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# Nutritional inhibition of genetically determined renal disease and autoimmunity with prolongation of life in *kdkd* mice

(calories/nephronophthisis/longevity/immunity/histopathology)

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ABSTRACT **Striking inhibition of development of renal disease and prolongation of lifespan have been achieved in *kdkd* mice by restricting their daily food intake. Restricting protein intake alone did not prolong life nor did it inhibit development of kidney disease. The *kdkd* nephronophthisis, although very different histologically from the renal disease of B/W mice, may also have immunological components. Like the immunologically based renal disease of B/W mice, renal disease in *kdkd* mice is decreased or eliminated histologically by dietary restriction, which inhibits development of autoimmunity directed toward the erythrocytes of these mice. Further analysis will be needed to elucidate the cause of progressive renal disease in both the *kdkd* and B/W models and to permit understanding of the profound influence of restriction of food intake on development and progression of these very different renal diseases.**

Recent studies have revealed that dietary restriction of fat, protein, or protein plus calories, have a profound influence on immunity, lifespan, and development of renal pathology in short-lived, autoimmunity-prone NZB or (NZB × NZW)$F_1$ (B/W) mice (1, 2). These studies have shown that such dietary restrictions will inhibit development and expression of autoimmunity, prevent development of splenomegaly, and delay involution, with aging, of the thymus and of immunologic functions in NZB and B/W mice. Both anti-DNA antibody and DNA complex formation as well as deposition of immunoglobulin and complement on the glomerular capillary membranes are strikingly inhibited (3, 4). Expression of xenotropic virus formation, however, does not appear to be inhibited by dietary restriction (5). In addition, dietary restriction can also profoundly influence the immunologic control mechanisms and development of spontaneously occurring suppressor lymphocytes in the spleens of short-lived, autoimmunity-prone B/W mice (3). Undernutrition also prolongs lifespan of long-lived mice (6) and is known to prolong life and reduce renal disease that occurs with aging in rats (7). We recently observed that the lifespan of B/W mice can be prolonged even when dietary restriction is imposed after the autoimmunity has begun to be expressed (8).

Lyon and Hulse (9) described a form of renal disease in mice similar to nephronophthisis of human beings, which develops as a function of autosomal recessive genetic inheritance. The responsible gene is located in linkage group X. The kidneys of the *kdkd* mice are normal at birth; progressive renal disease, however, appears early in life. Proteinuria is generally demonstrable by 10 weeks of age, and polydypsia soon follows. These mice regularly die between 7 and 9 months of age. In an effort to analyze further the influences of dietary restriction on development of renal disease, studies of the influence of dietary restriction on *kdkd* mutant mice have now been carried out and are reported herein.

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## MATERIALS AND METHODS

**Animals.** The *kdkd* mutant mice were obtained as a breeding stock from Mary Lyons (M.R.C. Radiobiology Unit, Harwell, U.K.). Mice were bred and maintained on a Purina Lab Chow diet at the University of Minnesota mouse colony by strict brother/sister mating procedures. Autoimmunity-resistant, inbred CBA/H and autoimmune-susceptible B/W mice, fed laboratory chow ad lib., were used in these analyses for comparison of histology and immunologic function with *kdkd* mice. These mice were obtained from the University of Minnesota mouse colony.

***In Vitro* Lymphocyte Stimulation Assay.** Details of this assay have been described (2). Nucleated spleen cells ($0.5 \times 10^6$), after being washed twice, were cultured in 0.2 ml of RPMI-1640 medium (Grand Island Biological Co., NY) containing 2% fetal calf serum and antibiotics, in triplicate wells of 2040 microtest II plates (Falcon Plastic, Oxnard, CA). Different dilutions of phytohemagglutinin (PHA) and concanavalin A (Con A) as T-cell mitogens, and lipopolysaccharide (LPS) as a B-cell mitogen, were used. Sixteen hours before harvest, 0.5 μCi of [*methyl*-$^3$H]thymidine (New England Nuclear, Boston, MA) were added, cultures were harvested on glass filter papers, and radioactivity was measured.

**Plaque Forming Assay (PFC).** Details of this assay have been described (10). In brief, each mouse was injected intraperitoneally with 0.2 $cm^3$ of a 20% suspension of sheep erythrocytes (SRBC) that had been washed four times in sterile saline. Four days later, spleen cells were obtained and PFC, plated in duplicates, were counted with the aid of an electronic colony counter (New Brunswick Co., NJ). The number of direct PFC per $10^6$ cells or per total spleen was calculated.

