Interaction of Inflammatory Cells and Oral Microorganisms

VII. In Vitro Polymorphonuclear Responses to Viable Bacteria and to Subcellular Components of Avirulent and Virulent Strains of *Actinomyces viscosus*

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Both virulent (V) and avirulent (AV) strains of *Actinomyces viscosus* T14 are capable of colonizing the oral cavity of gnotobiotic rats, but only T14-V causes destructive periodontal disease. The basis for this difference in *in vivo* pathogenicity has not been adequately defined. In the present study we compared the capacities of T14-AV and T14-V to provoke *in vitro* extracellular release of lysosomal constituents from human polymorphonuclear leukocytes (PMNs). In serum-free cultures, viable T14-V but not T14-AV stimulated discharge of PMN lysosomes. The release response was correlated with PMN phagocytic activity; thus, PMNs readily ingested T14-V but not T14-AV. To explain these differences in PMN-bacteria interactions, subcellular fractions of T14-AV or T14-V were incubated with PMNs. A crude, insoluble sonic extract derived from T14-V caused PMN lysosome release, but a similar fraction from T14-AV was inactive. However, following extensive washing and treatment with deoxyribonuclease or sodium dodecyl sulfate, cell wall fractions of T14-AV stimulated lysosome release. These procedures apparently removed an extracellular polysaccharide slime which is synthesized by T14-AV but not by T14-V. There was a significant reduction in the capacities of viable T14-V or cell wall fractions of T14-V or T14-AV to provoke PMN lysosome release when these agents were preincubated with a slime material isolated from T14-AV. This inhibitory influence of slime was overcome by the addition of fresh or heated (56°C, 30 min) serum to the PMN-bacteria cultures. The data suggest a relationship between the abilities of the avirulent and virulent strains of *A. viscosus* T14 to act as periodontal pathogens *in vivo* and to serve as stimuli for PMN lysosome release *in vitro*.

The initiation and progression of periodontal disease in humans is related to the accumulation of heterogeneous microbial plaques on tooth surfaces (21, 27, 29). Following oral implantation in conventional or gnotobiotic laboratory animals, periodontal destruction can be induced by certain bacteria derived from human plaque, including members of the genus *Actinomyces* (15, 28). It is not known, however, how whole plaque or plaque isolates produce disease, and various bacterial-mediated and host-mediated mechanisms have been proposed.

The influx of a substantial population of polymorphonuclear leukocytes (PMNs) is one of the characteristic features of periodontal disease (2). It has been postulated that these cells may serve as effectors of antibacterial defense and/or tissue injury in the disease process (3, 5, 10, 29). In previous studies we have shown that human

PMNs release lysosomal constituents into the culture medium upon *in vitro* contact with whole human plaque (32) or specific gram-positive (31) or gram-negative (C.-C. Tsai, B. F. Hammond, P. Baehni, W. P. McArthur, and N. S. Taichman, *J. Periodont. Res.*, in press) plaque isolates. In the present study we compare the response of PMNs to avirulent and virulent strains of *Actinomyces viscosus* T14. The virulent strain of *A. viscosus* (T14-V) was isolated from human dental plaque and can cause massive bone loss and destruction of periodontal tissues following oral implantation in gnotobiotic rats (14). The avirulent strain (T14-AV) is a spontaneous mutant of T14-V, retaining its capacity to colonize the oral cavity of rodents but no longer capable of causing periodontal lesions (14). The purpose of our experiments was to determine whether there is a correlation between the potentials of T14-

AV and T14-V to initiate disease in vivo and to influence PMN behavior in vitro.

MATERIALS AND METHODS

Propagation of T14-AV and T14-V. Both strains of T14 were originally isolated and generously supplied by S. S. Socransky (Forsyth Dental Center, Boston, Mass.). Microorganisms, derived from lyophilized stock cultures of T14-AV and T14-V, were grown aerobically in Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.) in 1-liter batches at 37°C for 24 h under stationary conditions. Bacteria were freshly harvested for each experiment by centrifugation ($10,000 \times g$, 4°C), washed three times in cold saline (0.85%) and resuspended in Hanks balanced salt solution supplemented with 0.1% gelatin (HBSS). Cell concentrations were appropriately adjusted with HBSS following direct counting in Petroff-Hausser chambers.

Preparation of cell wall fractions of T14-AV and T14-V. Various subcellular fractions of bacterial cell walls were prepared by adaptations of the procedures described by Wallinder and Neujahr (34).

