Published in final edited form as:
J Autoimmun. 2006 February ; 26(1): 1–6.

The mitochondrial and kidney disease phenotypes of *kd/kd* mice under germfree conditions

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Abstract

Interstitial nephritis occurs spontaneously in *kd/kd* mice, but the mechanisms leading to this disease have not been fully elucidated. The earliest manifestation of a phenotype is the appearance of ultrastructural defects in the mitochondria of mice as young as 42 days of age. To examine the influence of the environment on the phenotype, homozygous B6.*kd/kd* mice were transferred from specific pathogen-free (SPF) conditions to a germfree (GF) environment, and the development of the disease was observed. The GF state resulted in a highly significant reduction in the frequency of tubulointerstitial nephritis. In addition, GF conditions markedly reduced the appearance of the mitochondrial phenotype, with no sign of mitochondrial abnormalities in GF mice of up to 155 days of age. These results suggest that environmental factors are involved in the progression of all known manifestations of this disease phenotype.

Keywords

Autoimmunity; Germfree; Kidney disease; Mitochondria

1. Introduction

Germfree (GF) derivation has been an extremely useful procedure for providing insights into the underlying mechanisms involved in animal models of autoimmunity. In some studies the GF environment reduced the autoimmune response, whereas in others, it did not. For example, MRL-*lpr* mice showed a significantly reduced disease frequency in the GF environment [1], and there was a rather dramatic reduction in the sizes of lymph nodes and spleens of NZB mice that were raised in a GF environment [2]. The intestinal pathology characteristic of *IL-2*$^{-/-}$ mice reared in a specific pathogen-free (SPF) environment was not seen in their GF counterparts, although some of their other disorders, such as anemia and extraintestinal lymphoid deficiency, persisted. These observations supported the hypothesis the colitis in

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IL-2$^{-/-}$ mice was dependent on intestinal flora but that environment antigens were not responsible for the anemia and extraintestinal lymphoid hyperplasia [3].

The *kd/kd* mouse is homozygous for the "kidney disease" mutation, which occurred spontaneously in a CBA/CaH colony. Mutant homozygotes are apparently healthy for the first 8 weeks of life, but eventually die of progressive renal failure [4]. Histological examination of kidneys at earlier time points reveals a mononuclear cell infiltrate and tubular dilatation in cortical areas, which with time expands throughout the entire kidney [5–7]. Multiple autoimmune pathways are involved in this inflammatory response, as doubly mutant mice with both the *Rag-1*$^{-/-}$ and *kd/kd* genotypes develop nephritis as readily and as severely as *kd/kd* controls [8]. As the *Rag-1* gene is required for the diversity of both antibody and T cell receptor genes, this demonstrates that neither functional B nor T cells are required for this phenotype. Nevertheless, the slowly progressive nature of this disease suggests that environmental factors may influence disease progression.

In subsequent studies, we have shown that the molecular basis for this disease is based upon a missense mutation in a gene for a prenyltransferase-like mitochondrial protein (PLMP), which leads to mitochondrial abnormalities in the renal epithelial cells [10]. Ultrastructural abnormalities are also present in the podocytes of *kd/kd* mice, and these mice have additional features of collapsing glomerulopathy [11]. We now report that *kd/kd* mice raised under GF conditions have significant reductions in all known manifestations of the mutant phenotype, including the mitochondrial characteristics.

2. Materials and methods

2.1. Mice

The CBA/CaH-*kd/kd* strain derived by Lyon and Hulse [4] is no longer available. We therefore transferred the *kd* allele to the C57BL/6J (B6) background by selection for closely linked markers that were studied in a mapping experiment [9]. Homozygous mice obtained after 12 generations of backcrossing are designated B6.CBACaH(CAST)-kd/Upa (B6.*kd/kd)*. Mice in the specific pathogen-free (SPF) colony were fed the Lab-Diet 5010 Autoclavable Rodent Diet (PMI, Brentwood, MO), which has at least 23% crude protein and at least 4.5% crude fat. Mice on the calorie-restricted (CR) diet received the same chow, but were given approximately 50% of the normal caloric intake [12].