**Hematocrit and Coombs' Test.** Mice were bled with a heparinized capillary tube via retro-orbital plexus. The hematocrit was measured by standard methods, and the direct Coombs' analysis was carried out, using goat anti-mouse serum antibody (Meloy Lab, Springfield, VA.).

**Histology.** For histological study, the kidneys were stripped of capsule, bisected with a sharp razor blade, and placed in 10% neutral formalin for several days, embedded, sectioned, and stained with hematoxylin and eosin by the usual procedures.

**Dietary Studies.** To study the influence of diet, we placed 8- to 10-week-old male and female *kdkd* mice on the following standard defined diets, containing (wt/vol) casein (22%), starch

Abbreviations: PFC, plaque-forming cells; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; SRBC, sheep red blood cells; B/W, (NZB × NZW)$F_1$.

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Table 1. Mitogenic response of spleen cells to PHA, Con A, and LPS from *kdkd*, CBA/H, and B/W mice of different ages (mean cpm ± SEM)

| Strain | Age, month | Control | PHA (1 μg) | Con A (2.5 μg) | LPS (50 μg) |
|---|---|---|---|---|---|
| *kdkd* | 2 | 988 ± 233 | 131350 ± 12478 | 146320 ± 21650 | 70867 ± 4565 |
| *kdkd* | 6 | 622 ± 29 | 101630 ± 12528 | 112520 ± 13577 | 48588 ± 2800 |
| CBA/H | 2 | 596 ± 164 | 80030 ± 643 | 75971 ± 1116 | 53731 ± 2214 |
| CBA/H | 6 | 628 ± 191 | 102340 ± 8592 | 109420 ± 1500 | 58050 ± 1057 |
| B/W | 3 | 1758 ± 78 | 120520 ± 5012 | 164490 ± 2930 | 64320 ± 1019 |
| B/W | 6 | 1356 ± 49 | 110310 ± 4739 | 125570 ± 5243 | 52767 ± 2341 |
| B/W | 12 | 1142 ± 68 | 15380 ± 440 | 40164 ± 901 | 38382 ± 416 |

Data obtained with $0.5 \times 10^6$ cells/culture in triplicate from two to six individual spleens at optimal concentration of mitogen obtained after culturing for a total of 64 hr, including 16 hr of labeling each culture with 0.5 μCi of [$^3$H]dThd. Mice of all three strains used in these studies were maintained on Purina Lab Chow diet.

(33%), dextrose (33%), corn oil (5%), salt (4%), and vitamin mixture (2%), which is used regularly at our laboratory. To obtain a 6% protein diet, the casein level is reduced from 22% to 6%, and both starch and dextrose levels are increased to 41%. The details on the preparation of diet, source of ingredients, and feeding procedures have been described (1, 2). The following groups of *kdkd* mice were observed on different dietary regimens: group I received Purina Lab Chow ad lib.; group II received 22% protein diet, 16 cal/day; group III received 6% protein diet, 16 cal/day; and group IV received 22% protein diet, 8 cal/day. Mice of groups II, III, and IV were fed daily with fixed amounts of food per mouse. Animals on the restricted diet (group IV) were housed singly and given salt and vitamins in their rations equal to that given to animals in group II (16 cal/day).

## RESULTS

Table 1 records results comparing proliferative responses, obtained with optimal concentrations of the mitogens PHA, Con A and LPS, of *kdkd* and CBA/H at 2 and 6 months of age, and of autoimmunity-prone B/W mice at 3, 6, and 12 months of age. The *kdkd* mice showed vigorous proliferative cellular responses to each of the mitogens at 2 months of age when their kidneys were free of evidence of disease. A moderate decrease in the magnitude of these proliferative responses was recorded for the *kdkd* mice studied at 6 months of age, when renal disease was present in every mouse. By contrast, the CBA/H mice showed somewhat less vigorous proliferative responses to PHA and Con A at 2 months of age. CBA/H mice regularly lived longer than 2 years without any apparent abnormality of their kidneys.

Data are also presented in Table 1 comparing the spleen cell responses of B/W mice aged 3, 6, and 12 months. Although 3-month-old B/W mice showed vigorous responses to all three mitogens, an age-associated decline was apparent at both 6 and 12 months. However, compared to 6-month-old *kdkd* mice in which renal disease was regularly present, much lower responses to PHA and Con A were seen in B/W mice at 12 months of age when these mice had developed severe kidney disease of an autoimmune nature.