(i) **Crude bacterial cell sonic extracts.** Bacteria from 2-liter batches were washed three times in saline (0.85%), mixed with an equal volume of glass beads (Ballontini, Schwarz/Mann, Orangeburg, N.Y.), and subjected to sonic treatment for 30 min in a sonic disintegrator (MSE, Westlake, Ohio). After settling of the beads, the preparation was centrifuged to yield an opalescent soluble extract and an insoluble, crude cell wall sediment. The latter was washed three times in saline, and the soluble extract was dialyzed against large volumes of distilled water followed by lyophilization.

(ii) **DNase-treated bacterial cell walls.** Crude insoluble cell wall fractions (see above) were washed extensively in saline (0.1 M), followed by treatment with deoxyribonuclease (DNase; 6 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) in potassium phosphate buffer (0.05 M, pH 7.5) as outlined by Wallinder and Neujahr (34). The sediment was washed in water, lyophilized, and stored at -20°C.

(iii) **Trypsin-treated cell walls.** Crude cell walls treated with trypsin were prepared in the same fashion as described for DNase-treated cell walls with the addition of trypsin (bovine pancreas, 2 $\times$ crystallized, 0.5 mg/ml; Sigma Chemical Co.) to DNase-potassium phosphate buffer medium. The preparation was washed and dialyzed extensively in phosphate-buffered saline (0.1 M), lyophilized, and stored at -20°C.

(iv) **SDS-treated cell walls:** DNase-treated cell walls were suspended in a saturated solution of sodium dodecyl sulfate (SDS) at 37°C for 24 h, followed by repeated washing in distilled water. The preparation was lyophilized and stored at -20°C.

Preparation of extracellular polysaccharides from T14-AV. Isolation of the extracellular polysaccharide of T14-AV was accomplished by minor changes in the method detailed by Rosan and Hammond for the rodent strain of *A. viscosus* T6 (22). Briefly, bacterial culture supernatants were first dialyzed extensively against phosphate-buffered saline (0.1 M) and then subjected to acetone precipitation.

The resulting precipitate was further treated with trichloroacetic acid and then reprecipitated in acetone, followed by dialysis against distilled water. The dialyzed material (2 mg/ml) was then applied to a column (0.9 by 19.3 cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) previously equilibrated with 0.05 M phosphate buffer (pH 7.5) and eluted with the same buffer at a rate of 1 ml per 5 min. All fractions (1 ml) were monitored for *N*-acetyl glucosamine (1) and by Ouchterlony gel diffusion plates using rabbit antisera directed against whole cells of T14-AV. A single large hexosamine peak appeared at fraction no. 10 and disappeared at fraction no. 20. The appearance/disappearance of the hexosamine peak was paralleled by serological detection of the slime in gel diffusion. Fractions no. 10 through 19 were pooled, and the resulting preparation was a viscous gel rich in *N*-acetyl glucosamine (approximately 45%) with small amounts of glycerol and neutral sugars (glucose and galactose) and less than 10% protein. The preparation was lyophilized and stored at -20°C. The doses of extracellular slime employed in these experiments were expressed as dry weight.

Preparation of cell surface antigen containing 6-DOT from T14-V. A high-molecular-weight, specific cell wall antigen characteristic of T14-V was isolated as described by Hammond et al. (14). The soluble material is composed of two parts: (i) a polysaccharide containing 6-deoxytalose (6-DOT) as the major sugar (approximately 67% dry weight), which serves as the determinant of serological specificity, and (ii) a small peptide bearing some resemblance to peptidoglycan.

Preparation of PMNs. Human peripheral blood PMNs were isolated from heparinized venous blood of healthy donors by dextran sedimentation (Dextran T500, Pharmacia) followed by centrifugation on a Hypaque gradient (Lymphocyte Separation Medium; Bionetics Laboratory Products, Kensington, Md.) to yield a preparation containing 90 to 95% PMNs (32). Cells were suspended at a concentration of 20×10^6 per ml of HBSS.

Incubation of PMNs with microorganisms. Duplicate cultures of PMNs (0.5 ml, 10×10^6 PMNs) were exposed to varying concentrations of whole bacteria (5, 25, 50, and 100 bacteria per PMN) or to different subcellular bacterial fractions in a total volume of 1.0 ml of HBSS, using stoppered polypropylene centrifuge tubes (12-ml capacity, 10 by 15.8 mm; Nalge Sybron Corp., Rochester, N.Y.) as culture vessels. Tubes were continuously agitated at 37°C in a gyratory water bath shaker (150 rpm, model G86, New Brunswick Scientific Co., New Brunswick, N.J.) for 5 to 120 min. Controls consisted of PMNs in HBSS without bacteria or PMNs in HBSS plus serum without bacteria.

At the conclusion of the experiment, cultures were placed on ice for 10 min and then centrifuged to remove PMNs and bacteria or bacterial fractions (31). Culture supernatants were assayed for extracellular liberation of PMN constituents.