2.2. Germfree derivation

Pregnant B6.*kd/kd* mice were sacrificed and their gravid uteri were excised and passed into the GF isolator, where the pups were removed and fostered to GF BALB/c dams. Mice in the GF isolators were fed the LabDiet 5021 Autoclavable Breeder Diet (PMI, Brentwood, MO), which consists of at least 20.0% crude protein and at least 9% crude fat.

2.3. Histology

Kidneys were bisected, fixed in formalin and paraffin-embedded; 4 μm sections through the longitudinal axis of each kidney were prepared and stained with hematoxylin and eosin. The occurrence of interstitial nephritis was evaluated by one observer (MPM), blinded to the origin of the specimens, according to previously described methods [8]. Sections of kidneys in which there were small pockets of 5–10 mononuclear cells were not considered positive by this method, as small focal cellular infiltrates are occasionally also seen in non-mutant controls [8].

2.4. Electron microscopy

Kidney tissue samples were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C. After three cacodylate buffer washes, the samples were post-fixed with 2.0% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°C. After two additional sodium cacodylate washes and a wash in dH_2O, samples were stained with 2% aqueous uranyl acetate for 30 min at room temperature. Samples were rinsed again in H_2O subsequent to dehydration in graded ethanols, and infiltration and embedding in Embed-812 (Electron Microscopy Sciences, Fort Washington, PA). All sections were examined in a JEOL 1010 electron microscope, and digital images were recorded with a Hamamatsu camera system.

3. Results

A typical lesion seen in B6.*kd/kd* mice maintained under SPF conditions is shown in Fig. 1A, which is an H&E-stained section from a 153-day-old mouse. Fig. 1B shows a representative section from a GF B6.*kd/kd* mouse at 226 days of age. As shown in Table 1, although GF conditions did not completely prevent the occurrence of kidney disease in B6.*kd/kd* mice, there were significantly fewer positive animals at every age range that was tested, in comparison to B6.*kd/kd* mice raised under SPF conditions.

A subset of these mice was also evaluated for the mitochondrial phenotype by electron microscopy. Mutant (B6.*kd/kd*) and control (B6.*kd*/+ or B6) mice which had been raised under SPF conditions were sacrificed at various ages, and kidney sections were analyzed. The mitochondria from young B6.*kd/kd* mice at ages of 9 and 20 days were essentially normal (not shown). A section from the youngest B6.*kd/kd* mouse (42 days old) in which the mitochondrial phenotype was clearly identified is shown in Fig. 2A, along with a B6.*kd*/+ littermate control, Fig. 2B). A normal number of matrix granules were present in the B6.*kd*/+ control, but there was a deficiency of matrix granules in the B6.*kd/kd* mouse. Among the SPF B6.*kd/kd* mice that were evaluated at various ages over 50 days, most had essentially no matrix granules in the mitochondria. However, all of the GF mice that were studied by electron microscopy were normal (Table 2). Of the 11 SPF B6.*kd/kd* mice that were examined by electron microscopy, only one mouse (in the 50–100 day age group) had a normal phenotype with regard to the mitochondria, whereas all eight of the GF mice had normal mitochondria, and all of these were sacrificed above the age of 100 days ($X^2 = 15.35$; $p < 0.0001$). Representative sections are shown in Fig. 2C and D.

There were also overt histologic benefits of the GF environment related to podocyte morphology that can be demonstrated by electron microscopy. Fig. 3A shows a typical section from a 173-day-old B6.*kd/kd* SPF mouse with podocyte effacement as recently reported [11]. In contrast, in Fig. 3B is a section from a 218-day-old B6.*kd/kd* mouse raised under GF conditions, with no sign of podocyte effacement. The podocytes of eight additional GF mice, all over 100 days of age, were examined by electron microscopy, with no evidence of podocyte effacement.

Caloric restriction has been shown to abrogate the phenotype of CBA/CaH-*kd/kd* mice [12], so the same protocol was followed with the B6.*kd/kd* strain. As shown in Table 3, we have obtained the same result with the *kd/kd* genotype on the B6 background as was previously reported with CBA/CaH-*kd/kd* mice.