**PFC Response.** Table 2 summarizes the response of *kdkd*, CBA/H, and B/W mice to antigenic stimulation with SRBC. These data show that PFC in the spleen of *kdkd* mice, after stimulation by SRBC, showed responses comparable to those of CBA/H mice at both 2 and 6 months of age. No evidence of decline of this immunologic function with onset of the renal disease was observed in the *kdkd* mice. However, in the B/W mice, an age-associated decline in PFC response was present. Ten-month-old B/W mice with severe kidney disease always responded poorly to SRBC.

**Influence of Diet on Hematocrit and Coombs' Test.** Table 3 summarizes data analyzing hematocrit and Coombs' test responses of 7-month-old *kdkd* mice on different dietary regimens. The *kdkd* mice on diets I, II, and III developed anemia, accompanied in some mice by Coombs' positivity, which did not occur in mice on the calorie-restricted diet (diet IV).

**Influence of Diet on Survival of *kdkd* Mice.** Table 4 summarizes data reflecting the influence of diet on survival of *kdkd* mice. On diets I, II, and III almost all of the *kdkd* mice died of renal disease by 240 days of age. By 300 days all mice on these three dietary regimens had died. In striking contrast, 20 of 20 mice on the restricted-calorie diet were still alive by 240 days of age. At 260 days of age, 2 of these 20 mice were killed for kidney examination. Nine of these mice were continued on the low calorie diet (diet IV), while nine were switched to the higher calorie diet (diet II). Within 60 days of the start of this dietary regimen, at 300 days of age, 9 of 9 *kdkd* mice placed on the higher calorie regimen had developed renal disease and died,

Table 2. Development of PFC in *kdkd*, CBA/H, and B/W mice after immunization with SRBC *in vivo*

| Strain | Age, month | PFC (mean ± SEM) per $1 \times 10^6$ cells | PFC (mean ± SEM) per spleen |
|---|---|---|---|
| *kdkd* | 2 | 505 ± 46 | 44528 ± 5587 |
| *kdkd* | 6* | 755 ± 69 | 127610 ± 4469 |
| CBA/H | 2 | 520 ± 18 | 53492 ± 2965 |
| CBA/H | 6 | 599 ± 10 | 100960 ± 8692 |
| B/W | 2 | 2605 ± 251 | 338050 ± 21325 |
| B/W | 10* | 105 ± 15 | 10512 ± 872 |

In each group, three to five mice were maintained on Purina Lab Chow and immunized with 0.2 cm$^3$ of a 20% suspension of SRBC 4 days before spleen cells were obtained for the assay.
* All mice showed advanced renal disease at this age when killed.

Table 3. Hematocrit levels and Coombs' test at 210 days of age in *kdkd* mice maintained on different dietary regimens

| Diet* | No. of mice tested | Hematocrit, % | Coombs' test: Saline† | Coombs' test: Goat anti-mouse serum 1:5† | Coombs' test: Goat anti-mouse serum % positive |
|---|---|---|---|---|---|
| I | 12 | 42.8 ± 1.8 | 0/12 | 7/12 | 58.3 |
| II | 8 | 32.0 ± 3.2 | 0/8 | 5/8 | 62.5 |
| III | 5 | 37.1 ± 3.7 | 0/5 | 2/5 | 40.0 |
| IV | 7 | 50.1 ± 0.5 | 0/7 | 0/7 | 0 |

* See *Materials and Methods.*
† No. of mice positive/total no. of mice tested.

