Effect of co-aggregation on the pathogenicity of oral bacteria

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Summary. The pathogenicity of oral bacteria was studied by measuring the development of subcutaneous abscesses in mice after infection with *Actinomyces viscosus* and *Streptococcus mitis* either singly or as co-aggregated pairs. Heat-treated cells were also tested. The pathogenicity of the co-aggregates was examined in various viable and heat-treated combinations of the two bacterial species. More abscesses were formed by *A. viscosus* than *S. mitis* at all the bacterial concentrations tested. Also, abscess formation by co-aggregates of the two strains produced a higher percentage of abscess formation than those caused by infection with pure suspensions of *A. viscosus* or *S. mitis*. Co-aggregated cells were more resistant to phagocytosis and killing by neutrophils *in vitro* and *in vivo*. Furthermore, *A. viscosus* in co-aggregates were resistant to killing after engulfment by neutrophils. These results suggest that oral bacteria that are able to co-aggregate may resist phagocytosis, and this ability may be linked with pathogenicity.

Introduction

Bacterial infection often involves several species or genera of bacteria that behave in a co-operative fashion. Co-operative or synergic infections have been reported with facultative and anaerobic bacteria in a number of animal models^{1,2} including gingivitis,³ and nutritional interdependency has also been demonstrated clearly.⁴ It is well known that dental plaque is a complex assortment of bacteria held together both by bacterially synthesised polymers and host-derived salivary components. Plaque-associated bacteria, especially species of *Actinomyces* and *Streptococcus*, occur in human dental plaque and may contribute to various disease states including gingivitis,^{5,6} root surface caries and periodontitis.^{7,8} The involvement of *A. viscosus* in caries and periodontal disease, has been demonstrated in hamster and rat experimental models.^{1,9}

As dental plaque matures, a microbial succession occurs in which *Streptococcus* spp. dominate in the early stages followed by an increase in *Actinomyces* spp. and other related gram-positive bacteria.¹⁰ Inter-bacterial reactions between actinomyces and streptococci were first reported by Gibbons and Nygaard¹¹ who proposed that they were an important factor during the maturation of plaque. The results of other studies have shown extensive co-aggregations between strains of *A. viscosus* and *A. naeslundii* with oral streptococci such as *S. sanguis* and *S. mitis*.^{12,13} Also,

unusual morphological arrangements have been observed when filamentous bacteria, such as *Bacterionema* spp., form specific aggregates with oral streptococci in the shape of corn cobs.^{14,15} Previous reports have shown that co-aggregation of *Actinomyces* spp. and oral streptococci is a specific phenomenon mediated by cell-surface interactions between bacterial receptors.^{13,16,17}

Cervico-facial actinomycosis is an endogenously acquired infection, the source of bacteria being dental plaque, carious teeth and periodontal pockets.¹⁸ It is also well known that oral micro-organisms are present in bacteraemia and subacute bacterial endocarditis.¹⁹ However, there is little published information about the biological effect of co-aggregates of these bacteria. This study was performed to assess the pathogenicity of aggregates of *A. viscosus* and *S. mitis* in abscess formation and phagocytosis.

Materials and methods

Bacterial strains and culture conditions

S. mitis ATCC 9811 and *S. sanguis* H1 were used in this study. The latter is a reference strain for streptococcus co-aggregation group 2, and was kindly provided by Dr P. E. Kolenbrander (National Institute of Dental Research, USA). *A. viscosus* T14V was donated by Dr T. Koga (Department of Dental Research, National Institute of Health, Japan). A streptomycin-resistant strain (SMR) of *A. viscosus*

T14V was produced in our laboratory by a series of subcultures in Todd-Hewitt Broth (BBL) containing streptomycin (Meiji Seika, Tokyo, Japan) 2 mg/ml. Each culture was mixed with skimmed milk and stored frozen at -20°C . Cells were cultured anaerobically (CO_2 5%, N_2 95%) in Todd-Hewitt broth for 18 h at 37°C . Cells were harvested by centrifugation (1200 *g* for 20 min) and washed three times with buffer: 1.0 mM Tris (hydromethyl)-aminomethane, adjusted to pH 8.0; 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , NaN_3 0.02% and 0.15 M NaCl.¹³

Co-aggregation assay

Co-aggregation was assayed by the method of Cisar *et al.*¹³ Equal volumes (0.2 ml) of each cell suspension were mixed for 10 s on a vibrator and allowed to stand at room temperature for 10 min. The diameters of co-aggregates were measured microscopically with a micrometer insert (Olympus, Tokyo).