4. Discussion

The phenotype of the *kd/kd* mouse represents an interaction between immunological and non-immunological events, and raising these mice under GF conditions provided further insights

into disease pathogenesis. Matrix granules are normally present in mitochondria, and their disappearance from the mitochondria of renal tubular epithelial cells of *kd/kd* mice under SPF conditions is presumably not immunologically mediated. However, the current results demonstrate that this effect is influenced by a GF environment. One possible explanation for the effect of the GF environment in this case would be that intestinal flora are normally kept at bay through local immunity, which involves a certain baseline synthesis of cytokines, chemokines and other mediators. Release of soluble factors into the circulation leads to their systemic circulation and delivery to the kidney. Filtration and concentration in the urine could have profound effects on renal tubular epithelia, in a manner analogous to that found in a local immune response. This could potentially activate innate and adaptive immune responses. A precedent for this exists in inflammatory bowel disease, in which *E. coli* stimulate inflammation [13]. Furthermore, commensal bacteria activate Toll-like receptors [14,15], which have been linked to renal inflammation [16]. Another possible contributing factor is that cells with the *kd/kd* genotype are more prone to apoptosis than wild-type cells, and the GF environment is protective against whatever the stimulus is that normally induces these cells to become apoptotic.

The kidney disease phenotype can be prevented in *kd/kd* mice with either a CBA or B6 background by caloric restriction. Although the mechanism for caloric restriction-associated phenotypic changes is not well understood, there is considerable evidence to support the important role played by mitochondria [17–21]. The *kd* gene codes for a defective mitochondrial protein [10], so the function served by this protein is apparently affected by a GF environment. Reactive oxygen species (ROS) are generated by the mitochondrial electron transport chain, and these molecules are highly damaging to membrane lipids, mitochondrial and nuclear DNA, mitochondrial proteins, and other molecules. As caloric restriction reduces the generation of ROS, it has a variety of protective effects. The protective effect of the GF environment may be analogous to reduction in stress in the gut, leading to less cytokine and chemokine release into the circulation, with consequent less stress on renal epithelia. Either caloric restriction or a GF environment would reduce injury to mitochondria by unknown mechanisms. We therefore speculate that processes that stimulate mitochondrial energy demand may be involved in the initiation of the process. It is not yet clear how the defective gene product in *kd/kd* mice is related to this cascade. Previous data suggested that an increased amount of PLMP can be detected in the mitochondria of *kd/kd* kidneys [10], and we believe that this can be attributed to a compensatory increase in synthesis which results from reduced enzymatic activity. Studies with conditional knockouts are in progress which will hopefully resolve this issue.

Case reports of patients with tubulointerstitial nephritis who proved to have deletions or mutations in their mitochondrial DNA provide further evidence for the importance of these processes [22,23]. The *kd/kd* mouse will most likely be an extremely valuable model for investigating mitochondria-related mechanisms leading to both epithelial injury and to autoimmunity. In this regard, analysis of the conditions under which an inherited defect that is unrelated to the immune system triggers a lethal autoimmune reaction should be especially interesting.

Acknowledgements

We thank Drs Harry Rozmiarek and John Cebra for valuable support and advice. Dr Cebra's untimely death while this manuscript was in review has saddened all of us, and will surely have a negative impact on the field of gnotobiology. This study was supported by National Institutes of Health Grants DK 55852, P30-DK 50306 and RR12211; we thank the Morphology Core in the Center for Digestive and Liver Disease for histological preparations.

