Calorie restriction prevents the occlusive coronary vascular disease of autoimmune (NZW × BXSB)F1 mice

(reduced calorie intake/autoimmunity/cardiolipin autoantibodies)

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ABSTRACT Male (NZW × BXSB)F1 (W/BF1) mice develop systemic autoimmunity involving autoantibodies, thrombocytopenia, lupus nephritis, and coronary vascular disease (CVD) with myocardial infarction. To determine whether this murine lupus-associated CVD can be prevented by the reduction of dietary calories, male W/BF1 mice were separated into five experimental groups and fed either ad libitum (designated group A, n = 50), fed 32% fewer calories of an otherwise comparable diet (designated group B12, n = 20), or initially fed ad libitum and then switched to reduced calorie intake (RCI) feeding at ages 14, 17, or 22 weeks (designated B14, n = 10; B17, n = 20; or B22, n = 20). Occlusive CVD was prevented by RCI. Life-span was significantly extended among the early onset RCI cohorts, B12 and B14 (P = 0.0001 and P = 0.005), compared to group A mice. Mean anti-cardiolipin autoantibody titers and mean levels of circulating immune complexes were also lowered in RCI mice when all RCI mice were compared to ad libitum fed group A mice. Histological grades of both coronary vascular and glomerular lesions were significantly less than those of group A mice (P < 0.001). Immunoprecipitates indicative of immunoglobulin deposition within coronary or glomerular vascular walls were also substantially less than those of group A mice. These findings indicate a possible causal role for anti-cardiolipin autoantibody in development of autoimmune CVD in W/BF1 mice and suggest that regulating dietary calories can influence the mechanism involved in pathogenesis of autoimmune-associated CVD development.

Occlusive coronary vascular disease (CVD) contributes to the morbidity and mortality of patients with systemic lupus erythematosus (1, 2), perhaps as a consequence of endothelial injury by circulating immune complexes (CICs) and subintimal deposition of immune reactants (2) or the thrombogenic consequences of elevated titers of anti-cardiolipin autoantibody (aCL) (3–10).

Of the strains of mice that develop lupus-like syndromes, male (NZW × BXSB)F1 (W/BF1) mice have an unusually high incidence (~80%) of CVD (11–15). Male W/BF1 mice develop systemic autoimmunity beginning at 10 weeks of age that involves multiple autoantibodies to single- and double-stranded DNA (ssDNA and dsDNA), cardiolipin, and platelets and is manifested as progressive thrombocytopenia, lupus nephritis, and CVD (11–16). Although the major epicardial coronary arteries of male W/BF1 mice remain largely unaffected, multiple small arteries and intramyocardial arterioles develop endothelial and intimal proliferative thickenings, become stenotic, often develop occlusive thrombi, and are frequently surrounded by zones of myocardial necrosis (13, 14). The CVD of male W/BF1 mice lacks a substantial exudative inflammatory component, but immune reactants including IgM, IgG2, and IgG3 have been demonstrated within intramyocardial arteriole walls (15).

Limiting dietary calories to levels 25–35% less than those consumed ad libitum, while ensuring adequate consumption of all dietary essentials, delays or abrogates the development of systemic autoimmunity in autoimmune-prone strains of mice (17), including the (NZB × NZW)F1 (18–20), NZB (21, 22), and BXSB (23) strains. Reduced calorie intake (RCI) extends the healthful longevity of mice of each of these autoimmune-prone strains and prevents strain-specific pathology, including severe glomerulonephritis and vasculitis (18, 19). Whether RCI can prevent the development and reduce the severity of CVD in W/BF1 mice has not been addressed.

RCI suppresses the production of anti-DNA autoantibodies and the formation of CICs (20, 25) and delays or prevents the appearance of CD-5/Lyt-1+ B lymphocytes, a subpopulation linked to autoantibody formation (22). RCI has also been shown to lower maintenance rates of cellular proliferation within the intestine, skin, thymus, spleen, and lymph nodes (17, 21, 27), while preserving or even enhancing adaptive cellular proliferation such as in the regenerative hyperplasia of hepatocytes following partial hepatectomy (28) or as in the responses of lymphoid cells to antigens or phytohormones (17).

In the present report, we fed dietary cohorts of male W/BF1 mice two semipurified diets of comparable composition so that calorie intake differed by 32%. To determine whether the onset of CVD in W/BF1 mice can be blocked and the severity of vascular lesions that do develop can be minimized by RCI, dietary calories were restricted beginning when mice were either 6, 14, 17, or 22 weeks old. Mice were evaluated for development of anticardiolipin or anti-dsDNA autoantibodies, for levels of CICs, for manifestations and degree of coronary vascular and glomerular lesions, for the incidence of CVD, and for length of survival.

