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Differential gender effects of a reduced-calorie diet on systemic inflammatory and immune parameters in nonhuman primates

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Background and Objective: Dietary manipulation, including caloric restriction, has been shown to impact host response capabilities significantly, particularly in association with aging. This investigation compared systemic inflammatory and immune-response molecules in rhesus monkeys (*Macaca mulatta*).

Material and Methods: Monkeys on continuous long-term calorie-restricted diets and a matched group of animals on a control *ad libitum* diet, were examined for systemic response profiles including the effects of both gender and aging.

Results: The results demonstrated that haptoglobin and α1-antiglycoprotein levels were elevated in the serum of male monkeys. Serum IgG responses to *Campylobacter rectus*, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* were significantly elevated in female monkeys. While only the antibody to *Fusobacterium nucleatum* was significantly affected by the calorie-restricted diet in female monkeys, antibody levels to *Prevotella intermedia*, *C. rectus* and *Treponema denticola* demonstrated a similar trend.

Conclusion: In this investigation, only certain serum antibody levels were influenced by the age of male animals, which was seemingly related to increasing clinical disease in this gender. More generally, analytes were modulated by gender and/or diet in this oral model system of mucosal microbial challenge.

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The inflammatory response involves humoral and cellular responses to a given challenge. There has been increasing demand to assess the effects of aging on immune cell functions. It has been well documented that the numbers and function of T cells and B cells decrease progressively during aging (1), although the impact of aging on innate immunity remains to be clarified. Numerous studies in rodent models have documented a decline in immune responsiveness with age (2–5). In particular, these studies have indicated that advancing age produces a general depression in the adaptive immune response (5), which is accompanied by an increase in the production and release of reactive oxygen species, reactive nitrogen species and the activity of cyclooxygenase enzymes, with an accompanying increase in prostaglandin production (1,3,4). In addition, there appears to be an up-regulation of inflammatory cytokine gene expression with aging, including the cytokines tumor necrosis factor-α, interleukin-1, interleukin-6, interferon-γ and transforming growth factor-β (5–7). However, assessing the

impact of aging on cellular functions in humans is complicated by the effects of chronic diseases frequently observed in elderly persons. Thus, in human systems it continues to be a challenge to delineate the effects of aging vs. the effects of systemic or environmental conditions (8).

Caloric restriction of dietary intake has been shown to alter significantly a wide range of biological processes and, in particular, to attenuate age-related disease in rodent models of aging (4,8–11). This dietary manipulation has been demonstrated to attenuate the development of oxygen radical-induced cell damage, to maintain more robust host responses protecting against deleterious extrinsic and intrinsic challenges to normal cell, tissue and organ function, and to maintain general body-wide physiologic functions (12–25). Recent studies have interpreted these macro-observations at the molecular level by identifying that caloric restriction could stop aging-associated changes in the expression of numerous genes (12,13), including altering insulin-like growth factor 1, which is associated with age-related decreases in insulin sensitivity (20,26,27). Only recently have reports emerged regarding the potential for this dietary manipulation to alter physiologic parameters also in nonhuman primates, a species more closely related to humans than rodents (28–36). As many of these findings are similar to those seen in rodent models, the nonhuman primates may provide a valuable link between rodent studies of reduced-calorie diets and application of this approach to a human population.

Periodontal disease is a predominant chronic inflammatory disease of mankind (37–39) that is a consequence of oral infection, chronic inflammation and destruction of collagen and bone, and can be documented to occur naturally with aging in humans and nonhuman primates (37,40,41). The extent and severity of tissue destruction is affected by the magnitude and characteristics of the host response and may be modulated by environmental, systemic or genetic factors (38,39,42). Periodontal destruction is cumulative and not naturally reversible and thus it is unclear as to whether aging impacts the rate of disease progression or just reflects the accumulation of disease over time (41,43). The importance of periodontal disease as a model of host–bacterial interactions, inflammation and inflammatory disease lies in the ability to isolate and characterize bacterial and host factors from the oral cavity in a noninvasive manner and to correlate these changes with host tissue pathology. The nonhuman primate model has provided the essential bridge for understanding the interaction of the subgingival microbiota with the inflammatory/immune response targeted to selected members of this microbiota (44–48). Increasing evidence also suggests that these oral microorganisms can translocate to the systemic circulation and may routinely stimulate the reticuloendothelial and immune systems (49–51). Recent studies have provided clear evidence that the oral cavity can function as a nidus for a variety of potential medical problems (49,51,52). Several members of the periodontopathic microbiota have been found to be involved in other systemic infections, as well as in the induction of an acute-phase response (53). Increased levels of acute-phase proteins (e.g. C-reactive protein and haptoglobin) have been identified in adult patients with periodontitis and may reflect the infection and manifestations of acute and chronic inflammation that exist in the periodontium (53–56). Moreover, it was also evident that patients exhibiting the most severe disease had the greatest levels of each of the acute-phase reactants. In addition, a serum antibody response is observed in these localized periodontal infections. It has been suggested that this serum response may reflect a local gingival inflammatory/immune response to the bacteria. Thus, the systemic antibody response observed in periodontitis patients appears to result from specific elicitation of antibody to an infection with the microorganism (50,57,58).