Abscess development

Male mice (ddy) aged 4 weeks were obtained from Shizuoka Experimental Co. Ltd (Hamamatsu, Japan), and observed for 7 days before experiments. Only healthy mice whose weight indicated normal growth were used. Bacteria were cultured and centrifuged as described above and washed with 25 mM potassium phosphate buffer (pH 8.0) containing 25 mM NaCl. Inocula were adjusted to a density of (1.0×10^5) – (1.0×10^{10}) cfu/ml spectrophotometrically at 540 nm, and within 15 min, 0.1 ml was injected subcutaneously into the backs of the mice. The inocula for mixed cultures (0.1 ml) consisted of equal volumes (0.05 ml) of each species. Experiments were repeated four or five times with groups of five mice, and abscess formation was evaluated 2 days after inoculation. The mice were killed with ether and abscess sizes were measured with calipers. The pus samples collected from abscesses were cultured on brain-heart infusion (BHI) agar for 48 h anaerobically to confirm the purity of the original inoculum. Smears were also stained with Giemsa's solution for 15 min and examined microscopically. This protocol was used for viable and heat-treated cells (85°C for 30 min) of both individual and mixed species. Three kinds of co-aggregates were tested: viable *A. viscosus* T14V and *S. mitis*; viable *S. mitis* and heat-treated *A. viscosus* T14V; and viable *A. viscosus* with heat-treated *S. mitis*. Mixtures of heat-treated strains were also used.

Phagocytosis of co-aggregates by neutrophil polymorphonuclear leucocytes (PMNL)

In-vitro inhibition of phagocytosis. In the experiments on the inhibition of phagocytosis and the reduction of the bactericidal activity of PMNL by actinomycetes and streptococci, the ratio of test organisms that produced maximal co-aggregation with minimal free non-

aggregated cells was calculated. After spectrophotometric adjustment of each cell suspension to equal density, *A. viscosus* was mixed with *S. mitis* in the ratios of one part of *A. viscosus* to one, five or 10 parts of *S. mitis*. To separate non-aggregated and aggregated cells, each mixture was applied to the top of a glycerol column as described by Ochiai *et al.*,²⁰ 15 min after mixing. Step-gradient columns were prepared by the sequential addition of 1 ml of a 30, 10 and 5% glycerol layer. The mixture, consisting of discrete and aggregated cells was applied to the step-gradient, glycerol column and 0.5-ml fractions were collected from the bottom. After 10 min, non-aggregated cells remained in the top layer of the column and any aggregated cells that settled in the glycerol 30% layer were determined spectrophotometrically at 540 nm. The percentage aggregation was calculated in accordance with the method of McIntire *et al.*¹⁷ to obtain the optimum ratio of *S. mitis* to *A. viscosus* for co-aggregation to take place. The effect of co-aggregates on phagocytosis was investigated with PMNL induced by injecting 2 ml of oyster glycogen (0.1% in 2 mM phosphate buffered saline, pH 7.2; Sigma) into the abdominal cavity of each mouse.²¹ The mice were killed with ether 10–12 h later and peritoneal exudate cells were collected by the injection of RPMI-1640 Medium (Gibco, USA) supplemented with fetal calf serum 2% into the abdominal cavity. The RPMI-1640 was aspirated and the total number of cells was determined by haemocytometer (Kayagaki Irika Kogyo Co. Ltd, Japan) immediately after rubbing the abdomen. The number of PMNL present in peritoneal exudate cells was determined by Giemsa staining. Previous experiments showed that, with this technique, 95–98% of cells were neutrophils 10–12 h after inoculation, although this fell to 10–15% after 2 days. The number of peritoneal exudate cells was adjusted to 1.0×10^7 /ml, for use in experiments. The number of bacteria that adhered to and were phagocytosed by PMNL was determined by radiolabelling of bacteria. Todd-Hewitt Broth (30 ml), supplemented with 5.5 MBq of [^3H]-thymidine (New England Nuclear, Boston, USA) was inoculated with *A. viscosus* T14V or *S. mitis*. After anaerobic incubation at 37°C for 24 h, cells were harvested, washed with PBS, pH 7.2, and the turbidity was adjusted spectrophotometrically to 1.0×10^8 cfu. The cells were resuspended in the same volume of RPMI-1640 supplemented with fetal calf serum 10%. When co-aggregates were used, the ratio of *A. viscosus* to *S. mitis* was 1 to 10. Bacterial cells (0.1 ml) and PMNL (0.9 ml) were added to plastic dishes (3.5 cm in diameter; #25000, Corning Glass Works, USA) and incubated in a CO_2 incubator at 37°C for 30, 60 and 90 min. Samples collected at these times were stratified in Ficoll-Paque (Pharmacia LKB Biotechnology, Uppsala, Sweden). After centrifugation at 225 *g* for 15 min, the PMNL and sedimented bacterial cells i.e., those not taken up by phagocytes, were collected separately. After washing twice with PBS, Scintisol EX-H (Dojindo Laboratories, Kuma-

