Accelerated Alveolar Bone Loss in HLA-B27 Transgenic Rats: An Adult Onset Condition

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ABSTRACT. ***Objective.*** Patients with arthritis and Crohn's disease may be more susceptible to periodontitis associated alveolar bone loss (ABL). HLA-B27 transgenic (TG) rats spontaneously develop arthritis and colitis. Based on the hypothesis that TG rats would also be susceptible to ABL, we compared the naturally occurring ABL in TG and Fischer 344 wild-type (WT) rats.

Methods. Eighteen TG and 18 WT virgin female rats were used. Pairs (1 TG, 1 WT) were housed in suspended wire cages. At age 2.6, 6, and 11 months, 8, 5, and 5 pairs were sacrificed, respectively. ABL was measured as exposed molar root surface area (mm^2). Western blotting was used for analysis of serum reactivity against bacteria associated with arthritis, colitis, and periodontitis development.

Results. At 2.6 months of age, there was no difference in ABL between TG and WT rats. At 6 and 11 months ABL was significantly greater in TG animals by 28% and 53%, respectively. For TG rats, ABL was significantly different between the 3 age groups. For WT rats, ABL was not significantly different between 6 and 11 months. Western blotting revealed distinct TG serum reactivity against extracts of *Bacteroides vulgatus*, *B. fragilis*, *Prevotella intermedia*, and to a lesser extent against extracts of *B. forsythus*.

Conclusion. The accelerated ABL in HLA-B27 TG rats is an adult onset condition, independent of husbandry conditions or parity status. HLA-B27 rats exhibit strong immunoreactivity against bacteria implicated in arthritis, colitis, and periodontitis. (J Rheumatol 2002;29:1244–51)

Key Indexing Terms:

ALVEOLAR BONE LOSS	DISEASE MODELS	IMMUNOLOGY
HLA-B27	RATS	ANTIBODIES

HLA-B27 expression in humans has been associated with various inflammatory disorders, such as ankylosing spondylitis[1,2], reactive arthritis[1,3], juvenile spondyloarthropathy[4], and gastrointestinal (GI) inflammation (chronic colitis)[1,2]. HLA-B27 transgenic (TG) rats were developed by Hammer and colleagues[5,6] as an animal model of HLA-B27 associated disease. The HLA-B27 TG rat phenotype closely resembles the features of HLA-B27 associated human disease, as it includes generalized severe inflammatory reactions, spontaneous arthritis, and chronic GI inflammation[5,7].

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Supported by GenPharm International and the Loma Linda University Schools of Dentistry, grant 0313-4005 (DNT), and Medicine, grant 0316-6837 (HMF).

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Submitted June 29, 2001; revision accepted December 21, 2001.

Studies have suggested that humans susceptible to arthritis may be more susceptible to periodontitis or the associated alveolar bone and tooth loss[8-10]. Clinical reports have presented cases of extremely rapid alveolar bone loss (ABL) in patients with Crohn's disease[11,12]. Based on these observations, it was reasoned that HLA-B27 TG rats, a host susceptible to both arthritis and chronic GI inflammation, might be highly susceptible to periodontal ABL. This hypothesis was preliminarily tested in a previous study, where it was determined that conventionally housed, specific pathogen-free, female HLA-B27 retired breeders are prone to accelerated ABL, compared to age matched male or female wild-type (WT) retired breeders[13].

However, use of retired breeders maintained under conventional housing introduces several confounding variables for a study on ABL, such as the influence of husbandry conditions and hormonal changes, and potential differences in microbial flora. Conventional housing, i.e., use of wood chip bedding, can result in bedding impaction, a condition implicated in the ABL seen in rodent experiments[14,15]. The breeding (multiparous) status of the animals could result in changes in alveolar bone metabolism[16]. Further, differences in bacterial flora may affect ABL in rats[17], and account for differences in the manifestation of HLA-B27 associated diseases in this model[18]. We sought to resolve these confounding issues by comparing the naturally occurring ABL in virgin female HLA-B27 TG and WT rats housed in

suspended wire cages, under conditions that eliminate differences in bacterial flora. We also aimed to characterize possible differences between HLA-B27 TG and WT rats in serum reactivity against pertinent bacterial species.