This study utilized the accessibility and natural development of chronic inflammation and disease in the oral cavity to examine the effects of long-term dietary calorie restriction on inflammatory/immune responses in a human-like model system, the rhesus monkey.

Material and methods

Animals and diet

Eighty-three rhesus monkeys (*Macaca mulatta*), which are part of an ongoing study of caloric restriction and aging, were used in these studies (Table 1). These animals have been housed at the National Institutes of Health Animal Center (Poolesville, MD, USA). The ages of the monkeys ranged from 13–23 years for females and 16–33 years for males. The monkeys live in a controlled environment with a standard diet and are continually monitored with regard to health status. The calorie-restricted (CR) monkeys have been subjected to a 30% reduction in dietary caloric intake relative to control (CON) animals which started at 1–3 years or 4–16 years of age for adolescents and adults, respectively (33). The CR diet was supplemented with minerals and vitamins up to 100% daily allowance for CR animals. The diet was supplemented once weekly with fresh fruit for all monkeys. At the time of the current study, animals had been assigned to continuous long-term caloric restriction or control *ad libitum* diets for periods of 13–17 years.

Serum analyses

Blood was collected from all monkeys under ketamine or telazol anesthesia following an overnight fast, and then serum was separated and stored at −80°C until assay, when IgG to six oral bacteria was evaluated using an enzyme-linked immunosorbent assay, as described previously (59,60). Briefly,

Table 1. Age distribution of the nonhuman primate cohort in the study

Gender	Diet group	*n*	Mean ± SD
Female	CON	19	18.74 ± 1.29
	CR	16	16.94 ± 1.22
Male	CON	26	22.35 ± 1.21
	CR	20	22.70 ± 1.53

CON, control *ad libitum*; CR, calorie restricted.

Campylobacter rectus, Fusobacterium nucleatum, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Treponema denticola* and *Porphyromonas gingivalis* were grown in broth under anaerobic conditions, harvested by centrifugation, formalin-killed, washed and stored at −20°C for use as antigens (61).

Selected acute-phase reactants were quantified using enzyme-linked immunosorbent assay procedures developed in our laboratory (53,56,62). Specifically we examined the levels of C-reactive protein, haptoglobin, fibrinogen, α1-antiproteinase and α1-acid glycoprotein in serum samples from all animals.

Statistical analyses

In the primary analysis, the effects of age and caloric restriction were analyzed separately by gender because of different age distributions (Table 1). Age was modeled as a linear variable. In secondary analyses, data were submitted to a linear regression analysis in which gender was included in the model. The purpose of the secondary analysis was to verify the robustness of the results. Statistical analysis was performed using JMP (SAS, Inc., Cary, NC). Statistical significance was set at an alpha level of 0.05.

Results

Systemic acute-phase reactants

The levels of various acute-phase reactants were determined in serum obtained from each monkey and compared based upon gender and diet. Figure 1 demonstrates that the levels of haptoglobin and α1-acidglycoprotein were significantly higher in male than in female monkeys and were not affected by a caloric-restriction diet.

Systemic antibody responses to oral bacteria

Figures 2–5 show the levels of serum IgG in a group of oral bacteria commonly associated with periodontal disease (63,64). In Fig. 2 antibody levels to *A. actinomycetemcomitans* and *P. gingivalis* were significantly higher in the female monkeys compared with the male monkeys, with no effect of diet or age. In Fig. 3 the level of antibody to *P. intermedia* is shown to be significantly related to age in male animals, although the females did exhibit a trend toward higher levels of antibody, irrespective of diet. Figure 4 illustrates that serum IgG to *F. nucleatum* was significantly elevated by a caloric-restriction diet in the female monkeys only, and the levels increased significantly with age in the males and