Fig. 1. Abscess formation in mice 2 days after injection of cell suspension of live or heat-treated *A. viscosus* and *S. mitis*. Points represent the mean values from four or five different experiments with five mice in each group; vertical bars represent the SD. *A. viscosus* (○), *S. mitis* (△), heat-treated *A. viscosus* (●), heat-treated *S. mitis* (▲).

moto, Japan) was added and the radioactivity was measured in a Liquid Scintillating Counter LCS-673 (Aloka Co. Ltd, Japan).

In-vivo anti-phagocytic activity of co-aggregates. Test cell suspensions were made by adjusting both *A. viscosus* T14V and *S. mitis* 9811 cultures to 1.0×10^8 cfu/ml, then mixing 0.1 ml of the former with 0.9 ml of the latter; 0.5 ml of this suspension was injected into the abdominal cavity of mice. After 15, 30, 60 and 90 min, peritoneal exudate cells were collected from the abdominal cavity with a micro-capillary. Smears were made on glass slides, stained by Giemsa's solution for 15 min and examined at 1000 times magnification. Thirty fields were examined and the total number of neutrophils and the number with adherent and phagocytosed bacteria were counted microscopically.

Anti-bactericidal action of co-aggregates

S. mitis and streptomycin-resistant *A. viscosus* T14V-SMR were used in the experiment. Co-aggregation tests were performed on these strains by the method described above to determine if there was any difference between the parent strains and resistant strains in their ability to form co-aggregates. *A. viscosus* T14V-SMR and *S. mitis* either as individual cells or as co-aggregates, were inoculated into the abdominal cavity of mice that had been given an injection of oyster glycogen 0.1% 10–12 h before. 30 min later, the peritoneal exudate cells were collected by intraperitoneal injection of RPMI-1640. The PMNL were separated from bacterial cells with Ficoll-Paque as described earlier, washed twice, then incubated at 37°C for 60 min in RPMI-1640 containing gentamicin to kill adherent bacteria. The PMNL

suspension was adjusted to give a total volume of 2.0 ml and incubated at 37°C with shaking. A 0.2-ml sample was removed after 15, 30, 60, 90 and 180 min, added to distilled water (0.8 ml), then sonicated at 20 kHz for 30 s (Sonicater Model 5201, Ohtake Works, Tokyo, Japan) to disperse co-aggregates. After serial dilution in BHI broth, the samples were inoculated on to streptomycin-BHI agar plates, and cultured anaerobically at 37°C for 48 h. The number of *A. viscosus* T14V-SMR/sample was calculated, and the change with time determined.

Statistics

Statistical analysis was performed by Student's *t* test.