MATERIALS AND METHODS

Animals and experiment design. HLA-B27 transgenic and wild-type Fischer 344 female rats, 5–7 weeks old, were obtained from GenPharm International (Mountain View, CA, USA). Upon arrival, pairs of animals (one TG and one WT matched for age) were established and housed in suspended wire-mesh cages with no bedding. Pairs were established with the purpose of eliminating differences in flora between TG and WT rats by ensuring constant interchange of flora. Housing conditions were chosen to eliminate chewing and impaction of bedding and to minimize coprophagy. Animals were given standard laboratory rat chow and water ad libitum, and kept on a 12 h light cycle. Animals were weighed weekly and monitored for manifestations of the HLA-B27 transgenic phenotype; specifically, records were kept for time of onset of diarrhea, alopecia (hair loss), and arthritis. After 6 months of age animals were weighed monthly. A total of 36 animals (18 TG and 18 WT) were used; 8, 5, and 5 randomly chosen pairs of animals were sacrificed at the age of 2.6, 6, and 11 months, respectively. Animals were sacrificed by carbon dioxide inhalation and immediately decapitated. The study protocol was approved by the Loma Linda University Institutional Animal Care and Use Committee.

Bacteria and bacterial extracts. Bacteria used in this study were: *Porphyromonas gingivalis* W83, *Bacteroides fragilis* ATCC 25285, *B. forsythus* ATCC 43037, *B. vulgatus* ATCC 8482, and *Prevotella intermedia* ATCC 25261. All bacteria except *B. forsythus* were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) supplemented with hemin (5 μg/ml), vitamin K (0.5 μg/ml), and DL-cysteine (1%). *B. forsythus* was cultured in Trypticase soy broth containing hemin (5 μg/ml), vitamin K (0.5 μg/ml), and 0.001% N-acetylmuramic acid (Sigma Chemical Co., St. Louis, MO, USA). All cultures were incubated at 37°C in an anaerobic chamber in 10% H_2, 10% CO_2, 80% N_2. Recombinant *P. gingivalis* GroEL (HSP-60) was partially purified as described[19], and was a kind gift of Dr. H. Maeda.

Bacterial extracts were prepared twice, in identical manner, from different stocks. Five months elapsed between the 2 preparations. For extract preparation, bacteria were grown overnight in broth. Cells were recovered by centrifugation (8000 × g, 10 min, 4°C) then washed once with 0.25 M Tris buffer (pH 8.0) containing 10 mM Na-α-tosyl L-lysine chloromethyl ketone (TLCK) hydrochloride (Sigma) and resuspended in the same buffer. Cells were broken using the Mini-Beadbeater-8 Cell Disrupter (Biosepc Products, Bartelsville, OK, USA). The suspension was centrifuged at 20,000 × g, 4°C, for 10 min, and the supernatant fluid (crude extract) was used in Western blot analysis. Protein concentration was determined using the Bradford method.

Alveolar bone loss measurements. The method has been described in detail[13]. Animal heads were defleshed by mechanical and biological (*Dermestes maculatus* beetles) means, and then bleached. ABL measurements were performed on bleached and stained (1% methylene blue) skulls. Measurements of exposed molar root surface area (mm^2) on the buccal and palatal aspect of the maxillary right and the lingual aspect of the mandibular right were performed using a computer assisted image analysis system. The 2 maxillary measurements (buccal and palatal) were averaged to calculate mean maxillary bone loss, while animal ABL was calculated as the sum of the mean maxillary and the mandibular bone loss. All ABL measurements were performed after sacrifice of the last (11-month-old) group of rats, and the operator performing the measurements was blinded regarding age and type of animal. Coefficients of variation for ABL measurements using this method are under 5%[13].

To determine the possible influence of potential differences in mesiodistal dimension of the dentition on ABL measurements, the coronal mesiodistal distance (mm) was measured in 12 randomly chosen animals (6 TG and 6 WT, 3 each 6 months old and 3 each 11 months old), as described[13].

ABL rate (mm^2/month) was defined as

$$(ABL_2 - ABL_1)/(t_2 - t_1)$$

where ABL_2 and ABL_1 represent ABL values for age t_2 and t_1 (in months), respectively.