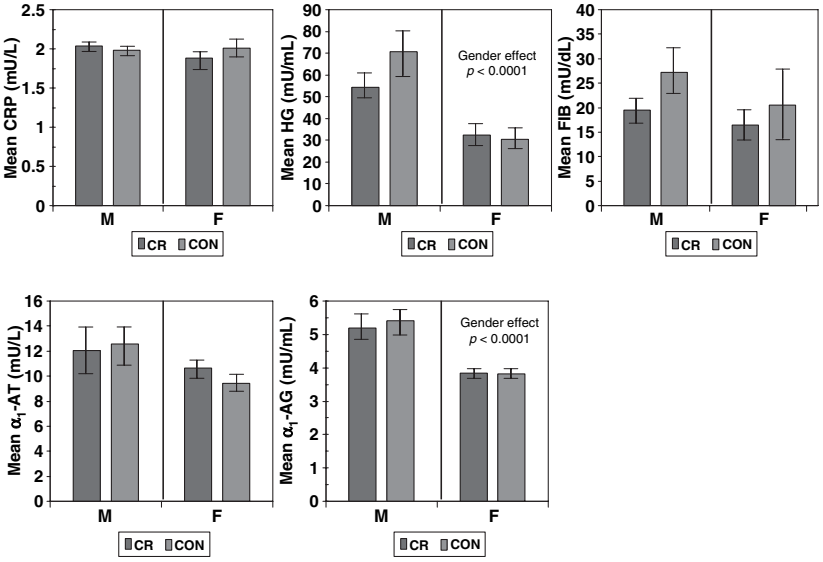

Fig. 1. Acute-phase reactants in serum from nonhuman primates, categorized based upon gender (F, female; M, male) and diet (CR, calorie restricted; CON, control *ad libitum*). The bars denote the mean levels of each mediator (HG, haptoglobin; FIB, fibrinogen; CRP, C-reactive protein; α_1-AT, α1-antiproteinase; α_1-AG, α1-acid glycoprotein) and the vertical brackets denote one standard error.

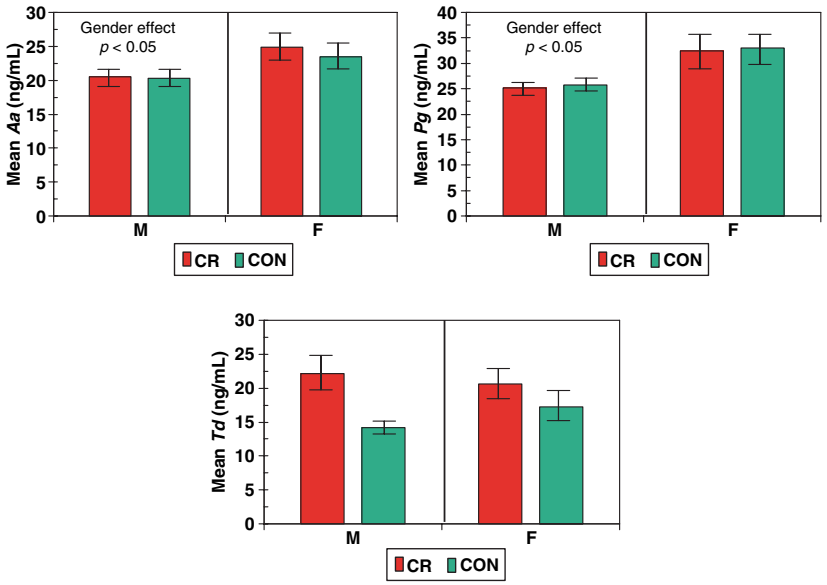

Fig. 2. Serum IgG level to *Actinobacillus actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*) and *Treponema denticola* (*Td*) in nonhuman primates categorized based upon gender and diet. The bars denote the mean levels of each mediator and the vertical brackets denote one standard error. CON, control *ad libitum*; CR, calorie restricted; F, female; M, male.

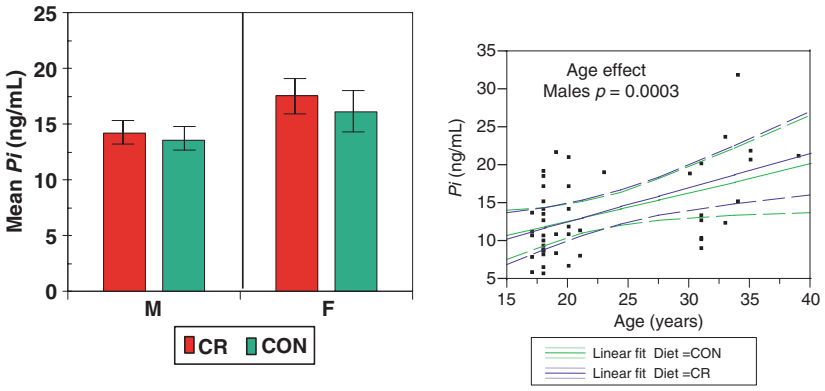

Fig. 3. Serum IgG levels to *Prevotella intermedia* (*Pi*) in nonhuman primates, categorized based upon gender and diet (left panel). The bars denote the mean levels of each mediator and the vertical brackets denote one standard error. Right panel: antibody levels related to age classified according to diet (green, control; blue, calorie restricted). The dashed lines denote the 95% confidence interval.