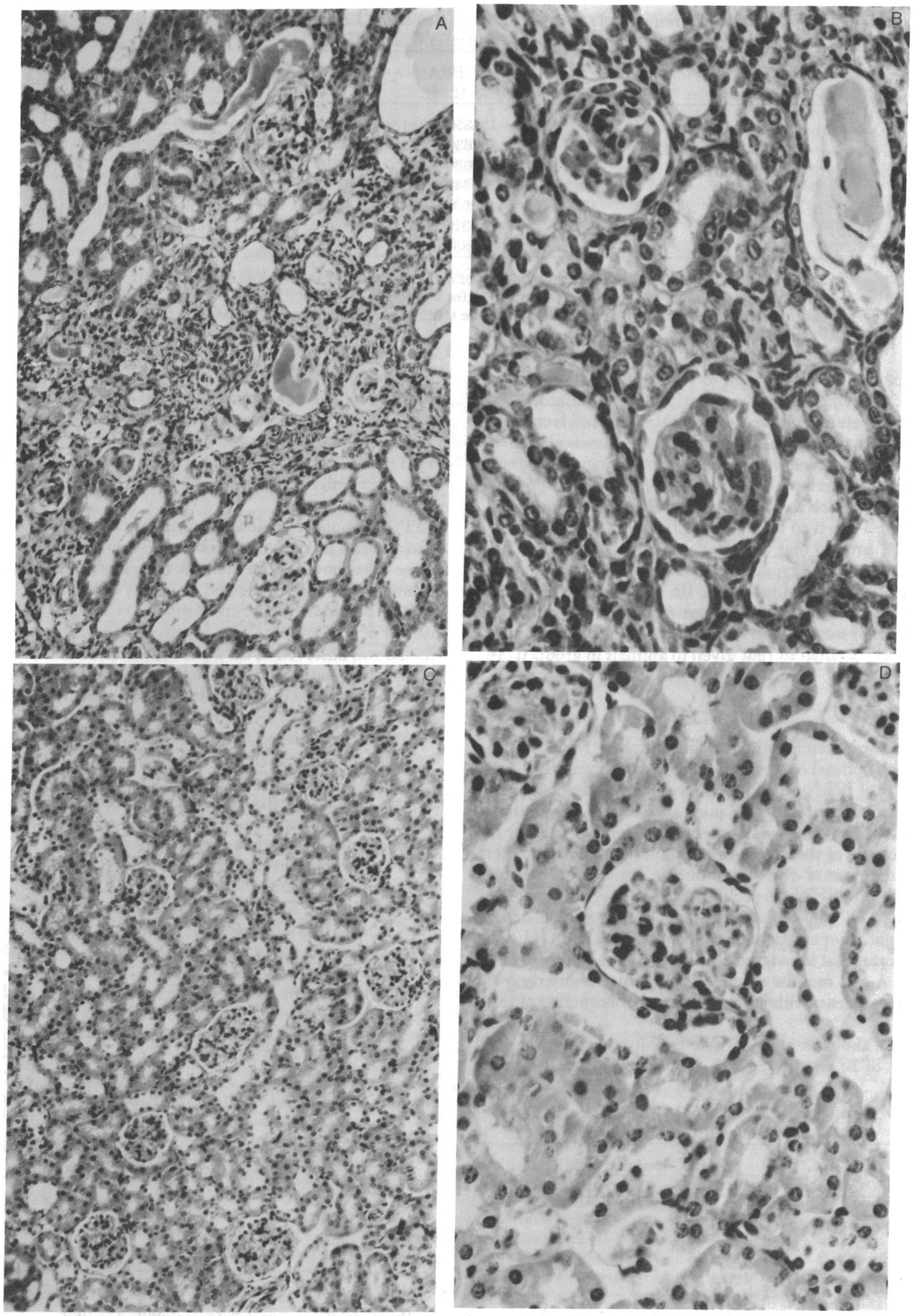


FIG. 1. Comparison of kidneys of *kdkd* mice on high and low food intake. (*A*) Low-power view of kidney of *kdkd* mouse, aged 7 months, fed a diet containing 16 cal/day. Note tubular dilation, atrophy of tubular cells, hyaline tubular casts, and striking mononuclear interstitial infiltration. (×100.) (*B*) Higher power view revealing interstitial infiltration. Glomerular and tubular abnormalities of *kdkd* mice fed higher calorie diet (16 cal/day). (×400.) (*C*) Kidneys of *kdkd* mouse fed low calorie diet (8 cal/day). (×100.) (*D*) Kidneys of *kdkd* mouse fed low calorie diet (8 cal/day). Note lack of interstitial infiltration and normal glomeruli and tubules. (×400.)