Human sera. The effects of serum on PMN-T14 interactions were evaluated by the inclusion of 5.0% (vol/vol) fresh or heated (56°C, 30 min) serum in the cultures. Autologous serum collected from the PMN

donor was used to modulate PMN reactions. Unless indicated otherwise, sera were added to bacteria or various bacterial fractions immediately prior to the addition of PMNs.

Extracellular release of PMN constituents. Lysozyme, β -glucuronidase, myeloperoxidase, and lactoferrin served as representative lysosomal markers and were estimated in cell-free PMN culture supernatants as described elsewhere (31, 32). Results were expressed in two ways: as a percentage of the total available activity in the PMN cultures or in terms of specific activity discharged into the cultures (32). In all calculations, the percentage or specific activity of products spontaneously released from control cultures (i.e., PMNs in HBSS alone or PMNs in HBSS plus serum alone) was subtracted from experimental values to provide net release of individual markers. Lysosome release from control cultures ranged from 2.0 to 8.0% of the total available activity in PMN suspensions.

As a reflection of cell viability, lactate dehydrogenase (LDH) was monitored in culture supernatants in selected experiments by using the Worthington assay kit (36) (Statzyme LDH; Worthington Diagnostics, Freehold, N.J.). Results were expressed in percentage of LDH release from stimulated cells when compared to maximal release from PMNs exposed to 1.0% Triton X-100.

Electron microscopy of PMN interaction with bacteria. PMN-bacteria interactions (25 microorganisms per PMN; 20-min incubation period at 37°C) were evaluated ultrastructurally by the methods previously reported (4).

Phagocytic activity of PMNs exposed to T14-AV or T14-V was estimated by counting at least 200 PMNs in sections from each culture and recording the number of cells showing evidence of ingesting one or more bacteria.

Additional procedures will be described below.

RESULTS

PMN lysosome release in response to virulent and avirulent strains of *A. viscosus* T14. In the absence of added serum, viable T14-V provoked dose-dependent extracellular release of PMN lysosomes but, by comparison, T14-AV

cells were inactive (Table 1). Although data are not presented in this report, PMN lysosome release by T14-V varied with the duration (5 to 120 min) of the experiment; on the basis of the results obtained it was decided that an incubation period of 60 min at 37°C could serve as standard conditions in all experiments unless otherwise indicated. T14-V failed to trigger PMN lysosome release if incubations were carried out at 4°C instead of 37°C or in the presence of 0.001 to 0.0001 M sodium iodoacetate (Sigma Chemical Co.). Varying amounts of bacterial growth culture supernatants (concentrated by lyophilization to 1/10 original volume) were incubated with PMNs; these fluids failed to induce lysosomal discharge. Under conditions where PMNs liberated lysosomal markers, there was no corresponding extracellular release of LDH (Table 1). Neither strain of T14-AV or T14-V in itself was capable of absorbing or inactivating detectable amounts of any of the lysosomal markers used in this study; this was determined by incubating known concentrations of various markers with T14-AV or T14-V for 60 min at 37°C. Lysosomal marker recoveries were almost 100% in all instances.

Effects of serum on PMN lysosome release in response to T14-AV and T14-V. The inclusion of either fresh or heated autologous serum (5%, vol/vol) in the incubations had significant effects on PMN lysosome release in response to viable bacteria. Whereas T14-AV cells were relatively ineffectual stimuli for PMN lysosome release in the absence of serum, these bacteria prompted vigorous reactions in the presence of serum (Fig. 1). Serum also significantly enhanced the capacity of T14-V to provoke lysosome release (Fig. 2). In general, fresh serum seemed to be more potent than heated serum as a modulator of these reactions (Fig. 1, 2). It was apparent, however, that even in the

TABLE 1. Extracellular release of PMN constituents in response to *A. viscosus* T14-AV and T14-V^a

No. of bacteria per PMN	Percent enzyme release			
	Lysozyme	β -Glucuronidase	Myeloperoxidase	Lactate dehydrogenase
Avirulent strain				
5	<1.0	<1.0	<1.0	<1.0
25	1.4 $\pm$ 0.5	<1.0	<1.0	<1.0
50	2.4 $\pm$ 0.2	<1.0	<1.0	<1.0
100	2.8 $\pm$ 0.3	<1.0	<1.0	<1.0
Virulent strain				
5	12.7 $\pm$ 1.3	9.5 $\pm$ 0.9	12.7 $\pm$ 1.0	<1.0
25	18.3 $\pm$ 4.9	25.3 $\pm$ 1.2	17.4 $\pm$ 1.2	<1.0
50	34.7 $\pm$ 2.6	32.7 $\pm$ 2.5	21.1 $\pm$ 1.6	<1.0
100	42.0 $\pm$ 5.3	37.9 $\pm$ 0.9	31.0 $\pm$ 3.4	<2.0

^a Human PMNs were incubated with bacteria for 60 min at 37°C in the absence of serum. Results represent mean $\pm$ standard error of mean for 15 separate experiments.

