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A novel murine model for chronic inflammatory alveolar bone loss

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Background and Objective: Chronic inflammatory bowel disease (IBD) demonstrates some similarities to the dysregulated chronic immunoinflammatory lesion of periodontitis. Trinitrobenzene sulphonic acid (TNBS) and dextran sodium sulphate (DSS) administered to rodents have been shown to elicit inflammatory responses that undermine the integrity of the gut epithelium in a similar manner to IBD in humans. The objective of this study was to evaluate the ability of these chemicals to elicit periodontal inflammation as a novel model for alveolar bone loss.

Material and Methods: Mice were treated by oral application of TNBS twice a week, or with DSS in the diet over a period of 18 weeks. Alveolar bone loss was assessed on the defleshed skull using morphometric measures for area of bone resorption.

Results: The TNBS-treated animals tolerated oral administration with no clinical symptoms and gained weight at a similar rate to normal control animals. In contrast, DSS exerted a systemic response, including shortening of colonic tissue and liver enzyme changes. Both TNBS and DSS caused a localized action on periodontal tissues, with alveolar bone loss observed in both maxilla and mandibles, with progression in a time-dependent manner. Bone loss was detected as early as week 7, with more severe periodontitis increasing over the 18 weeks ($p < 0.001$). Young (7-month-old) and old (12-month-old) mice with severe combined immunodefiency were treated with TNBS for a period of 7 weeks and did not develop significant bone loss.

Conclusion: These data show that oral administration of TNBS or DSS provokes alveolar bone loss in concert with the autochthonous oral microbiota.

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There is accumulating evidence for an increasing incidence of chronic diseases in the human population (1–3). Additionally, many of these diseases clearly result from chronic inflammation that contributes to eventual loss of function of cells, tissues or organs (4–6). In this regard, the chronic inflammation occurring in inflammatory bowel disease (IBD) has many similarities to the chronic immunoinflammatory response in the oral cavity that destroys the soft and hard tissues of the periodontium (i.e. periodontitis), potentially resulting in exfoliation of the teeth (7).

In mice, trinitrobenzene sulphonic acid (TNBS) given rectally or dextran sodium sulphate (DSS) orally elicit gastrointestinal inflammatory responses, linking with the natural microbiota of the murine gut (8–10). Dextran sodium sulphate acts to undermine the epithelial barrier and as an immune cell activator, resulting in innate immune damage to the tissues. Trinitrobenzene sulphonic acid appears to function as a hapten to modify autologous proteins and induce a T cell-mediated response, resulting in autoimmune-like inflammatory responses (11). These

compounds also up-regulate reactive oxygen species (ROS), creating a reproducible model of inflammatory bowel disease (8,12,13). Chronic inflammatory responses at all sites in the body result in the production of both inflammatory mediators and high levels of these ROS in the local microenvironment of the inflamed tissue. Ample evidence in IBD (12,14) and more limited data in periodontitis (15–17) provide support for a role of ROS in the clinical presentation of these mucosal diseases.

Rodent models of periodontal disease generally have been developed using exogenous oral infections with human pathogens, i.e. bacteria not part of the animals commensal oral microbiota, in an attempt to mimic some of the chronic inflammatory responses and resulting alveolar bone loss observed in humans. Alternatively, some iterations of these models have evaluated changes in the periodontium following gingival injection of antigens (18,19) or microbial stimulants (20–25). While these models have provided a range of data and new knowledge concerning host responses and tissue destruction, a concern remains regarding the nature of the triggering of periodontitis in humans via commensal bacteria that appear to change to opportunistic pathogens in the subgingival sulcus vs. the microbial approach used in the rodent models. Since the gingival tissues also represent a mucosal surface that is subjected to a complex commensal microbial challenge, we attempted to translate the mucosal immunopathological findings of IBD models to the oral cavity.

The implementation of the DSS and TNBS models would enable studies of osteoimmunological interactions in the oral cavity, by targeting the innate immune system with DSS and the T cell-mediated immune responses with TNBS. We tested the hypothesis that challenge of the periodontium with TNBS or DSS, agents that are known to elicit chronic inflammation and IBD in murine models, will stimulate periodontitis in these animals. This is the first study to document the oral mucosal disease triggered by these compounds and should provide seminal data for evaluation of the potential contribution of this model to studies of gingival inflammation and subsequent alveolar bone loss in periodontitis.

