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The Use of Rodent Models to Investigate Host-Bacteria Interactions Related to Periodontal Diseases

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Abstract

Even though animal models have limitations they are often superior to *in vitro* or clinical studies in addressing mechanistic questions and serve as an essential link between hypotheses and human patients. Periodontal disease can be viewed as a process that involves four major stages: bacterial colonization, invasion, induction of a destructive host response in connective tissue and a repair process that reduces the extent of tissue breakdown. Animal studies should be evaluated in terms of their capacity to test specific hypotheses rather than their fidelity to all aspects of periodontal disease initiation and progression. Thus, each of the models described below can be adapted to test discrete components of these four major steps, but not all of them. This review describes five different animal models that are appropriate for examining components of host-bacteria interactions that can lead to breakdown of hard and soft connective tissue or conditions that limit its repair as follows: the mouse calvarial model, murine oral gavage models with or without adoptive transfer of human lymphocytes, rat ligature model and rat *Aggregatibacter actinomycetemcomitans* feeding model.

Keywords

animal model; bacteria; bone; cytokine; inflammation; infection; mouse; murine; osteoclast; periodontitis; rat

Introduction

Animal models are necessary to prove cause and effect relationships and to test the potential of novel therapeutics. In periodontal disease human studies are limited by the difficulty in establishing causal relationships between various factors and periodontal breakdown. Although animal models including those of periodontal disease are not perfect the limitations in addressing mechanistic questions are usually not as severe as those *in vitro* or clinical studies. The periodontium is a complex set of tissues that is chronically exposed to large numbers of bacteria (Paster, et al., 2006; Pihlstrom, Michalowicz, & Johnson, 2005). There are many

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uncertainties about which models are appropriate due in part to open questions about the initiation and progression of periodontitis. It is well known that periodontal diseases are initiated by bacteria, yet there is still controversy about which bacteria actually initiate the events that lead to irreversible breakdown of periodontal tissues (Pihlstrom, Michalowicz, & Johnson, 2005; Sakamoto, Umeda, & Benno, 2005). Much attention has been spent on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, which have been linked to adult periodontitis and localized aggressive periodontitis, respectively (Fine, et al., 2006; Holt & Ebersole, 2005). However, there is only indirect evidence that these bacteria are important periodontal pathogens. Similarly it is not known whether they represent important constituents in a polymicrobial infection, and if the infection is polymicrobial what other bacteria participate. The choice of bacteria has important implications as does the decision to study a single or polymicrobial infection.

It is recognized that the host response plays an important role in periodontal tissue breakdown and that it is likely to involve both innate and acquired immunity (Assuma, et al., 1998; Baker, et al., 1999; Delima, et al., 2002; Teng, et al., 2000). One of the most important uncertainties regarding periodontal disease is its chronic nature. By analogy, periodontal diseases may be like a visit from unwanted relatives. They may move in for visits that are short with each visit representing an acute insult that accumulates so that it appears to be chronic over time. Alternatively the relatives may move next door representing a relatively constant mild insult that becomes problematic with time. The former is consistent with the burst model of periodontal breakdown and the latter is consistent with the slow continuous model (Cohen & Ralls, 1988; Socransky, et al., 1984). To this day it is not known which either or both models are accurate (Gilthorpe, et al., 2003). If the burst model is more accurate it is also not known whether the burst lasts for days or weeks. And if the chronic model is more appropriate it is not known how long the chronic destructive period lasts. In fact, these two scenarios are not mutually exclusive. This is relatively important since the time frame in which periodontal breakdown takes place is not known and hence, assumed advantages of a given model regarding chronicity may be illusory. Another significant issue is whether the response represents a primary or secondary exposure to a pathogen. In some models such as the oral gavage and A.a rat feeding model exposure to the pathogen occurs over a period of several weeks suggesting that there is secondary exposure. Similarly, in the rat ligature model the ligature facilitates invasion of endogenous flora. It may be presumed that the response represents a secondary exposure since it is likely that the animals have been previously exposed to their endogenous flora. In the calvarial and airpouch models there is a capacity to specifically examine primary and secondary responses. A secondary response can be initiated by immunizing the mice prior to inoculation while a primary response would be initiated without prior immunization.

Rodent models have several useful features for investigating molecular mechanisms involved in the pathogenesis of infection-driven inflammatory diseases. Indeed, there is considerable background information on mouse and rat immune systems and a wide range of immunologic and cellular reagents available. Mouse models are particularly informative in studying down stream events associated with the host because of the number of transgenic mice and mice with targeted genetic deletions. Both mouse and rat models provide considerable insight when examining the impact of overlying systemic issues such as diabetes.

In designing studies that assess the various steps required for infection and periodontal disease several parameters need to be considered. It is clear that the infectious process occurs in several distinct but interconnected steps. Examining each step independently and then interdependently can provide a unique window into the disease process. Many lessons have been learned from experiments using rats and hamsters designed to assess the role of *Streptococcus mutans* in a caries infection (Fitzgerald & Keyes, 1960). In a serendipitous finding Keyes discovered that albino hamsters were caries free as compared to golden hamsters

who were caries prone (Keyes, 1959). After isolating a *S. mutans*-like, acid producing micro-organisms, the authors were able to show transferring feces (containing *S. mutans*) from golden hamster cages to cages containing albino hamsters would result in newly formed carious lesions when these albino hamsters were given a soft sucrose containing chow diet (Keyes, 1960). These studies were then supplemented by other studies using a large number of different *in vivo* and *in vitro* models to investigate mechanisms by which environmental factors (fluoride, etc) and host factors (saliva, plaque fluid, etc) can modulate caries (Aoba, 2004; Caufield, Li, & Dasanayake, 2005). These elegant experiments illustrate that; a) animal models can determine the role of both the appropriate host and a pathogenic microbe in a transmissible infection, b) models are useful in understanding the mechanisms by which a bacterium can cause disease, c) models are useful in understanding how the host can modify the disease process. With some modification the lessons learned from caries models can be applied to animal studies of periodontal disease.

The limitation of all animal models is that there is no single model that represents all aspects of human periodontal disease. On the other hand, mechanistic questions cannot be typically addressed in human studies due to important ethical considerations. Thus, animal studies are critical for establishing cause and effect relationships and just as importantly, for initial tests of principle for the development of advanced therapeutics. The goal here is not to define which model comes closest to human disease since periodontal diseases have multiple components but rather to better define how various models can be used to provide insight into mechanisms of periodontal diseases. Thus, the most important issue is whether a given model is suitable for studying a specific hypothesis. Most animal models of periodontal disease tend to focus on specific clinical features of gingivitis and periodontitis or the growth of potential etiologic bacteria *in situ*. However, in other disease systems, a series of models have emerged that emphasize measurement of the host response to various challenges. Several of these have been adapted for use as models to study processes relevant to host-bacteria interactions that shed light on similar processes that take place in the periodontium. The impact of bacteria on the host can be studied at several levels including colonization, host-bacteria interactions, loss of connective tissue attachment, bone resorption and coupling, the process whereby bone formation occurs following resorption to limit bone loss. It may be that one model is not suitable to study all aspects. For example, if the goal is to examine the potential of gingipains to reduce cytokine levels in connective tissue through degradation it may not be necessary to examine the complicating effect of bacterial colonization. Due to the complicating effect of multiple variables (e.g. colonization, connective tissue invasion, etc) the hypothesis may be tested most directly by injecting known levels of bacteria or bacterial mutants lacking gingipains directly into connective tissue rather than to relying on a more complex model where the variables are difficult to control. In some cases the goal maybe to study bacterial genes needed in early biofilm formation so that periodontal breakdown may be irrelevant. The selection of a model should therefore be optimized to the goals of the study and should not necessarily reflect all aspects of periodontal disease. That said, an appropriate model is likely to provide more information than *in vitro* studies where complex cellular interactions are typically ignored or human studies where mechanisms are difficult to prove. Thus, we propose that one should not evaluate animal models with respect to whether they are closest to human periodontal disease, but whether they are appropriate for testing a specific hypothesis.

Calvarial Model

The calvarial or scalp model was developed by Brendan Boyce to study the effect of cytokines on osteoclastogenesis (Boyce, et al., 1989). It has been adapted to study the effect of bacteria on bone resorption (Zubery, et al., 1998) or host-bacteria interactions in connective tissue *in vivo* (Graves, et al., 2001; Leone, et al., 2006). In this model a stimulus is injected directly into the connective tissue overlying the calvarial bone in a small volume (e.g. 25 ul) of carrier.

Classic inflammatory events occur including the rapid expression of pro-inflammatory cytokines within a few hours and the recruitment of PMNs within 24 hours (Graves, et al., 2005). Bone resorption is induced within three to five days depending upon the size of the stimulus (He, et al., 2004; Li, et al., 2002b). If the stimulus is small there is an inflammatory event which resolves with relatively little tissue destruction. On the other hand, a large inoculum can create a perturbation large enough to induce a soft tissue wound and considerable bone resorption that subsequently undergoes repair. New bone formation can then be examined as part of the healing process and can be measured eight to twelve days following inoculation of the stimulus (He, et al., 2004; Liu, et al., 2006a).Thus, a potentially important use of this model is the ability to examine coupling, the process of reparative bone formation that occurs after bone resorption. The precise time frame in which events are measured may be modified by the strain of mouse (Battaglino, et al., 2007). The use an outbred mouse strain, CD-1 avoids some of the extremes of inbred strains. Thus, the calvarial model is useful in examining the sequence of inflammation, destruction and repair. This may be particularly important in periodontal disease where the net loss of tissue may be related to loss of balance between destruction of bone and connective tissue and the degree of repair that occurs. A summary of hypotheses that have been tested in the calvarial model and potential uses of the model are described in Table 1.

It is well known that bacterial products are chemotactic for leukocytes. One of the important questions that has previously been addressed using the calvarial model is the relative contribution of bacterial products or induced host cytokines in stimulating recruitment of inflammatory cells [[ref]]. Thus, the calvarial model was used to test the hypothesis that TNF-α induced by P. gingivalis played a more significant role in the formation of a PMN infiltrate than the direct effects of bacterial products. This was tested by inoculation P. gingivalis into the connective tissue overlying calvarial bone in wild type or knockout mice lacking TNF receptors and measuring the impact of osteoclast formation (Fig 1). The results indicate that there were significantly fewer cells osteoclasts in the absence of TNF receptor signalling compared to the normal mice representing a substantial and significant decrease. This indicates that the TNF activity plays a critical role in bone resorption induced by *P. gingivalis*. TNF activity also played an important role in the formation of an inflammatory infiltrate and fibroblast cell death stimulated by *P. gingivalis* (Graves, et al., 2001). Furthermore, the results are consistent with data obtained with canine and non-human primate models, which indicate that the host response plays an essential and vital role in periodontal disease destruction and is likely to be more important than the direct effect of bacteria in the loss of connective tissue and bone (Assuma, et al., 1998;Offenbacher, et al., 1992;Williams, et al., 1985). These experiments also illustrate the advantage of murine models; a large number of genetically modified mice are available.

