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SECRETION OF CYTOKINES IL-1β, IL-6 AND TNF-α FROM MOUSE MACROPHAGES UPON CO-INCUBATION WITH DIFFERENT SPECIES OF ORAL TREPONEMA

The Thesis in Oral Molecular Biology

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ABSTRACT

Periodontal disease is probably caused by bacteria in dental plaque. Oral spirochetes are among the putative periodontal pathogens associated with periodontitis. Besides bacterial challenge, the host response to the bacteria is thought to be of fundamental importance in disease initiation and progression. Host cells respond to bacteria in a variety of ways, including production of soluble protein mediators called cytokines. Cytokines are cell regulators with major influences on different effector cells involved in inflammation.

Experiments reported here studied the release of cytokines IL-1β, IL-6 and TNF-α from mouse macrophages when grown in the presence of oral treponemes. Supernatants were harvested at 2, 8, 24, and 48 hours after macrophages were co-incubated in tissue culture media with different species of oral treponema. *Porphyromonas gingivalis* was used as the positive control, based on the evidence in the literature suggestive of its ability to stimulate cytokine production from macrophages. Negative controls consisted of macrophages cultured in media without bacteria. It was also determined whether viable bacteria were necessary to elicit cytokine secretion. Bacteria were killed by treatment with penicillin and streptomycin for 48 hours in the presence of oxygen. They were then co-incubated with macrophages, and the supernatants were harvested at the same time intervals as above. The cytokine concentrations in cell-free supernatants were determined by enzyme-linked immunosorbent assay by reference to standard curves.

Results showed that there were substantially greater amounts of IL-6 released from mouse macrophages when co-cultured with *Treponema denticola* strain ST10 than unstimulated controls. On the other hand, only small amounts were detected from supernatants stimulated with *Treponema pectinovorum*, *Treponema vincentii* and *Porphyromonas gingivalis* when compred to unstimulated controls. The presence of *Treponema socranskii* subspecies *buccale* and *Treponema socranskii* subspecies *paredis*, as well as *Treponema denticola* serovar C did not elicit IL-6 release. TNF-α secretion was greatly increased when macrophages were co-cultured with *T. denticola* strain ST10, *T. vincentii*, *T. pectinovorum*, *T. denticola* serovar C or *P. gingivalis*. Smaller, but significant increases in TNF-α were noted from co-cultures with both subspecies of *T. socranskii*. No IL-1β release was detected in any experiments. There were differences in kinetics of IL-6 and TNF-α release. Both cytokines were low at 2 hours, and increased at 8 and 24 hours. Concentration of IL-6 continued to increase between 24 and 48 hours, whereas TNF-α leveled off or even slightly decreased between 24 and 48 hours.

These findings demonstrated that some but not all oral treponemes are capable of stimulating macrophages to release proinflammatory cytokines. Different secretion levels of these cytokines and their biological activities, induced by oral treponemes, might be important in the onset and progression of periodontal diseases.

LIST OF ABBREVIATIONS

ATCC: American Type Culture Collection, Bethesda, MD

BSA: Bovine Serum Albumin

CO2: Carbon Dioxide

DMEM: Dulbecco's Minimal Essential Media

ELISA: Enzyme Linked Immuno-Sorbent Assay

IL: Interleukin

KDa: Kilodalton

LPS: Lipopolysaccharide

MMP: Matrix Metalloproteinase

PBS: Phosphate Buffered Saline

PGE2: Prostaglandin E2

RPM: Rotations Per Minute

TNF-α: Tumor Necrosis Factor-α

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1. INTRODUCTION

1.1. Spirochetes

Periodontal diseases are the most prevalent cause of tooth loss in adults (1). Bacteria or their components and metabolic products present in dental plaque are thought to be important etiologic factors in bacterial periodontal pathosis (2).

Spirochetes are among the bacteria most commonly found in subgingival plaque associated with destructive periodontal diseases (3-6). In recent years, some species of oral spirochetes have been found in subgingival plaque associated with healthy gingiva (7,8). Oral spirochetes have been observed within gingival tissues in several forms of periodontal disease, including acute necrotizing ulcerative gingivitis (9-11), adult periodontitis (12,13), and juvenile periodontitis (14,15). The presence of large numbers of spirochetes seems to be indicative of active periodontal disease. In adult marginal periodontitis they can constitute 40-56% of the flora and actually be the predominant type of organism present (16). Higher frequency of development of periodontal disease has been associated with sites that harbored spirochetes in adjacent dental plaque (17).

While mechanisms of tissue destruction have not been completely defined pure strains of oral spirochetes express a variety of potentially virulent factors, including proteolytic enzymes, metabolic products and endotoxins. The fibronectin coat found on some spirochetes provides

protection against phagocytosis (18). Some spirochetes show an ability to attach to host epithelial cells (19). This attachment is likely to be an initial step in tissue invasion that contributes to the disease process (20,21).

Oral spirochetes belong to the genus Treponema. Several oral treponemes have been isolated in pure culture, including *Treponema denticola*, *Treponema socranskii*, *Treponema pectinovorum*, and *Treponema vincentii*. Several different serotypes or subspecies of *T. denticola*, *T. socranski* and *T. vincentii* have been identified (22-24).

Treponemes may initiate disease as they attach to epithelial cells and make their way through underlying tissues. It is likely that sulcular epithelium of gingiva is the first tissue through which plaque bacteria enter the host. Once treponemes cross epithelium and basal lamina, they may enter the connective tissue where the host defense cells would attempt to limit their penetration and localize the infection. Initial steps of treponemal invasion through tissues are likely followed by the appearance of antigen-presenting cells that start further host immune responses. Thus, substances from periodontopathogenic bacteria could initiate and drive the inflammatory response, and their continued presence in tissue might lead to promotion and maintenance of inflammation.

1.2. Cytokines and inflammation

The cascade of inflammation involving cellular and humoral immunity is regulated in part by cytokines, which are endogenous host

molecules that mediate the inflammatory process. Cytokines play a major role as proinflammatory mediators in periodontal disease. There is growing evidence that these molecules are involved in amplification and perpetuation of inflammation as well as contributing to tissue destruction (25). Of several possible host degradative pathways, considerable interest has focused on cytokines not only as mediators of host defense but also as mediators of periodontal tissue destruction. It has been shown that cytokines can promote bacterial growth following tissue destruction caused by inflammation and that bacterial enzymes can activate or inactivate cytokines (26).

The host response to spirochetes and their virulent factors might be an important determinant of the onset and progression of periodontal disease (27,28). Epithelial cells and fibroblasts have the ability to produce cytokines in the presence of bacteria, viruses and parasites (29-31). Among the many different cell types that have been shown to synthesize cytokines, macrophages are likely to be among the most efficient producer cells.

1.3. Macrophages

Macrophages originate in the bone marrow from stem cells. The most immature population in the bone marrow that exhibits macrophage characteristics are the monoblasts. Upon division, monoblasts give rise to promonocytes and monocytes in the bone marrow. From there, monocytes

enter the circulation. In response to stimuli such as infection, monocytes migrate into the tissues and organs where they differentiate into macrophages. The ability of macrophages to phagocytose particles represents the first step in the series of events that ultimately lead to the elimination of invading microorganisms. Macrophages have been shown to release cytokines following bacterial, viral or chemical stimulation (28,32-34). Macrophages derived from a mouse cell line can be used as a model for macrophage cell functions.

Macrophages represent the ubiquitously-distributed population of fixed and circulating phagocytes that express a variety of functions other than phagocytosis, including cytokine production, killing of microbes, and processing and presentation of antigens to lymphocytes. Expression and release of proinflammatory cytokines and chemokines from macrophages enhances site-directed immigration of leukocytes into inflamed tissue. Gradients of cytokines and chemokines contribute to vascular adhesion, direct transendothelial migration, and movement through the extracellular matrix (35).

It has been previously demonstrated that an infection of monocytes with Influenza-A virus (33) or Coxsackievirus-B3 (34) can induce the secretion of tumor necrosis factor-alpha (TNF-α), and interleukins one (IL-1) and six (IL-6). Expression and release of proinflammatory cytokines was strongly enhanced by lipopolysaccharide (LPS), which is a powerful inducer of a variety of immunological mediators (34,35). More specifically, it has been shown that lipoproteins derived from spirochetes like *Treponema pallidum* and *Borrelia burgdorferi* are potent macrophage

activators and inducers of proinflammatory cytokines (36). *Borrelia burgdorferi* also appears to be a strong inducer of chemokines, which may significantly contribute to inflammation and tissue damage observed in Lyme disease (37). These observations indicate that the interaction of spirochetes with host cells may induce the production and release of cytokines and chemokines.