Material and methods

Animals

Mice of the BALB/c strain (11–12 weeks old; Harlan Laboratories, Indianapolis, IN, USA) were housed in micro-filter-top cages in an American Association of Accreditation of Laboratory Animal Care (AAALAC) certified Laboratory Animal Research Resource Facility at the University of Kentucky Medical Center. On day 0 of the experiment, mice were weighed and ear-punched for appropriate identification. They were placed in a room maintained at 22°C with a 12 h–12 h light–dark cycle and fed rodent chow and water *ad libitum*.

Severe-combined-immunodeficiency mutation (C.B-17 SCID) mice, which are similar to BALB/c mice except that they carry the Igh-1b allele from the C57BL/Ka strain and lack both T and B cells due to a defect in variable diversity joining gene recombination (do not mount an antibody response to immunogenic material), originated from Taconic Inc. (Hudson, NY, USA), were reared in the knockout rodent facility using autoclaved micro-filter-top shoebox cages and bedding, and provided with sterilized drinking water and irradiated mouse diet (Purina, Harlan Laboratories) *ad libitum*. The animals were either 7 (similar to age of the BALB/c mice) or 12 months old (aged mice) at the time of the TNBS challenge.

This experimental study was approved and performed in accordance with the guidelines for Institutional Animal Care and Use Committee (IACUC). All cages were only opened in a biosafety hood to minimize contamination. The mice were monitored daily for comfort, food and water intake, and for clinical symptoms and survival. Animals were weighed weekly. At each time point, all mice were weighed and some were killed to provide samples.

The TNBS and DSS models

After 1 week of acclimation, the animals were randomly divided into three groups. The BALB/c mice were treated biweekly with dextran sodium sulphate (2% DSS; Biochemical International Chemical Inc., Framingham, MA, USA) in the diet followed by 1 week of abstinence, repeated for a period of 7–18 weeks. Trinitrobenzene sulphonic acid (TNBS, 2.5 mg solution; Sigma-Aldrich, St Louis, MO, USA) was delivered via a micropipette tip (100 μL) orally twice a week. Animals received fresh food and water three times a week. At each time point (0,7,12,14 and 18) five animals were killed. Normal control animals received a normal diet and sham challenge (sucrose) (13,26). The SCID mice were similarly treated with TNBS or sham challenge. These animals tolerated TNBS for a period of 7 weeks before they started to lose weight and develop clinical symptoms.

Tissue collection

At each time point (0,7,12,14,18 weeks), the animals were euthanatized by overdose halothane inhalation, chest and abdominal cavity exposed and tissue dissected. Liver tissue was excised, flash frozen in liquid nitrogen and kept at −80°C for further analysis. Liver cystine concentration was measured, in Dr Theresa Chen's laboratories, using high-performance liquid chromatography as described previously (26). The colonic tissue was removed and flushed out with phosphate-buffered saline (Sigma Aldrich, St Louis, MO, USA). The severity of tissue changes was determined by measurement of tissue wet length and weight (27).

Analysis of alveolar bone

Five mice from each group were euthanatized by over dose halothane inhalation, chest and abdominal cavity exposed and tissue dissected at each time point. The mouse head was removed and gingival tissue excised. The skulls were autoclaved for 5 min, the alveolar bones separated from the skull

and hemisected. Mandible and maxilla were defleshed, treated overnight with 0.3% H_2O_2, cleaned and stained with 0.5% methylene blue for 45 s.

Recent reports indicate that alveolar bone loss in mice can be accurately quantified using microscopic morphometry, histomorphometry or microcomputed tomography with no significant variations in outcomes (28). Therefore, we used a morphometric approach for the analyses. Digital photographs were prepared under stereomicroscopy on a custom-made stage-holder with the jaws angled to enhance visualization of the cemento-enamel junction (CEJ) and bone level, as described previously (29). The images were analysed using NIH ImageJ Program (http://rsbweb.nih.gov/ij/) with enhancements for estimation of the area of alveolar bone loss. Using the CEJ of the teeth and the horizontal bone level, the area of bone loss was measured on buccal and lingual surfaces for each segment. Means for the mandibles, maxilla and total were determined and used for comparisons between treatment and control groups. The area pixel readings for the bone loss were converted to units of millimetres squared for CEJ–alveolar bone measurements by inclusion of a standard calibrator into each digital image.

