Effects of Age and Oral Disease on Systemic Inflammatory and Immune Parameters in Nonhuman Primates

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Periodontal disease is the predominant chronic inflammatory disease of humanity (37, 38, 78, 82) and has been noted to occur naturally with increasing age in humans and nonhuman primates (36, 63, 69, 88). This oral disease is an outcome of complex oral infections, chronic immunoinflammatory responses, and resulting destruction of soft and hard tissues of the periodontium (37, 78, 80, 82, 84). In both humans and nonhuman primates, the extent of disease is predicted to be controlled by the quality and quantity of the host response and likely is modulated by systemic disease (48), environmental stressors (6, 76, 85), and the genetic backgrounds of the individuals (3, 70, 84).

The oral microbial characteristics of subgingival biofilms in younger and older individuals demonstrate differences in composition and complexity, which have been suggested to contribute directly to the microbial infections that trigger the destructive disease of oral tissues that occurs during aging (4, 35, 49, 53, 67, 83). It is clear that levels of gram-negative periodontal pathogens increase with age, although studies of young humans and nonhuman primates demonstrate that many microorganisms associated with periodontal pathogenesis are acquired early in life and become integrated into the commensal autochthonous oral microbial ecology (9, 29, 30, 56). However, it remains unclear how the age of the host impacts recognition of and response to these oral microorganisms.

Increasing evidence also suggests that these microorganisms can translocate from the oral cavity into the systemic circulation, enabling routine stimulation of the reticuloendothelial and immune systems, albeit generally in the absence of clinical symptoms of bacteremia (17, 19, 58, 65, 74, 77). Recent studies have provided clear data that the oral cavity can function as a nidus for a variety of potential medical problems (33, 42, 75). Bacterial infections frequently provide a strong stimulus for a systemic acute-phase response manifested by the increased production of some 25 plasma proteins (18, 22). Increased levels of acute-phase proteins have been identified in adult periodontitis patients and appear to reflect both the infection and the acute and chronic inflammation that exists in the periodontium (18, 39, 55). At the same time, it is clear that a serum antibody response to these localized infections exists and that it results from specific elicitation of antibody to an infecting microorganism (19, 24, 40, 41, 46, 79).

Periodontal disease has been effectively used as a model of host-bacterium interactions, inflammation, and chronic inflammatory diseases, particularly for the ability to longitudinally describe bacterial and host factors in the oral cavity and to correlate changes in these factors with pathological changes in...
the juxtaposed host tissues. The nonhuman primate model has provided a model with which to critically define the interaction of the subgingival microbiota with the host inflammatory/immune response in the maintenance of gingival homeostasis or the exacerbation of a chronic inflammatory process, leading to progression of the disease (20, 22, 59, 62, 68). This study described the characteristics of systemic inflammatory mediators and serum antibody responses to oral bacteria in nonhuman primates as functions of age and in relation to clinical measures of periodontitis. The accessibility of oral tissues and the development of chronic inflammation in the oral cavity in response to microbial biofilms will provide tools for examining the ontogeny of inflammatory/immune processes as related to disease expression in this animal model.

**MATERIALS AND METHODS**

**Animals and diet.** Rhesus monkeys (*Macaca mulatta*) (*n* = 208), housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies; 112 of these animals were females, and 66 were males. The age of the animals ranged from 0.75 to >25 years, and they have been housed in a large community representing 3 or 4 generations with many individual family units based on a matriarchal family lineage. An additional group of 30 animals (age range, 0.8 to 2.8 years) raised under specific-pathogen-free (SPF) conditions was also evaluated and included 24 female and 6 male monkeys. The CPRC’s SPF Program is a source of rhesus monkeys free of B virus (herpesvirus simiae or cercopithecine herpesvirus type 1), simian type D retrovirus, simian immunodeficiency virus, and simian T-lymphotropic virus 1. The monkeys are fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified; Harlan Teklad). The diet is supplemented with fruits and vegetables, and water is provided ad libitum in an enclosed corral setting. This protocol was approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico.
Oral clinical parameters. All animals were examined, while anesthetized, by the same periodontal investigator in this study. The periodontal examination was conducted using a Maryland probe (William’s markings) on the facial, mesiobuccal, and distobuccal aspects of all teeth, excluding the canines and third molars. The examination included probing pocket depth (PD), clinical attachment level (CAL), plaque index, and gingival bleeding index (bleeding on probing [BOP]) (13). The plaque index characterizes the extent and quantity of tooth-associated bacterial plaque. PD and CAL measures were made using a calibrated probe and are measures of the extent and severity of periodontal pathology. The presence and degree of bleeding upon gentle periodontal probing provided a measure of the presence of inflammation within the periodontal tissues.