Blood collection. Whole blood was obtained by cardiac puncture at sacrifice. The blood was allowed to clot at 4°C overnight, and the serum was collected after centrifugation. Serum samples were aliquoted and stored at –70°C until further testing. Serum from all 10 animals (5 TG/WT pairs) sacrificed at 6 months was available for analysis by Western blotting.

Western blotting. Electrophoresis of bacterial cell extracts, including molecular weight standards (SeeBlue® prestained standard, Invitrogen™, Carlsbad, CA, USA), was performed using NuPage® Novex 4–12% Bis-Tris gels (Invitrogen™) with MOPS buffer. Equivalent amounts of protein were loaded in each lane and the proteins were separated under reducing conditions at 200 V in a minigel system (Xcell Sure Lock™, Invitrogen™). Proteins were then transferred to nitrocellulose membrane at 15 V for 20 min using a semi-dry trans-blot apparatus (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes (BioTrace®NT, Gelman Sciences) were used immediately for immunoblotting or stored at –20°C until required.

For immunoblotting, nitrocellulose membranes were blocked in TBST buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20) containing 5% nonfat dry milk. Membranes were then probed for 60 min with an appropriate dilution of primary antibody (TG or WT rat serum; 1:2000–1:4000) in TBST containing 5% nonfat dry milk. After washing with TBST, membranes were incubated with 1:5000 peroxidase coupled goat anti-rat IgG secondary antibody (Zymed Laboratories, San Francisco, CA, USA) for 30 min in TBST containing 5% nonfat dry milk. Membranes were washed for 45 min with frequent changes of TBST. Enhanced chemiluminescence detection of the proteins (Renaissance®, NEN Life Science Products, Boston, MA, USA) was performed with Biomax™ ML (Eastman Kodak, Rochester, NY, USA). Negative controls included omission of primary or secondary antibody.

Data management and statistical analysis. Analysis of monthly weight data was performed by repeated measures ANOVA. Significance level for differences between time points (age in months) was set at $p < 0.0011$, to account for multiple comparisons (Bonferroni modification). ABL data and mesiodistal dimension of dentition data were analyzed by nonparametric tests (Mann-Whitney U and Kruskal-Wallis). Significance level for rejection of the null hypothesis was set at $\alpha = 0.05$.

RESULTS

Growth and HLA-B27 related systemic manifestations. Both TG and WT animals thrived under the experimental conditions, evident by the growth (weight) curves (Figure 1). One of the 11-month-old TG animals died of unknown causes 2 weeks prior to the scheduled sacrifice. Repeated measures ANOVA indicated there was a significant effect ($p < 0.0001$) for age (2–11 months) and type (TG and WT), with significant interaction between the 2 variables. Animal weight reached a plateau (no weight significantly different from any subsequent weight) at 8 (TG) or 9 (WT) months of age. TG rats weighed significantly more than WT rats at 7, 8, 9, and 10 months (Figure 1).

TG animals developed diarrhea, alopecia, and arthritis, in that order. Such conditions were not observed in WT animals at any time. At 2.6 months, 83% (15/18) of the TG

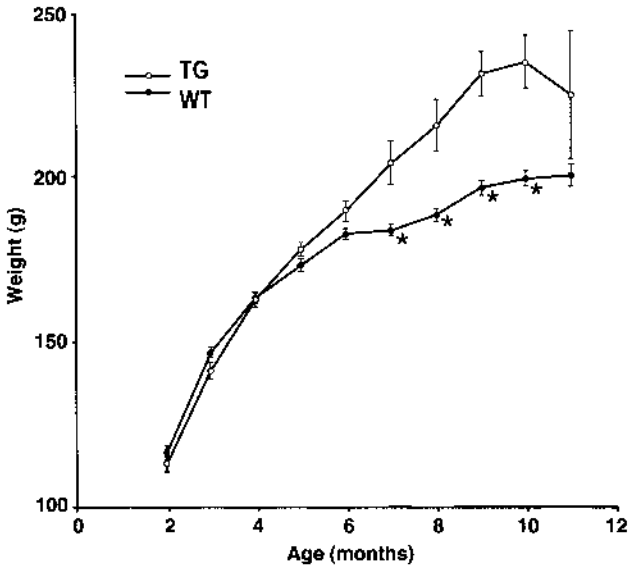

Figure 1. Growth curves for HLA-B27 transgenic (TG) and wild-type (WT) Fischer 344 rats (mean ± SE). Mean values from n = 18 (2 mo), n = 10 (3–6 mo), and n = 5 (7–11 mo) animals per group. *Significant difference between TG and WT at the indicated time points.

animals had developed diarrhea, while none had developed alopecia or arthritis. By the age of 6 months, 90% (9/10), 40% (4/10), and 10% (1/10) of TG rats had developed diarrhea, alopecia, and arthritis, respectively. At 11 months, 100% (5/5) of the TG animals had developed all 3 conditions.