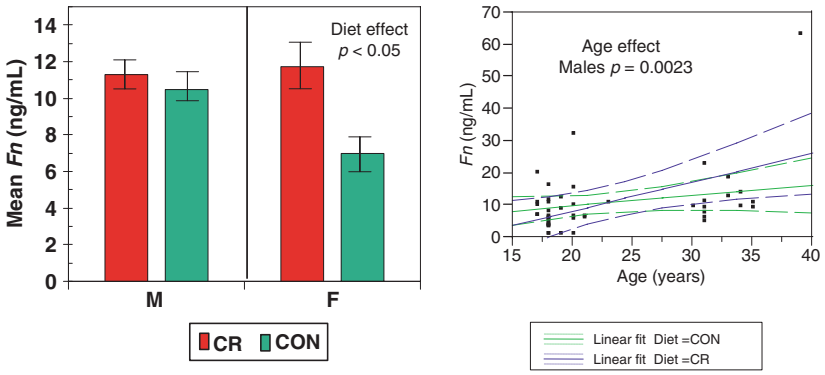

Fig. 4. Serum IgG levels to *Fusobacterium nucleatum* (*Fn*) in nonhuman primates, categorized according to gender and diet (left panel). The bars denote the mean levels of each mediator and the vertical brackets denote one standard error. Right panel: antibody levels related to age, classified according to gender (green, female; blue, male). The dashed lines denote the 95% confidence interval. CON, control *ad libitum*; CR, calorie restricted; F, female; M, male.

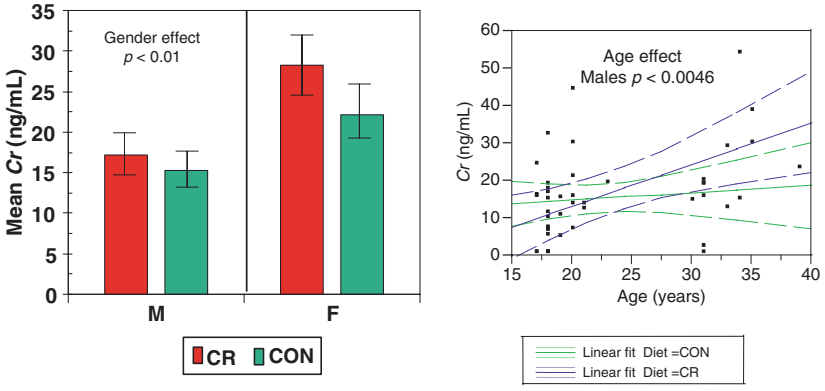

Fig. 5. Serum IgG levels to *Campylobacter rectus* (*Cr*) in nonhuman primates, categorized based upon gender and diet (left panel). The bars denote the mean levels of each mediator and the vertical brackets denote one standard error. Right panel: antibody levels related to age, classified according to gender (green, female; blue, male). The dashed lines denote the 95% confidence interval. CON, control *ad libitum*; CR, calorie restricted; F, female; M, male.

was unrelated to diet. Figure 5 shows that the serum antibody levels to *C. rectus* were significantly elevated in female monkeys compared with male monkeys, and these levels increased significantly in male monkeys with age, unrelated to diet.

Discussion

This investigation described the characteristics of systemic inflammatory and immune responses of a nonhuman primate cohort related to a calorie-restricted diet. This dietary manipulation has been demonstrated to contribute towards potential therapeutic outcomes related to biologic processes adversely affected by aging (10,11,15,16). Caloric restriction has been shown to minimize the decline in specific immune functions (1,5), as well as to attenuate destructive inflammatory responses (4). Various physiologic parameters (4,8,14,25,35,65) and hormonal changes (thyroid hormones, melatonin and dehydroepiandrosterone sulfate) (19,35,66,67) that are related to improved aging have been reported in nonhuman primates on a long-term caloric-restriction diet.