Statistical analysis

All results are expressed as means ± SEM unless otherwise stated. Data were accordingly evaluated using a Mann–Whitney *U*-test or a one-way analysis of variance on ranks (ANOVA), followed by an appropriate *post hoc* test (the Tukey–Kramer multiple comparisons test) using GraphPad instat version 3 for Windows (GraphPad Software; San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

In this investigation, a low dose of DSS was added into the diet for 1 week intervals. When the clinical features of the gut disease became apparent, the DSS was removed and the diarrhea and bloody stool resolved after 1–2 days. In a different group of animals, TNBS was applied orally twice a week. To evaluate any systemic responses in treated animals, we measured body weight, wet colonic length and the concentration of cystine in the liver. The animals tolerated administration of either compound and demonstrated a generally normal weight gain over the 18 week study period (Table 1). The TNBS-treated animals did not display clinical systemic effects and maintained normal colonic length, with no statistical difference from control animals (89% of control colonic length), while the colonic length in DSS animals was significantly shortened compared with the normal control animals (58%) and TNBS-treated animals ($p < 0.001$), consistent with the occurrence of gastrointestinal symptoms during the experimental protocol (Fig. 1). We also measured liver cystine as a marker of the systemic changes. Animals treated with TNBS did not show any significant differences in the levels of this tissue marker compared wtih the normal control animals (90% of control), while the liver cystine levels were decreased significantly in the animals treated with DSS compared with control animals (60%) or TNBS-treated mice ($p < 0.001$; Fig. 1). However, there was no substantial morbidity or mortality in either of the treatment groups during the 18 week period.

Table 1. Body weights of mice

	Weight (g)		Weight gain (%)
	Week 0	Week 18	
Control	20.3 ± 0.5	26 ± 0.8	30 ± 3
TNBS	19.9 ± 0.3	25.5 ± 0.8	28 ± 5
DSS	20.3 ± 0.4	24.4 ± 0.6	20 ± 2

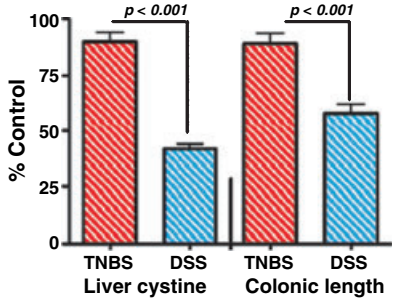

Fig. 1. Percentage amount of liver cystine and colonic length normalized according to sham control values (i.e. cystine, 327 ± 0.6 nmol/mg and colonic length, 117 ± 0.6 mm) after 18 weeks of treatment. Note that TNBS exerted its effect locally, with no significant difference between TNBS and control values.

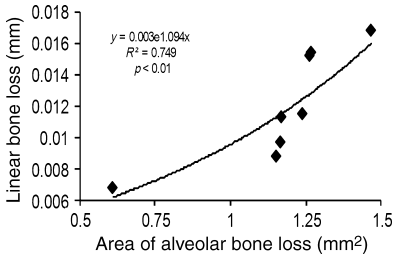

Fig. 2. Linear correlation between total bone loss with two measures/molars (mm) compared with our technique (mm^2) in randomly selected mandibles.

We initially compared evaluation of alveolar bone loss by measuring the total area of maxillary/mandibular bone loss expressed as millimetres squared with a technique that measured linear dimensions of bone loss at six sites (mesial, distal edge) of each molar tooth in the individual quadrants. Our findings indicated a significant correlation between these bone loss measures (Fig. 2).

Significant alveolar bone loss was observed in all four quadrants in the oral cavity of the treated animals after 18 weeks (Fig. 3), with no statistically difference between the extent of bone loss in the mandibles compared with the maxilla. No major differences were noted between bone loss on the right or left side in treatment groups or control animals (data not shown). The TNBS and DSS treatments caused significant alveolar bone loss in a time-dependent manner. Significant bone loss was detected as early as week 7 and progressed to a more severe periodontitis by week 18 (Fig. 4). No significant difference was noted between the level of alveolar bone loss in mice treated with either TNBS or DSS.