Serum analyses. Blood was collected from all animals, serum was prepared, and levels of immunoglobulin G (IgG) antibodies to seven oral bacteria were evaluated using an enzyme-linked immunosorbent assay (ELISA) as we have described previously (14, 15). Briefly, Campylobacter rectus, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Treponema denticola, Tannerella forsythia, and Porphyromonas gingivalis were grown under anaerobic conditions in mycoplasma broth base with the addition of appropriate additives as we have reported previously (23). The bacteria were harvested by centrifugation, formalin killed, washed, and stored at −20°C for use as antigens (14, 15).

Selected systemic inflammatory biomarkers were quantified using ELISA procedures developed in our laboratory (C-reactive protein [CRP] [21]). Luminex Beadlyte technology was used for interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), and RANTES (Upstate, Temecula, CA) and for matrix metalloproteinase 1 (MMP-1), MMP-2, and MMP-9 (R&D Systems, Minneapolis, MN). Commercial ELISA kits were used for prostaglandin E2 (PGE2) (Assay Design, Ann Arbor, MI), lipopolysaccharide binding protein (LBP; Cell Sciences, Canton, MA), and bactericidal permeability-inducing factor (BPI; Cell Sciences, Canton, MA) in serum samples from all animals.

Statistical analyses. An analysis of variance (ANOVA) with post hoc testing was used for the various continuous variables, including clinical parameters and
Systemic inflammatory mediators. The levels of various systemic inflammatory mediators were determined in serum samples from each animal and segregated based on the age of the animal: young (<3 years), adolescent (3 to 8 years), adult (8 to 15 years), or aged (>15 years). Figure 1 summarizes the levels of the various inflammatory mediators with aging in this cohort of animals. CRP, LBP, and MCP-1 levels were significantly decreased in the young animals, and MCP-1 levels were elevated in the aged group. BPI, RANTES, MMP-1, and MMP-9 levels were all significantly elevated in the young and adolescent animals compared to adult and aged monkeys.

Figure 2A and B show comparisons of the levels of these inflammatory mediators in the sera of young animals within the large group cohort versus those detected in the sera of animals of similar ages maintained under SPF conditions. The results showed elevated levels of PGE₂, CRP, BPI, MMP-1, and MMP-9 in the sera of the SPF animals compared to the young animals raised under standard housing conditions.

Systemic antibody responses to oral bacteria. Figure 3 shows the levels of serum IgG antibodies to a group of oral bacteria commonly associated with periodontal disease (1, 34, 44) in the various age groups of nonhuman primates. The results show significantly lower antibody levels in the group of young animals. The adult and aged animals routinely demonstrated significantly elevated levels of antibodies to the individual species, with minimal differences between these age groups.

Figure 2C provides an analysis of the serum antibody levels in the young animals housed under standard conditions compared to the young SPF animals. There were few differences in serum antibody levels between these groups; the SPF animals had levels at least as high as those of the standard group of animals.

Systemic responses and clinical parameters. No clinical differences were observed between the male and female animals, although significant increases in disease parameters of BOP (0.75 ± 0.2 versus 0.95 ± 0.15 units; P < 0.05), PD (2.50 ± 0.10 versus 3.15 ± 0.40 mm; P < 0.04), and CAL (0.25 ± 0.05 versus 0.55 ± 0.15 mm; P < 0.05) were noted in the aged animals. The younger groups of animals demonstrated negligible plaque, inflammation, or gingival tissue changes. We stratified the adult and aged animals into two groups based on the bleeding index (mean, ≥0.9 or <0.9 units) or on mean pocket depth (≥3 mm or <3 mm). When the animals were grouped based on these clinical parameters, the differences in systemic responses were greater in animals with poorer oral health.