1.4. Cytokines associated with periodontal disease

Cytokines stongly associated with periodontal disease include IL-1, TNF-α and IL-6. Several investigators have demonstrated that IL-1β, IL-6 and TNF-α were increased in periodontal tissue from diseased sites compared with healthy sites. Significant reduction in total amounts of these cytokines was noted following effective periodontal treatments, indicating relevance to the severity of periodontal disease (38-42). There was a significant correlation between tissue levels of IL-6 and the severity of periodontal inflammation (43).

It is likely that cytokine release modifies the inflammatory reaction and tissue destruction (26-29,33). Therefore, putative periodontopathic oral treponemes or their products may stimulate monocytes/macrophages to synthesize cytokines. These cytokines may potentiate their own expression and mediate a shift in the properties of various host cells, such as fibroblasts. The fibroblastic properties that may be altered in response to cytokine stimulation include cell proliferation, phagocytosis, collagen

synthesis, synthesis of proteolytic enzymes such as matrix metalloproteinase (MMP) and their corresponding tissue inhibitors. Factors effecting cytokine-mediated collagen metabolism by fibroblasts play an important role in the pathogenesis of microbial-induced periodontal disease (25,28).

There is significant homology between mouse and human IL-1β, IL-6 and TNF-α. Mature human and mouse IL-1β share approximately 75% amino acid sequence identity, and human IL-1β is capable of activating murine cell lines. In comparison, mouse IL-6 and human IL-6 exhibit approximately 65% and 42% sequence identity at the nucleotide and the amino acid levels, respectively. Human and mouse IL-6 are equally active on mouse cells. Human and murine TNF-α show approximately 79% homology at the amino acid level and exhibit cross-reactivity between the two species.

1.4.1. Interleukin-1

In a recent review, Bevilaqua et al. discussed the role and properties of IL-1 (44). IL-1 is a cytokine with two distinct forms, designated IL-1 alpha (IL-1α) and IL-1 beta (IL-1β), which are encoded by separate genes. Although the two forms have only 27% amino acid homology, they bind with comparable affinity to a common receptor found on many cell types. Both proteins are produced by a large variety of cells in response to stimuli such as those produced by inflammatory agents,

infections, or microbial endotoxins. The proteins are synthesized as 31 kDa precursors that are subsequently cleaved into proteins with molecular weights of approximately 17.5 kDa. IL-1β is processed by IL-1β-converting enzyme. IL-1β is a potent pro-inflammatory cytokine and induces a wide variety of biological activities in different cell types.

Two distinct types of IL-1 receptors have been identified and cloned from human and mouse cells. The IL-1 type I receptor is an 80 kDa transmembrane protein with demonstrated IL-1 signaling function. The type II receptor is a 68 kDa membrane protein with a relatively short cytoplasmic tail and has no signaling function. IL-1β amplifies and perpetuates inflammation and tissue destruction. IL-1β also acts on endothelial cells to increase attachment of neutrophils and monocytes, aiding in recruitment of these cells into the sites of inflammation.

Increased IL-1β concentrations have been identified in gingival crevicular fluid from periodontally diseased sites (45,46). IL-1β is believed to induce many pro-inflammatory or catabolic effects that may contribute to destruction of bone and surrounding connective tissue found around teeth with periodontitis (46). IL-1β can enhance protease production in many cell types and can stimulate synthesis of prostaglandin E2 (PGE2) by macrophages and by gingival fibroblasts (47). PGE2 is a vasodilator and a cofactor involved in increased vascular permeability occurring at sites of inflammation as well as acting as a mediator in bone demineralization (48).

1.4.2. Interleukin-6

IL-6 is a multifunctional protein that appears to be directly involved in responses that occur after infection or injury and may be very important in regulating the acute phase response. IL-6 plays important roles in host defense, acute phase reactions, immune response and hematopoiesis. IL-6 has been variously called interferon-β2, 26-kDa protein, B-cell stimulatory factor-2 (BSF-2), hybridoma/plasmocytoma growth factor, hepatocyte stimulating factor, cytotoxic T-cell differentiation factor, and macrophage-granulocyte inducing factor 2A (MGI-2A). The IL-6 designation was adopted after these variously named proteins were found to be identical on the basis of their amino acid and coding nucleotide sequences. IL-6 is expressed by the variety of normal and transformed cells including fibroblasts, activated T-cells, B-cells, activated monocytes/macrophages, hepatocytes, astrocytes, keratinocytes, vascular endothelial cells, and various tumor cells. It acts upon a variety of cells, including fibroblasts, myeloid progenitor cells, T cells, B cells and hepatocytes. The production of IL-6 is up-regulated by numerous signals including mitogenic and antigenic stimulation, LPS, IL-1, IL-2, TNF, and viruses. IL-6 expression in monocytes is inhibited by IL-4 and IL-13. IL-6 induces multiple effects. It appears to interact with IL-2 in proliferation of lymphocytes (49), and also potentiates the proliferative effect of IL-3 on multipotential hematopoetic progenitors (50).

IL-6 levels were elevated in gingival connective tissue adjacent to unresolved periodontal defects after initial therapy (51). IL-6 may

correlate with the severity of periodontal disease (52). IL-6 is elevated at sites of refractory periodontitis compared to sites of stable advanced periodontitis (53), which suggests that it could be a diagnostic marker for sites with active periodontal disease.

1.4.3. Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α), also called cachectin, is produced by macrophages, neutrophils, activated lymphocytes, astrocytes, endothelial cells, smooth muscle cells and some transfected cells. TNF-α occurs as a secreted, soluble form and as a membrane-anchored form, both of which are biologically active. The naturally occurring form of TNF-α is glycosylated, but non-glycosylated recombinant TNF-α has comparable biological activity. The active native form of TNF-α is a trimer. There are two types of receptors for TNF-α and virtually all cell types show the presence of one or both of these receptor types making this cytokine potentially a very important modulating factor on numerous different cell types in the process of disease progression.

TNF-α has extremely pleiotropic effects due to the ubiquity of its receptors. It has the ability to activate multiple signal transduction pathways and induce or suppress the expression of the wide number of genes. TNF-α is involved in activation of inflammatory leukocytes, modification of vascular permeability, induction of bone resorption,

mediation of resistance to infections and tumor growth, as well as mediation of the inflammatory response (25,26,28,33).

1.5. Purpose

It is uknown whether the presence of oral treponemes leads to secretion of cytokines from macrophage cells. Therefore, the purpose of this research was to determine whether the presence of specific oral treponemes leads to release of the proinflammatory cytokines from murine monocyte/macrophage cells.

A second goal was to determine whether different strains of oral treponemes stimulate different levels of IL-1β, IL-6 and TNF-α secretion in murine macrophage cells compared to macrophages in culture alone and to compare the extent of cytokine release by oral treponemes with release occurring in the presence of *P. gingivalis*.

A third objective was to compare release of cytokines from murine macrophages in presence of viable and non-viable oral treponemes.

Findings from this research will hopefully contribute to our understanding of the pathogenesis of periodontal disease associated with certain oral treponemes.

2. HYPOTHESES

Ho: Co-incubation of oral treponemes with mouse macrophage cells does not induce secretion of cytokines.

Ha: Oral treponemes induce mouse macrophage cells to secrete cytokines.

Ha1: Different species of oral treponemes result in different levels of cytokine secretion in mouse macrophage cells.

Ha2: There is a time-dependent release of cytokines after mouse macrophage cells have been co-incubated with oral treponemes.

3. MATERIALS AND METHODS

3.1. Pilot study

Preliminary studies were conducted to determine whether the model consisting of eukaryotic cells incubated with oral treponemes was appropriate for testing the hypotheses.

The first objective was to determine whether the selected macrophage cell line was able to secrete cytokines upon co-incubation with oral treponemes and positive control bacteria. Different cell lines were tested under the same conditions to find the line with best and most consistent cytokine secretion. Tested cells included epithelial cell line ATCC #CCL17/KB from a human oral epidermoid carcinoma, ATCC mouse macrophage cell line #TIB71/RAW 264.7, and ATCC mouse macrophage cell line #TIB-186. Fresh human leukocytes including macrophages were also examined. Positive controls in pilot experiments included LPS from *Escherichia coli*, as well as whole cells of *Borrelia burgdorferi* and *Porphyromonas gingivalis*.

Observations from pilot studies also served to determine the number of bacteria and the number of cells required to stimulate the secretion of cytokines. The length of co-incubation of bacteria with eukaryotic cells was also determined in pilot studies. These studies were also necessary to define the optimal ranges of standard curves and conditions for ELISA assays, including concentrations of capture and detection antibodies, as

well as lengths of incubation times for different steps involved in these assays.

Overall, pilot studies provided observations for optimal standard conditions used in the subequent studies to test the hypotheses.

3.2. Macrophages

There were no human macrophage cell lines available in the ATCC catalog at the time of this work; therefore, a mouse macrophage/monocyte cell line was selected. The cell line ATCC #TIB-71/RAW 264.7 was selected. This macrophage cell line originates from mice and is transformed with Abelson leukemia virus. Viability of macrophages was determined by their appearance under an inverted-phase light microscope. Cells were screened by observing the attachment to the flask and confluence of the cell layer before bacterial co-incubation as well as at each time interval when supernatants were harvested. Attachment to the flask as well as the ability of the cell to spread-out cell were considered criteria for cell viability. Macrophages that were detached and balled-up floating in tissue culture media were considered non-viable.