FIG. 1. Effect of autologous serum (5%, vol/vol) on PMN lysozyme release in response to *A. viscosus* T14-AV. Bacteria were incubated with PMNs for 60 min at 37°C. Symbols: (○) bacteria alone; (□) bacteria plus fresh autologous serum; (Δ) bacteria plus heated (56°C, 30 min) autologous serum.

presence of serum T14-V was still more effective than T14-AV in provoking PMN lysosome release (Fig. 1 and 2). LDH was not released from PMNs exposed to bacteria in the presence of serum.

Phagocytosis of viable bacteria by PMNs. As evaluated by quantitative electron microscopy, T14-V but not T14-AV cells were readily phagocytosed by PMNs in the absence of serum (Fig. 3 and 4; Table 2). There was an appreciable enhancement of PMN ingestion of both T14-AV and T14-V when serum was included in the incubations (Fig. 5, Table 2). Phagocytosis of bacteria by PMNs was associated with degranulation of lysosomes into phagocytic vacuoles (Fig. 4 and 5).

Effect of extracellular bacterial polysaccharide (slime) on PMN responses to T14-V. In the absence of added serum, the extracellular slime isolated from T14-AV markedly depressed PMN lysosome release in response to T14-V. Varying amounts of this material (0.1 ng to 100 μg) were preincubated with T14-V at 37°C for 60 min prior to the addition of PMNs; extremely small concentrations of slime (1.0 to 10 ng) produced significant inhibition of PMN release (Table 3). On the other hand, if the slime was added simultaneously with or 15 min after the presentation of T14-V to PMNs, there was little or no significant inhibition of lysosome release (data not shown). This suggested that slime had to bind to or coat bacteria to demonstrate inhibition of PMN-T14-V interaction. The following experiment was carried out to test this hypothesis. T14-V cells were preincubated with or without slime at 37°C for 30 min. Bacteria

were subsequently washed twice in cold HBSS and then exposed to PMNs for an additional 30 min at 37°C. Likewise, PMNs were preincubated with or without slime at 4 or 37°C for 30 min; subsequently, twice-washed PMNs were exposed to viable untreated T14-V for 30 min at 37°C. The results are shown in Fig. 6. It is apparent that bacteria pretreated with slime were no longer able to trigger significant PMN lysosome release. On the other hand, PMNs pretreated with slime still vigorously responded to untreated T14-V.

Fresh or heated autologous serum was capable of neutralizing the inhibiting influence of slime on PMN reactions to T14-V, as shown in the following experiments. T14-V cells were pretreated with slime for 60 min. Subsequently, PMNs together with fresh or heated serum were added to the culture tubes and, in the presence of serum, slime-treated bacteria served as potent triggers for PMN lysosome release (Table 3). The slime preparation had no demonstrable effects on the extinction values for known concentrations of various lysosomal markers.

Effects of subcellular bacterial components on PMN lysosome release. (i) Crude bacterial sonic extracts. Varying concentrations of crude, insoluble sediment derived from sonically disrupted T14-V were capable of inducing PMN lysosomal but not LDH release (Fig. 7). Identical amounts of a similar material

FIG. 2. Effect of autologous serum (5%, vol/vol) on PMN lysozyme release in response to *A. viscosus* T14-V. Bacteria were incubated with PMNs for 60 min at 37°C. Symbols: (○) bacteria alone; (□) bacteria plus fresh autologous serum; (Δ) bacteria plus heated (56°C, 30 min) autologous serum.

derived from T14-AV did not trigger significant PMN reactions (Fig. 7). Electron microscopic evaluation of the crude insoluble sediments indicated that they contained various particles (e.g., ribosomes, cell membranes, etc.), but it was an impression that these preparations were clearly enriched with bacterial cell walls. Soluble fractions derived by sonic disruption of either T14-AV or T14-V were not capable of provoking detectable PMN release.

(ii) **DNase-treated bacterial cell walls.** DNase-treated cell wall preparations from T14-

AV or T14-V provoked dose-dependent PMN lysosome release in the absence of added serum (Table 4). The release response was variably enhanced upon the addition of either fresh or heated autologous serum to the cultures, generally with fresh serum being more potent than heated serum (Table 4). DNase-treated bacterial cell wall fractions did not provoke LDH release, nor did these materials cause lysosome discharge when incubations were carried out at 4°C instead of 37°C or in the presence of 0.001 M sodium iodoacetate.