MATERIAL AND METHODS

Animals. Male W/BF1 mice and female BALB/c mice (The Jackson Laboratory) were maintained in American Association for Accreditation of Laboratory Animal Care-accredited, specific pathogen-free conditions in accordance with ref. 29.

Abbreviations: W/BF1, (NZW × BXSB)F1; CVD, coronary vascular disease; RCI, reduced calorie intake; aCL, anti-cardiolipin autoantibody; CIC, circulating immune complex; dsDNA, double-stranded DNA; ALP, alkaline phosphatase; PAS, periodic acid/Schiff reagent.

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Semipurified Diets. Mice fed semipurified diet ad libitum throughout life consumed ≈10.5 kcal/day (1 kcal = 4.18 kJ) and were designated group A. Four other groups of mice were restricted in dietary calories by 32% and consumed ≈7.1 kcal/day. RCI mice were fed ad libitum prior to the initiation of RCI, which was imposed gradually with a 10% reduction in calories beginning when mice were either 6, 14, 17, or 22 weeks old. Groups of RCI mice were designated groups B6, B14, B17, and B22 to indicate age at initiation of RCI. Composition of the two semipurified diets has been presented in detail elsewhere (28). All dietary constituents were obtained from ICN Biochemicals. Diets were low in dietary fat (≈6% of total calories), differed in the level of total calories available, but were otherwise similar. Amounts of essential dietary constituents in each diet were determined with regard to the gram and calorie consumption of mice fed ad libitum. The diet fed RCI mice was further enriched so that although less food in grams and fewer calories were consumed by RCI mice, equivalent amounts of vitamins, minerals, essential fatty acids, and 30% of calories as protein were consumed by all mice. Approximately 60% of total calories in both diets were derived from carbohydrates. All mice were fed twice weekly and weighed weekly. Food consumption was determined for individually housed RCI mice and on a cage basis for pair-housed ad libitum fed mice by weighing the food offered and reweighing the remaining food at the end of the feeding interval.

Circulating Autoantibodies to dsDNA. Calf thymus dsDNA (Sigma) was used to coat each well of a microtiter plate. After blocking with bovine serum albumin, 50 μl of serum diluted 1:80 was incubated in DNA-coated wells for 1 hr at 37°C. Plates were washed, alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG was added, the ALP reaction was developed, and the optical absorbance was measured at 405 nm, plotted on a standard curve, and recorded as values from 1 to 100 units (30).

Circulating aCLs. Anticardiolipin antibodies were detected by solid-phase enzyme immunoassay as described (31). Briefly, 50 μl of a cardioplin solution, 10 μg of cardiolipin per ml (Sigma) in 0.01 M phosphate-buffered saline (pH 7.4), was incubated in each well of a microtiter plate for 12 hr at 4°C. Serum samples diluted 1:80 (50 μl) were added and incubated for 1 hr, ALP-conjugated goat anti-mouse IgG (100 μl) was added, and the ALP reaction was developed and read as described above (30).

CICs. Determination of CIC levels was made using an anti-mouse C3 F(ab')2 (Cappel Laboratories) solid-phase immunoassay as described (32). Anti-mouse C3 F(ab')2 (10 μg/ml, 200 μl per well) was added to microtiter wells and incubated at 4°C overnight. To determine CIC levels, a 1:80 dilution of serum was added and incubated at 37°C for 60 min. ALP-conjugated rabbit anti-mouse IgG (200 μl, 1:1000) was added, and the ALP reaction was developed and read as described above (30).

Histological Analysis. Formalin-fixed tissues were sectioned at 2–4 μm and stained with either hematoxylin/eosin or periodic acid/Schiff reagent (PAS). Each heart and kidney were cut transversely into three blocks, and from each block serial sections were prepared. The degree of CVD and myocardial infarction was classified from 0 to 3+, according to the method used by Berden et al. (15). Grade 1 indicates minimal PAS-positive deposits along and within one coronary vascular wall; grade 2 indicates PAS-positive deposits with narrowing of the lumen in two or three coronary vessels; grade 3 indicates four or more affected coronary vessels in the section. Myocardial infarctions were often associated with CVD grades 2 and 3. The degree of glomerulonephritis was classified from 0 to 3+, using a grading scale slightly modified from methods previously reported (15). Grade 1 indicates thickening and increased cellularity of the mesangium; grade 2 indicates increased mesangial plus increase of glomerular cellularity, inflammatory infiltrates, and presence of capsular adhesions; grade 3 indicates severe glomerular destruction with tubular cast formation.

Immunohistochemistry and Immunofluorescence. Heart sections were evaluated for the deposition of immunoglobulin within microvasculature using either biotinylated goat anti-mouse IgG or IgM, an avidin-biotin-peroxidase complex, and diaminobenzidine as substrate in a Vectostain kit (Vector Laboratories) (33). Kidney sections were evaluated for glomerular IgG or IgM deposition using standard methods of immunofluorescence and fluorescein-conjugated anti-mouse IgG or IgM.