Alveolar bone loss. Descriptive statistics for ABL results are provided in Table 1. At 2.6 months of age there was no difference in ABL between TG and WT rats ($p = 0.67$, Mann-Whitney). Within the 6 and 11 month age groups, ABL was significantly greater in TG compared to WT animals ($p < 0.02$ and $p < 0.01$, respectively). Results remained significant when analyzed separately for each jaw (maxilla or mandible; data not shown). The greater ABL in 6 and 11-month-old TG rats compared to age matched WT rats was readily evident by gross examination (Figure 2).

ABL was significantly different between the 3 age groups for both WT ($p < 0.002$, Kruskal-Wallis) and TG ($p < 0.001$, Kruskal-Wallis) animals. Figure 3 shows ABL data (mean ± SE) to illustrate the temporal aspect. The rate of ABL between months 2.6 and 6 was 1.18 mm^2/month for TG and 0.64 mm^2/month for WT rats. Between months 6 and 11 the corresponding ABL rates were 0.61 and 0.16 mm^2/month.

Table 1. Alveolar bone loss. Data are mean ± SD, in mm^2 (median; range).

Age, mo	Female Transgenic (TG) Rats	Female Wild-type (WT) Rats
2.6	5.09 ± 0.59 (n = 8) (4.84; 4.62–6.05)	4.93 ± 0.67 (n = 8) (4.85; 4.13–6.07)
6	9.09 ± 1.46* (n = 5) (8.77; 7.85–11.61)	7.11 ± 0.89 (n = 5) (7.18; 5.69–7.93)
11	12.12 ± 2.30** (n = 5) (11.29; 9.3–14.69)	7.91 ± 0.43 (n = 5) (8.08; 7.16–8.24)

* $p < 0.02$ from age matched WT animals (Mann-Whitney U test); ** $p < 0.01$ from age matched WT animals (Mann-Whitney U test).

To exclude the remote possibility that differences in ABL (surface area) were due to significant differences in the dimension of the dentition, the mesiodistal dimension was measured. The mean ± SD value for 6-month-old (n = 3 per group) TG and WT was 6.33 ± 0.30 mm and 6.12 ± 0.25 mm, respectively ($p = 0.38$, Mann-Whitney), while for 11-month-old (n = 3 per group) TG and WT it was 6.25 ± 0.23 mm and 6.17 ± 0.25 mm, respectively ($p = 0.38$, Mann-Whitney). For each of the animal types (TG or WT) there was no difference between 6 and 11-month-old animals ($p \geq 0.75$, Mann-Whitney).

Western blotting. Western blotting revealed that both TG and WT sera exhibited reactivity against a wide range of *B. forsythus* proteins, although the pattern of recognized proteins differed between the 2 types of animals (Figure 4). TG sera, in contrast to WT sera, reacted strongly against *B. fragilis*, *B. vulgatus*, and *P. intermedia* proteins, most prominenty in the 45–48 kDa range (Figure 4). The reactivity pattern against *B. forsythus* also included proteins in the 45–48 kDa range (Figure 4). WT sera, and to a much lesser extent TG sera, exhibited weak reactivity against *P. gingivalis* GroEL (HSP-60). These results were consistent among the 5 independently housed pairs: all 5 of the 5 TG animals aged 6 months exhibited the TG reactivity pattern seen in Figure 4. Also, 5 of the 5 WT cagemates exhibited the WT reactivity pattern seen in Figure 4. In addition, 2 of the 5 WT animals exhibited strong reactivity to a high molecular weight (> 150 kDa) *B. vulgatus* protein (data not shown). The Western blotting results were also consistent between the 2 sets of bacterial extracts (data not shown), while no reactivity was observed in any negative control (data not shown).