3.3. Bacteria

The following strains of oral treponemes were used in these experiments:

1. *Treponema socranskii* subspecies *buccale* (American Type Culture Collection, Bethesda, MD (ATCC #35534)
2. *Treponema socranskii* subspecies *paredis* (ATCC #35535)
3. *Treponema vincentii* (ATCC #35580)
4. *Treponema pectinovorum* (ATCC #33768)
5. *Treponema denticola* serovar C (ATCC #35404)
6. *Treponema denticola* serotype D strain ST10 (Virginia Polytechnic Institute and State University)

Porphyromonas gingivalis was a generous gift from Dr. J. Craig Baumgardner, Oregon Health Sciences University School of Dentistry. *Porphyromonas gingivalis* was selected as the positive control because of its ability to stimulate cytokine secretion in vitro (54).

3.4. Cytokines

ELISA assays (see section 3.6.) were used to measure IL-1ß, IL-6 and TNF-α concentrations in supernatants. Standard curves were consistent in every experiment and were obtained by using recombinant murine cytokine proteins (R&D Systems Inc., Minneapolis, MN) in concentrations predetermined in the pilot study.

3.5. Co-Cultivation Experiments

Macrophage cells were grown in Dulbecco's minimal essential medium (DMEM) (Sigma Chemical Co., St. Louis, MO), supplemented with 1% penicillin/streptomycin (Corning Inc., Corning, NY) and incubated at 37°C in 5% CO_2 and 95% air. Once the cells reached confluence, they were harvested using gentle scraping with a rubber spatula to detach them from the flasks (Corning Inc., Corning, NY). Cells were then seeded into 4 x 6 culture plates (Polysciences, Inc., Warrington, PA) in DMEM supplemented with 1% rifampin (Corning Inc., Corning, NY). Rifampin was used as antibiotic because treponemes are resistant to it.

Oral treponemes were grown in culture tubes containing 10 ml of spirolate broth (Becton Dickinson Micrbiology Systems, Cockeysville, MD) supplemented with 10% (v/v) fetal bovine serum (Sigma Chemical Co., St. Louis, MO). All treponemes were maintained at 37°C under anaerobic conditions (BBL Gas Pak Plus, Becton Dickinson Microbiology Systems). In order to maintain actively growing cultures for experiments, some treponemes were subcultured into fresh medium. Actively growing cultures of each treponeme were utilized for experiments. The concentrations of treponemes and *P. gingivalis* were estimated by darkfield microscopy using a Petroff-Hausser bacteria-counting chamber (Hausser Scientific Partnership, Horsham, PA). An inoculum of each suspension containing $10x10^6$ bacteria per well was placed in separate sterile microfuge tubes (1.5 ml), centrifuged for 10 minutes at 20°C at

14,000 RPM. After centrifugation old medium was removed and bacteria were resuspended in 2 ml of DMEM with rifampin. Each well in the culture plate containing a confluent layer of macrophage cells was drained of its original culture medium and immediately inoculated with a 2 ml aliquot of treponeme suspension. The negative control wells were not co-cultured with bacteria, but contained 2 ml of DMEM with rifampin. *Porphyromonas gingivalis* was used in positive control wells. Triplicate wells were used for 2-, 8-, 24- and 48-hours supernatants for each of the six treponemes, positive and negative control.

Macrophages were co-cultivated with bacteria and negative control at 37°C at 5% CO2 and 95% air. Supernatants were harvested at intervals of 2, 8, 24 and 48 hours from individual wells for each time interval. Supernatants were placed in sterile microfuge tubes (1.5 ml) and centrifuged for 10 minutes at 14,000 RPM to sediment cells and bacteria. The cell-free supernatants were separated in 0.5 ml aliquots, placed in sterile microtubes (1 ml) and frozen at -80°C. After the experiment was completed they were thawed for ELISA analysis.

3.6. Experimental design

Co-culture experiments were repeated twice on different days. Each experiment consisted of triplicate co-cultures for each of the treponemes as well as the positive and negative controls. A total of six supernatants were collected for each variable at 2, 8, 24 and 48 hour time points.

Finally, randomly selected supernatants were tested for cytokine concentration in four independent ELISA assays consisting of duplicate wells for each supernatant.

3.7. Cytokine Assays

The standard curve represents the relationship between absorbancy at 405 nm of the solution and the concentration of a cytokine in it. Standard curves were established using known concentrations of recombinant murine IL-1ß, IL-6 or TNF-α proteins (R&D Systems Inc., Minneapolis, MN).

Standard curves were defined by plotting the absorbancy at 405 nm from the spectrophotometer on the Y-axis and plotting the corresponding cytokine concentration on the X-axis. Concentrations of recombinant IL-6 and TNFα used for standard curves were 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml and 0.312ng/ml. Concentrations of recombinant IL-1β used for standard curves were 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml and 0.312ng/ml. All standard curves were generated using duplicate wells for each concentration and were repeated in each assay. Mean absorbancies were calculated to represent each value plotted on the curve. For each assay new standard curves were established. In order to use standard curves to interpret the concentrations of supernatants, the curves had to be consistent with previous assays and fall within the linear range established by the range of standards.

Capture antibodies for IL-1ß, IL-6 and TNF-α (R&D Systems Inc., Minneapolis, MN) were used in the sandwich ELISA assays to capture cytokines from the supernatants. Monoclonal anti-mouse IL-1ß capture antibody was produced by a murine hybridoma derived from a mouse immunized with purified *E. coli*-derived recombinant mouse IL-1ß. Monoclonal anti-mouse IL-6 antibody was produced by a murine hybridoma cell line derived from a mouse immunized with Cos 7-produced recombinant mouse IL-6. Polyclonal anti-mouse TNF-α capture antibody was produced in goats immunized with purified *E. coli*- derived recombinant mouse TNF-α.

Capture antibodies were prepared in phosphate buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO) at concentrations of 4 µg/ml for IL-1β; 1µg/ml for IL-6; and 2.4 µg/ml for TNF-α. Antibodies were coated onto walls of ELISA wells at 100 µl/well and incubated overnight at room temperature. Unbound capture antibodies were removed by rinsing three times with PBS. Unreactive sites were blocked at room temperature with 200 µl/well of a solution of PBS supplemented with 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO). After 60 minutes incubation, the blocker solution was rinsed from wells with PBS.

Captured cytokines were detected on ELISA plates using biotinylated anti-mouse IL-1ß, IL-6 and TNF-α antibodies (R&D Systems Inc., Minneapolis, MN). These antibodies were produced in goats immunized with purified *E. coli*-derived recombinant mouse IL-1ß, IL-6 and TNF-α, respectively.

Alkaline Phosphatase-Avidine (Zymed Laboratories Inc., San Francisco, CA) reagent was used to detect the amount of cytokine binding using para-nitro-phenyl-phosphate (Sigma Chemical Co., St. Louis, MO) as the photoactive substrate.

Concentrations of cytokines were determined with the capture antibody-amplified enzyme immuno-assay technique outlined below.

Recombinant IL-1β,IL-6 and TNF-α were used in serial dilutions in DMEM for generation of standard curves. Frozen cell-free culture supernatants stored at -80°C were thawed and placed into wells. All incubations were done in duplicate wells containing 100 µl/well for 2 hours and were maintained at room temperature. Supernatants that exhibited optical density values above the interpretable range of the standard curve were diluted with DMEM to 1:2 or 1:4 and tested again. Wells were then washed three times with PBS containing 0.05% Tween 20 (v/v) followed by PBS alone.

Biotinylated anti-IL-1ß/IL-6/TNF-α antibodies (R&D Systems Inc., Minneapolis, MN) were prepared in PBS plus 0.1% BSA. Antibodies were prepared at concentrations of 0.1µg/ml for anti-IL-1β; 1µg/ml for IL-6; and 0.2 µg/ml for TNF-α. All wells on one plate received 100 µl/well of corresponding biotinylated antibody. After two hours incubation at room temperature plates were washed three times with PBS solution containing 0.05% Tween 20 (v/v).

Alkaline Phosphatase-Avidine was prepared in PBS plus 1% BSA and 0.05% Tween 20 (v/v). Each well received 100 µl, and was incubated

at room temperature for 30 minutes. Plates were then washed three times with PBS solution containing 0.05% Tween 20 (v/v).

Para-nitro-phenyl-phosphate (Sigma Chemical Co., St. Louis, MO) in diethanolamine buffer (Sigma Chemical Co., St. Louis, MO) served as the substrate. Hundred µl of was added to each well and incubated for 15 to 30 minutes in the dark.