Results

Abscesses caused by non-aggregated cells

The results for pure bacterial suspensions injected subcutaneously into the backs of mice are shown in fig. 1. Abscesses developed in all animals inoculated with $\geq 1.0 \times 10^7$ cfu of *A. viscosus* T14V but this was reduced to 18.5% when an inoculum of 1.0×10^6 cfu was used. Abscesses developed in all mice inoculated with 1.0×10^9 cfu of *S. mitis*, in 50% with 1.0×10^8 cfu, but no lesions were observed at concentrations of $\leq 1.0 \times 10^7$ cfu. These abscesses were smaller than those produced by *A. viscosus* T14V. Heat-treatment of both *A. viscosus* T14V and *S. mitis* reduced the numbers of abscesses that formed compared with abscess formation with viable cells (fig. 1). Bacterial cells were present in all the stained smears of pus and

Fig. 2. The effect of co-aggregation of *A. viscosus*, a, 1.0×10^6 ; b, 1.0×10^5 and various concentrations of *S. mitis* on abscess formation in mice. Strains were injected after co-aggregates had formed. Points represent the mean values from four or five different experiments with five mice in each group; vertical bars represent the SD. *A. viscosus* + *S. mitis* (○), *A. viscosus* + heat-treated *S. mitis* (●), heat-treated *A. viscosus* + *S. mitis* (△), heat-treated strains of *A. viscosus* and *S. mitis* (▲).

the test species were isolated from the abscess samples initiated by viable bacterial suspension.

Abscesses caused by co-aggregates

When the diameter of co-aggregates was measured microscopically, live cells formed much larger co-aggregates than heat-treated cells. Depending upon cell concentrations, the diameters of viable cell aggregates were at least 30 μm . When co-aggregates of *A. viscosus* T14V (1.0×10^6 cfu) and various concentrations of *S. mitis* were injected into mice, abscesses developed in 96.1% and 91.2% of animals for inocula of 1.0×10^7 cfu and 1.0×10^6 cfu, respectively (fig. 2a). This compares with values of 18.5% and 0% when *A.*

viscosus and *S. mitis*, respectively, were tested in pure culture at the same concentrations. However, in the case of co-aggregation of 1.0×10^5 cfu of *A. viscosus* and various concentrations of *S. mitis*, the abscess development rate remained at a level similar to that for pure cultures of *S. mitis* (fig. 2b). In studies of co-aggregates formed by viable *A. viscosus* T14V (1.0×10^6 cfu) and heat-treated *S. mitis* (1.0×10^8 and 1.0×10^7 cfu), significantly more abscesses developed in mice than in experiments with pure cultures of *A. viscosus* (1.0×10^6 cfu). In experiments with various concentrations of *S. mitis* and 1.0×10^6 cfu of heat-treated *A. viscosus*, abscess formation was less than when co-aggregates containing viable cells of *A. viscosus* were used. No significant differences in abscess

Table. Inhibitory effect of bacterial co-aggregation on phagocytosis

Strain	Incubation time (min)	Bacterial cells associated with PMNL			Non-phagocytosed bacterial cells			Total percentage of recovered† bacterial cells
		Bq	(SD)*	Percent	Bq	(SD)*	Percent	
<i>S. mitis</i> 9811	0	11.5	(3.1)	1.4	711.3	(45.1)	86.7	88.1
	30	88.7	(24.2)	10.8	651.4	(66.5)	79.4	90.2
	60	247.7	(84.5)	30.2	520.0	(84.4)	63.4	93.6
	90	262.4	(79.7)	32.0	496.8	(61.1)	60.6	92.6
<i>A. viscosus</i> T14V	0	10.9	(3.4)	4.0	215.2	(14.5)	79.1	83.1
	30	35.2	(11.4)	12.9	184.1	(38.9)	67.7	80.6
	60	57.1	(9.8)	21.0	178.9	(24.5)	65.8	86.8
	90	73.4	(12.4)	26.9	147.4	(18.4)	54.2	81.1
<i>A. viscosus</i> + <i>S. mitis</i>	0	17.7	(7.2)	2.1	704.4	(82.1)	83.1	85.2
	30	50.0	(10.1)	5.9	951.8	(65.4)	76.9	82.8
	60	75.3	(12.7)	8.9	604.3	(74.9)	71.3	78.3
	90	97.5	(21.1)	11.5	587.4	(68.4)	69.3	81.3

* Total Bq of bacterial cells labelled with [³H]-thymidine were; 820.4 Bq/1.0 × 10⁷ cfu for *S. mitis* 9811, 272.0 Bq/1.0 × 10⁷ cfu for *A. viscosus* and 847.6 Bq/1.0 × 10⁷ cfu for the co-aggregated cells. See *Materials and methods*.

