1000 ml

The lysis buffer was prepared by dissolving the above constituents in distilled water and the solution was then sterilized by filtration through a 0.45 μm Sterifil D-HA filtration unit (Nihon Millipore, Kogyo K.K., Yonezawa, Japan) and stored at 4°C. A fresh solution had to be prepared at least once a week to minimize any possible damage to the PMNLs.

Acridine Orange Stain/Buffer

Sources: BDH-British Drug Houses, Laboratory Chemicals, Poole England, and Hopkin and Williams Ltd., Essex, England

The citric acid-sodium hydroxide buffer was prepared from a 0.1 M solution of citric acid (BDH, 21 g/l) and 0.1 M solution of sodium hydroxide (BDH). A buffer with a pH of 3 was prepared by mixing 100 ml of the citric acid solution with 54 ml of the sodium hydroxide solution. At the same time a stock solution of 1 % acridine orange (Hopkin and Williams) was made in distilled water. One millilitre of the acridine orange was then added to 39 ml of the citric acid sodium hydroxide buffer to give a final concentration of 0.025 % acridine orange. The stain was then filter sterilized through a 0.45 µm Sterifil D-HA Filtration unit (Nihon Millipore, Kogyo, Japan) to remove any debris. For safety reasons a face mask and disposable gloves were worn when handling acridine orange. Both buffer and acridine orange were stored at 4°C and used up to four weeks.

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