Another approach to identifying the functional role of specific molecules in the host response to bacteria involves inhibitors to investigate cause and effect relationships. For example, TNF and caspase inhibitors have been used to establish the role in TNF and apoptosis in orchestrating the events that follow a *P. gingivalis*-induced wound (Al-Mashat, et al., 2006; Liu, et al., 2006a).. Likewise, this model is useful in examining reparative bone formation following bone loss. For example, the injection of LPS causes bone resorption followed by repair with net bone loss due to uncoupling. When fluoxetine is given, the amount of bone formation is sufficiently enhanced so that there is no net bone loss, demonstrating the anabolic effect of fluoxetine (Battaglino, et al., 2007). If bone resorption and formation had not be measured separately, the simplest interpretation of the results would have been that fluoxetine blocks bone resorption. However, in this example, fluoxetine affected coupling so that reparative bone formation was the principle factor affected rather than resorption.

A second advantage of the calvarial model is the ability to deliver a precise amount of inoculum and to examine qualitative changes in the response to different levels of the same stimulus. This has been investigated in the calvarial model using mice with targeted deletions as well as inhibitory antibodies. It has been shown that high doses of enteric LPS induce osteoclastogenesis that is substantially mediated by IL-1 and TNF receptor signaling. Lower doses of LPS in contrast, induce osteoclastogenesis through other pathways that are independent of TNF and IL-1 and involve IL-11 (Chiang, et al., 1999; Li, et al., 2002a).

In addition to studying bone repair the calvarial model can be used to examine soft tissue healing. As mentioned above, the inoculation of a sufficiently large dose of bacteria causes an inflammation-induced soft tissue wound that follows that classic steps of healing including the migration of fibroblasts into the wounded site, proliferation, production of extracellular matrix and reorganization (Liu, et al., 2004). If the bacteria are killed by formaldehyde fixation the inflammation-induced wound is sterile and healing is not affected by the ability to kill bacteria. We have used this model to quantify differences in healing in diabetic and normoglycemic mice and found that one of the principle differences is a higher rate of fibroblast apoptosis that is related to high levels of TNF production in diabetic animals (Liu, et al., 2006a). In fact, when fibroblast apoptosis is reduced with a caspase inhibitor there is a significant improvement in soft tissue healing (Al-Mashat, et al., 2006). It is striking that elevated fibroblast apoptosis has been implicated in poor healing that occurs in diabetic wounds (Darby, et al., 1997). It is noteworthy that loss of fibroblasts is one of the most distinctive cellular changes that occurs in progressing periodontal disease (Zappa, et al., 1992). In patients with periodontitis, fibroblasts have one of the highest rate of apoptosis in the gingiva and are observed predominantly in areas where inflammatory cells have been recruited (Koulouri, et al., 1999). Thus, studies in the calvarial model point to potential novel difference in the response to a bacteria-induced injury in diabetic animals. In addition, the results of increased osteoblast and fibroblast apoptosis with concomitantly reduced numbers of these cells in diabetics is similar to results obtained in other diabetic complications. For example, neuronal apoptosis is increased in diabetic neuropathy, cardiomyocytes apoptosis in cardiomyopathy associated with diabetes, mesangial cell apoptosis in diabetic nephropathy and microvascular cell apoptosis in diabetic retinopathy (Graves et al, 2006).

By immunizing mice against a bacterium, the calvarial model can be used to examine events in connective tissue when the acquired immune response is added to the innate response (Leone, et al., 2006). When *P. gingivalis* is inoculated there is increased expression of several cytokines including gamma interferon, interleukin 6, macrophage inflammatory protein 2, and monocyte chemoattractant protein 1. Immunization against *P. gingivalis* enhances the mRNA levels of all of these cytokines. Interestingly, greater cytokine and chemokine expression is associated with an enhanced and prolonged inflammatory cell infiltrate ($P < 0.05$), increased connective tissue destruction, greater osteoclastogenesis, increased bone loss, enhanced expression of proapoptotic genes and higher levels of fibroblast apoptosis ($P < 0.05$). The results indicate that activation of acquired immunity increases the destructive events stimulated by *P. gingivalis* in part, through upregulating the innate immune response. These studies illustrate the utility of the calvarial model in examining specific aspects of the host-bacteria interactions in a connective tissue environment that retains complex cellular interactions.

Experimental periodontitis in humanized NOD/SCID gavage model

Since most human periodontal pathogens are not habitants in mice and laboratory mouse strains, rendering *in-vivo* study of human periodontal disease difficult; humanization of mouse models provides an excellent opportunity to investigate the underlying processes or mechanisms regarding specific aspects of immune-bacteria interactions related to aspects of periodontal pathogenesis. We summarize here some specific hypotheses tested in humanized

immunodeficient NOD/SCID model in order to study human immunity-mediated pathology *in vivo*. Despite being considered exogenous pathogen(s) in mice under study, the secondary (or recall) immune responses and periodontal tissue inflammation against *A. actinomycetemcomitans* infection can be readily observed and measured in humanized model using oral gavage approach (see Table-2).

Immuno-deficient severe combine immuno-deficient (SCID) mice provide a unique *in-vivo* model to study human lympho-hematopoietic development. Humanization of this model is achieved by engraftment of human peripheral blood leukocytes (HuPBL) into SCID mice (Mosier, et al., 1988). An advantage of this mouse model is the ability to directly assess the function of human lymphoid cells (i.e., $CD4^+$ & $CD8^+$ T-cells and B-cells) *in vivo*. However, there are limited engraftment rates for other human cell lineages such as IgA^+B-cells, granulocytes and neutrophils. In order to establish and boost T-cell immune responses, the host can be treated with anti-NK (sialo-GM1) antibody that depletes NK cells to increase xenograft acceptances and chimerism (Sandhu, et al., 1994; Shpitz, et al., 1994). However, the depletion of NK cells with the antibody results in a much shorter life span (<4-5 wks; (Hozumi, et al., 1994), which limits long term *in vivo* studies. HuPBL-SCID mice can be used to test the protective effect of vaccines (i.e., for *P. gingivalis* infection) and to identify human B cell epitopes for immune protection against *Streptococcus mutans*-induced dental caries (Choi, et al., 1998; Senpuku, et al., 1996). The fidelity of the adoptive transfer is supported by findings that HuPBL from aggressive periodontitis patients placed into SCID mice produce increased anti-leukotoxin IgG production *in vivo* (Shenker, et al., 1993).

Another strain of SCID mice, NOD (non-obese diabetic)/SCID has been generated, which is immuno-deficient with multiple defects in innate and adaptive immunity and lacking functional B and T cells, NK cells, and hemolytic complement activity (Shultz, et al., 1995). A prerequisite of achieving high engraftment rate in humanized NOD/SCID mice involves screening of donor HuPBL; successful adoptive transfer of HuPBL-NOD/SCID mice can reach as high as 40-70% of cells in lymphoid organs/tissues such as the spleen (Mosier, et al., 1991; Rohane, et al., 1995). This is significantly higher than that detected in HuPBL-SCID mice, where endogenous mouse NK cells would attack the xenografts. It should be noted that both humanized SCID and NOD/SCID mice may not be useful to study the initiation of periodontal infection, as the majority of human lymphocytes transferred to the mouse *in vivo* exhibit a memory phenotype (being $CD45RO^+$). Therefore, they are not suitable to study the early interactions between antigen presenting cells, other innate immune cells and naïve T-cells that are important in the initial stages of disease pathogenesis.

Oral infection of mice in this model is achieved by gavage without prior antibiotic treatment (Baker, Evans, & Roopenian, 1994; Teng, et al., 1999). Gavage with *A. actinomycetemcomitans* results in the formation of inflammatory infiltrates in the periodontal pocket and gingival connective tissue area consisting of T and B lymphocytes and $CD14^+$ monocytes/macrophages. Like most of the small mouse models reported, the jaw samples can be examined by histology to assess bone loss, formation of inflammatory infiltrates and by immunohistochemistry to identify cell types involved (Teng, et al., 2000; Teng, et al., 1999). Early alveolar bone loss can be observed around 5 weeks with peak loss at the end of 7-8 weeks. T-cells can be collected and isolated from local mucosal tissues or cervical lymph nodes as described previously (Teng, 2002b) and re-stimulated *in vitro* to further examine their responses and phenotypes. The humanized SCID or NOD/SCID models can also be used to examine immunization strategies or to test the contribution of specific aspects of lymphocyte function *in vivo*. For example, human B- or T-cells harvested from HuPBL-SCID or NOD/SCID mice can be screened by various techniques. This approach has been used to identify genes expressed in *A. actinomycetemcomitans* that are associated with aggressive periodontitis such as CagE-homologue and OPM-1 (Teng & Hu, 2003; Teng & Zhang, 2005). Alternatively,

the induction of genes in lymphocytes can be examined by various mRNA profiling or proteomic techniques (see Table-2; and Teng *et al*., unpublished data).

SCID mice infected with periodontal pathogens (i.e., *P. gingivalis*) produce significantly less alveolar bone loss than the immuno-competent wild-type mice (Baker, et al., 1999; Baker, Evans, & Roopenian, 1994). Similar findings occur in humanized NOD/SCID mice (Teng, et al., 2000; Teng, et al., 1999), suggesting the common features and validity of both model systems. Thus, the humanized NOD/SCID chimeras offer a unique approach to study specific aspects of periodontal pathogenesis such as cytokine profiling and a detailed analysis of lymphoid cell recruitment and participation in alveolar bone loss *in vivo* (Teng, et al., 2000; Teng & Hu, 2003; Teng & Zhang, 2005; Zhang & Teng, 2006). This model has been used to demonstrate that RANKL-mediated osteoclastogenesis and activation plays a significant role in alveolar bone loss *in vivo* (Teng, 2002a; Teng, et al., 2000; Teng, Mahamed, & Singh, 2005; Zhang & Teng, 2006). Similarly, by use a specific inhibitor it was shown that diabetic NOD mice that manifest characteristics of human type-1 diabetes exhibit bone loss that is RANKL dependent (Mahamed, et al., 2005). This suggests that the humanized mice can serve as a useful model to assess the efficacy of experimental therapeutics for specific aspects of periodontal pathogenesis related to bone loss *in vivo* (see Table-2 & Fig. 2). For instance, engraftment of human fetal liver or thymic tissues into NOD/SCID facilitates testing human MHC-I & II-restricted immune responses against specific infections as well as super-antigens (Melkus, et al., 2006). Lymphocyte recruitment *in vivo* may also be tested in humanized NOD/SCID mice in which the mice or the engrafted lymphoid tissue may be genetically modified to express specific genes (e.g. chemokines or chemokine receptors) to study dynamic processes in the acquired immune response (Shultz, Ishikawa, & Greiner, 2007).