† Total percentage recovery of bacterial cells compared with original suspension.

Fig. 3. The effect of bacterial co-aggregates on the ability of PMNL to phagocytose *in vivo*. Points represent the mean values from four different experiments with three mice in each group; vertical bars represent the SD. *S. mitis* (▲), *A. viscosus* (●), co-aggregates of *A. viscosus* and *S. mitis* (○). Values for co-aggregates were significantly lower than with *A. viscosus* or *S. mitis* alone (*, $p \leq 0.01$).

formation were found between the inoculation of a single heat-treated species and the combinations of heat-treated species. Raised spherical abscesses, containing yellow and sticky pus, resulted from viable bacterial injections, whereas flat abscesses with an indistinct margin and containing watery or serous pus were formed by heat-treated strains.

Anti-phagocytic action of cell aggregates

The number of cells phagocytosed *in vitro* by PMNL was measured by counting the number of isotope-labelled bacterial cells. In the case of pure suspensions of *S. mitis*, 10.8% and 30.2% of the total number of cells were associated with phagocyte after 30 and

60 min, respectively (table). In contrast, the number of *A. viscosus* T14V cells phagocytosed after 30 and 60 min was 12.9% and 21.0% respectively, and even after 90 min the number was only 26.9%. When co-aggregates of *A. viscosus* and *S. mitis* cells were used, the number of phagocytosed cells was 5.9% and 11.5% after 30 and 90 min. These percentages were one-third of the values for pure cultures of *S. mitis* and approximately one-half of these for *A. viscosus* cells.

When *S. mitis* 9811 was injected into mice, 65.1% of PMNL had phagocytosed bacteria 15 min after inoculation, and 82.5% after 60 min (fig. 3). For *A. viscosus* T14V the percentage of cells with phagocytosed bacteria was 62.1%, 66.2% and 68.2% after 15, 30 and 90 min respectively.

Fig. 4. Survival ratio of *A. viscosus* in PMNL. Co-aggregated and non-aggregated *A. viscosus* T14V in PMNL were recovered and total cfu were counted after cultivation. Points represent the mean values from four different experiments with seven mice in each group; vertical bars represent SD. ○, *A. viscosus* (85.7 SD 25.3 cfu at 0 min); ▲, co-aggregates of *A. viscosus* and heat-treated *S. mitis* (67.5 SD 21.2 cfu at 0 min); ●, co-aggregates of *A. viscosus* and *S. mitis* (24.1 SD 15.3 cfu at 0 min). Values for viable co-aggregates of *A. viscosus* and *S. mitis* were significantly higher than those for pure cultures of *A. viscosus* (*, $p \leq 0.01$), and co-aggregates of *A. viscosus* and heat-treated *S. mitis* (†, $p \leq 0.01$).

When co-aggregates of *A. viscosus* and *S. mitis* were inoculated into the abdominal cavity of mice after co-aggregation, the number of PMNL with phagocytosed bacteria was 42.4% after 15 min, 48.2% after 30 min and 56.2% after 90 min (fig. 3). A significant difference ($p \leq 0.01$) was found between the co-aggregate and the pure culture groups of *A. viscosus* and *S. mitis* *in vivo*.

Bactericidal action of neutrophils on co-aggregates

PMNL were collected 30 min after the intra-abdominal inoculation of viable cells of *A. viscosus* T14V and after incubation for various time periods in a CO₂ incubator the number of viable bacteria within phagocytes was assessed by culture. Following incubation for 15 min *in vitro*, 50.2% of *A. viscosus* T14V were viable and this viability declined to 31.6% after 180 min (fig. 4). However, the viability of *A. viscosus* cells present in co-aggregates of *A. viscosus* and *S. mitis* were as high as 72.3% 15 min after the start of incubation *in vitro* and fell to 61.8% after 180 min, (approximately 1.9-fold that for *A. viscosus* cells alone). When co-aggregates of *A. viscosus* and heat-treated *S. mitis* cells were used, 44.2% of the *A. viscosus* cells were viable after incubation for 180 min *in vitro*.