DISCUSSION

We examined the naturally occurring alveolar bone loss in virgin female HLA-B27 transgenic (TG) and wild-type (WT) rats housed in suspended wire cages. Pairs of TG and WT rats (cagemates) were established upon arrival of the animals and were maintained for the duration of the experiment in order to eliminate differences in flora between TG and WT animals[20,21]. In view of the experimental design, it is reasonable to postulate that any differences between TG and WT rats can be attributed to the genetic differences (expression of HLA-B27) between these animals rather than any differences in flora. The Fischer 344 rat, which constitutes the genetic background of this transgenic line and is the indicated wild-type control, is considered to be resistant to ligature induced periodontitis[22], i.e., resistant to the effects of the endogenous flora. Fischer 344 rats are also

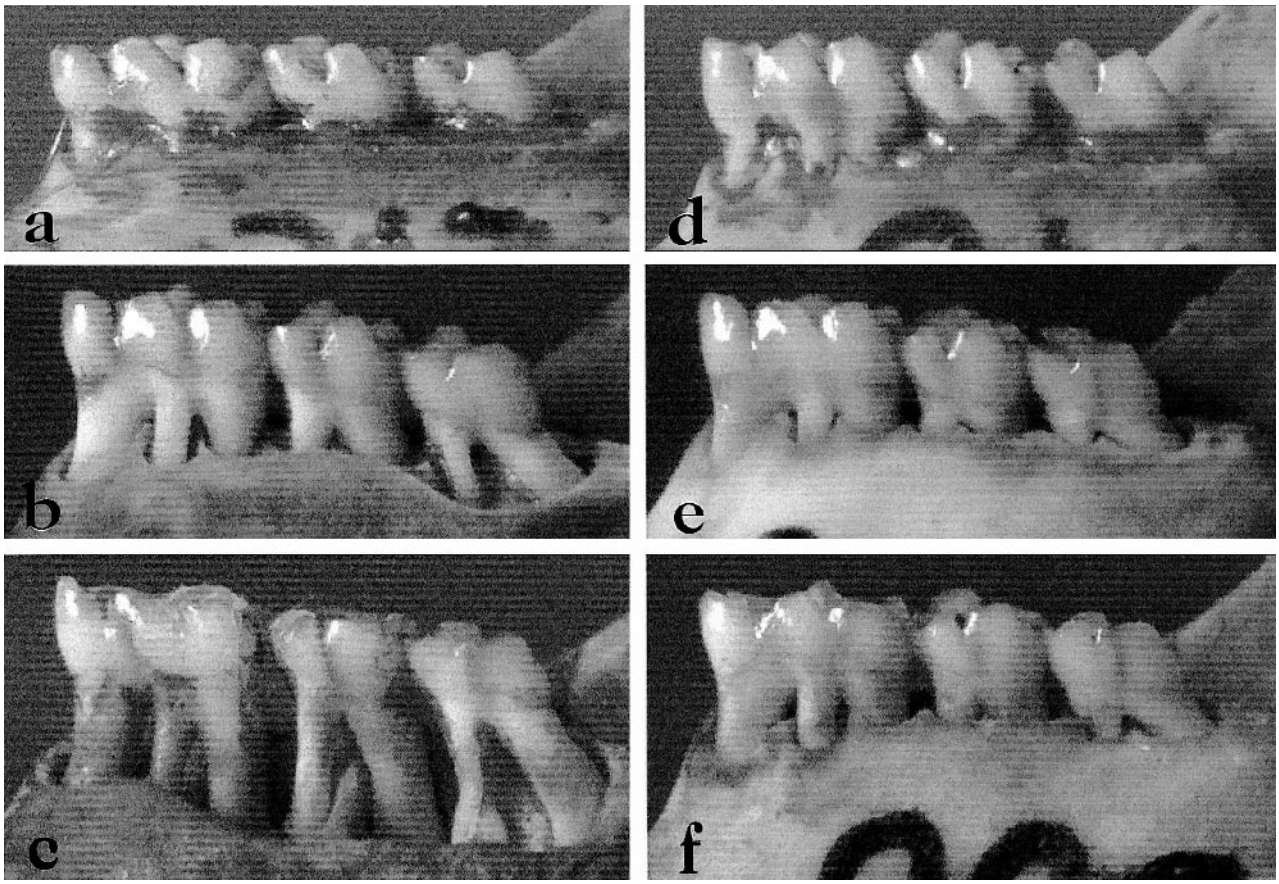

Figure 2. Alveolar bone loss (mandibular lingual aspect) in HLA-B27 transgenic (TG) and wild-type (WT) rats. Representative TG (panels a–c) and WT (panels d–f) animals aged 2.6 (a, d), 6 (b, e), and 11 (c, f) months. Animal pairs represented here (cagemates) were chosen to mirror the mean alveolar bone loss value of their respective group. Photographs were taken for illustration purposes only and were not used for measurements.