Oral gavage model of experimental mouse periodontitis

The introduction of human strains of bacteria by oral gavage and subsequent impact on the periodontium has been studied in various rodent models (Chang, et al., 1988; Klausen, et al., 1991). A mouse periodontitis model was developed in which oral gavage with *P. gingivalis* induces reproducible alveolar bone loss (Baker, et al., 1999; Baker, Evans, & Roopenian, 1994; Baker, Dixon, & Roopenian, 2000; Lalla, et al., 1998). Bone loss induction in this model has been replicated using other periodontal pathogens, including *A. actinomycetemcomitans* (Garlet, et al., 2006), *Tannerella forsythia* (Sharma, et al., 2005) and *Porphyromonas gulae*, an animal periodontal pathogen considered equivalent to human *P. gingivalis* (Hardham, et al., 2005). Typically, a susceptible mouse strain, such as BALB/cByJ, is orally administered a viscous suspension (2% carboxymethylcellulose) containing bacteria. Reproducibility and predictability is enhanced by prior treatment with antibiotics to reduce the oral flora and with repeated inoculations of the implanted organism (*e.g*., three times at 2-day intervals with 10^9 CFU). Substantial bone loss typically takes more than 4 weeks to develop and can be measured histologically, by morphometric analysis as shown in Figure 3, or by microcomputed tomography (Garlet, et al., 2006; Hart, et al., 2004; Wilensky, et al., 2005). For bone loss assessment, the investigators most often examine the bone around the maxillary molars since induction of bone loss in the mandible is slower, whereas incisors are not included in the assessment due to their continuous eruption. In many reports mice are euthanized six weeks after the final infection dose (Baker, et al., 1999; Baker, et al., 2000; Yu, et al., 2007), although it seems reasonable that antecedents could be investigated at earlier time points. In one analytical approach the buccal and lingual gingival tissue surrounding the six maxillary molars are dissected for determination of cytokine induction at the mRNA (Hart, et al., 2004) and/or the protein level (Garlet, et al., 2006) and bone loss quantified by macro- or microscopic examination (Baker, et al., 2000; Baker, Dixon, & Roopenian, 2000; Burns, et al., 2006; Garlet, et al., 2006).

In the oral gavage model *P. gingivalis* is undetectable prior to infection but can be recovered from the mouse oral cavity during the infection period (Baker, Dixon, & Roopenian, 2000). The ability of *P. gingivalis* to establish an oral infection is facilitated by prior antibiotic-mediated suppression of the murine oral flora and may exploit inter-bacterial interactions with early colonizers as has been proposed for the human oral cavity (Daep, et al., 2006; Jeon, et al., 2005). Although vendors may claim that mice are pathogen-free, this does not necessarily mean that they are free of potential periodontal pathogens. In fact, at least one mechanism whereby *P. gingivalis* may contribute to periodontitis is perhaps by influencing inter-bacterial interactions so that it modifies the endogenous subgingival biofilm to become destructive. Such alterations may function synergistically to promote colonization, persistence and virulence of multi-species microbial populations which may thereby acquire enhanced virulence. This is a distinct possibility given that the oral gavage approach does not constitute a monoinfection model (unless mice are germ-free). Indeed, the antibiotic treatment transiently suppresses but does not eliminate the normal oral flora, which gradually re-emerges. Additionally, *P. gingivalis* may modify the host response in ways that favor pathogen survival and disease development.

The oral gavage model has been used to establish proof of principle in vaccine development and to examine the impact of different components of the host response. For instance, immunization against RgpA and Kgp gingipain peptides or intact RgpA gingipain reduces *P. gingivalis*-induced bone resorption (Gibson & Genco, 2001; O'Brien-Simpson, et al., 2005). In an early study, Baker et al showed a greater degree of *P. gingivalis*-induced bone loss in immunocompetent mice than in immunodeficient (SCID) mice lacking both T and B cells (Baker, Evans, & Roopenian, 1994). In a follow-up study by the same group, mice deficient in MHC class II-responsive $CD4^+$ T cells displayed increased resistance to *P. gingivalis*-induced bone loss, compared to normal mice or mice deficient in MHC class I-responsive $CD8^+$ T cells (Baker, et al., 1999). These findings indicate that at least certain aspects of the host response contribute to periodontal tissue destruction. Moreover, the severity of induced bone loss in this periodontitis model is dependent upon both the genetic background of the mice and the particular *P. gingivalis* strain used, suggesting that the interplay of host and microbial factors determines disease activity (Baker, et al., 2000; Baker, Dixon, & Roopenian, 2000).

Because of the complexity of this model there are instances where results need to be interpreted with caution because genetic modifications in mice could affect a number of parameters including colonization, invasion, downstream destructive events, repair or all four. In this regard, the observation that SCID mice display resistance to *P. gingivalis*-induced bone loss (Baker, Evans, & Roopenian, 1994) could be interpreted to suggest that innate immunity may sufficiently protects against periodontitis, although the study was not specifically designed to test this hypothesis. This is consistent with another report that periodontitis-susceptible IL-17-receptor-knockout mice have reduced recruitment of neutrophils to the periodontium, which may be interpreted to mean that neutrophils represent a critical protective component of the host response (Yu, et al., 2007). An alternative interpretation is that genetic modification indirectly leads to a more pathogenic flora as discussed in the same study (Yu, et al., 2007).

Whereas more studies are warranted to conclusively associate the innate response with protection, it should be borne in mind that pathogens in general have evolved strategies to exploit and undermine innate immunity (Finlay & McFadden, 2006). In this context, the oral gavage model appears to be appropriate for investigating aspects of immune evasion in periodontitis. Specifically, it was recently shown that TLR2 activation leads to enhanced *P. gingivalis* persistence when inoculated into chambers implanted into the abdomen and TLR2-deficient mice display increased resistance to *P. gingivalis*-induced periodontal bone loss compared to normal controls (Burns, et al., 2006). Although the precise underlying mechanisms

are uncertain, at least partly, they may involve the ability of *P. gingivalis* to co-opt TLR2 inside-out signaling for activation of CD11b/CD18 outside-in signaling which inhibits IL-12 p70 production (Hajishengallis, Ratti, & Harokopakis, 2005; Hajishengallis, et al., 2006; Harokopakis & Hajishengallis, 2005). Since IL-12 p70 plays a key role in intracellular bacterial clearance, this mechanism may presumably undermine the ability of the host to control an infection (Trinchieri, 2003). In support of this notion, in vivo blockade of CR3 leads to enhanced IL-12 p70-mediated clearance of *P. gingivalis* and inhibition of its capacity to cause periodontal bone loss in the oral gavage model (Hajishengallis, et al., 2007). Moreover, oral infection of mice with *P. gingivalis* expressing a mutant version of fimbriae that interacts poorly with CR3 leads to severely attenuated bone loss, compared to the wild-type control (Wang, et al., 2007). Interestingly, these in vivo findings from the oral gavage model are correlated with the in vitro virulence characteristics of the tested strains or the in vitro effects of CR3 blockade. Therefore, the oral gavage model seems appropriate for addressing hypotheses related to putative immune evasion strategies, identified from in vitro host-pathogen mechanistic studies.

The oral gavage model was moreover successfully used to mechanistically link *P. gingivalis*-induced periodontitis to systemic conditions. For example, oral infection of hyperlipidemic mice with *P. gingivalis* mice accelerates atherosclerotic plaque formation (Gibson, et al., 2004; Lalla, et al., 2003). Moreover, *P. gingivalis*-induced alveolar bone loss is enhanced in diabetic mice unless the receptor for advanced glycation end products (RAGE) is blocked (Lalla, et al., 1998; Lalla, et al., 2000). It becomes obvious that the oral gavage model is useful for addressing a wide variety of hypotheses related to periodontal pathogenesis, ranging from the role of the host response to virulence traits of suspected periodontopathogens and the interconnection of those factors with systemic parameters. A summary of such tested hypotheses is presented in Table 3 along with additional future uses of this model.

A. actinomycetemcomitans Rat Feeding Model

Colonization is a critical process in infection and dependent on surface related, tissue specific adhesins that interact with host receptors. The *A. actinomycetemcomitans* rat model of periodontal disease is particularly well suited to examine aspects of bacterial behavior that promote colonization and initiation of periodontal disease (see Figure 4). Scannapieco and colleagues demonstrated that clinical isolates of *A. actinomycetemcomitans* when grown on agar had a rough surface colonial morphology which attached with great avidity when grown in broth (Scannapieco, et al., 1987). Further when these rough wild-type strains were passaged in the laboratory, the adherent strains often converted to a smooth, gum drop like colony that no longer attached to surfaces. These characteristic are seen in many *A. actinomycetemcomitans* strains in the ATCC culture collection. Moreover, it appears that conversion is irreversible and thus smooth colonies do not have the capacity to revert to their original rough adherent phenotype (Fine, et al., 1999). In order to investigate the significance of rough versus smooth strains of a suitable infectious model is needed, as described below.