The absorbancy at 405 nm was read with a spectrophotometer. The average of duplicate optical density readings was calculated for the standard curves of IL-1ß, IL-6 and TNF-α and then plotted as semi- log curves with concentration on the X-axis and the absorbancy at 405 nm on the Y-axis. The average of duplicate optical density values for each culture supernatant was calculated and the concentrations of IL-1ß, IL-6 and TNF-α were then extrapolated from the straight part of the appropriate standard curve. For diluted samples, compensation for the dilution was done by multiplying the reading by the dilution factor.

3.8. Viability of bacteria

It was of interest to determine whether viable and non-viable treponemes induced different cytokine responses when co-cultured with macrophages. *Treponema denticola* ST10, *T. pectinovorum* and *T. vincentii*. were grown as described in section 3.4. Old media was removed by centrifugation. Treponemes were resuspended in DMEM tissue culture

After 48 hours, viability of treponemes was determined using darkfield microscopy and by subculturing the sample back into OMIZ media and incubating them in carbon dioxide. All the treponemes were sub-cultured in the second experiment from the co-culture supernatants to determine their viability. Viability of treponemes was determined by their ability to replicate in subculture and their motility.

3.9. Data analysis

3.9.1. Quantitative ELISA

Standard concentrations for each cytokine were incorporated into each ELISA plate and log concentrations were plotted against corresponding optical densities using CA-cricket Graph. Concentrations were calculated from linear portions of standard curves using Microsoft Excel. The average concentration was calculated from replicate observations for each time interval, in each assay, for each bacteria and the negative control.

3.9.2. Statistical comparisons

The Student's t-test was used to compare mean concentrations of each cytokine pooled over all four time intervals. Mean concentrations of each cytokine produced in each experimental group were compared to the negative control for each assay. In addition, individual p-values calculated from each of the four independent assays for each cytokine were placed in tables in order to illustrate the consistency of results among the assays.

Concentrations of IL-6 derived from co-cultures with macrophages and either viable or antibiotic-treated *T. denticola* ST10, *T. vincentii* or *T. pectinovorum* were compared to the negative control by Student's t-test.

Concentrations of TNF-α from co-cultures with macrophages and either viable or antibiotic-treated *T. denticola* ST10 and *T. pectinovorum* were compared to the negative control by Student's t-test.

Concentrations of each cytokine produced at each time interval were also plotted in figures to illustrate the kinetics of secretion of the individual cytokines over time.

4. RESULTS

4.1. Pilot study

The macrophage cell line ATCC #TIB-71/RAW 264.7 was chosen as the most appropriate model for these experiments. These macrophages were the most reliable, with replicable release of TNFα and IL-6. Unfortunately, they did not release detectable levels of IL-1β in any of the experiments even with *P. gingivalis* or LPS. The epithelial and other mouse macrophage cell lines tested did not secrete any of the three cytokines in several experiments upon co-incubation with different bacteria or LPS. Fresh human macrophages produced large amounts of IL-1β when co-cultured with *T. vincentii*; however, due to the presence of polymorphonuclear cells in the culture we were reluctant to attribute secretion of IL-1β solely to the macrophages. Polymorphonuclear leukocytes are capable of releasing IL-1β and TNF-α in response to lipopolysaccharides from periodontopathic bacteria (55).

The optimal cytokine concentrations for the standard curves ranged from 20 ng/ml to 0.6125 ng/ml each for IL-6 and TNF-α. Both standard curves for cytokines plateaued between 10 ng/ml and 20 ng/ml; therefore, values between 10 ng/ml and 0.6125 ng/ml were used for the interpretable ranges. The standard curve for IL-1β plateaued between 5 and 10 ng/ml; thus, standard concentrations between 5 ng/ml and 0.6125 ng/ml were used for the interpretable range.

4.2. Viability of bacteria and macrophages

All bacteria examined under the microscope were motile replicating before they were separated from their media and co-incubated with macrophages. Bacteria were sub-cultured from 2, 8, 24 and 48 hours supernatants to observe their viability during the experiment. Although most of them were motile in subcultures, they did not tend to replicate. Surprisingly, some of the treponemes that had been treated in 10% antibiotic penicillin/streptomycin solution appeared slightly motile. However, the majority of the treated treponemes were non-motile and they appeared less numerous than their non-treated equivalents. Therefore, it could be speculated that treated treponemes were non-viable during co-incubation. on-treated treponemes failed to qualify as fully viable due to their lack of ability to replicate in subculture after being co-cultured with macrophages in DMEM.

Macrophages attained monolayer confluency within 3-4 days. Cells were firmly attached to the bottom of the flasks or wells and could only be removed by gentle scraping and were therefore considered viable. After the final supernatants were harvested at 48 hours, macrophages exhibited the same monolayer and attachment characteristics as at the beginning of the experiment.

4.3. Cytokine secretion

Negative controls produced no IL-1ß or IL-6, and very low levels of TNF-α. Statistical analysis was based on comparisons of values produced by the positive control and the six groups co-cultured with oral treponemes to the values produced by the negative controls. Statistically significant values were considered at $p<0.0001$ and $p<0.05$.

4.3.1. Interleukin-6

A highly significant increase ($p<0.0001$) in production of IL-6 compared to negative control was observed upon co-incubation with *T. denticola* ST10. *Porphyromonas gingivalis* also resulted in a statistically significant ($p<.05$) increase in secretion. The highest levels of IL-6 were observed in supernatants from co-cultures with *T. denticola* ST10. Co-culture of macrophages with the other five species of treponemes resulted in increased but not significant secretion of IL-6 when compared to negative controls (Figure 1).

In the first IL-6 ELISA assay the optical density values for 24- and 48-hours supernatants from co-cultures with *T. denticola* ST10 were too high to interpret the corresponding IL-6 concentration from the standard curve. ELISA assays were then repeated four times and IL-6 concentrations for 24- and 48-hours supernatants were determined in 1:2 and 1:4 serial dilutions of culture supernatants for 24-and 48-hours

supernatants, respectively. The elevated levels of IL-6 secretion upon co-culture with *T. denticola* ST10 were consistent in four ELISA assays including supernatants from both experiments (Table 1). Co-cultures with the other oral treponemes produced consistent but not significant increases in IL-6 when compared to negative control (Table 1). *Porphyromonas gingivalis,* however, resulted in less consistent levels in the four assays.

Concentrations of IL-6 in *P. gingivalis* and *T. denticola* ST10 terated cells increased over the time intervals, starting with levels comparable with bacteria-free control and gradually increasing, reaching the highest level at 48 hours. Kinetic characteristics for IL-6 secretion were similar for *P. gingivalis* and *T. denticola* ST10 (Figure 3).

Co-incubation with both dead ($p<0.05$) and live ($p<0.0001$) *T. denticola* ST10 resulted in significantly elevated concentration of IL-6 when compared to negative control. Average values from co-cultures with dead bacteria were approximately 40% lower than those from live bacteria (Figure 4); however, the kinetic characteristics over the time intervals were similar (Figure 6). Treated *T. vincentii* and *T. pectinovorum* resulted in slightly higher IL-6 secretion from macrophages than the same live strains and differences were statistically (Figure 4).

4.3.2. Tumor necrosis factor-alpha

Detectable levels of TNF-α were observed in supernatants from co-cultures with all oral treponemes (Figure 2). A statistically significant increase ($p<0.0001$) in release of TNF-α when compared to negative control was consistently observed after co-incubation with *P. gingivalis*, *T. denticola* ST10, *T. denticola* serovar C and *T. vincentii* (Table 2). Co-incubation with *T. socranskii* subspecies *buccale* and *T. pectinovorum* resulted in statistically significant secretion of TNF-α in two and three of four assays, respectively. *T. socranskii* subspecies *paredis* induced very low levels of TNF-α release from macrophages in only one of four assays at $p<0.05$. (Table 2).

Release of TNF-α displayed a different kinetic profile than did IL-6 over the time intervals. TNF-α concentrations increased faster initially and then leveled between 24 and 48 hours. Kinetic characteristics of TNF-α secretion were similar for *P. gingivalis* and *T. denticola* ST10 (Figure 3).

Co-incubation with both treated ($p<0.0001$) and live ($p<0.0001$) *T. denticola* ST10 and *T. vincentii* resulted in a significantly elevated concentration of TNF-α when compared to the negative control. Average TNF-α concentrations from co-cultures with dead bacteria were approximately 20% lower than those from live bacteria (Figure 5), however the kinetic characteristics over the time intervals were similar (Figure 6).

4.3.3. IL-1β

Despite consistently optimal IL-1β standard curves IL-1β was not detected in the supernatants from co-culture experiments.