In the current study, the nonhuman primate model was used to examine the effect of a calorie-restricted diet on systemic inflammatory and antibody responses to oral commensal and opportunistic bacterial pathogens. Periodontal disease is a complex microbial infection in which similarities have been observed between humans and nonhman primates (48,54). This oral infection elicits a chronic immunoinflammatory lesion that destroys soft and hard tissues, resulting in destruction of the periodontium (37,68–70). While the extent and severity of periodontal disease is related to aging (41,71), it is unclear whether this finding represents a cumulative expression of years/decades of challenge to the tissues or an exacerbated disease process reflecting altered aging processes measured at a molecular level. Periodontal disease provides a model of host–bacterial interactions, inflammation and adaptive immune responses that can be used to examine nutritional and aging

changes in the oral cavity. In addition, ample evidence has demonstrated that these local oral infections also stimulate a systemic inflammatory and humoral immune response (50,53,58, 72–77).

We have previously reported an age-associated increase in periodontal disease in nonhuman primates (Reynolds M, G. Branch-Mays, D. Dawson III, K.F. Novak, J. Mattison, J. Gunsolley, D. Ingram, M. Lane, G. Roth, and M.J. Novak. Effects of dietary calorie restriction on inflammatory disease in a nonhuman primate model. Submitted). Periodontal disease was more prevalent in the male nonhuman primates, with a more dramatic effect related to aging. The current study suggested that characteristics of the systemic host responses were consistent with these disease findings. Systemic inflammatory mediators were significantly greater in male nonhuman primates compared with female nonhuman primates. Human studies have shown that increased severity/extent of periodontitis results in higher serum levels of these host response molecules (53,72,78,79). Thus, it was expected that the males would have elevated levels of these mediators. Although the male monkeys on a long-term caloric-restriction diet had generally lower levels across the profile of acute-phase reactants, this difference was not statistically significant. These outcomes are consistent with cross-sectional and longitudinal observations of human populations demonstrating elevated levels of acute-phase reactants in periodontitis and a decrease in these mediators after mechanical and anti-inflammatory therapies (53,72,78,79). Because these systemic inflammatory responses have been suggested to reflect and/or contribute to chronic inflammatory diseases (e.g. cardiovascular and diabetes), the contribution of chronic periodontitis to these systemic biomolecules has been suggested to be a biologic link between oral and systemic diseases (80).

In humans, both the specificity and levels of serum antibody responses to oral pathogens are clearly related to periodontal disease (50,58,77,81,82). Both antibody frequency and level increase with increasing severity of periodontal disease, and various studies have demonstrated that these serum antibody levels will be elevated following mechanical therapy and will correlate with response to treatment (50,83–86). Moreover, changes in serum antibody to selected oral pathogens appear to occur following emergence of the microorganisms in oral biofilm samples and prior to the identification of progressing disease (50,87). These findings suggest that the humoral immune response in local tissues, reflected in the systemic circulation, is probably an important component of the host's responses attempting to re-establish homeostasis by controlling the challenge of these extracellular bacterial pathogens. Interestingly, we observed significantly elevated antibody to these oral pathogens in female monkeys who displayed less periodontal inflammation and disease than the male monkeys (Reynolds M, G. Branch-Mays, D. Dawson III, K.F. Novak, J. Mattison, J. Gunsolley, D. Ingram, M. Lane, G. Roth, and M.J. Novak. Effects of dietary calorie restriction on inflammatory disease in a nonhuman primate model. Submitted). The antibody responses also appeared to be generally elevated with caloric restriction, with the most substantive impact in females. These results suggest a gender-specific differentiation of responses oriented towards a potentially destructive inflammatory response in males vs. a protective adaptive immune response in females. This type of observation has a basis in existing data demonstrating inherent gender-based variations in levels of immunoglobulins (88–90) and other host response biomarkers (91,92). Subsequent studies, implementing a longitudinal, prospective design creating a ligature-induced periodontal challenge in these animals, should help to clarify the dynamics of the relationship of periodontal disease with these response profiles. Lastly, of the analytes measured, serum antibody levels demonstrated some positive correlations with aging, primarily in the males, which was consistent with increased clinical parameters of periodontal disease in this group.

These cross-sectional observations provide a snapshot of host serum acute-phase and antibody responses in nonhuman primates. The response profiles supported an inherently different response pattern in monkeys that was gender determined, and demonstrated differences in the genders with respect to the impact of caloric restriction on these systemic responses. The results of further analyses will be used to establish in more detail the interaction of oral clinical presentation and these responses, demonstrating the usefulness of the oral cavity as a model for aging studies of host–bacterial interactions.

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