Seminal experiments by Mekalanos and colleagues highlighted differences seen when microbes were removed from their natural environment and grown in the laboratory (Mahan, Slauch, & Mekalanos, 1993). Use of promoter-reporter constructs established differences in gene expression between *in vivo* as compared to *in vitro* bacterial gene expression. This has led to identification of novel virulence gene sequences in the bacteria tested (Mahan, Slauch, & Mekalanos, 1993). To test this concept in *A. actinomycetemcomitans*, a smooth laboratory strain of *A. actinomycetemcomitans* was placed in the mouths of rats to determine if growth in an animal host could lead to conversion of smooth to rough by expression or upregulation of genes in the smooth strain. To make this comparison, wild-type *A. actinomycetemcomitans* was passaged in broth to achieve a smooth, afibrillar, minimally adherent isogenic *A. actinomycetemcomitans* mutant. Both wild type and smooth *A. actinomycetemcomitans* strains

were made rifampicin resistant so that they could be readily identified in the mouths of rats. The results indicated that the smooth strain could survive in some animals for a 2-3 week period but not more than 4 weeks. On the other hand the rough adherent strain was found over the 12 week experiment period and in some cases 7 months after inoculation. No reversion was seen in any strain. Thus none of the isogenic smooth strains recovered from the mouths of rats were found to be rough and none of the rough strains converted to smooth even though rough strains gave rise to the smooth strains in the laboratory in less than 4 passages (Fine, et al., 2001).

At about the same time it was discovered that avid attachment of *A. actinomycetemcomitans* to tooth surfaces related to a group of genes called the *tad* genes (for tight adherence) (Kachlany, et al., 2001). The assumption was that these genes were capable enabling *A. actinomycetemcomitans* to attach to teeth of rats because the *tad* genes conferred on these *A. actinomycetemcomitans* strains, the ability to bind to abiotic and biological surfaces. A study testing this hypothesis showed that knocking out these *tad* genes and thus creating the equivalent of a smooth strain, prevented the inoculated strains from colonizing and inducing bone loss. In contrast the *A. actinomycetemcomitans* wild type strain colonized, developed antibody to *A. actinomycetemcomitans*, and stimulated bone loss. Each animal that was colonized by *A. actinomycetemcomitans* showed an increase in antibody titer and bone loss; while the *A. actinomycetemcomitans* knock-out strains had neither antibody titer or bone loss (Schreiner, et al., 2003).

Lessons learned from these experiments indicated that it is important to consider the existing flora, tagging the exogenous inoculated species, the housing of animals, the methods of recovery and the methods of feeding of animals. Experiments designed to add exogenous *A. actinomycetemcomitans* into an existing oral flora requires that the existing flora be reduced to facilitate colonization of the implanted strain. We also found it beneficial to use a tag so that the implanted strain could be distinguished from the existing flora. This is particularly useful to distinguish between rough and smooth forms of *A. actinomycetemcomitans*. Thus when tested against the existing flora of the host animal only the implanted rifampicin resistant strain would survive in rifampicin containing culture media since no other isolates obtained from the normal oral flora could survive. In this manner the organism recovered is derived from the organism implanted. In addition to these considerations, housing of animals is a critical issue in this a colonization and transmission model of disease. In this case separate cages are required to avoid transmission of microorganisms from one animal to another. In feeding experiments where some animals are more aggressive than others, co-habitating can lead to inadvertent transmission of microbes via, licking, touching etc. Further great pains should be taken in delivering a standard dose over a standard period of time (Fine, et al., 2001). Mixing a given amount of *A. actinomycetemcomitans* in with the animal feed and caging animals separately allowed for a more quantitative estimate of the dosage given and the colonization efficiency.

More recently we have become aware of the fact that both species and tissue specificity can be critical issues with respect to the choice of an animal model. With respect to *A. actinomycetemcomitans*, wild type strains have been shown to have a narrow host range that includes humans, old world monkeys, cows and rats when tested against buccal epithelium (Fine, et al., 2005). Human and old world monkey specificity was found to be due to two *A. actinomycetemcomitans* adhesions. Thus, *A. actinomycetemcomitans* adhesions Aae and ApiA, in addition to species specificity, demonstrated a narrow range of tissue tropism that limited attachment to buccal epithelial cells and tongue and not to alveolar, nasal, skin, or, vaginal epithelium. Moreover, wild type *A. actinomycetemcomitans* binds to rat buccal epithelial cells and a review of the literature indicates that *A. actinomycetemcomitans* is a normal inhabitant of a species of rice rat making the rat an appropriate model for studies of *A. actinomycetemcomitans* colonization and infection (Fine, et al., 2005). However, *A. actinomycetemcomitans* has difficulty colonizing the oral cavity of mice. Thus, the mouse is

not an appropriate model to study *A. actinomycetemcomitans* colonization and studies with *A. actinomycetemcomitans*-induced bone loss in mice should keep in mind that the infection is transient and as a result, self-limiting. While Actinobacillus actinomycetemcomitans, recently renamed Aggregatibacter actinomycetemcomitans, has been isolated from the mouths of rats (MacDonald et al., 1959; Shaw et al., 1961) the exact species was only tentatively identified. Moreover, in our feeding model experiments, an extensive survey of the mouths of the Sprague Dawley rats prior to feeding, failed to reveal any pre-existing *A.a*. As such, animals used in this rat feeding model were infected by an exogenous source of Aa. Nevertheless, the ability of rat buccal cells and salivary coated rat teeth to bind *A.a in vitro*, coupled with the finding of Actinobacillus in the mouths of laboratory rodents (Simpson and Simmons, 1980) suggests that, unlike mice, *A.a* can infect and inhabit the mouths of rats. As a result one can extrapolate and suggest that *A.a* is well suited to cause an indigenous infection (Macdonald et al, 1959) which can be altered by antibiotic administration (Shaw et al, 1961).

To overcome this issue of tissue and species specificity, Finlay and coworkers in a diarrhea model have used *Citrobacter rodentium*, an enteropathogenic rat microbe with a similar phenotype to *E. coli*. As a non-invasive Gram-negative species of bacteria that colonizes the intestine of rodents and causes diarrhea these studies have advanced the understanding of human intestinal disease. These bacteria colonize by direct contact with the intestinal tissue making it feasible to test adhesin/receptor interactions. It is also not surprising that different strains of rats may have different levels of susceptibility to infection and bone loss. Preliminary data from our laboratory suggests that three different strains of rats show different levels of bone loss even if the same *A. actinomycetemcomitans* strain and the same method of inoculation of *A. actinomycetemcomitans* is used. This may well reflect differences in rat strains manifested as an expression of host genetic variability that could result in strain variation in production of cytokine and in the robustness of the innate or acquired immune responses as has been shown in mice (Adelman, Cohen, & Yoshida, 1978; Darville, et al., 1997; Gemmell, et al., 2000). A sumary of hypotheses that have been tested and potential future uses of the *A.a* rat feeding model are described in Table 4.

Rat Ligature Model

The placement of ligatures around teeth to initiate periodontal tissue loss has been used in a various types of animals. The ligature leads to greater accumulation of plaque and ulceration of the sulcular epithelium facilitating invasion of connective tissue. Although this model was once commonly used with non-human primates it is used less frequently due to the expense and difficulty in carrying out experiments with these animals. Because many of the same series of events occur in the rat as in the non-human primate there has been renewed interest in the rat ligature model, particularly since it is simpler to work with and less expensive (Rovin, Costich, & Gordon, 1966). In the rat, loss of attachment and bone occurs predictably in a 7 day period (Bezerra, et al., 2002; Bezerra, et al., 2000; Lohinai, et al., 1998; Nowotny & Sanavi, 1983) although investigators have conducted experiments over much longer periods of time (Kuhr, et al., 2004; Nociti, et al., 2001; Rovin, Costich, & Gordon, 1966; Weiner, DeMarco, & Bissada, 1979). Like human periodontitis, alveolar bone loss in the ligature model is dependent upon bacteria. In gnotobiotic rats placement of ligatures does not induce significant gingival inflammation or periodontal bone loss (Rovin, Costich, & Gordon, 1966). Topical treatment with chlorhexidine reduces bone resorption and antibiotics reduce loss of attachment and loss of bone, supporting the role of bacteria in initiating destruction in this model (Kenworthy & Baverel, 1981; Weiner, DeMarco, & Bissada, 1979). In contrast, increasing gram negative bacterial burden enhances osteoclastogenesis and bone resorption (Samejima, Ebisu, & Okada, 1990). A summary of hypotheses that have been tested in the rat ligature model are described in Table 5 in addition to examples of hypotheses that could be tested in the future.

Like human periodontitis the destructive phase of ligature-induced experimental periodontitis is associated with a host response as shown by formation of an inflammatory infiltrate in the gingiva prior to bone resorption (Gyorfi, et al., 1994). When the host response is diminished by inducing endotoxin tolerance bone loss is decreased (Samejima, Ebisu, & Okada, 1990). The functional role of the inflammatory response is further demonstrated by decreasing periodontal destruction in rats treated with prostaglandin inhibitors. The COX-1/COX-2 inhibitor, indomethacin reduces gingival inflammation, osteoclast formation and bone loss in this model (Bezerra, et al., 2000). The COX-2 inhibitor, meloxicam, has a similar effect (Bezerra, et al., 2000). Low dose doxycycline, which inhibits MMP activity, also reduces ligature enhanced alveolar bone loss (Bezerra, et al., 2002). The role of cytokines in the disease process is demonstrated by enhanced bone loss when ligature-induced periodontitis is augmented by application of IL-1 or TNF (Gaspersic, et al., 2003; Koide, et al., 1995). And lastly, the ligature-induced model is sensitive to systemic effects such as smoking, so that rats exposed to cigarette smoke have greater bone loss and higher MMP-2 activity than ligated rats not exposed to cigarette smoke (Cesar Neto, et al., 2004). Similar results have been obtained when ligated rats were injected with nicotine (Benatti, et al., 2003; Nociti, et al., 2001).

An advantage of the rat ligature model is the availability of systemic disease models. For example, the type-2 Zucker diabetic fatty (ZDF) rat can be used to examine the impact of diabetes. The Zucker diabetic fatty rat develops obesity and insulin resistance, which results in diabetes by eight weeks of age (Clark, Palmer, & Shaw, 1983; Peterson, 1995). When ligature-induced periodontal bone loss is induced in the ZDF rats and normoglycemic littermates, diabetes increases the intensity and duration of the inflammatory infiltrate, enhances osteoclastogenesis and activity and increases bone loss (Liu, et al., 2006b). Furthermore, by removing the ligatures, a healing response ensues in which coupling may be examined. When tested, the amount of new bone formation following resorption was 2.4 to 2.9 fold higher in normoglycemic versus diabetic rats. This suggests that the amount of bone formed after resorption, which represents “coupling” a process where bone mass is maintained, is deficient in the diabetic animals and is illustrated in Figure 5. Thus, diabetes may have more net bone loss because resorption is greater and coupling is less. Thus, this model is useful in investigating mechanisms by which diabetes could aggravate periodontal tissue loss including more persistent inflammation, greater bone resorption and decreased reparative bone formation leading to uncoupling and greater net bone loss.