(iii) **SDS-treated bacterial cell walls.** SDS-treated cell wall fractions from either T14-AV or T14-V prompted vigorous lysosome release in the absence of serum (Table 5). LDH release was not detected under these circumstances.

(iv) **Trypsin-treated bacterial cell walls.** After treatment with trypsin there was a substantial reduction in the capacity of bacterial cell wall preparations to stimulate PMN lysosome release (Table 6). However, in the presence of added serum, trypsin-treated cell walls caused PMNs to release lysosomal constituents (Table 6).

(v) **6-DOT antigen of T14-V.** 6-DOT-enriched polysaccharide derived from T14-V did not provoke PMN lysosome release in the absence or presence of serum (data not shown).

TABLE 2. *Percentage of PMNs exhibiting phagocytosis of A. viscosus T14-AV and T14-V^a*

Stimuli	Percentage of PMNs exhibiting phagocytosis ^b	
	T14-AV	T14-V
Bacteria alone	2.0 ± 0.81	25.0 ± 3.1
Bacteria plus fresh serum ^c	60.5 ± 1.25	65.5 ± 3.1
Bacteria plus heated serum ^c	17.5 ± 5.9	44.5 ± 5.8

^a PMNs were exposed to bacteria (25 per PMN) for 20 min at 37°C.

^b At least 200 PMNs were counted in each specimen. Results are expressed as mean ± standard error.

^c Serum: 5% (vol/vol) fresh or heated (56°C, 30 min) autologous PMN donor serum.

TABLE 3. *Effect of bacterial slime on PMN lysozyme release in response to A. viscosus T14-V*

Agents tested			PMN lysozyme release (μg/ml) at no. of T14-V per PMN ^a :			
Slime ^b	Fresh serum ^c	Heated serum ^c	5	25	50	100
—	—	—	2.0 ± 0.9	15.8 ± 5.6	27.9 ± 6.4	34.3 ± 3.4
+	—	—	0.7 ± 0.7	4.5 ± 2.3	12.7 ± 3.2	17.1 ± 2.2
—	+	—	11.9 ± 0.7	48.2 ± 12.9	80.9 ± 1.7	85.2 ± 2.7
+	+	—	10.0 ± 2.8	48.1 ± 4.9	69.5 ± 13.0	61.1 ± 11.7
—	—	+	7.6 ± 3.8	22.8 ± 3.8	33.7 ± 5.9	52.2 ± 11.5
+	—	+	13.8 ± 4.8	23.5 ± 6.2	39.4 ± 3.8	55.4 ± 6.2

^a PMNs were incubated with bacteria for 60 min at 37°C. Results are expressed as mean ± standard error of 12 separate experiments.

^b Extracellular slime (10 ng) derived from *A. viscosus* T14-AV was preincubated with T14-V for 60 min at 37°C.

^c Fresh or heated (56°C, 30 min) autologous serum (5%, vol/vol) was added to bacteria immediately prior to addition of PMNs.

FIG. 3. PMNs incubated (20 min, 37°C) with viable T14-AV in the absence of autologous serum. Numerous microorganisms can be identified in the extracellular milieu, but none has been phagocytosed by the PMNs in this section. Note the abundance of cytoplasmic granules (lysosomes) in the PMNs. The bar in the lower right-hand corner of the electron micrographs represents 1 μm.

FIG. 4. PMNs exposed (20 min, 37°C) to viable T14-V in the absence of autologous serum. The PMN in this section has ingested several microorganisms, which are localized within phagocytic vacuoles. A reduction in the number of PMN cytoplasmic granules is apparent.

FIG. 5. PMNs incubated (20 min, 37°C) with viable T14-AV in the presence of autologous fresh serum (5%, vol/vol). The cell has phagocytosed many bacteria, which are found within phagocytic vacuoles. The cytoplasm of the PMN contains relatively few lysosomal granules.