Discussion

It has been reported that saliva and dextran induce aggregation in *S. mutans*, *S. sanguis*, *S. mitis* and *A.*

viscosus.²²⁻²⁴ These species exhibit both homotypic and heterotypic aggregation with the latter being related to cell-to-cell interactions between the adhesin-receptor complementary pairs on the two cell types.^{12,13,17} As the biological significance of microbial co-aggregation *in vivo* has not been studied previously, we investigated pathogenicity by means of an experimental animal abscess model. Actinomyces are pathogenic bacteria that cause chronic inflammatory lesions in man and animals.^{25,26} Engel *et al.*²⁷ have reported that *A. viscosus* releases various extracellular inflammatory substances that cause increased migration of PMNL and accelerate tissue destruction. They also reported that inoculation of *A. viscosus* cells caused a high rate of abscess development. The size of the bacterial aggregates has also been shown to be an important factor in the development of experimental actinomycotic lesions in mice.²⁸ To clarify the role of co-aggregation in our experiments, bacterial strains were selected for their ability to form co-aggregates after heat treatment, thus reducing the possible effect of soluble inflammatory factors from viable microorganisms. Co-aggregates between *A. viscosus* and *S. mitis* (fig. 2a and b) exhibited greater pathogenicity than that shown by single bacterial cells (fig. 1). Although *A. viscosus* had a lower ability to co-aggregate with heat-treated *S. mitis*, the rate of abscess development was still higher than when *A. viscosus* was tested alone. As heat-treated *S. mitis* is only weakly pathogenic (fig. 1), mixtures of heat-treated strains of actinomyces and streptococci did not produce significant effects on abscess development (fig. 2). These results suggest that the pathogenicity of *A.*

viscosus is increased by co-aggregation even with heat-treated *S. mitis*.

As differences in pathogenicity were observed between single cells and aggregates of *A. viscosus*, studies were performed to elucidate this mechanism in the early stages of bacterial infection. It was found that *A. viscosus* was more resistant to phagocytosis than *S. mitis* in pure culture, and when actinomyces formed part of the co-aggregates their uptake by PMNL was decreased further both *in vitro* and *in vivo* (table and fig. 3). Verbugh *et al.*²⁹ reported that in studies with *Staphylococcus aureus*, opsonisation with antibodies was important in phagocytosis by PMNL. Passo *et al.*³⁰ also reported the bactericidal affect of PMNL on *A. viscosus* and plaque bacteria in the presence of fresh human serum. Specific antibodies or fresh serum were not added to our experiments, and this may explain the difference found in the percentage reduction in bacterial counts of *A. viscosus* between Passo *et al.*³⁰ and our study. Previous reports have demonstrated clearly that phagocytosis of actinomyces by PMNL was mediated by the lectin associated with type-2 fimbriae.³¹ In the present study it is possible that *A. viscosus* could evade phagocytosis by forming co-aggregates with *S. mitis* which interferes with the direct interaction between fimbriae and receptors on PMNL. As aggregates are larger in size than single

cells, it is assumed that they are not as easily phagocytosed. However, various enzymes and other unknown substances that reverse aggregation may exist in host tissues and, if active, they could break up aggregates thus allowing the phagocytosis of single or small groups of cells to proceed. Further studies are required to clarify the effect of opsonisation on phagocytosis and bactericidal action on co-aggregated bacteria.

It is well known that co-aggregation amongst gram-positive bacteria contributes to the accumulation of dental plaque on tooth surface. In recent years, many investigators have demonstrated that the gram-negative bacteria that co-aggregate with *Actinomyces* spp. in the gingival crevice appear to be major pathogenic species in periodontitis.³²⁻³⁶ These inter-generic cell-to-cell interactions may have an active role in the bacterial colonisation of the gingival crevice and probably confer some protection against phagocytosis. Thus, co-aggregation may help to explain the pathogenesis of actinomycosis, and also of periodontal disease, and if aggregates of plaque bacteria enter the blood stream it may play a role in the development of focal infection.

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