Airpouch/ Chamber Models

The Airpouch Model was originally developed in mice and rats by the Willoughby group to study the function of synovial membrane (Edwards, Sedgwick, & Willoughby, 1981). To produce an airpouch, 5 cc of air is injected subcutaneous in the back of a mouse or rat near the midline. A second injection is usually made after 3 days to maintain the patency of the airpouch. After 6 days, the airpouch will have become filled with fluid and it is lined by epithelium. The model was used extensively in the rheumatoid arthritis (RA) literature to investigate the dynamics of synovial inflammation and the initiation of inflammation. Many of these studies were seminal in the identification of mast cells and histamine as important players in the early inflammatory response (Sin, et al., 1986). The model also proved useful developing clinical correlates for the role of cytokines, chemokines and lipid mediators in the pathogenesis of RA (Ferrandiz & Foster, 1991).

More recently, the murine airpouch model has been adapted for the evaluation of potential pharmacologic agents. Injection of proinflammatory agents including irritants, cytokine, or bacteria into the airpouch elicits a quantifiable inflammatory response measured as number of inflammatory cells, secretion of cytokines, or other common parameters (Sin, et al., 1986) (Hachicha, et al., 1999) adapted Willoughby’s model to assess neutrophil responses to TNFα

and IL-1β and how the acute inflammatory response is modified by lipoxins and aspirin triggered lipoxins. The model has the advantage of allowing both analysis of the exudate fluid and the epithelial lining of the airpouch. Lipoxins and their aspiring triggered analogs induced a profound dampening of acute inflammation demonstrating a dose response reduction in the number of leukocytes in the exudate as well as a reduction of leukocytes in the epithelial lining. The experiment is performed in the viable whole animal, which reduces *in vitro* artifact.

The airpouch model was adapted for periodontitis by (Pouliot, et al., 2000) to assess the acute inflammatory response to *Porphyromonas gingivalis (P.g.).* This report provides an excellent example of the benefit of whole animal experiments to minimize *in vitro* artifact. In previous work (Van Dyke, et al., 1982) had reported that *P.g.* produced a factor that inhibited neutrophil chemotaxis prompting the suggestion that *P.g.* was evading the host response by inhibiting its first line of defense. However, injection of *P.g.* into the airpouch induced a profound neutrophil infiltrate. In retrospect, it is likely that gingipains were degrading either the chemotactic agent or the neutrophil receptor for the agent, but importantly, it is now clear that this does not appear to occur *in situ* and *P.g.* is apparently a strong pro-inflammatory stimulus. Another important observation relevant to the pathogenesis of periodontitis was also uncovered in this model. The major source of PGE_2 in periodontal tissues has been widely held to originate from monocyte/macrophages; these experiments demonstrated that neutrophils are a rich source of PGE_2 in the inflammatory lesion.

The shortcoming of the airpouch model is the duration of the experiment; it is not practical to conduct a chronic inflammation experiment with the airpouch with maintenance of the airpouch being the limiting factor. This problem was solved in part by the use of tissue cage and chamber models. The use of the airpouch and chamber models to test specific hypotheses is described in Table 6 along with potential future uses of this model.

The mouse subcutaneous chamber model was adapted for use in periodontal studies by C. Genco et al. (Genco, et al., 1991). Originally used to study immungenicity and strain diversity of *Neisseria gonorrhoeae* in mice (Wong, et al., 1979), the method was also used to assess the pathogenesis of foreign body infections in guinea pigs (Zimmerli, et al., 1982). Teflon tissue cages have also been used in guinea pigs (Schenkein, 1989) and New Zealand White Rabbits (Dahlen & Slots, 1989). The chamber is a coiled stainless steel wire that is surgically implanted subcutaneously in the back of the mouse (or other animal) and the wound is allowed to heal for ten days. Like the airpouch model, the interior of the coil become epithelialized allowing for the injection of bacteria or other substances into the lumen of the chamber. Fluid can be aspirated from the chamber at intervals or the entire chamber can be excised and histology performed.

The chamber model and the tissue cage variants used as periodontal models have, for the most part, assessed the virulence of bacteria. It has the advantage over abscess models of allowing assessment of host parameters as well as bacterial colonization, growth and tissue damage. Genco et al. were able to demonstrate development of an acquired immune response to *P.g.* injected into the chamber and to demonstrate strain differences within the bacteria (Genco, et al., 1991). Dahlen and Slots (Zimmerli, et al., 1982) obtained similar results with the tissue cage in rabbits. Schenkein and co-workers evaluated the role of complement in clearance of *P.g.* (Schenkein, 1989).

The long term chamber model was also effectively used in immunization studies (Genco, et al., 1992). Mice were immunized with non-invasive and invasive strained of heat-killed *P.g.* followed by chamber challenge with invasive strains. The investigators were able to demonstrate that immunization with any strain provided protection against tissue damage and invasion by invasive (virulent) strains. Interestingly, immunization did not prevent

colonization of the chamber by any *P.g.* strain, but it did prevent tissue damage and spread of the colonizing bacteria. In another related experiment, Hu, et al, demonstrated that activation of the kinin system is involved in the breach of the vascular barrier that allows for dissemination of *P.g.* The investigators demonstrated that *P.g.* in the chamber caused leakage through the epithelial barrier and activation of the kinin system resulted in more severe dissemination, while blocking the kinin system prevented dissemination of *P.g.*

The strength of the airpouch and chamber models in small animals is clearly the whole animal aspect for the measurement of host response parameters. The strength of the model is enhanced, particularly in mice, by the availability of genetically modified knock-out or transgenic animals that can be used in the experiments. For short term, acute inflammation experiments, the strength of the airpouch model is its simplicity and ease of use with no requirement for surgical interventions. The major advantage of the chamber models is the ability to perform long term experiments that can be adapted to immunization and chronic inflammation and pathogenesis experiments.

Concluding Remarks

Rodent models have provided a great deal of information about the process of periodontal disease. The rat ligature model using gnotobiotic rats demonstrated that bacteria play an essential role in initiating gingival inflammation and periodontal bone loss. The oral gavage and A. actinomycetemcomitans rat models have established that certain bacterial virulence factors are essential in promoting an infection that causes periodontal bone loss. That the acquired immune response contributes significantly to periodontal tissue destruction induced by transient A. actinomycetemcomitans or P. gingivalis infection has been demonstrated in the gavage model using mice that are genetically modified or modified by adoptive transfer of human lymphocytes. Mechanisms by which diabetes affects the response to P. gingivalis by prolonging inflammation, enhancing the death of fibroblasts and osteoblasts and by interfering with bone coupling has been shown in the calvarial model and in the ligature model of periodontal bone loss. Much of this critical insight into mechanisms through which oral pathogens lead to connective tissue and bone loss could have been obtained in human studies and would have been considerably more difficult or expensive in other animal models. Moreover, *in vivo* models come closer to providing an accurate assessment of cell behavior compared to *in vitro* models. For example, the response of host cells to bacteria is much more likely to be accurately depicted by *in vivo* models. This is true in spite of the fact that the *in vivo* setting in animals is not identical to the human periodontium. It is therefore the conclusion of the authors that well designed animal models are essential to understanding the complex processes of periodontal diseases.

Clinical Relevance

Scientific rationale

Animal models are widely used to establish cause and effect relationships. However no animal model recapitulates all aspects of disease initiation and progression. The purpose of this review is to illustrate hypotheses that can be addressed in different rodent models to provide insight into processes that occur in human periodontal diseases.

Principal findings

Different rodent models are well suited to address specific questions that are important in understanding host-bacteria interactions. Insights can be applied to design studies using animal models with specific end points in mind so that the strength of a particular model is utilized. Thus, more than one model may be needed to provide a better understanding of different steps in host/microbial interactions that lead to pathogenic developments. Important steps that may

be investigated include the successful colonization of a susceptible host, bacterial invasion into vulnerable tissue, host-bacterial interactions that lead to an inflammatory response that may ultimately stimulate tissue destruction and finally a repair process that may mitigate some of the tissue damage which has occurred. With this approach in mind, animal models can provide data that would be impossible to obtain from humans because in an animal model a particular process can be examined at many points in time with interventions that establish cause and effect relationships. In this manner a better understanding of discrete steps in disease initiation or progression can be obtained.

Implications for practice

Animal models are particularly well suited for studying cause and effect relationships and for initial studies of safety and efficacy of new treatments. A better understanding of animal models will allows a more judicious match of a model with the hypothesis or therapy to be tested.