To explore the requirement for an intact host immune system in this model of alveolar bone loss, we examined SCID mice, which lack both T and B cell responses. The SCID mice tolerated oral TNBS application for a

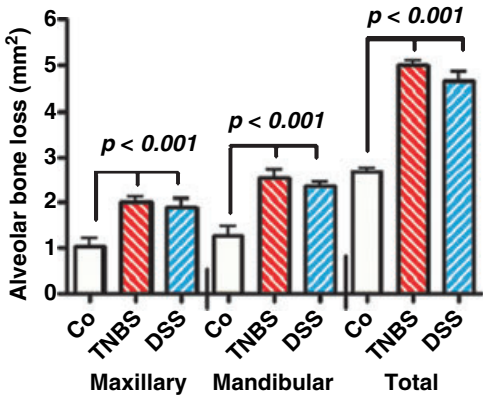

Fig. 3. Significant bone resorption at the levels of maxillary, mandibular and total quadrant after 18 weeks of treatment with TNBS or DSS ($p < 0.001$).

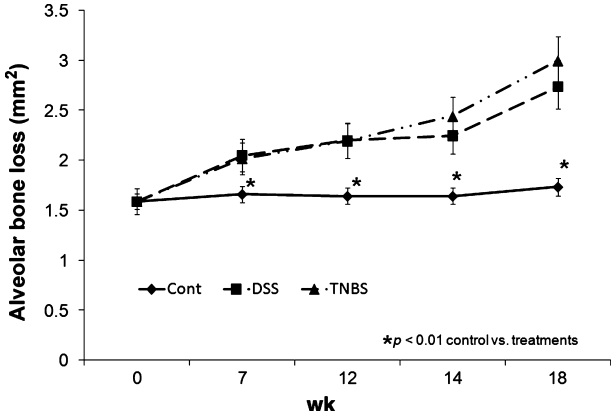

Fig. 4. Extent of the alveolar bone resorption. Significant mandibular bone loss was detected as early as week 7 and progressed to a more severe periodontitis by week 18. The *y*-axis denotes area of bone loss (in mm^2). ($n = 5$/group).

period of 7 weeks before beginning to demonstrate weight loss and becoming moribund. However, they showed no significant bone loss when treated with TNBS at 7 months of age vs. sham control animals [wild-type, 0.62 mm^2 (30%) vs. SCID, 0.14 mm^2 (7%) bone loss], comparable to the age of wild-type BALB/c mice, or at 12 months of age, consistent with aged animals (Table 2).

Table 2. Alveolar bone loss (in mm^2) in SCID mice

	Age 7 months	Age 12 months
Baseline	2.13 ± 0.13	2.14 ± 0.05
Control*	2.14 ± 0.05	2.18 ± 0.11
TNBS*	2.27 ± 0.13	2.34 ± 0.12

*Alveolar bone loss was assessed 7 weeks after initiation of control sham or TNBS treatment.

Discussion

This study demonstrates the induction of alveolar bone loss in BALB/c mice dosed orally with TNBS or DSS over an extended time period of 18 weeks. Periodontitis is a chronic immunoinflammatory disease with progressive loss of attachment of gingival tissues, reflecting destruction of the periodontal ligament and adjacent supporting alveolar bone. The chronic inflammation of periodontitis is initiated by a complex subgingival biofilm comprised of commensal bacteria and microorganisms that represent opportunistic pathogens (21). In response to periodontal pathogens, polymorphonuclear leucocytes and other inflammatory and gingival resident cells can release destructive oxygen free radical (15,16,20), proteinases (30) and other factors that can damage host tissues (17,31,32). These molecules induce damage to gingival tissue and periodontal ligament, and elicit osteoclastic bone resorption (17,33–35).

Our data indicate that mice tolerated the treatments for up to 18 weeks with no mortality or significant clinical side-effects. In modifying the protocol for the use of these inflammatory agents, we were able to examine the effects of challenge with TNBS on alveolar bone loss, presumably resulting from a localized inflammatory response in the periodontium, and challenge with DSS, which appears to exert its effect in a more generalized and systemic manner. These agents historically have been used to cause inflammation of the intestine, as models of chronic idiopathic inflammatory bowel disease (IBD), principally ulcerative colitis and Crohn's disease (8–10,14,26,36). Human IBD is a chronic immunoinflammatory condition mediated by aberrant immune responses to the luminal bacterial antigens by activated $CD4^+$ T cells, not dissimilar from the host–microbial interactions that can occur in the oral cavity, resulting in inflammation and tissue destruction. Murine models of IBD (12, review) have also used chemical induction of acute and chronic inflammation in the gut to evaluate molecular mechanisms of disease. Dextran sodium sulphate, a sulphated polysaccharide compound, is commonly given in water for 1 (acute) to 6 weeks (chronic) to elicit destructive inflammation of the gut, similar to ulcerative colitis (12,26,37,38) Exposure of animals to DSS induces inflammation and macrophage activation commensurate with activation of innate immune mechanisms, with subsequent loss of epithelial integrity, and increases in the gram-negative microbiota of the colon (9,10). Our published data, as well as others, show increased activation of nuclear factor-κB responsive genes (e.g. tumor necrosis factor α) and decreased antioxidant activities in these inflammatory models, suggesting the importance of ROS activation in digestive system inflammation (26,36–39). Previous studies demonstrate that TNBS delivered rectally to BALB/c mice induced similar