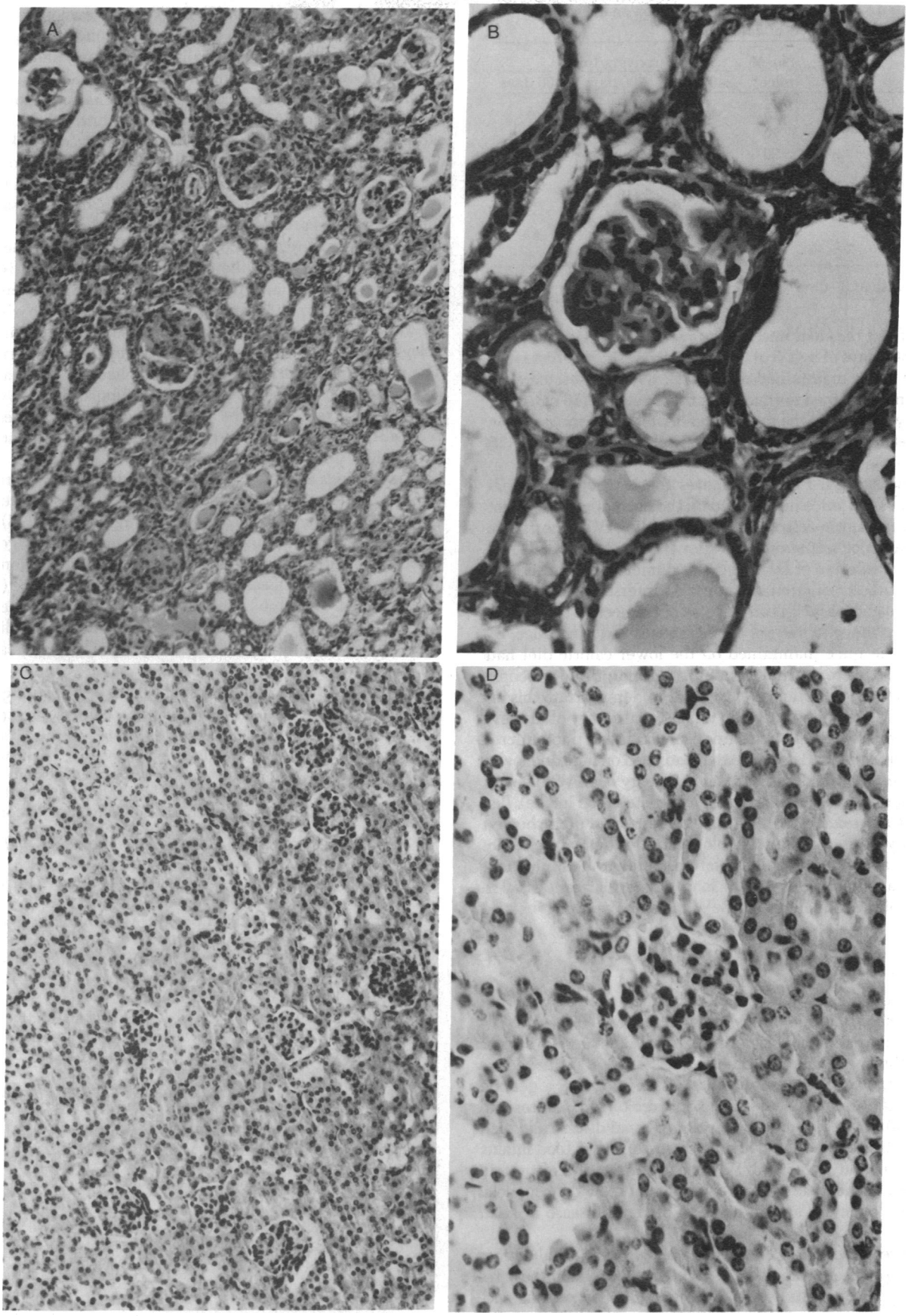


FIG. 2. Comparison of kidneys of (NZB × NZW)$F_1$ mice on high and low food intake. (*A*) Low-power view showing proliferative nephritis, striking glomerulosclerosis, tubular hyaline casts, and dilation. Interstitial infiltration is minimal. (×100.) (*B*) Higher power view revealing tubular dilation, tubular cell atrophy, and glomerulosclerosis of 10-month-old mouse on high calorie diet (20 cal/day). (×400.) (*C*) Lower power view revealing virtually normal kidney of 10-month-old B/W mouse fed lower calorie diet (10 cal/day). (×100.) (*D*) Higher power view of normal tubules and glomeruli of 10-month-old B/W mouse fed low calorie diet (10 cal/day). (×400.)