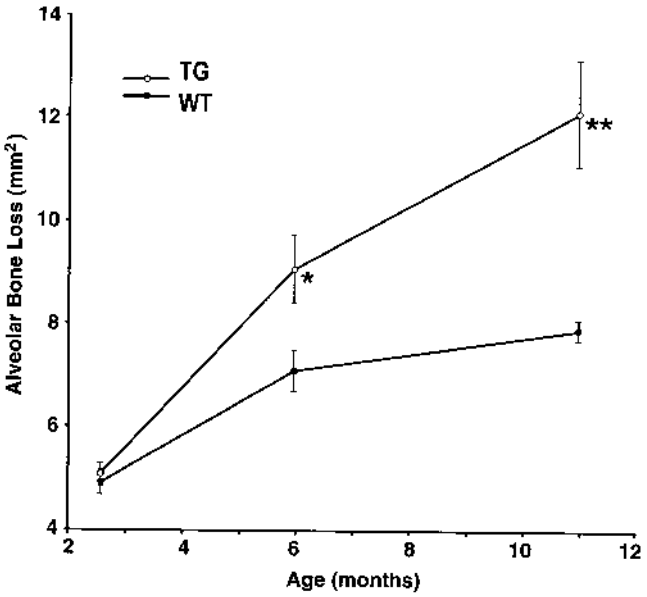

Figure 3. Alveolar bone loss (mean ± SE) in virgin female HLA-B27 transgenic (TG) and wild-type (WT) rats maintained in suspended wire cages. Mean values from n = 8 (2.6 mo), n = 5 (6 mo), and n = 5 (11 mo) animals per group. *p < 0.02, **p < 0.01 significant difference between TG and WT at the indicated time points.

relatively resistant to the induction of arthritis and other inflammatory diseases[23,24]. Therefore, expression of the human HLA-B27 gene converts a resistant animal to a highly susceptible one.

In this context, it should be noted that there are no reports linking HLA-B27 expression with periodontitis in humans. Case reports have linked inordinately rapidly progressive ABL with Crohn's disease[11,12], and a single epidemiological study has reported association between inflammatory bowel disease (IBD; ulcerative colitis and Crohn's disease, both conditions associated with HLA-B27 expression) and higher prevalence (but not severity) of periodontal disease in humans[25]. Flemmig, *et al* acknowledged that their convenience cross-sectional sample of patients with IBD undergoing therapy (no information regarding treatment regimens was provided) may have biased the results[25]. The lack of reported association between periodontitis and HLA-B27 notwithstanding, evidence from other rodent models of genetically determined susceptibility to chronic colitis (e.g., accelerated ABL in IL-10 knockout mice[26]) suggests that the relevance of the HLA-B27 rat model to the human condition may reside in the described association of aggressive periodontal bone loss with Crohn's disease cases[11,12] and/or the