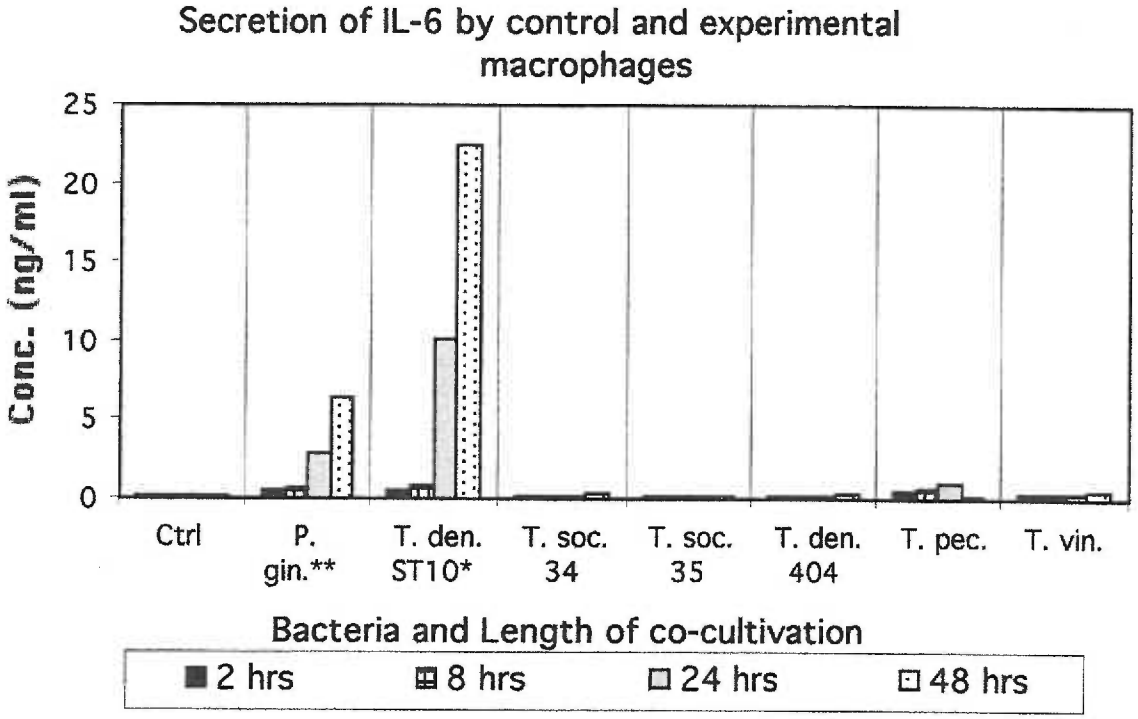

Figure 1: Secretion of IL-6 by control macrophages and experimental macrophages stimulated by *P. gingivalis* or oral treponemes. Murine macrophages from ATCC were incubated with bacteria indicated for 2, 8, 24 and 48 hours. After incubation, supernatants were harvested and assayed for IL-6 by ELISA. The data represent mean values (significantly different from the levels of the control unstimulated cells at *$p<0.0001$, **$p<0.05$).

Legend:

T. den. ST10 = *T. denticola* serotype D strain ST10

T. soc. 34 = *T. socranskii* subspecies *buccale* (ATCC #35534)

T. soc. 35 = *T. socranskii* subspecies *paredis* (ATCC #35535)

T. den. 404 = *T. denticola* serovar C (ATCC 335404)

T. pec. = *T. pectinovorum* (ATCC #33768)

T. vin. = *T. vincentii* (ATCC #35580)

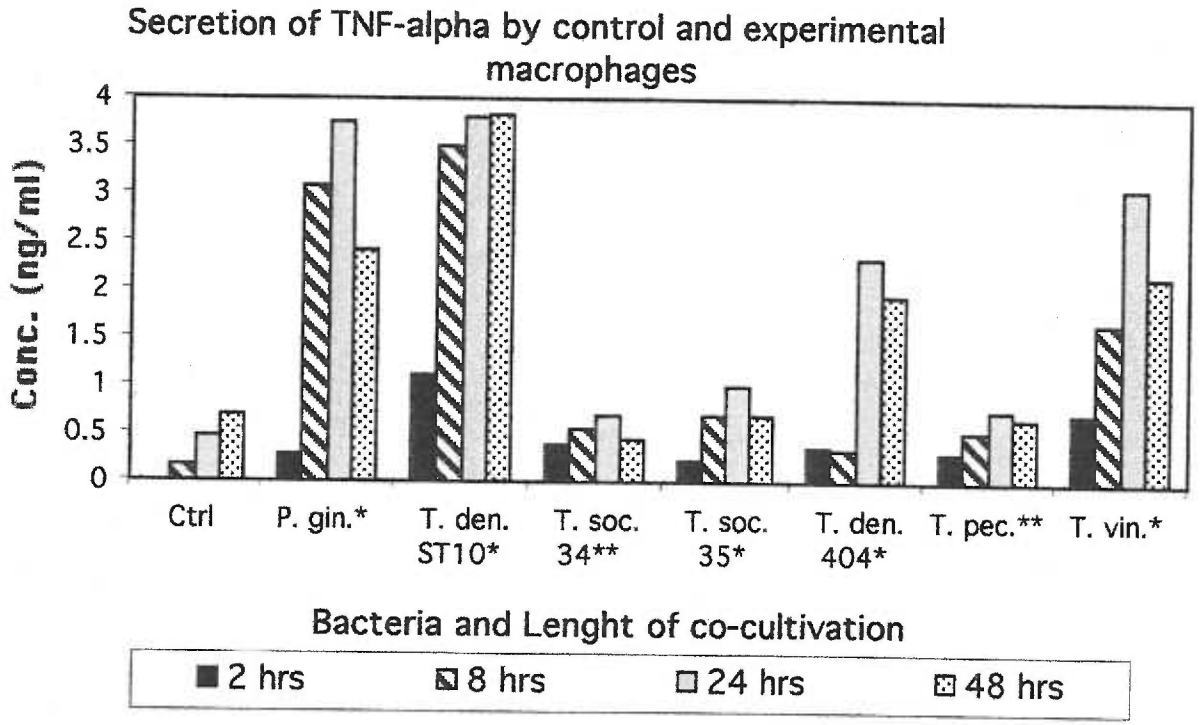

Figure 2: Secretion of TNF-α by control macrophages and experimental macrophages stimulated by oral treponemes or *P. gingivalis*. Murine macrophages from ATCC were incubated with bacteria indicated for 2, 8, 24 and 48 hours. After incubation, supernatants were harvested and assayed for TNF-α by ELISA. The data represent mean values (significantly different from the levels of the control unstimulated cells at *p<0.0001, **p<0.05).

Legend:

T. den. ST10 = *T. denticola* serotype D strain ST10

T. soc. 34 = *T. socranskii* subspecies *buccale* (ATCC #35534)

T. soc. 35 = *T. socranskii* subspecies *paredis* (ATCC #35535)

T. den. 404 = *T. denticola* serovar C (ATCC 335404)

T. pec. = *T. pectinovorum* (ATCC #33768)

T. vin. = *T. vincentii* (ATCC #35580)

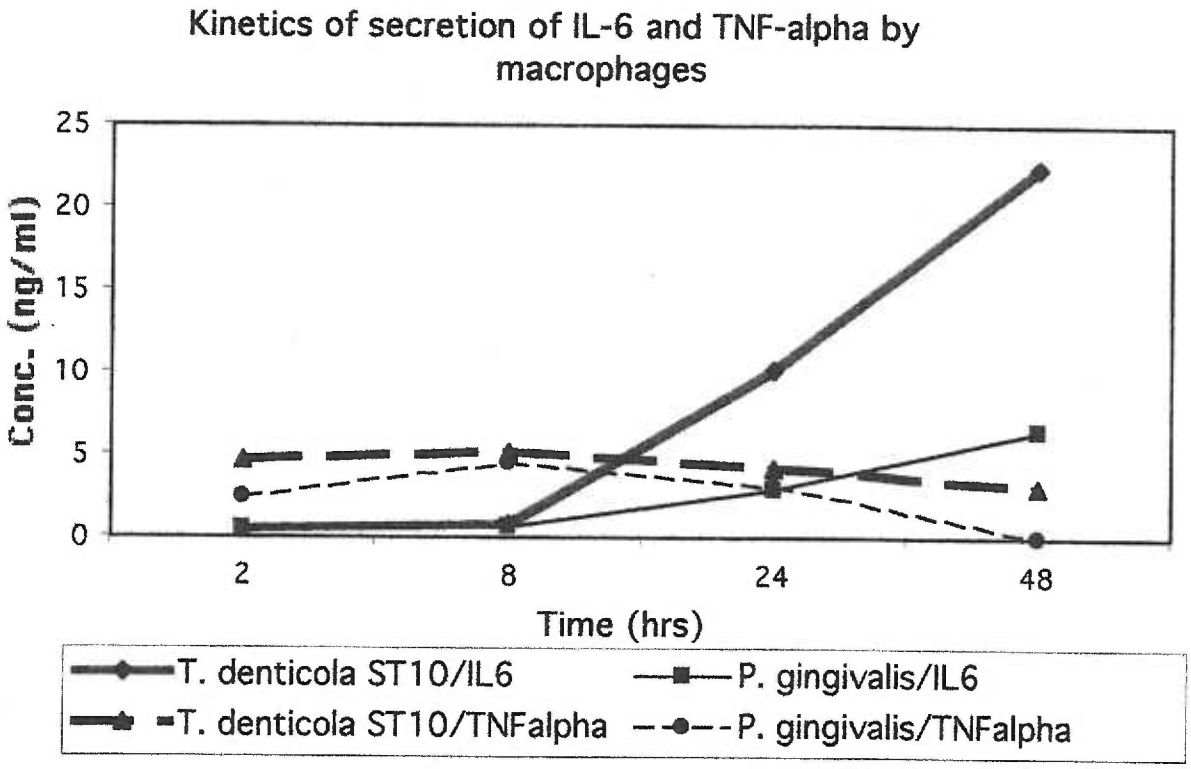

Figure 3: Kinetics of secretion of TNF-α and IL-6 by macrophages at 2, 8, 24 and 48 hours as observed for stimulation with *T. denticola* ST10 or *P. gingivalis*. *Treponema denticola* ST10 is representative of all oral treponemes that were associated with elevated cytokine production and showed the similar kinetic profile over time but displayed different levels of TNF-α and IL-6.

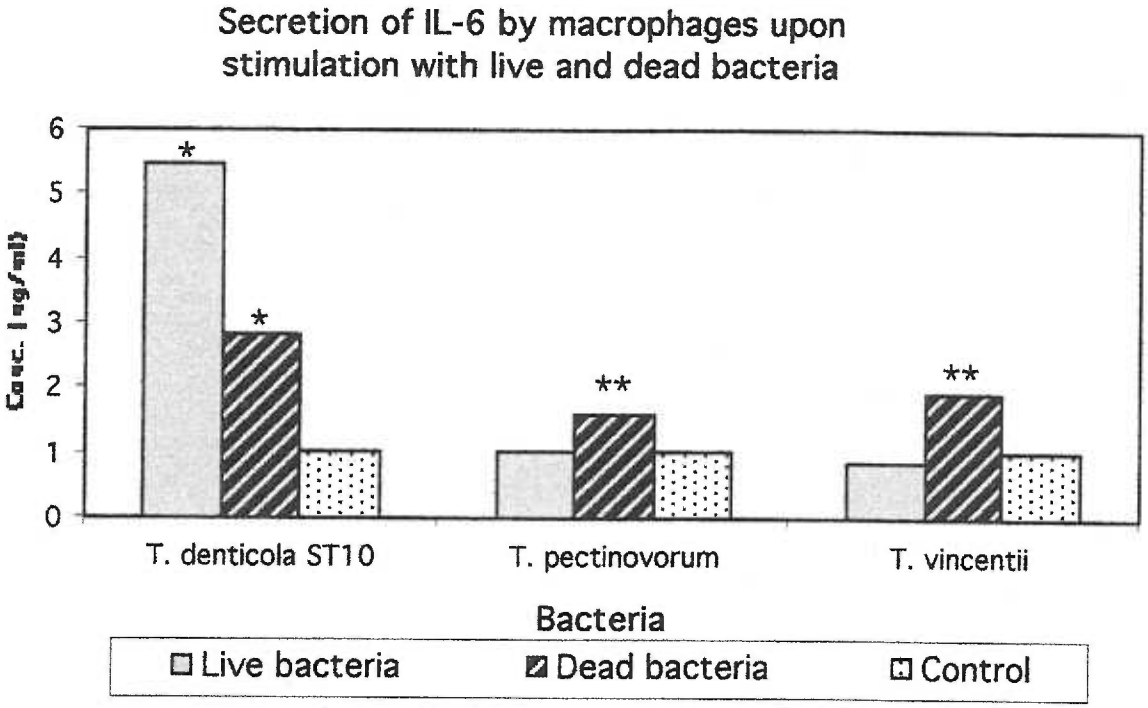

Figure 4: Secretion of IL-6 by control macrophages and experimental macrophages stimulated by live and dead oral treponemes. Experimental macrophages were incubated with *T. denticola* ST10, *T. pectinovorum*, and *T. vincentii*, both live or dead bacteria. After incubation, supernatants were harvested at 2, 8, 24, and 48 hours and assayed for IL-6 by ELISA. The data represent means of the highest values for each pair (significantly different levels of IL-6 secretion when stimulated by live or dead bacteria at *$p<0.0001$, **$p<0.05$).

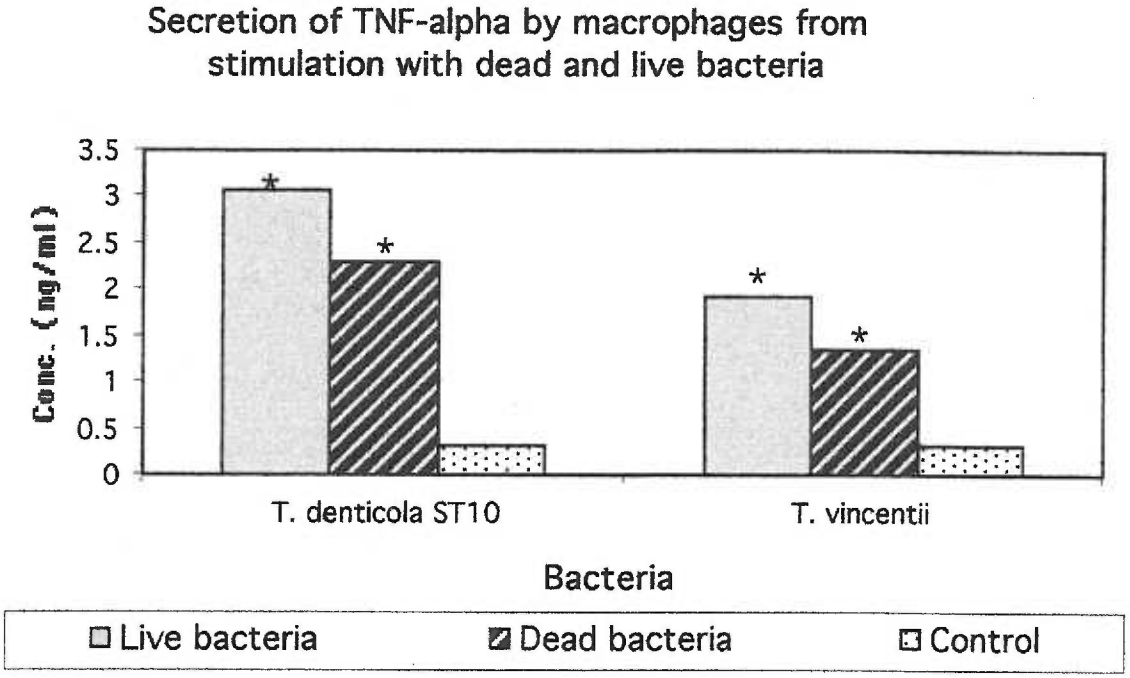

Figure 5: Secretion of TNF-α by control macrophages and experimental macrophages stimulated by live and dead oral treponemes. Experimental macrophages were incubated with *T. denticola* ST10, *T. pectinovorum* and *T. vincentii*, either live or dead bacteria. After incubation, the supernatants were harvested at 2, 8, 24, and 48 hours and assayed for TNF-α by ELISA. The data represent means of the highest values for each pair (significantly different levels of TNF-α secretion when stimulated by live or dead bacteria at *$p<0.0001$, **$p<0.05$).