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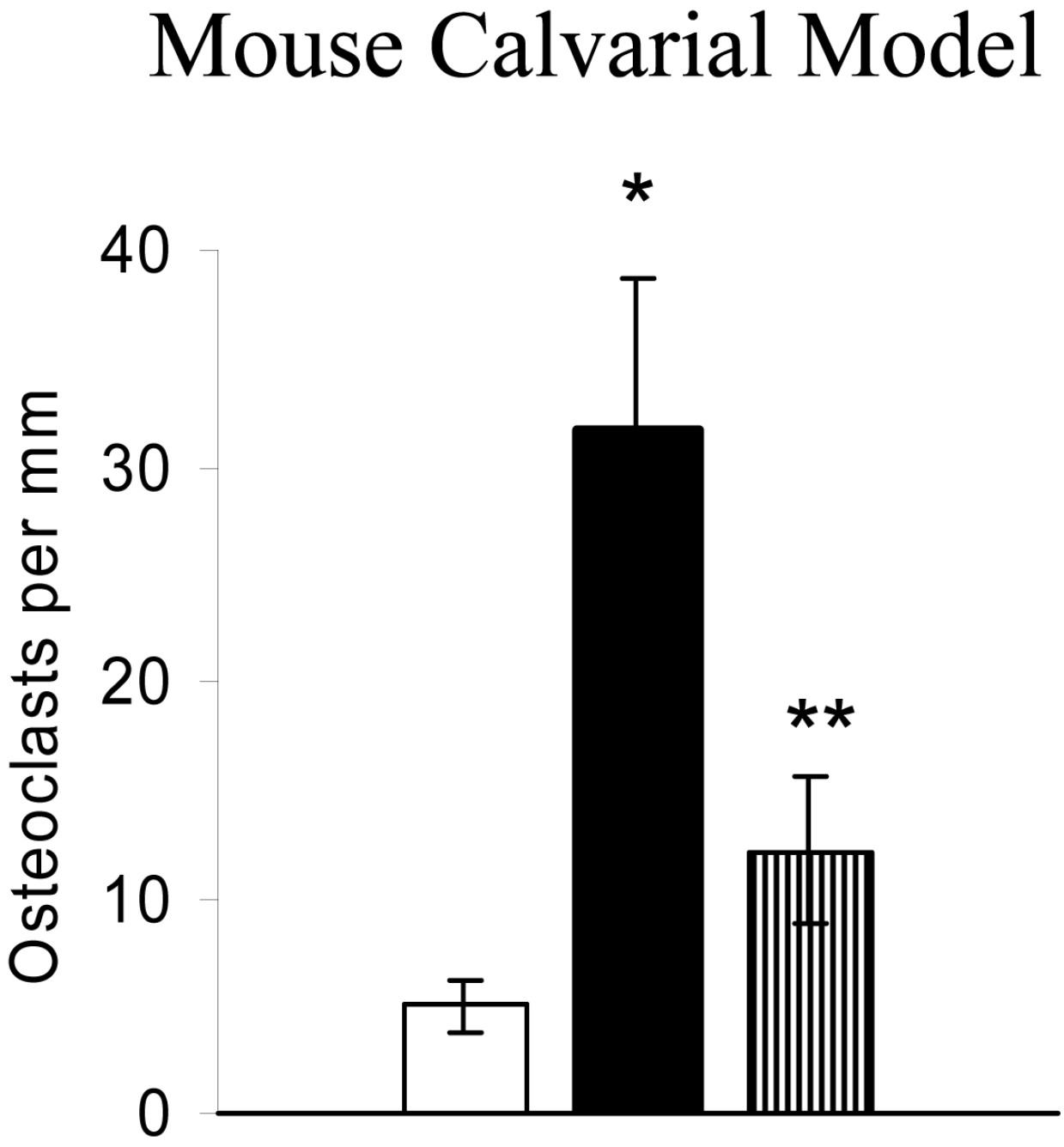

Fig 1.

Use of the calvarial model to demonstrate that *P. gingivalis*-induced osteoclastogenesis *in vivo* is largely modulated through TNF. *P. gingivalis* or vehicle alone was inoculated into the scalp of TNFRp55$^{-/-}$/p75 $^{-/-}$ or control mice. Osteoclasts were identified as TRAP positive multinucleated cells lining bone and quantified from sections taken five days after *P. gingivalis* inoculation. * indicates $p<0.05$ for P.g. vs. vehicle alone, ** indicates $p<0.05$ for TNFR$^{-/-}$ vs. WT. This figure was previously published in (Graves, et al., 2001).

Aa-HuPBL-NOD/SCID Mouse Model

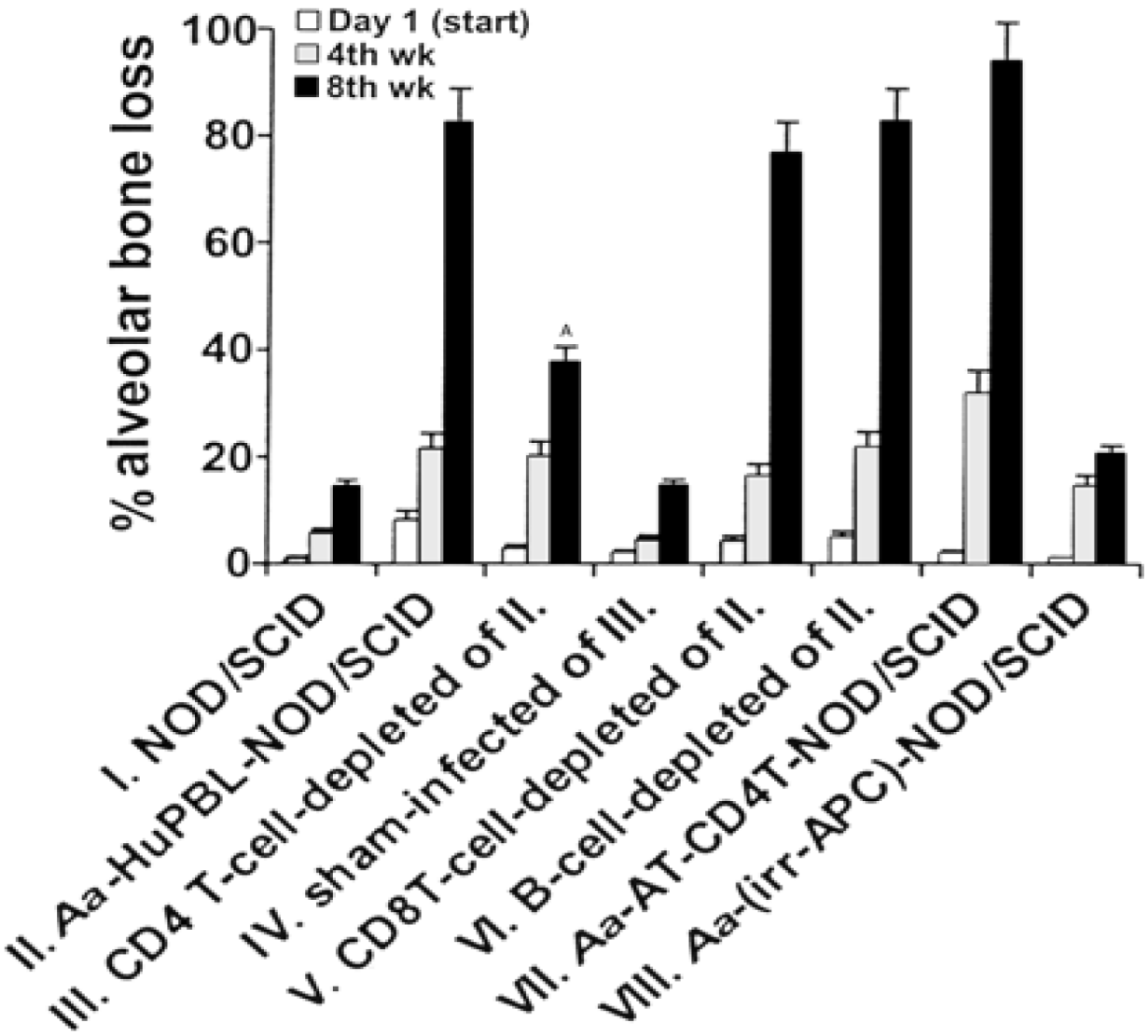

Fig 2.

Control of alveolar bone loss by *A. actinomycetemcomitans* (*A.a*)-reactive $CD4^+$T-cells. Groups of mice as indicated were either left untreated or inoculated with *A.a.* $CD4^+$T, $CD8^+$T, or B cells were depleted from mice using antibody and complement as described (Teng *et al*. 2000). Data shown are the mean (± SD) of alveolar bone loss by time up to 8 weeks, expressed as % of bone loss detected in positive control, *A.a*-infected BALB/c mice (as 100%). Group I, sham-infected, nondepleted NOD/SCID mice (*n* = 10); group II, *A.a*-infected, nondepleted HuPBL-NOD/SCID mice (*n* = 32); group III, *A.a*-infected, $CD4^+$T cell-depleted NOD/SCID mice (*n* = 20); group IV, sham-infected, $CD4^+$T cell-depleted NOD/SCID mice (*n* = 9); group V, *A.a*-infected, $CD8^+$T cell-depleted NOD/SCID mice (*n* = 12); group VI,

A.a-infected, B cell-depleted NOD/SCID mice (n = 16); group VII, *A.a*-infected NOD/SCID mice bearing adoptively transferred *A.a*-reactive $CD4^+$T cells (AT-CD4T) plus autologous MO/MQ as APCs (n = 10); group VIII, *A.a*-infected NOD/SCID mice bearing adoptively transferred autologous MO/MQ as APCs (irr-APC; n = 6). Statistical differences for bone loss between group III and groups II, IV, V, and VI were significant ($p<0.005$).

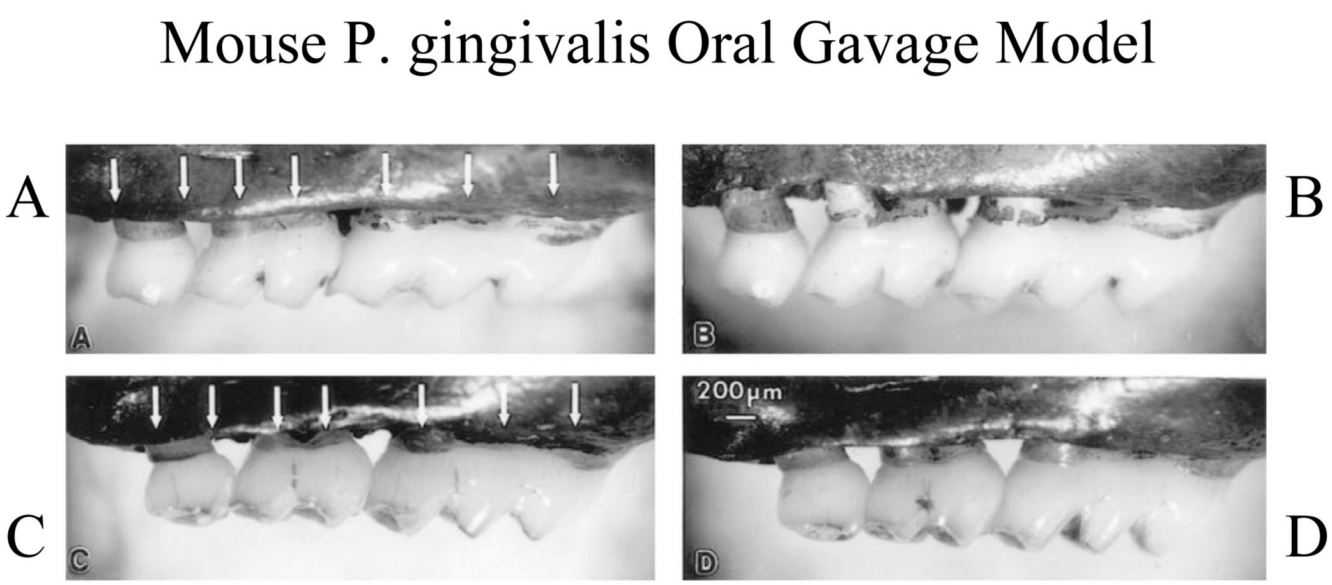

Fig 3.