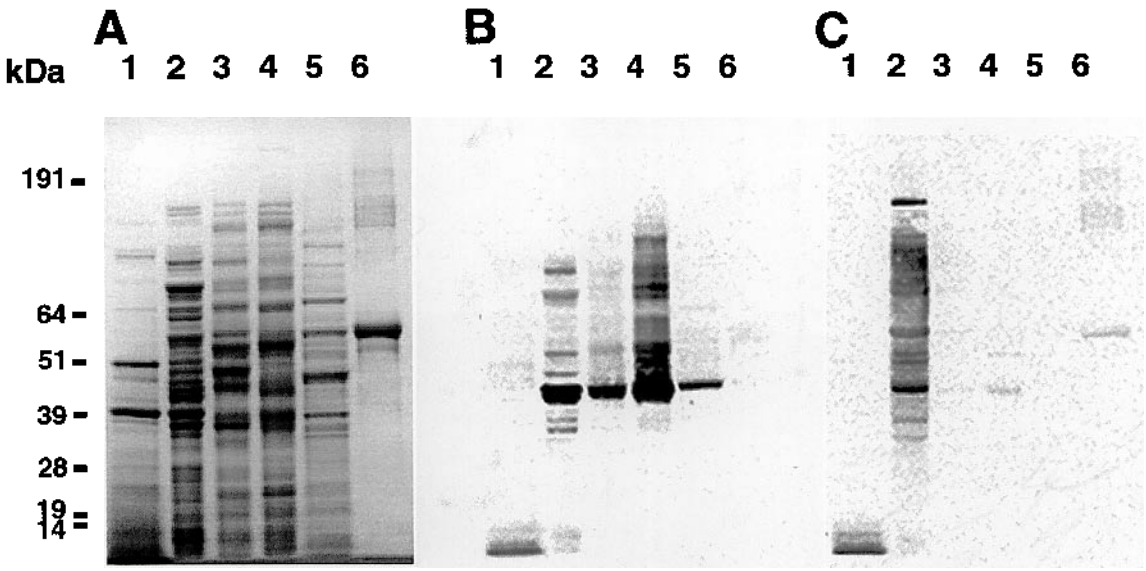

Figure 4. Western immunoblot analysis using serum from a representative virgin female HLA-B27 transgenic rat aged 6 months (panel B) and its age and sex matched wild-type cagemate (panel C). A: Simply Blue™ stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis results of bacterial crude extracts. Relative position of molecular weight standards is marked on the left margin. Lanes were loaded with 20 μg of protein each and correspond to extracts of *P. gingivalis* (lane 1), *B. forsythus* (lane 2), *B. fragilis* (lane 3), *B. vulgatus* (lane 4), *P. intermedia* (lane 5), and partially purified *P. gingivalis* GroEL (HSP-60) (lane 6). Panels B (TG rat serum) and C (WT rat serum): immunoblots of the bacterial extracts under identical conditions; primary antibody (rat sera) at 1:4000 dilution, secondary antibody at 1:5000 dilution; lanes as per panel A.

reported association between arthritis and periodontal bone loss[10].

Both TG and WT animals thrived under the chosen experimental conditions. From 7 months onward, TG rats gained weight at a greater rate, perhaps as a result of reduced activity due to arthritis. As food consumption was not monitored, increased caloric intake cannot be excluded as a reason for the increased weight gain. The incidence of clinically evident manifestations of HLA-B27 associated diseases observed in this study is consistent with the incidence of diarrhea[6], arthritis[6], and alopecia[27] reported in large numbers of conventionally housed females of this particular transgenic line.

TG and age matched WT virgin female rats maintained under identical conditions exhibit significantly different ABL. At 2.6 months (79 days) of age, a time corresponding to young adulthood, there was no difference in ABL between TG and WT rats. However, by 6 months of age TG animals had experienced significantly elevated ABL compared to WT animals. These results indicate that the accelerated ABL manifested in TG rats is an adult onset, age dependent condition, which does not precede the appearance of GI complications. This suggests that development of elevated ABL may be related to the development of the GI condition. Our working hypothesis is that the severe GI inflammation (diarrhea) results, possibly through elevation of circulating catabolic cytokine levels[28], in systemic osteopenia that can contribute to elevated ABL. Such a sequence of events in the HLA-B27 transgenic rat model would be consistent with the reported development of osteopenia/osteoporosis in humans with IBD[29,30], rheumatoid arthritis[31], or ankylosing spondylitis[32] and the reported association between osteopenia/osteoporosis and ABL[33-35]. The implication of the same proinflammatory cytokines, such as interleukin 1 and tumor necrosis factor, in all these conditions (IBD[36,37], arthritis[38,39], ankylosing spondylitis[40,41], osteoporosis[38,42], periodontitis and associated ABL[43,44]) provides a potential unifying pathogenetic mechanism for the reported associations among these inflammatory conditions.

These results (virgin females, suspended wire cages) are in accord with the results of a previous study (retired breeders, conventional housing) from this laboratory[13]. TG rats, at least at 6 months of age and older, exhibit higher ABL. The level of difference between TG and WT animals (percentage increase in ABL), at both 6 and 11/12 months, is also consistent between the 2 studies. Collectively, these results indicate that the susceptibility of TG rats to accelerated ABL is independent of husbandry conditions and parity status. Studies on the natural history of ABL in male TG rats would be of interest, as they could provide further evidence supporting the lack of hormonal influence on ABL.