Table 4. Influence of dietary restriction on longevity of *kdkd* mice

| Cal/ day | Protein, % | No. of mice* | Survivors/total 240 days | 300 days | 500 days |
|---|---|---|---|---|---|
| I. Ad lib. | 17 | 15 | 4/15 | 0/15 | 0 |
| II. 16 | 22 | 20 | 1/20 | 0 | 0 |
| III. 16 | 6 | 20 | 2/20 | 0 | 0 |
| IV. 8 | 22 | 20 | 20/20 | 0/9 (16 cal) | 0 |
| | | | | 9/9 (8 cal) | 5/9 |

* Started with at 60 days.

while 0 of 9 of the *kdkd* mice on the lower calorie diet had died. Even at 500 days of age, 5 of 9 mice on the restricted diet were still alive. Mice maintained on diet III, which restricted only protein and contained normal amounts of calories (16 cal/day), died early, just as did the mice on ad lib. intake of mouse chow or on a defined diet with higher protein and normal calories (16 cal/day).

**Histopathology.** Fig. 1 compares the histology in the 7-month-old *kdkd* mice maintained on the normal regimen (diet II) and the calorie-restricted regimen (diet IV). The figure shows that mice maintained on diet II (Fig. 1 *A* and *B*) had extensive formation of hyaline casts, glomerular sclerosis, and dramatic tubular dilation and damage. Interstitial infiltration with round cells as well as an increase of connective tissue were also present in the kidneys of these normally fed *kdkd* mice. By contrast, the mice maintained on the lower calorie diet had kidneys that were normal, or almost normal, in microscopic appearance (Fig. 1 *C* and *D*). In order to demonstrate this influence of dietary restriction on the histopathology of kidneys in *kdkd* mice, we have, for comparison, included photomicrographs of kidneys of B/W mice that had been on high and lower calorie diets (3). Fig. 2 shows renal pathology of B/W mice fed diet II (normal calories) and diet IV (restricted food intake). Just like the *kdkd* mice, the B/W mice on restricted diets showed essentially normal kidney histology at 10 months of age. By contrast, advanced tubular dilation, tubular hyalinization, glomerular sclerosis, and wire loop glomerular lesions were features of the kidneys of the B/W mice on the higher calorie diet.

## DISCUSSION

In this study, we show once again dramatic prolongation of life and striking inhibition of development of renal disease by dietary manipulation, i.e., lowering food intake from 8 weeks of age. In this case, the renal disease that has been influenced is the progressive nephronophthisis that develops on a genetic basis in *kdkd* mice. These mice, kept free of renal disease up to 8 months of age, promptly developed renal disease and died within a 2-month period when placed on a higher food intake. As observed earlier with B/W mice (1), limitation of protein intake as a single dietary constituent did not show the beneficial effect on longevity and prevention of renal disease in *kdkd* mice.

The present findings on the genetically based renal disease of this experimental model are strikingly parallel to those revealed in our prior studies of the influence of diet on longevity and renal disease in NZB and B/W mice (1–3). The pathogenesis of the renal disease in the two strains, however, is thought to be very different (9). B/W mice develop renal disease as the consequence of a profound immunological perturbation which, although genetically based, is associated with a persistent xenotropic virus infection, development of autoimmunity, and immunological assault by antigen–antibody complex deposition in the glomerular membranes of the renal capillaries (11). The pathogenesis of the progressive kidney disease in the *kdkd* mouse, although still obscure, does not have the histological and immunohistochemical characteristics that are usually associated with the immunologic injury considered fundamental to early death in the B/W model.

Our findings of regular Coombs' positive anemia in the *kdkd* mice, the moderate decline of lymphocyte proliferative responses with aging, and the striking beneficial influence of dietary restriction on longevity raise the question of whether the *kdkd* nephronophthisis might be associated with immunological abnormality correctable in part by dietary restriction. The interstitial nephritis, involving lymphocytes, plasma cells, and mononuclear cells, and the dramatic tubular damage which progresses so rapidly in the well-fed *kdkd* mice suggest the possibility that an immune response, perhaps to tubular antigens, might underlie this renal disease.

Our findings and those of others linking dietary restriction to prolongation of life in long-lived mice (12), prolongation of life and inhibition of renal disease with aging in rats (13), prolongation of life and maintenance of immunologic vigor, inhibition of autoimmunity and immunologic involution, and prevention of renal disease in autoimmunity-prone mice (3, 14) as well as prevention of mammary cancer in C3H mice (15), demand that these striking salutary influences of low dietary intake be thoroughly studied and analyzed in both cellular and molecular terms.

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