Statistical Analysis. Development of systemic autoimmunity was compared by Kaplan–Meier analysis, using a Bonferroni adjustment. Rather than using a nominal level of 0.05 to indicate marginal significance, a more conservative P value of 0.005 was used to identify important mortality differences. Statistical analysis of autoantibody levels was performed using paired t tests. Histological grades were compared using the Mann–Whitney U test.

RESULTS

Physical Parameters. Mice fed semipurified diet at either calorie intake level grew and gained body weight (Table 1). Mice restricted in calories consumed 7.1 kcal/day and were leaner and lighter in body weight than ad libitum fed controls.

### Table 1. Influence of calorie intake on W/BF1 mean body weights

<table>
<thead>
<tr>
<th>Group</th>
<th>6</th>
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<th>20</th>
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</tr>
<tr>
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<td>22.2</td>
<td>27.0</td>
<td>26.8</td>
<td>24.4*</td>
<td>26.7*</td>
<td>26.6*</td>
</tr>
<tr>
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<td>24.3</td>
<td>27.7</td>
<td>29.8</td>
<td>24.5*</td>
<td>25.0*</td>
<td>24.8*</td>
</tr>
<tr>
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<td>24.8</td>
<td>27.7</td>
<td>28.8</td>
<td>26.3*</td>
<td>25.7*</td>
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*P < 0.001 compared to body weight of age-matched group A mice.

![Fig. 1. Kaplan–Meier analysis of mortality attributable to systemic autoimmunity of W/BF1 mice fed ad libitum (A) (n = 50), fed ad libitum until 14, 17, or 22 weeks of age (B14 (n = 10), B17 (n = 20), or B22 (n = 20)) and then reduced in calorie intake (B) or fed RCI levels from 6 weeks of age B6 (n = 20). *P < 0.0001; †P < 0.005.](image-url)
Fig. 2. (Legend appears at the bottom of the opposite page.)
which consumed 10.5 kcal/day. Body weights of RCI B6, B14, B17, and B22 mice were significantly lower than those of group A mice when the mice were 10, 15, 20, 25, and 35 weeks of age, respectively (P < 0.001).

Incidence of Fatal Autoimmunity. A significant reduction in death attributable to systemic autoimmunity occurred among group B6 mice compared to all other groups (P = 0.005 against group A, P = 0.006 against B14, P = 0.002 against B17, P = 0.001 against B22) (Fig. 1). Reducing calories beginning when W/BF1 mice were 14 weeks old reduced their mortality compared to that of group A mice (P = 0.005). Ninety percent of group B6 mice and 60% of group B14 mice survived beyond the age of 40 weeks. Delaying the initiation of RCI increased the incidence of fatal systemic autoimmunity among W/BF1 mice. Mortality of group B17 mice was only marginally less than group A mice (P = 0.06). Mice of group B22 developed fatal systemic autoimmunity at a rate comparable to that of ad libitum fed group A controls (P = 0.34). Fatal systemic autoimmunity developed in 50% of mice in groups A, B17, and B22 when mice were 20, 22, and 19 weeks old, respectively.

Incidence and Histopathology of CVD and Glomerulonephritis. Hearts and kidneys from mice were serially sectioned and examined. Immunologically based lesions within the microvasculature of the kidney and coronary vessels were evident. Lesions of murine lupus-associated CVD occurred typically within ventricular walls and consisted of endothelial cell proliferation, PAS-positive deposition within the coronary arterioles, frequent thrombosis, and myocardial necrosis (Fig. 2). The mean grade of CVD lesions for all RCI mice, groups B14-22, was 0.52 ± 0.66 and was significantly less than that of ad libitum fed group A mice, which had a mean score of 2.00 ± 1.00 (P < 0.001; mean ± SD) (Fig. 3). A subintimal brown precipitate within the coronary vascular walls, indicative of IgG and IgM subintimal deposition, was evident in immunohistological preparations of heart sections from mice with CVD. This precipitate was more abundant in heart sections from group A mice compared to those of RCI mice (Fig. 2).

Within the kidneys, PAS-positive thickening of glomerular tufts, mononuclear infiltrates, increased mesangial and glomerular cellularity, and capsular adhesions were evident. The mean grade of glomerular lesions for all RCI mice, groups B14-22, was 0.83 ± 0.77 and was significantly less than that of ad libitum fed group A mice, which had a mean score of 2.37 ± 0.92 (P < 0.001; mean ± SD). Intense immunofluorescence, indicating deposition of IgG or IgM, was evident in the affected glomeruli of group A mice, while immunofluorescence of glomeruli in kidneys of RCI mice was faint or not observed (Fig. 2).