clinical features to human Crohn's disease, predominantly a T-helper 1 (Th1) activity in the mucosal $CD4^+$ T cell population and transmural infiltration of inflammatory mononuclear cells (40). These colonic inflammatory responses may result from covalent binding of the haptenizing agent to autologous host proteins with subsequent immune reactivity towards TNBS-modified self-antigens (11).

Numerous animal models have been used to evaluate the clinical, microbiological and immunological aspects of this oral disease in attempts to recapitulate features of human periodontal disease (20–22). Specifically, studies using rodents (mice, rats and hamsters) have elicited disease via placement of ligatures in the gingival sulcus around the molar teeth (41), which increases biofilm accumulation as well as disrupting the gingival epithelium and enhancing osteoclastogenesis and bone loss. In alternative models, these animals are orally infected with select human pathogens in an attempt to document the virulence potential of these species in rodents (20,23). This approach has also enabled the use of genetically manipulated mice to focus on individual components of the host response and describe their role in the disease process (42,43). More recently, researchers in various laboratories have performed gingival tissue injections of microorganisms (18) or their products (44,45) to elicit periodontitis. However, since current models to examine molecular aspects of alveolar bone loss in rodents generally use an oral bacterial infection or challenge with microbial products derived from human bacteria that are not a part of the oral autochthonous microbiota of rats or mice, we suggest that additional models of alveolar bone loss would assist our ability to understand the molecular mediators of tissue destruction in periodontitis. We demonstrated that the bone loss involves both maxillary and mandibular areas. Finally, TNBS treatment, which primarily mimics the induction of an autoimmune type of destructive inflammation, and DSS treatment, which undermines the integrity of the epithelium, as well as activating various inflammatory cells, both elicited significant bone loss.

Previous studies have proven that DSS and TNBS exert their inflammatory effects through up-regulation of ROS (26,36–38,46). The role that these reactive intermediates play in triggering/regulating molecular aspects of the inflammatory and innate immune responses in oral tissues remains to be determined.

T cell functions are also a critical portion of the periodontal milieu at sites of alveolar bone loss caused by the oral microbial biofilms (45,47,48). Mice deficient in major histocompatibility complex (MHC) class II-responsive $CD4^+$ T cells when infected with *Porphyromonas gingivalis* exhibited decreased bone loss, but no change in bone loss was detected in mice deficient in MHC class I-responsive $CD8^+$ T cells or $NK1^+$ T cells (49). Mice lacking the cytokines interferon-γ or interleukin-6, both Th1 cytokines, also demonstrated decreased bone loss (50). Studies in T cell-deficient or adoptively transferred rats have demonstrated the characteristics of T cells in periodontal bone loss in rodents (43,51,52). Finally, after an oral infection with *P. gingivalis*, SCID mice exhibited considerably less bone loss compared with immunocompetent mice, suggesting a crucial role for host responses in the disease process (49). In our model, SCID mice showed no significant difference in bone loss between sham- and TNBS-treated animals, in younger or older animals. These results illustrated that alveolar bone loss in this model may be instigated and/or regulated by T- and B-cell-dependent responses, supporting the suggestion that exaggerated bone loss cannot progress in the absence of host responses in this model.

In conclusion, these data indicate that intermittent oral administration of TNBS or DSS induced alveolar bone resorption in a time-dependent manner. The findings suggest that these chemicals probably caused similar alterations of host responses at oral mucosal surfaces as occur in the gastrointestinal tract, and provide a useful model system for examining molecular aspects of destructive periodontal inflammation leading to bone loss.

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