Periodontal bone loss in the oral gavage model. Assessment of bone levels is made by measuring the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at the seven indicated buccal sites on the three molars on the left side and the symmetrical seven sites on the right side of the maxilla. To determine bone loss, the 14-site total CEJ-ABC distance for each infected mouse is subtracted from the mean CEJ-ABC distance from the group of sham-infected mice. (A) Sham-infected BALB/cByJ mouse (B) *P. gingivalis*-infected BALB/cByJ mouse demonstrating increased CEJ-ABC distances at the molar sites, compared to sham infection. (C) Sham-infected C57BL/6J mouse (D) *P. gingivalis*-infected C57BL/6J mouse demonstrating negligible bone loss compared to sham infection, due to genetically-determined resistance to bone loss. Originally published by Baker PJ, Dixon M, Roopenian DC, 2000.

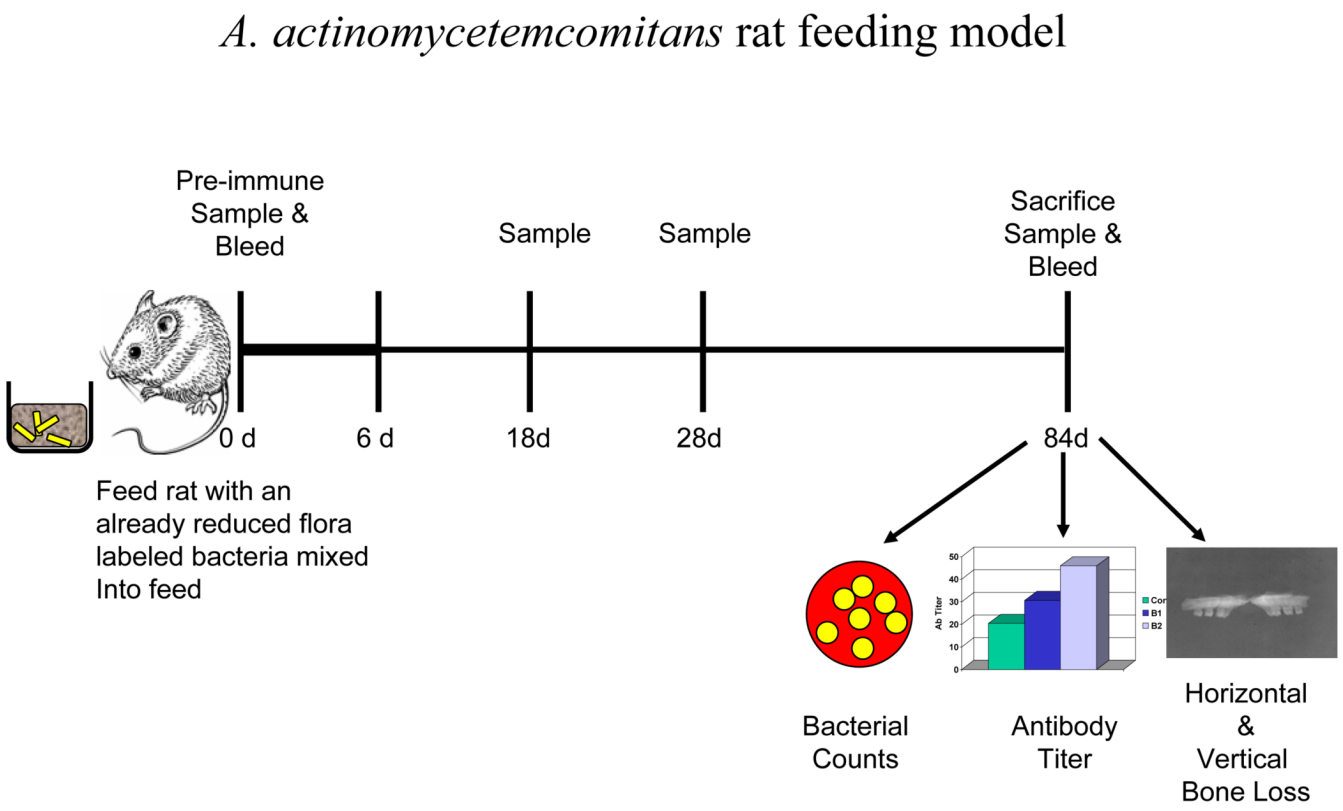

Fig. 4.
Time line for studying *A. actinomycetemcomitans* colonization and bone loss in the rat.

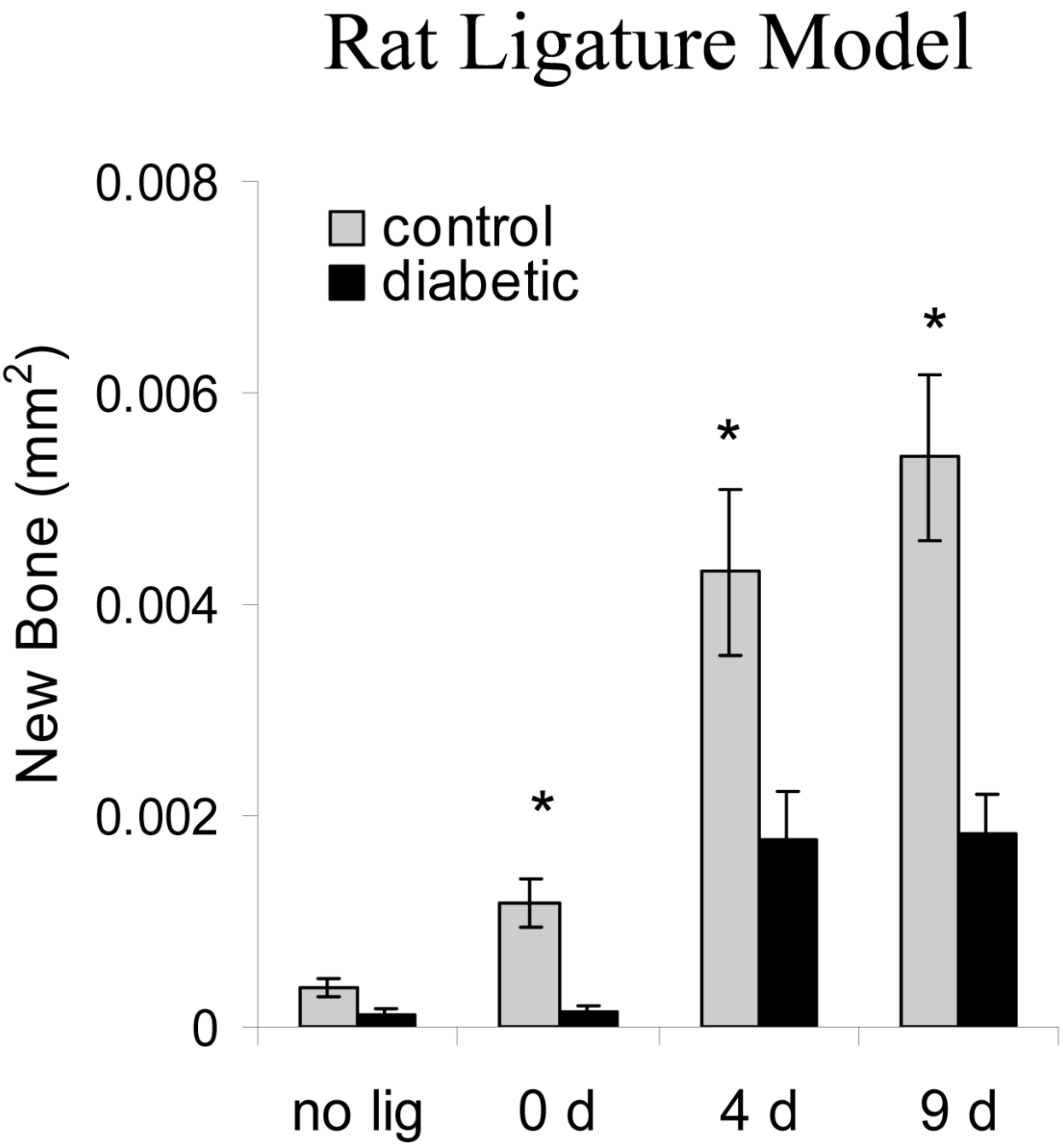

Fig 5.

Diabetes inhibits reparative alveolar bone formation following resorption. To induce periodontal bone loss ligatures were placed around the second molar teeth of type 2 diabetic (ZDF) and normoglycemic matched littermates. After 7 days ligatures were removed which allowed coupling to proceed involving the formation of new bone. The amount of bone formed following resorption was significantly less in the diabetic group suggesting that net bone loss may be greater in the diabetic group due to the failure to form new alveolar bone following an episode or resorption. * indicates significant difference between diabetic and normoglycemic control rats ($P<0.05$). This figure was previously published (Liu, et al., 2006b).

Table 1

Hypotheses Tested in the Calvarial Model

Impact of the host response on hard and soft tissue damage	
IL-1 induces osteoclastogenesis and bone resorption	(Boyce, et al., 1989)
Periodontal pathogens induce osteoclastogenesis and bone resorption partly through induction of prostaglandins	(Zubery, et al., 1998)
P. gingivalis induces an inflammatory infiltrate and osteoclastogenesis indirectly via TNF	(Graves, et al., 2001)
Periodontal pathogens P. gingivalis and A. actinomycetemcomitans induce greater cytokine expression than a commensal bacterium, S. gordonii.	(Cutler & Teng, 2007)
Activation of the acquired immune response significantly increases expression of innate immune cytokines and an inflammatory infiltrate stimulated by P. gingivalis.	(Leone, et al., 2006)
Bacterial virulence factors	
Low dose P. gingivalis LPS induces osteoclast formation through IL-11 and prostaglandins.	(Li, et al., 2002b)
High dose P. gingivalis LPS induces osteoclasts through IL-1 and TNF activity.	(Chiang, et al., 1999)
P. gingivalis fimbriae are pro-inflammatory but do not play an essential role in the innate immune response to P. gingivalis in connective tissue.	(Graves et al, 2005b)
P. gingivalis LPS, fimbriae and intact bacteria exhibit a qualitative different stimulation of the innate immune response.	(Zhou et al, 2005)
LPS indirectly stimulates fibroblast apoptosis through TNF activity	(Alikhani, et al., 2003)
Impact of systemic disease (diabetes)	
Diabetes types 1 & 2 both cause prolonged inflammation in response to P. gingivalis	(Graves, et al., 2005; Naguib, et al., 2004)
Diabetes enhanced apoptosis limits the repair of a bacteria induced wound	(Al-Mashat, et al., 2006)
Diabetes enhanced TNF increases fibroblast & osteoblast cell death initiated by P. gingivalis, which significantly interferes with repair of connective tissue and bone.	(Liu, et al., 2006a)
Diabetes interferes with the coupling of bone resorption and formation following bacteria induced bone loss	(Al-Mashat, et al., 2006)
Potential and Future Uses of This Model	
To investigate the role of specific virulence factors by genetic manipulation of bacterial genes in stimulating or evading a host response in connective tissue in vivo.	
To investigate the role of specific genes in mounting an innate immune response to oral bacteria in connective tissue in vivo using genetically modified mice.	
To investigate the role of specific genes in mounting an acquired immune response to oral bacteria in connective tissue in vivo using genetically modified mice.	
To investigate host mechanisms or systemic factors that modulate repair following bacteria induced injury to connective tissue.	
To investigate mechanisms or systemic factors that affect osseous repair following bacteria induced bone resorption	