DISCUSSION

In previous studies we found that whole bacterial plaque (32) as well as certain gram-positive (31) or gram-negative (Tsai et al., in press) microbial isolates from plaque could provoke PMN lysosome release in vitro. Of the gram-positive bacteria tested in those experiments, only microorganisms which were able to induce periodontal disease in gnotobiotic animals also proved to be capable of provoking exocytosis of PMN lysosomes in vitro in the absence of added serum

(31). On the other hand, gram-positive plaque bacteria which did not produce periodontal lesions in gnotobiotic animals failed to stimulate PMN lysosome release unless serum was included in the tissue culture medium (18, 31). This correlation between the capacity of gram-positive plaque isolates to cause periodontal disease in vivo and PMN lysosome discharge in vitro has been confirmed and explored in greater detail in the present communication. Thus, the virulent strain of *A. viscosus* T14, T14-V, is able to incite periodontal syndromes upon oral implantation in appropriate animals (14) and con-

FIG. 6. Effects of preincubating T14-V or PMNs with extracellular bacterial slime (derived from T14-AV) on PMN lysozyme release. (A) (■) T14-V (50 bacteria per PMN) were preincubated (37°C) with slime (10 ng), washed, and then exposed to PMNs; (□) control, i.e., bacteria preincubated in HBSS. (B) (■) PMNs preincubated (37°C) with slime (10 ng), washed, and subsequently exposed to T14-V (50 bacteria per PMN); (□) control, i.e., PMNs preincubated in HBSS. (C) (■) PMNs preincubated (4°C) with slime (10 ng), washed, and subsequently exposed to T14-V (50 bacteria per PMN); (□) control, i.e., PMNs preincubated in HBSS.

FIG. 7. Percentage of PMN lysosome release in response to crude sonic sediments of T14-AV (○) and T14-V (□). Bacterial preparations were incubated with PMNs at 37°C for 60 min.

TABLE 4. PMN lysozyme release in response to DNase-treated cell wall fractions of *A. viscosus* T14-AV and T14-V^a

Prepn ^b	Percent lysozyme release from cell wall fractions (mg) ^c				
	0.025	0.05	0.125	0.25	0.5
T14-AV					
Alone	1.8 ± 1.4	18.6 ± 8.0	16.3 ± 3.5	25.3 ± 4.0	31.3 ± 5.9
Plus fresh serum	18.8 ± 7.3	42.4 ± 19.2	54.2 ± 20.8	80.9 ± 16.2	ND
Plus heated serum	10.4 ± 1.2	36.8 ± 8.7	37.6 ± 8.5	48.7 ± 5.5	ND
T14-V					
Alone	1.9 ± 1.4	10.8 ± 2.6	14.1 ± 3.5	21.5 ± 2.2	24.1 ± 3.5
Plus fresh serum	7.9 ± 3.4	15.9 ± 3.2	56.7 ± 25.1	64.1 ± 23.4	ND
Plus heated serum	11.4 ± 0.1	31.0 ± 9.9	32.4 ± 10.9	26.3 ± 3.2	ND

^a DNase-treated cell wall preparations were incubated with PMNs for 60 min at 37°C.

^b Bacterial preparations were incubated with PMNs in the absence or presence of 5% (vol/vol) fresh or heated (56°C, 30 min) autologous serum.

^c Mean ± standard error of 14 experiments. ND, Not determined.

sistently causes in vitro PMN lysosome release without the need of serum. On the other hand, the avirulent mutant, T14-AV, of this microorganism is no longer able to induce disease in vivo, nor does it promote PMN release in vitro without serum.

The PMN response triggered by viable T14-V was not associated with indications of cytotoxic effects on PMNs. This conclusion is based upon the observation that lysosome release was never accompanied by significant release of LDH into the culture fluids. Pilot studies also demonstrated that PMNs interacting with T14-V failed to be stained by trypan blue. Lysosome release in response to T14-V was dependent upon metabolic changes in PMNs, since the reaction failed to occur at 4°C or in the presence of the glycolytic poison, sodium iodoacetate. It would appear that phagocytosis of T14-V was one prerequisite for the release reaction; conversely, T14-AV were not readily ingested by PMNs.

TABLE 5. PMN lysosome release following exposure to SDS-treated cell wall fractions of *A. viscosus* T14-AV and T14-V^a

Cell wall fraction (mg)	Percent release of lysosomal marker ^b :		
	Lysozyme	β -Glucuronidase	Myeloperoxidase
T14-AV			
0.1	8.5	5.3	12.7
0.5	13.8	15.8	17.5
1.0	21.6	22.5	17.8
T14-V			
0.1	8.0	10.8	0
0.5	13.3	23.3	10.1
1.0	14.8	22.5	11.4

^a PMNs were exposed to SDS-treated bacterial cell wall fractions for 60 min at 37°C.

^b Mean of three experiments.