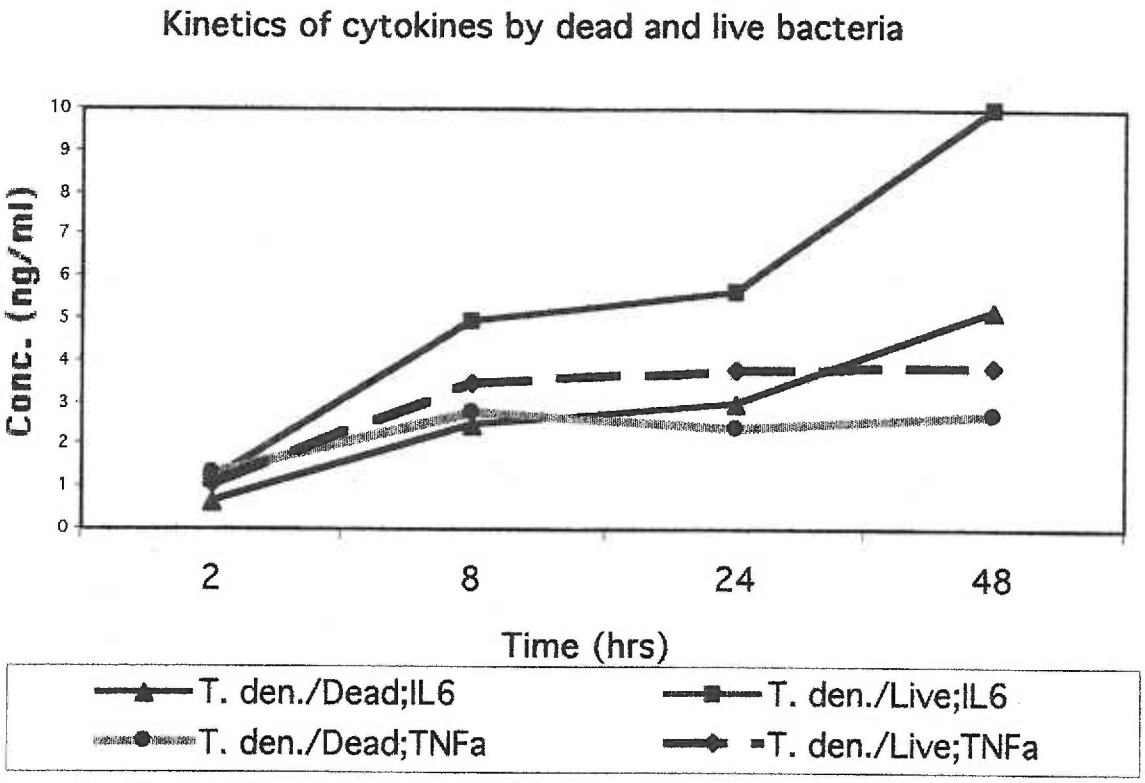

Figure 6: Kinetics of secretion of TNF-α and IL-6 by experimental macrophages at 2, 8, 24 and 48 hours following stimulation by live or dead *T. denticola* ST10. *Treponema denticola* ST10 is representative of all oral treponemes that were associated with elevated cytokine production and showed the similar kinetic profile over time but displayed different levels of TNF-α and IL-6.

Legend:

T. den. = *T. denticola* serotype D strain ST10

Significance of differences between control and experimental macrophages for IL-6 secretion in four assays

IL-6	*P. gin.*	*T. den.* ST-10	*T. soc.* #33534	*T. soc.* #33535	T. den. #35404	*T. pec.* #33768	*T. vin.* #35580
Assay 1	0.9999	<.0001*	0.997	1.000	1.000	0.015**	0.777
Assay 2	0.456	<.0001*	0.926	0.935	0.036**	0.996	0.905
Assay 3	0.719	0.021**	1.000	0.599	1.000	1.000	1.000
Assay 4	0.029**	<.0001*	1.000	1.000	0.948	1.000	0.998

Table 1: P values for four independently repeated assays comparing levels of secretion of IL-6 by control macrophages to macrophages stimulated by *P. gingivalis* or one of six oral treponemes. Experimental macrophages from ATCC were incubated with bacteria for 2, 8, 24 and 48 hours. After incubation, supernatants were harvested and assayed for IL-6 by ELISA assay. The data represent averaged values for all four time intervals for each bacteria compared to negative control (significantly different from the levels of the control unstimulated cells at *$p<0.0001$, **$p<0.05$).

Significance of differences between control and experimental macrophages for TNF-α secretion in four assays

TNF-α	*P. gin.*	*T. den.* ST-10	*T. soc.* #33534	*T. soc.* #33535	T. den. #35404	*T. pec.* #33768	*T. vin.* #35580
Assay 1	<.0001*	<.0001*	0.057	.0003**	<.0001*	<.0001*	<.0001*
Assay 2	<.0001*	<.0001*	0.942	0.053	<.0001*	<.0001*	<.0001*
Assay 3	<.0001*	<.0001*	0.703	0.569	<.0001*	0.943	<.0001*
Assay 4	<.0001*	<.0001*	.0019**	<.0001*	<.0001*	.0002**	<.0001*

Table 2: P values for four independently repeated assays comparing levels of secretion of TNF-α by control macrophages to macrophages stimulated by *P. gingivalis* or one of six oral treponemes. Experimental macrophages from ATCC were incubated with bacteria for 2, 8, 24 and 48 hours. After incubation, supernatants were harvested and assayed for TNF-α by ELISA assay. The data represent averaged values for all four time intervals for each bacteria compared to negative control (significantly different from the levels of the control unstimulated cells at *p<0.0001, **p<0.05).

5. DISCUSSION

Periodontal diseases are inflammatory diseases that are believed to be of bacterial etiology. Bacteria probably affect the disease process directly with their toxins, and indirectly by stimulating and eliciting host response. The ability of macrophages to phagocytose particles represents an early step in the series of events that ultimately lead to the elimination of intruding microorganisms and cellular debris (56). Internalization of different substances by macrophages is mediated through distinct surface receptors that recognize carbohydrates on microbial surfaces and other specialized receptors that recognize apoptotic cell surfaces from surrounding degrading tissue.

In infectious diseases including gingivitis and periodontitis, the interaction of microorganisms with host cells can directly induce release of cytokines in an attempt to keep infection limited (34,35,36). Oral treponemes may possess the ability to stimulate cytokine-mediated responses by interacting directly or indirectly with different receptors on macrophages or following phagocytosis. Secretion of cytokines might occur in an immediate or delayed fashion after contact with bacteria. An immediate response might result in production of cytokines that are related to acute cellular and vascular responses, and delayed responses might include cytokines that influence antigen-specific humoral responses. The hallmark of inflammatory tissue reactions is the

recruitment and activation of leukocytes. Cytokines with chemotactic activities play a pivotal role in mediating these events.

It has been previously shown that macrophages are capable of producing several different cytokines including IL-1β, IL-6 and TNF-α (34,35,36,37). However, little is known about the production of cytokines by macrophages in the presence of oral spirochetes. In this study we have found increased production of IL-6 and TNF-α in the presence of some oral treponemes, but not others. We were unable to detect secretion of IL-1β following contact with any bacteria or LPS.

5.1. Failure to detect Interleukin-1β

Higher levels of IL-1β were found in gingiva of patients with periodontal disease compared with healthy patients (57,58), suggesting that this cytokine is involved in mediating the disease. Lymphocytes and macrophages from inflamed periodontal tissues contain high levels of IL-1 messenger RNAs (59,60). A positive correlation between IL-1β levels and attachment loss has been reported (61). Gingival mononuclear cells and macrophages (62,63) were shown to produce IL-1 after stimulation with putative periodontal bacteria. No evidence could be found in the current literature that the mouse macrophage cells used as a model in the present study produced IL-1β upon co-incubation with oral treponemes or *P. gingivalis*. In most studies fresh human macrophages are used as the model. We observed significantly elevated levels of IL-1β from fresh

human macrophages after challenging them with oral spirochetes; however separation of macrophages from other white blood cells on a routine basis was not feasible.

Failure to detect IL-1β secretion from mouse macrophages in this study could be due to several factors. The most likely one that has also been observed in previous studies (58,64), is the failure of macrophages to synthesize IL-1β and TNF-α simultaneously. In tissue samples from sites with periodontal disease human macrophages produced high levels of IL-1β but much less TNF-α. The two cytokines have coordinated expression by macrophages upon stimulation with LPS or bacterial components (58). There is evidence that IL-1β and TNF-α can be differentially regulated and that macrophages are not always capable of secreting both mediators simultaneously (64). High TNF-α production observed in this study suggests that the presence of oral treponemes elicited TNF-α rather than IL-1β synthesis in macrophages.

There is also the possibility that, unlike human cells, oral treponemes do not stimulate mouse macrophages to increase IL-1β synthesis. Also transformation of the mouse cells may alter their characteristics. Although macrophages posses a variety of heterogenic receptors on their surfaces that lead to intracellular responses, it is possible that this particular cell line does not express the specific receptor or lacks intracellular signaling to regulate IL-1β production. It is also possible that the macrophages might respond to the relatively lower number of oral treponemes by producing IL-6 and TNF-α, but not IL-1β.

Since IL-1β is known as an early cytokine in host response it could be speculated that by not eliciting its production, oral treponemes might be capable of avoiding the initial host defense and therefore, be able to penetrate further into host tissues, at least in mice.

In this model, failure to detect IL-1β could not be attributed to detection methods, as the standard curves and tested unknowns on the same ELISA plate consistently produced detectable and replicable results.

5.2. Interleukin-6

TNF-α has been shown to have an effect on IL-6 production in keratinocytes (65). Therefore, IL-6 production by macrophages in inflammation is likely to be increased and follow an increased secretion of TNF-α. In this study we initially observed low levels of IL-6 that showed a tendency to increase concurrently with increased TNF-α secretion. This might reflect on the involvement of TNF-α in initiating, and IL-6 in maintaining, the inflammatory process associated with the disease.