Consistent with the lack of differences in clinical presentation related to gender, no differences in serum inflammatory mediators or serum antibody levels were observed between the genders (data not shown). The results in Fig. 4 show the differences in inflammatory mediator levels between the groups stratified by gingival bleeding. No significant differences in these serum response analytes were observed between the groups. However, in Fig. 5, it can be noted that levels of antibody to *P. gingivalis* and the sum of antibodies to the seven target bacteria were significantly increased with greater gingival bleeding. In contrast, Fig. 6 and 7 depict significant differ-
ences in multiple inflammatory mediators (BPI, IL-8, MMP-1, MMP-2) and serum antibodies to F. nucleatum, P. gingivalis, T. forsythia, and the sum of antibodies with more-severe destructive disease, i.e., greater pocket depth.

**DISCUSSION**

Evaluation of inflammatory and immune responses has provided evidence of ontogenic development of the immune system (2, 5, 52, 61, 64, 71, 81), as well as alterations in various host response parameters that are affected by aging (12, 28, 60, 87). However, while changes in oral health with aging similar to those reported for humans (88) have been reported to occur in various nonhuman primate species (7), little information on the biologic underpinnings of these clinical differences has been provided. While systemic inflammation, often resulting from bacterial sepsis, is frequently related to negative clinical outcomes with both morbidity and mortality, the systemic inflammatory response can also accomplish at the whole-organism level what the local inflammatory response is designed to do: that is, utilize disparate, nonspecific effector molecules to ameliorate potential tissue damage by noxious agents, including infecting bacteria (18). However, the characteristics of this ancient response system have generally been evaluated in adult individuals and have been related to sepsis, neoplastic changes, and responses to chronic diseases (18). This study demonstrated the patterns of selected systemic inflammatory molecules in young individuals and demonstrated specific changes in these levels with aging. Of particular note were the significantly elevated levels of BPI, RANTES, and both MMP-1 and MMP-9 in the younger groups of animals. This was unex-

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**FIG. 4.** Acute-phase reactants and inflammatory mediators in serum samples from nonhuman primates stratified on the basis of a low (<0.9) (n = 32) or high (≥0.9) (n = 9) mean index score for mouth bleeding upon probing. Bars represent group means; error bars, 1 standard deviation. PGE2, BPI, RANTES, MMP-2, and MMP-9 are measured in ng/ml; LBP and CRP are measured in μg/ml; and IL-8, MCP-1, and MMP-1 are measured in pg/ml (as in Fig. 1).

**FIG. 5.** Serum IgG antibodies to individual oral bacteria and total antibodies to this battery (SUM) in nonhuman primates stratified on the basis of a low (<0.9) or high (≥0.9) index score for mean bleeding upon probing. Bars represent mean antibody levels; error bars, 1 standard deviation. Statistical differences are depicted on the graph. For bacterium abbreviations, see the legend to Fig. 2.
expected, with minimal previous evidence of this type of response except that reported in obese children and adolescents (86) and a general concept of a lack of systemic challenge in children in the absence of clinical changes, but it is consistent with the maturation of host responses in young animals that have to cope with a range of environmental challenges to naïve mucosal surfaces. Testing this concept, we compared these systemic responses in young animals housed under standard conditions with those in comparably aged SPF monkeys. The results showed that the SPF animals often had elevated levels of the mediators compared to normal nonhuman primates. While there are likely various explanations for these differences, one existing theory, the “hygiene hypothesis,” suggests that the increasing incidence of asthma and other allergic diseases in the human population results from a lack of sufficient ontogenic development or “training” of the immune system in the young, who are then less able to effectively distinguish a noxious challenge later in their development (27, 50, 66, 89). Irrespective of the basis, these data demonstrate significant differences in response profiles of the inflammatory and innate immune systems during aging.