Evidence is presented to focus attention on extracellular bacterial polysaccharides, i.e., slime, as an important determinant of the interaction between PMNs and the bacteria employed in this study. T14-AV synthesizes an extracellular slime material which in this study impedes bacterial engulfment and subsequent activation and release of PMN lysosomal constituents. When compared with untreated T14-V, T14-V pretreated with the slime preparation was less vigorous in triggering PMN lysosome release. Presumably, the polysaccharide-rich material binds to T14-V and interferes with or blocks attachment and phagocytosis by PMNs. These conclusions seem reasonable in view of reports by other investigators indicating that capsular polysaccharides inhibit PMN phagocytosis to encapsulated strains of a variety of microorganisms such as *Pseudomonas aeruginosa* (23, 24) and *Streptococcus pneumoniae* (35). In some instances, capsular glycoproteins bind to PMNs (17), but, in our experiments, we found no evidence that slime had any direct effects on PMNs. When PMNs rather than bacteria were pretreated with slime the leukocytes still responded to T14-V. This indicates that slime does not bind to PMNs but coats microorganisms, thereby "masking" substances needed for attachment to membrane receptors on PMNs. An analogous conclusion has been offered by Kozel (16), who found that cryptococcal polysaccharides inhibited phagocytosis by binding to yeast rather than to the surface of macrophages. Pertinent to this is the fact that removal of the slime from subcellular fractions of T14-AV allows these stimuli to provoke PMN reactions. Crude sonic sediments of T14-AV did not cause lysosome release, presumably because much of the polysaccharide material still remained on the cell wall portion of this fraction.

TABLE 6. PMN lysozyme release in response to trypsin-treated cell wall fractions of *A. viscosus* T14-AV and T14-V^a

Prepn ^b	Percent lysozyme release from cell wall fraction (mg) ^c :			
	1.0	2.5	5.0	10.0
T14-AV				
Alone	0	2.2 $\pm$ 2.1	5.1 $\pm$ 3.9	5.5 $\pm$ 2.9
Plus fresh serum	38.4 $\pm$ 2.9	34.5 $\pm$ 3.4	34.4 $\pm$ 10.5	42.4 $\pm$ 0.9
Plus heated serum	7.5 $\pm$ 1.2	16.5 $\pm$ 9.0	30.4 $\pm$ 11.1	ND
T14-V				
Alone	0	4.9 $\pm$ 3.3	5.9 $\pm$ 4.0	1.9 $\pm$ 0.7
Plus fresh serum	38.4 $\pm$ 3.3	51.8 $\pm$ 6.9	43.9 $\pm$ 8.0	43.2 $\pm$ 5.2
Plus heated serum	19.3 $\pm$ 4.1	21.4 $\pm$ 2.1	19.7 $\pm$ 0.4	21.1 $\pm$ 1.3

^a Trypsin-treated bacterial cell wall preparations were incubated with PMNs for 60 min at 37°C.

^b Bacterial preparations were incubated with PMNs in the absence or presence of 5% (vol/vol) fresh or heated (56°C, 30 min) autologous serum.

^c Mean $\pm$ standard error of five experiments. ND, Not determined.

Extensive washing and treatment of the T14-AV crude sonic sediment with DNase or with DNase and SDS removed most of the slime, as verified by immunodiffusion and immunoelectrophoresis using rabbit antisera against slime.

Autologous serum exerted profound effects on PMN interactions with both T14-AV and T14-V. This was most readily appreciated with viable T14-AV, which did not trigger lysosome release without serum. When either fresh or heated serum was placed into the incubations, T14-AV cells were more readily ingested by the leukocytes, which then released lysosomes but not LDH extracellularly. Similarly, serum amplified PMN responses to T14-V. These observations were anticipated because in earlier studies we found that serum was able to modulate PMN phagocytosis and lysosome discharge in response to numerous "active" and "inactive" plaque bacteria (18; Tsai et al., in press). There are several mechanisms whereby serum could influence PMN-bacteria interactions. The presence of antibodies to dental plaque bacteria, including *A. viscosus*, has been demonstrated in human serum (11, 25), and it is likely that the combination of anti-*A. viscosus* antibodies with various determinants (including slime) on either T14-AV or T14-V leads to bacterial opsonization and facilitation of phagocytosis. Since fresh serum was usually more powerful than heated serum in potentiating lysosome release, this suggests that activation of the complement system may be relevant in amplifying PMN reactions to plaque bacteria. Sequential activation of the alternative complement pathway may be directly precipitated by *A. viscosus*, and the classical pathway could be triggered by the formation of bacteria-antibody complexes (33). In either event, the generation of soluble, biologically active complement split products could lead to opsonization of bacteria and/or to activation of PMN lysosome release-inducing peptides (i.e., C5a) [33; C. C. Tsai, N. S. Taichman, W. P. McArthur, and P. Baehni, *J. Dent. Res.* 57(Special Issue A):755, 1978].