Development of aCLs. The sera of mice were evaluated for the presence of circulating autoantibodies to dsDNA or cardiolipin and for the presence of CICs (Fig. 4). Mean titers of autoantibodies that reacted with dsDNA (P < 0.05) or cardiolipin (P < 0.01) and levels of CICs (P < 0.01) among RCI mice were lower than those of ad libitum fed group A mice.

DISCUSSION
The potential therapeutic benefits of modifying dietary composition or calorie intake on the progression of autoimmune disease in humans have not been effectively addressed, typically evaluating dietary modulation of rheumatoid arthritis or occasionally systemic lupus erythematosus (34–39). The potential of RCI in preventing CVD has not been investigated.

Experimentally, limiting dietary calories delays the onset and arrests the progression of systemic autoimmunity in short-lived autoimmune-prone strains of mice (17–26). In the current study, we determined that RCI could prevent CVD in W/BF1 mice, and, in those RCI mice where lesions developed, the histological grade of those lesions was less severe. Immunohistological evidence of subintimal deposition of immunoglobulin in the coronary vessels of RCI mice was reduced compared to substantial immunoprecipitates present in the coronary vasculature of ad libitum fed mice. This abrogation of CVD development was accompanied by lower

Fig. 3. Individual histological scores of CVD lesions in hearts or of glomerulonephritis in kidneys from mice of each dietary cohort. Hearts and kidneys from group A mice were collected, evaluated, and graded upon autoimmune-based death of each mouse when 15–30 weeks old. Hearts and kidneys of group B6-22 mice were collected at euthanasia of mice when 40 weeks old. Criteria for scoring are given in the text.

Fig. 2 (on opposite page). (A) Macroscopic appearance of representative hearts from group A (Left) and group B6 (Right) mice showing severe, multiple, necrotic myocardial infarctions in hearts from ad libitum fed group A mice and normal appearance of heart from RCI mice. (x3.5.) (B) Representative histopathologic section of CVD, grade 3, from a group A mouse. Note PAS-positive deposits within the media of the coronary arterial wall, presence of thrombus, and presence of minor inflammatory infiltrate. (x410; PAS.) (C) Representative histological section of an unaffected heart from RCI group B6 mouse. (x410; PAS.) (D) Immunohistochemical preparation of a representative section of heart from a group A mouse showing substantial subintimal brown precipitate in several small coronary arterioles, indicating the subintimal deposition of IgG. (x260.) (E) Immunohistochemical preparation of a representative section of heart from a group B6 mouse showing scant or no subintimal brown precipitate. (x260.) (F and G) Immunofluorescent preparation of representative kidney sections from a group A mouse showing substantial glomerular deposition of IgG (F) and absence of fluorescent signal in a glomerulus from a group B6 mouse (G). (x415.)
mean anti-cardiolipin and dsDNA autoantibody levels and reduced levels of CICs.

Both coronary and glomerular autoimmune-based lesions of W/BF1 mice progress rapidly, early in life, so that delaying the imposition of RCI until mice are >17 weeks of age did not impair the progression to more severe vascular pathology that develops with age in mice of this strain. The mechanism by which early-onset RCI abrogates this aggressive vascular pathology has not been determined. Our previous studies have shown that transplantation of T-cell-depleted bone marrow (TCMD) from autoimmune-resistant strains of mice prevents CVD in W/BF1 recipients, showing that the autoimunity of W/BF1 mice is attributable to genetic defects that reside within marrow stem cells (30). Conversely, transplantation of W/BF1 TCMD to autoimmune-resistant mice regularly induced systemic autoimmunity with CVD and glomerulonephritis in the recipients, and development of CVD in recipient mice was accompanied by elevated titers of aCL (30). Since, in the present report, reducing calorie intake was associated with the human titers of aCL, and reduced incidence and severity of CVD, the protective abrogation of CVD development by RCI in W/BF1 mice could be the result of actions of the marrow stem cells that prevent the production of elevated aCL titers.

The mechanism by which RCI increases healthful longevity and impairs the development of autoimmune disease has not been elucidated, but it may involve neuroendocrine, immunologic, and metabolic systems (40) with effects on rates of cellular proliferation and responsiveness (17, 21), levels of free radical generation and capacity for detoxification (41, 42), and the efficiency of enzymatic repair of DNA (43). To our knowledge, the prevention of CVD by limiting calorie intake has not been reported previously. Early-onset calorie restriction not only abrogated the development of CVD but also inhibited all aspects of W/BF1 autoimmunity. Further, the RCI W/BF1 mice thrived. The accessibility and malleability of dietary variables, and the abundant experimental evidence for RCI’s role in preventing and frequently reversing autoimmune disease, make it imperative that further evaluation of the role of the diet, especially calorie intake level, in the prevention of autoimmune disease be elucidated.

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