There was considerable heterogeneity in IL-6 release from macrophages in response to different oral treponemes. The reason for these differences is not known and requires further investigation. It could be related to different surface characteristics among oral spirochetes. Contact with *T. denticola* ST10, and to the lesser degree *T. pectinovorum* and *T. vincentii*, led to an increased production of IL-6 in macrophages.

The local release of IL-6 is likely to further enhance immuno-inflammatory processes by enhancing T-cell and B-cell activation and proliferation (66-68). Greater IL-6 production may help to explain the more prolonged nature of periodontal disease associated with bacteria in disease-associated plaque. By encouraging differentiation of cytotoxic lymphocytes, IL-6 may promote basal cell lysis and lead to further tissue destruction. IL-6 also stimulates keratinocyte proliferation, which is likely to play an important role in wound healing and thickening of epithelium.

5.3. Tumor necrosis factor-alpha

TNF-α has extremely pleiotropic effects due to the ubiquity of its receptors. After being released from macrophages it can further activate multiple signal transduction pathways by inducing or suppressing the expression of a wide number of genes. The local release of TNF-α is likely to play a pivotal role in initiating immune and inflammatory processes in periodontal disease.

TNF-α levels were significantly increased in the presence of *T. vincentii*, *T. pectinovorum* and *T. denticola* ST10 and *P. gingivalis*. These three oral treponemes have been associated with periodontal disease (69). TNF-α synthesis was slightly elevated in the presence of other oral treponemes included in this study. The different levels of TNF-α secretion in the presence of different oral treponemes is suggestive of differences in the virulence potential of bacteria involved in periodontitis. Macrophages

activated by bacteria produce TNF-α, IL-1β, MMPs and PGE2 (37). These mediators target host cells to initiate tissue catabolism and provide signals that trigger fibroblastic apoptosis, MMP release and the expression of homing receptors for lymphocytic recruitment (47).

5.4. Oral spirochetes associated with disease

Speculation about invasiveness and destructive potential of different oral treponemes could be extrapolated from their ability to attach to cell surfaces, or the lack thereof (19). Treponemes that attach to cell surfaces could be more likely to elicit cytokine response by interacting with cell surface receptors or invading host cells. All serotypes of *T. denticola* attached to human epithelial cell monolayers (19). On the other hand, *T. vincentti* showed low attachment and *T. socranskii* showed varying degrees of attachment to epithelial cells (19). These properties could be reflective of the underlying virulent potential of these oral treponemes.

Potentially important information can be drawn also from the presence of certain oral treponemes in plaque from diseased sites but low prevalence of them in plaque from healthy sites. *Treponema vincentii* and *T. pectinovorum* were detected only in plaque from subjects with periodontal disease and were not found in plaque from disease-free subjects (69). *Treponema denticola* has been observed in low numbers in plaque from children and young adults with healthy periodontal tissues

(70), and in much higher number in plaque from sites of adult periodontitis (7,71). Although *T. socranskii* has been detected more frequently in plaque from adults with periodontitis, it has also been detected in plaque from adults in periodontal health (8). On the other hand *T. socranskii* and *T. denticola* were detected in dogs with pocket probing depths ≥5 mm in significantly greater proportions compared to healthy sites (72). *Treponema socranskii* in our study did not result in high increase of cytokine production from macrophages. There was only slight increase in TNF-α secretion associated with this oral treponema.

5.5. Viability of bacteria

Bacterial lipopolysaccharides (LPS) most commonly trigger cytokine release. Purified LPS results in higher cytokine release in monocytes than do whole bacteria (36).

In this study oral treponemes were incubated in 10% penicillin/streptomycin solution and incubated in atmospheric oxygen for 48 hours. The combination of these two antibiotics and an aerobic environment should have created an unfavorable condition for growth and survival of presumably obligate anaerobic spirochetes. Treated *T. denticola* ST10, *T. vincentii* and *T. pectinovorum* appeared mostly non-motile; only few of them showed slight motility under darkfield microscopical examination. Despite compromised viability the presence of treated treponemes resulted in increased secretion of cytokines at only

slightly lower levels than non-treated counterpartners. These results are suggestive that treponemes, viable or not, posses the ability to induce cytokine secretion. This is most likely due to surface components or to their by-products.

The mechanisms by which spirochetes activate macrophages to produce cytokines have not been fully clarified. Some spirochetes like *B. burgdorferi* lack LPS (73) and therefore LPS can not be responsible completely for the inflammatory response.

5.6. Acute and chronic response

Subclinical gingivitis begins to develop as an acute inflammatory response in connective tissue within 2-4 days of plaque accumulation (74). The major cell populations recruited to these sites are predominantly neutrophils, although lymphocytes and mononuclear cells are present as well (19). These cells have the ability to produce local factors which affect the bone remodeling. After 4-7 days, inflammatory cells infiltrate the connective tissue and up to 70% of the collagen may be lost. Acute reaction is an initial step in the cascade of immune responses that can lead to apoptosis or chronic disease with constant presence of cytokine secretion and inflammation. In the persistent presence of pathogens the disease usually progresses from acute to chronic. Inflammatory cells accumulate further and connective tissue continues to be destroyed.

Periodontitis presents clinically mostly in the chronic state with mild to moderate inflammation and without acute symptoms. In chronically diseased sites inflammatory infiltrates are dominated by mononuclear cells, suggesting a lymphocyte-independent pathogenesis in which cells of the innate response and pro-inflammatory mediators play an important role. In an attempt to limit the infection, host cells secrete cytokines to attract cells and molecules that are involved in both humoral and cell immune responses.

There was a distinct difference between early and delayed reaction of macrophages to co-incubation with oral treponemes. Macrophages exhibited a slightly increased cytokine release after being co-cultured with bacteria for only 2 hours. Concentrations of TNF-α increased at 8 hours and peaked at 24 hours. At 48 hours the TNF-α synthesis slowed down, suggesting an early response. On the other hand, IL-6 increased sharply at 24 hours and continued to increase at 48 hours, suggestive of a more delayed response.

The time couse of cytokine synthesis suggests that cells initially react with higher TNF-α secretion, but as they cannot eliminate the bacteria, secrete lesser amounts of TNF-α and higher amounts of IL-6 as a chronic response to the persisting pathogen's presence. Decrease in TNF-α production at 48 hours could also correlate with decreasing viability of bacteria with increasing time of incubation. Bacteria are more likely to be vital and motile at earlier time periods in tissue culture media, since they are subjected to aerobic conditions. This speculation is supported by our observations of decreased viability of treponemes from subcultures

originating from 48-hour supernatants. TNF-α protein could potentially be less stable over time and its decrease over time might be due to its degradation. It is less likely that macrophages were affected by the length of experiment and that their ability to produce cytokines was compromized. Throughout the experiment macrophages appeared attached and viable under the microscope.

6. FUTURE DIRECTIONS

Numerous studies have been able to demonstrate that monocytes and other cell types respond to microbial challenge with increased IL-1β secretion (33,35,37,62,63). There is also strong evidence that IL-1β is increased at sites with periodontitis (45,53,59,61). Both the above findings support the hypothesis that macrophages could be reasonably expected to secrete IL-1β in the presence of putative oral treponeme species. To eliminate the possibility that the failure to detect IL-1β in this study is because this particular macrophage cell line can not produce IL-1β, the experiment should be repeated on another eukaryotic macrophage cell line or fresh blood macrophages.

An increasing body of evidence supports the concept that host-produced prostaglandin E2 mediates much of the tissue destruction that occurs in the periodontal diseases. Goodson reported that gingival biopsies from gingivitis patients contained prostaglandin E2 levels that were 10 times higher than those in healthy patients (76). Most of PGE2 is secreted by LPS-activated gingival macrophages (63). PGE2 is considered to be one of the most important cytokines associated with bone resorption and collagenolysis (60,61). Based on this evidence it would be interesting to include measurements of PGE2 secretion from macrophages upon co-incubation with oral treponemes.

Other oral treponemes found commonly in plaque could be included in future experiments. Treponemes like *T. amylovorum* and *T. maltophilum* were detected in plaque from healthy and diseased sites (69).

Periodontal tissues also include epithelium and connective tissue. Bacteria associated with periodontal disease interact with many different cell types. Polymorphonuclear neutrophils are predominant in the initial host response to bacterial insult. There have been no studies to date reporting the response of epithelial cells, fibroblasts or PMN cells upon co-incubation with oral spirochetes. These cell types could also be included in future experiments.

7. CONCLUSIONS

Mouse macrophages are able to synthesize IL-6 and TNF-α in response to the presence of certain oral treponemes. These two cytokines may play an important role in immuno-inflammatory diseases, including periodontitis and wound healing.

Differences in the production of these cytokines in the presence of various bacteria may help explain the different virulent characteristics of particular oral treponemes and their role in pathogenesis of periodontitis.

The kinetics of cytokine secretion is suggestive of a mild initial response with low concentrations of cytokine secretion, followed by increased release of both cytokines over time in the persisting presence of pathogens.

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