Our studies demonstrate that soluble fractions derived by sonic disruption of T14-AV or T14-V or as a result of extraction of a 6-DOT-enriched antigen from T14-V are inactive in promoting PMN lysosome discharge. The function of 6-DOT antigen in explaining the pathogenicity of T14-V is not known, but our studies indicate that this material is not relevant to stimulating PMN lysosome secretion. Unlike soluble fractions, various particulate preparations of bacterial cell walls proved to be potent stimuli for lysosome release. It is interesting that trypsin treatment of otherwise active bacterial cell wall fractions markedly reduced the capacity of these

agents to provoke release in the absence of serum. This suggests that the enzyme removed peptides critical to the binding mechanism of bacterial cell walls to leukocyte receptors. However, the addition of serum to this fraction resulted in substantial restoration of PMN lysosome release-inducing potentials. Under these circumstances we presume that serum serves in a similar capacity to modulate release, as described earlier.

Numerous investigators have shown that cell walls from certain bacteria can provoke PMN and macrophage lysosome release in vitro and have speculated that related events in vivo may help to explain the development of chronic inflammatory lesions associated with the persistence of undigestible bacterial cell walls in lesions (6, 7, 20, 26). Since bacterial cell walls can be seen in dental plaque and within leukocytes contacting plaque (4, 10, 32), it may be reasonable to presume that bacterial fragments, in addition to whole bacteria, can serve as stimulants of PMN lysosome release in periodontal disease (12, 32). In this regard it is pertinent to point out that homogenates of various strains of *Actinomyces* are also capable of stimulating lymphoid blastogenesis [J. J. Baker, S. P. Chan, B. F. Hammond, and S. E. Mergenhagen, *J. Dent. Res.* 54(Special Issue A):178, 1975], inducing secretion of leukocyte inhibition factors from mononuclear cells [W. P. McArthur, A. Orloff, and G. Tawil, *J. Dent. Res.* 57(Special Issue A):247, 1978] and producing cytopathic changes in fibroblast cultures (8).

In infections caused by several slime-coated parasites, one generally visualizes that extracellular capsular polysaccharides or slimes serve to enhance microbial virulence (16, 19, 23, 24). This may be the consequence of numerous adverse effects on host defense systems such as impediment of PMN phagocytic and microbiocidal activities (see above). In terms of the development of periodontal disease, the present study suggests that bacterial slime may not be detrimental and indeed may be "protective" insofar as potential consequences of bacteria-leukocyte interaction in the gingival crevice are concerned. Recall that T14-V, which is devoid of slime, is capable of inducing periodontal disease in gnotobiotic animals, whereas slime-coated T14-AV no longer induces similar lesions. As summarized elsewhere, there are many reasons for suspecting that PMNs may be effectors of tissue injury in periodontal disease by virtue of their capacity to release lysosomes in the gingival environment (3, 32). It becomes evident that the slime on T14-AV renders these microorganisms "unattractive" to PMNs (as reflected by low PMN phagocytic activity), and hence little or no ly-

lysosome release occurs in response to these bacteria in the absence of serum. Conversely, T14-V readily interacts with PMNs and provokes lysosome release under similar conditions. In light of these considerations, one would anticipate that gingival infection caused by T14-AV would be associated with less tissue damage and inflammation than infection by T14-V. This projection does not, of course, take into account the possible modulating influences of serum in the gingival crevice. Thus, the exudation of serum components into the crevice may tend to minimize relative differences between the lysosome release-inducing potentials of different strains of *A. viscosus* T14. Even if this proves to be the case, our data indicate that T14-V is still more effective than T14-AV in activating PMN release in the presence of serum.

There are other possible ways in which T14-AV slime could theoretically serve as a determinant in the development of periodontal infections. Brecher and van Houte [J. Dent. Res. 57(Special Issue A):789, 1978] have demonstrated that T14-AV and T14-V readily colonize the oral cavity following oral inoculation in gnotobiotic animals, but, when compared to T14-V, relatively few T14-AV adhere onto enamel surfaces. Thus, the slime may prevent or limit establishment of bacterial colonies on strategic areas of the tooth surface critical to the initiation of periodontal disease. Lastly, slime may explain why T14-AV, as opposed to T14-V, fails to trigger lymphocyte blastogenesis in vitro [Baker et al., J. Dent. Res. 54(Special Issue A):178, 1975]. If the secretion of lymphokines from lymphocytes is involved in the periodontal disease process, then T14-AV may not be capable of activating this effector mechanism.

Periodontal disease is not a single disorder but an exceedingly complex series of chronic inflammatory diseases. It is likely that no single bacterial species or host effector system will explain the etiology and pathogenesis of all or even one of these disease entities. Our experiments lend credence to the hypothesis that PMN-bacteria interaction in the gingival crevice may be one interesting chapter in these disorders.

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