Nonhuman primates have historically been utilized to evaluate infectious agents (8, 32, 47, 54) and biologic processes (10, 16, 31, 51) associated with various human diseases. This is related to homologies in a range of host responses between humans and the other primate species, as well as species tropisms for infectious agents that cross human and nonhuman primate lines (26, 43, 73). These similarities extend to the microbial ecology and host responses in the oral cavity related to microbial biofilms that trigger periodontal disease (20, 22, 45, 62, 68). We observed that levels of serum antibody to various bacteria associated with periodontal-disease biofilms were significantly lower in the youngest animals. As is noted in humans and nonhuman primates, the extent of disease was increased in the aged group, although the antibody levels were similar for the adult and aged animals. This is consistent with the early acquisition and accumulation of these species as part of the commensal microbiota of the oral cavity and an association of these bacteria as etiologic triggers of periodontal pathology related to aging. We have also characterized the effects of aging on naturally occurring periodontitis, and we use a specific ligature-induced model of specific challenge to the oral cavity to describe acute responses of mucosal tissues during aging. In addition, this model has allowed us to document gender and diet effects on local and systemic inflammatory and immune responses that are altered with aging (25).

FIG. 6. Acute-phase reactants and inflammatory mediators in serum samples from nonhuman primates stratified on the basis of a low (<3 mm) (n = 25) or high (≥3 mm) (n = 16) mean pocket depth in the mouth. Bars represent group means; error bars, 1 standard deviation. Statistical differences are depicted on the graph. PGE$_2$, BPI, RANTES, MMP-2, and MMP-9 are measured in ng/ml; LBP and CRP are measured in µg/ml; and IL-8, MCP-1, and MMP-1 are measured in pg/ml (as in Fig. 1).
We then addressed specific questions regarding these systemic responses and the expression of chronic periodontal infections and inflammation in the oral cavities of adult and aged animals. As is noted in humans and nonhuman primates, the extent of disease was increased in the aged group, with no gender differences in expression of disease. This is in contrast to our findings with *M. mulatta* raised in individual housing with ad libitum feeding. Our previous results demonstrated that aged males exhibited significant weight gain, demonstrated various biologic parameters of unhealthy aging, and exhibited significantly greater periodontal disease than similarly aged females (unpublished data). Placing the males on a calorie-restricted diet decreased the disease to a level similar to that of the female cohort. The group of animals in this study was housed in a large corral that permitted constant exercise, required food-scavenging behaviors, and permitted natural competition among the various strata of colony members. Thus, the older males were much more physically fit than the sedentary singly housed animals, which may have translated into a preservation of oral health for aging males comparable to that for females.

We also stratified the animals, irrespective of age, based on the clinical presentation of gingival bleeding, a measure of local mucosal inflammation, and on pocket depth as an indicator of local challenge resulting in destructive disease. These subgroups were then evaluated for the patterns of systemic inflammatory and antibody responses. The results demonstrated rather minimal differences in the systemic responses related to the level of gingival inflammation in these adult and aged monkeys. In contrast, multiple significant differences were observed in both the inflammatory mediators and the levels of antibodies to oral bacteria in the animals with more severe periodontitis. These data for the nonhuman primates support similar data from humans indicating that the tissue destruction associated with chronic periodontitis enhances challenge of the systemic circulation with the potential to alter the function of the vascular and/or distant tissues (11, 48, 57, 72).

The literature is generally lacking on the use of nonhuman primates to elucidate the ontogenic development of the inflammatory, innate, and adaptive immune system. The oral cavity provides a readily accessible model of these host response changes at mucosal surfaces that interface with an evolving microbial ecology. This study described oral clinical findings and systemic responses in nonhuman primates and described differences in these measures that occurred from young through aged animals. The results from these initial studies of this primate colony provide a basis for the use of this robust resource to test hypotheses regarding the local and systemic ontogeny of innate and adaptive immune responses in relationship to the acquisition, adaptation, and evolution of the microbial ecology at this mucosal surface.

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