Table 2
Experimental periodontitis in humanized NOD/SCID gavage model

T-cells significantly contribute to periodontal bone loss and tissue inflammation	(Teng, et al., 1999) (Teng, et al., 2000) (Teng, 2002b)
The cytokine, RANKL plays an important role in periodontal bone loss and its production is associated with both Th1 & Th2 responses during periodontal pathogenesis	(Teng, et al., 2000) (Teng, 2002b) (Mahamed et al., 2005)
OPG administration significantly abolishes alveolar bone destruction *in vivo*; IFN-γ treatment increases periodontal bone loss while treatment with IL-10 reduces alveolar bone loss	(Teng, Mahamed, & Singh, 2005) (Zhang & Teng, 2006)
The antigen CagE found on *A. actinomycetemcomitans*, which is pro-apoptotic, plays a significant role in periodontal tissue and bone loss	(Teng & Hu, 2003) (Teng, 2003) (Teng & Zhang, 2005)
$CD11c^+$ dendritic cells can participate in modulating inflammation-induced bone loss by acting as osteoclast precursors	(Alnaeeli, Penninger, & Teng, 2006) (Alnaeeli, et al., 2007)
Potential and Future Uses of This Model **i.** To investigate mechanisms by which diabetes exacerbates periodontal bone loss. **ii.** To identify susceptibility genes that lead to enhancing periodontal bone loss. **iii.** To examine the role of leukocyte subsets in promoting periodontal inflammation and alveolar bone loss.	

Table 3

Hypotheses Tested in the Oral Gavage Model

	References
Genetic basis for host susceptibility or resistance to periodontal disease	
Different genetic mouse strains display differential susceptibility to *P. gingivalis*-induced periodontal bone loss.	(Baker, 2005)
Il1b, *Tnf*, and *Stat6* are associated with susceptibility, whereas *Il15* and *Selp* with resistance to *P. gingivalis*-induced periodontal bone loss	(Hart, et al., 2004)
Role of specific cytokines or antimicrobial molecules in periodontal disease	
Increased susceptibility of IL-10 knockout mice to *P. gingivalis*-induced periodontal bone loss	(Sasaki H, et al., 2000)
IL-17-receptor-knockout mice display increased susceptibility to *P. gingivalis*-induced periodontal bone loss	(Yu, et al., 2007)
Inducible nitric oxide synthase (iNOS)- knockout mice exhibit increased periodontal bone loss in response to *P. gingivalis* infection	(Alayan, et al., 2006)
Identification of potential virulence factors	
Wild-type *P. gingivalis*, but not a FimA-deficient mutant, induces periodontal bone loss and accelerates atherosclerosis.	(Gibson, et al., 2004)
Diminished induction of periodontal bone loss by *P. gingivalis* mutant expressing FimA fimbriae lacking the FimCDE accessory components.	(Wang, et al., 2007)
Diminished induction of periodontal bone loss by *P. gingivalis* mutants lacking expression of Kgp and/or RgpB.	(Pathirana, et al., 2007)
Diminished induction of periodontal bone loss by *T. forsythia* mutant lacking BspA adhesin.	(Rai, et al., 2005)
Immune evasion through exploitation of innate receptors	
TLR2 deficiency attenuates *P. gingivalis*-induced periodontal bone loss.	(Burns, et al., 2006)
Blockade of complement receptor-3 inhibits *P. gingivalis*-induced periodontal bone loss.	(Hajishengallis, et al., 2007)
Periodontal disease connection with systemic diseases	
Oral infection with *P. gingivalis* accelerates atherosclerosis.	(Gibson, et al., 2004) (Lalla, et al., 2003)
P. gingivalis-induced periodontal bone loss is enhanced in diabetes.	(Lalla, et al., 1998)
Blockade of RAGE (receptor for advanced glycation end products) suppresses periodontal bone loss in diabetic mice.	(Lalla, et al., 2000)
Proof-of-principle immunization studies to identify candidate vaccine antigens	
Systemic immunization using RgpA and Kgp gingipain peptides protects against *P. gingivalis*-induced periodontal bone loss	(O'Brien-Simpson, et al., 2005)
Systemic immunization using intact RgpA protects against *P. gingivalis*-induced periodontal bone loss.	(Gibson & Genco, 2001)
Oral immunization with FimA-expressing *S. gordonii* vector protects against *P. gingivalis*-induced periodontal bone loss.	(Sharma, et al., 2001)
Potential and Future Uses of This Model	
To determine the role of pattern-recognition receptors in protection or susceptibility to periodontitis using genetically modified mice.	
To determine the impact of aging in periodontal disease susceptibility using young and aged mice.	
To investigate the protective potential of specific antagonists of disease-promoting mechanisms (*e.g.*, destructive inflammation, bacterial evasion of immunity).	
To investigate the role of specific bacterial genes in interbacterial co-adherece and biofilm formation.	

Table 4

Hypotheses Tested *A. actinomycetemcomitans* rat feeding model

Ability of microbe delivered via oral feeding to induce an infection.	(Fine, et al., 2001) (Schreiner, et al., 2003)
Ability of microbe to attach, colonize, and integrate into an established (albeit reduced) oral flora.	(Fine, et al., 2001) (Schreiner, et al., 2003)
Recovery of microbe from animal and analysis of traits unique to its growth in that animal as compared to growth seen in laboratory cultures of same organism.	(Fine, et al., 2001) (Schreiner, et al., 2003)
Microbial attachment factors essential for colonization of animal tooth and tissue Can be analyzed	(Schreiner, et al., 2003) (Sharma, et al., 2001)
Microbial attachment and colonization can be related to antibody response and bone loss.	(Burckhardt & Guggenheim, 1980 (Schreiner, et al., 2003; Sharma, et al., 2001)
Analysis of effect of antagonistic or symbiotic organisms on growth and survival of test microbe	(Hillman, et al., 2000)
Potential and Future Uses of This Model	
To investigate how mutants in known *A.a* virulence traits (i.e. leukotoxin, cytolethal distending toxin) effect colonization, antibody responses and bone loss.	
To identify relationship between specific and non-specific adhesions on attachment and colonization.	
To determine how different strains of *A.a* and other “pathogens” colonize in the rat feeding model.	
To determine how different strains of rats are affected by *A.a* colonization; which strains are more susceptible to colonization; does colonization correlate to antibody response and bone loss.	
To determine the immunopathological response in animals colonized by *A.a* in different rat species	

Table 5

Hypotheses Tested in the Rat Ligature Model

Bacteria play an essential role in initiating gingival inflammation and periodontal bone loss	(Rovin, Costich, & Gordon, 1966)
Chlorhexidine and antibiotics reduce bone resorption induced by oral bacteria	(Kenworthy & Baverel, 1981),(Weiner, DeMarco, & Bissada, 1979)
A Gram negative bacterial burden enhances osteoclastogenesis and bone resorption	(Samejima, Ebisu, & Okada, 1990)
Endotoxin tolerance reduces periodontal bone loss	(Samejima, Ebisu, & Okada, 1990)
Cox-1 and -2 contribute to gingival inflammation, osteoclast formation and bone loss	(Bezerra, et al., 2000) (Bezerra, et al., 2002)
Application of IL-1 or TNF enhance periodontal bone loss	(Gaspersic, et al., 2003; Koide, et al., 1995)
Cigarette smoke or nicotine enhances alveolar bone loss	(Cesar Neto, et al., 2004) (Benatti, et al., 2003; Nociti, et al., 2001)
Diabetes prolongs gingival inflammation, increases osteoclast activity and enhances fibroblast and osteoblast cell death.	(Al-Mashat, et al., 2006)
Diabetes interferes with osseous coupling limiting reparative bone formation following periodontal bone resorption.	(Al-Mashat, et al., 2006)
Potential and Future Uses of This Model	
To investigate systemic conditions that modulate loss of connective tissue attachment or alveolar bone	
To investigate the role of specific factors in the loss of attachment or alveolar bone by the use of well defined molecules including inhibitors.	
To investigate mechanisms that affect coupling of alveolar bone resorption and formation.	
mRNA or protein profiling of factors modulated during the progression of alveolar bone loss.	

Table 6

Hypotheses Tested in Airpouch/ Chamber Models

Eicosanoids control TNFα expression in acute inflammation	(Ferrandiz & Foster, 1991)
Lipoxins and ATL regulate chemokines and cytokines and damped acute inflammation	(Hachicha, et al., 1999)
Lipoxin analogues dampen the neutrophil response to P.g. Neutrophils are a rich source of PGE_2	(Pouliot, et al., 2000)
P.g. induces an acquired immune response	(Genco, et al., 1991)
Serologic diversity of *Neisseria* can provide protective immunity	(Wong, et al., 1979)
P.g. evades phagocyte clearance by failing to activate or degrading complement	(Schenkein, 1989)
Immunization with P.g. protects against disseminating infection	(Dahlen & Slots, 1989) (Genco, et al., 1992)
P.g. activates blood kinin pathways to escape the vasculature creating a disseminating infection.	(Hu, et al., 2006)
Potential and Future Uses of This Model	
Determine the role of pharmacologic agents in acute and chronic inflammation.	
Assess the role of putative virulence factors of single bacteria and mixed infection in acute and chronic inflammation	
Using transgenic animals, determine the role of specific host pathways, enzymes, receptors and ligands in the acute and chronic inflammatory response	