ABL rates obtained in this study differ from those reported previously[13]. When the published retired breeder data[13] were analyzed again to calculate ABL rate between 6 and 12 months, the ABL rate was 1.34 mm^2/month for TG and 0.63 mm^2/month for WT female rats. Thus an almost 4-fold relative increase in ABL rate for TG versus WT rats was

observed in the present study, compared to a 2-fold relative increase in the previous study. The ABL rate differences between the present and the previous study[13] suggest that husbandry and parity may have a much greater relative influence on WT rats. Collectively, these findings suggest that both TG and WT female rats older than 6 months experience increased levels of ABL when maintained under conditions conducive to periodontal bone loss (bedding impaction). These findings do not exclude the possibility that pregnancy and multiparity also contribute to greater ABL[16,33].

HLA-B27 transgenic rats, an inflammation-prone host susceptible to both arthritis and chronic colitis, were found to be highly susceptible to periodontal ABL. This is consistent with the reported susceptibility of arthritis patients to periodontitis and/or the associated alveolar bone and tooth loss[8-10], as well as the reported rapid ABL in cases of Crohn's disease[11,12]. Taken together, these findings support the concept that a host response characterized by a strong inflammatory component is associated with elevated periodontal tissue destruction[45,46].

Of the HLA-B27 associated manifestations in this animal model, some depend on the presence of commensal gut flora and some do not[18,47]. For example, arthritis and colitis do not develop in germ-free TG animals, while skin and male genital lesions do occur[47]. It remains to be proven whether the severe ABL seen in TG rats is dependent on the presence of commensal oral flora. In this context, it should be noted that the oral environment of these animals was never manipulated, in contrast to what is required for the induction of ABL in other rodent models[14,15].

We also aimed to characterize possible differences between TG and WT rats in serum reactivity against pertinent bacterial species. Serum from TG rats exhibited distinct reactivity against *B. fragilis*, *B. vulgatus*, *P. intermedia*, and to a lesser extent against *B. forsythus*. TG sera reacted strongly against proteins in the 45–48 kDa range. *Bacteroides* species in general, and *B. vulgatus* in particular, have been implicated in the development of arthritis and colitis in this animal model[18,48]. The similarity in reactivity against the various bacteria tested suggests the possibility of an antibody response against shared antigen(s). The identity of such antigen(s) is not known at this point. Evidence suggests that patients with arthritis[49-51], IBD[52-54], and periodontitis[19,55,56] exhibit elevated immune responses to heat shock proteins of bacterial origin; in certain instances, the bacteria in question have been putative periodontal pathogens[19,51,55,56]. Although the present results exclude the *P. gingivalis* GroEL (HSP-60) as a candidate, further exploration of the identity of the antigens may still prove that other heat shock proteins are involved in this host response.

The HLA-B27 transgenic rat is used as an animal model to study the pathogenesis and treatment of several conditions, e.g., arthritis, colitis, psoriasis, and alopecia. Our results indicate that the severe alveolar bone loss spontaneously occurring in HLA-B27 transgenic rats is an adult onset, age dependent, husbandry and parity independent condition. Because of these attributes, HLA-B27 transgenic rats could prove to be a very informative model for the study of alveolar bone loss pathogenesis, including the potential interrelationship(s) with systemic conditions such as arthritis and inflammatory bowel disease.

ACKNOWLEDGMENT

We thank the following for their generous help. Dr. Francis Roy, Loma Linda University (LLU), for expert technical assistance with Western blotting. Dr. Hiroshi Maeda, Okayama University, Okayama, Japan, for *P. gingivalis* GroEL (HSP-60). Dr. Casey Chen, University of Southern California, Los Angeles, for *P. intermedia*. Dr. Kenneth Godowski, Atrix Laboratories Inc., Fort Collins, CO, USA, for *B. forsythus*. Dr. Paul McMillan, LLU, for making available his histometric equipment. Dr. Grenith J. Zimmerman, LLU, for expert statistical advice. Richard Tinker and Richard Cross, LLU, for graphic and photographic support, respectively.

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