References

1. Maldonado MA, Kakkanaiah V, MacDonald GC, Chen F, Reap EA, Balish E, et al. The role of environmental antigens in the spontaneous development of autoimmunity in MRL/*lpr* mice. J Immunol 1999;62:6322–30. [PubMed: 10352243]
2. East J, Branca M. Autoimmune reactions and malignant changes in germ-free New Zealand Black mice. Clin Exp Immunol 1969;4:621–35. [PubMed: 4891243]
3. Contractor NV, Bassiri H, Reya T, Park AY, Baumgart DC, Wasik MA, et al. Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnotobiotic IL-2-deficient mice. J Immunol 1998;160:385–94. [PubMed: 9551995]
4. Lyon MF, Hulse EV. An inherited kidney disease of mice resembling human nephronophthisis. J Med Genet 1971;8:41–8. [PubMed: 5098070]
5. Neilson EG, McCafferty E, Feldman A, Clayman MD, Zakheim B, Korngold R. Spontaneous interstitial nephritis in kdkd mice. I. An experimental model of autoimmune renal disease. J Immunol 1984;133:2560–5. [PubMed: 6384368]
6. Kelly CJ, Korngold R, Mann R, Clayman M, Haverty T, Neilson EG. Spontaneous interstitial nephritis in kdkd mice. II. Characterization of a tubular antigen-specific, H-2K-restricted Lyt-2^+ effector T cell that mediates destructive tubulointerstitial injury. J Immunol 1986;136:526–31. [PubMed: 2416810]
7. Sibalic V, Fan X, Wuthrich RP. Characterization of cellular infiltration and adhesion molecule expression in CBA/CaH-*kdkd* mice with tubulointerstitial renal disease. Histochem Cell Biol 1997;108:235–42. [PubMed: 9342617]
8. Hancock WW, Tsai T-L, Madaio MP, Gasser DL. Cutting edge: multiple autoimmune pathways in *kd/kd* mice. J Immunol 2003;171:2778–81. [PubMed: 12960297]
9. Dell KM, Li Y-X, Peng M, Neilson EG, Gasser DL. Localization of the mouse kidney disease (kd) gene to a YAC/BAC contig on Chromosome 10. Mammalian Genome 2000;11:967–71. [PubMed: 11063251]
10. Peng M, Jarett L, Meade R, Madaio MP, Hancock WW, George AL Jr, et al. Mutant prenyltransferase-like mitochondrial protein (PLMP) and mitochondrial abnormalities in *kd/kd* mice. Kidney Int 2004;66:20–8. [PubMed: 15200409]
11. Barisoni L, Madaio MP, Eraso M, Gasser DL, Nelson PJ. The *kd/kd* mouse is a model of collapsing glomerulopathy. J Am Soc Nephrol 2005;16:2847–51. [PubMed: 16120817]
12. Fernandes G, Yunis EJ, Smith J, Good RA. Nutritional inhibition of genetically determined renal disease and autoimmunity with prolongation of life in *kdkd* mice. Proc Natl Acad Sci U S A 1978;75:2888–92. [PubMed: 275859]
13. Schuppler M, Lotzsch K, Waidman M, Autenrieth IB. An abundance of *Escherichia coli* is harbored by the mucosa-associated bacterial flora of Interleukin-2-deficient mice. Infect Immun 2004;72:1983–90. [PubMed: 15039318]
14. Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol 2002;14:103–10. [PubMed: 11790539]
15. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229–41. [PubMed: 15260992]
16. Anders H-J, Banas B, Schondorff D. Signaling danger: Toll-like receptors and their potential roles in kidney disease. J Am Soc Nephrol 2004;15:854–67. [PubMed: 15034087]
17. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science 1996;273:59–63. [PubMed: 8658196]
18. Gabbita SP, Butterfield DA, Hensley K, Shaw W, Carney JM. Aging and caloric restriction affect mitochondrial respiration and lipid membrane status: an electron paramagnetic resonance investigation. Free Radic Biol Med 1997;23:191–201. [PubMed: 9199881]
19. Lass A, Sohal BH, Weindruch R, Forster MJ, Sohal RS. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. Free Radic Biol Med 1998;25:1089–97. [PubMed: 9870563]

20. Gredilla R, Sanz A, Lopez-Torres M, Barja G. Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. FASEB J 2001;15:1589–91. [PubMed: 11427495]

21. Lopez-Torres M, Gredilla R, Sanz A, Barja G. Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. Free Radic Biol Med 2002;32:882–9. [PubMed: 11978489]

22. Szabolcz MJ, Seigle R, Shanske S, Bonilla E, DiMauro S, D'Agati V. Mitochondrial DNA deletion: a cause of chronic tubulointerstitial nephropathy. Kidney Int 1994;45:1388–96. [PubMed: 8072250]

23. Zsurka G, Ormos J, Ivanyi B, Turi S, Endreffy E, Magyari M, et al. Mitochondrial mutation as a probable causative factor in familial progressive tubulointerstitial nephritis. Hum Genet 1997;99:484–7. [PubMed: 9099838]

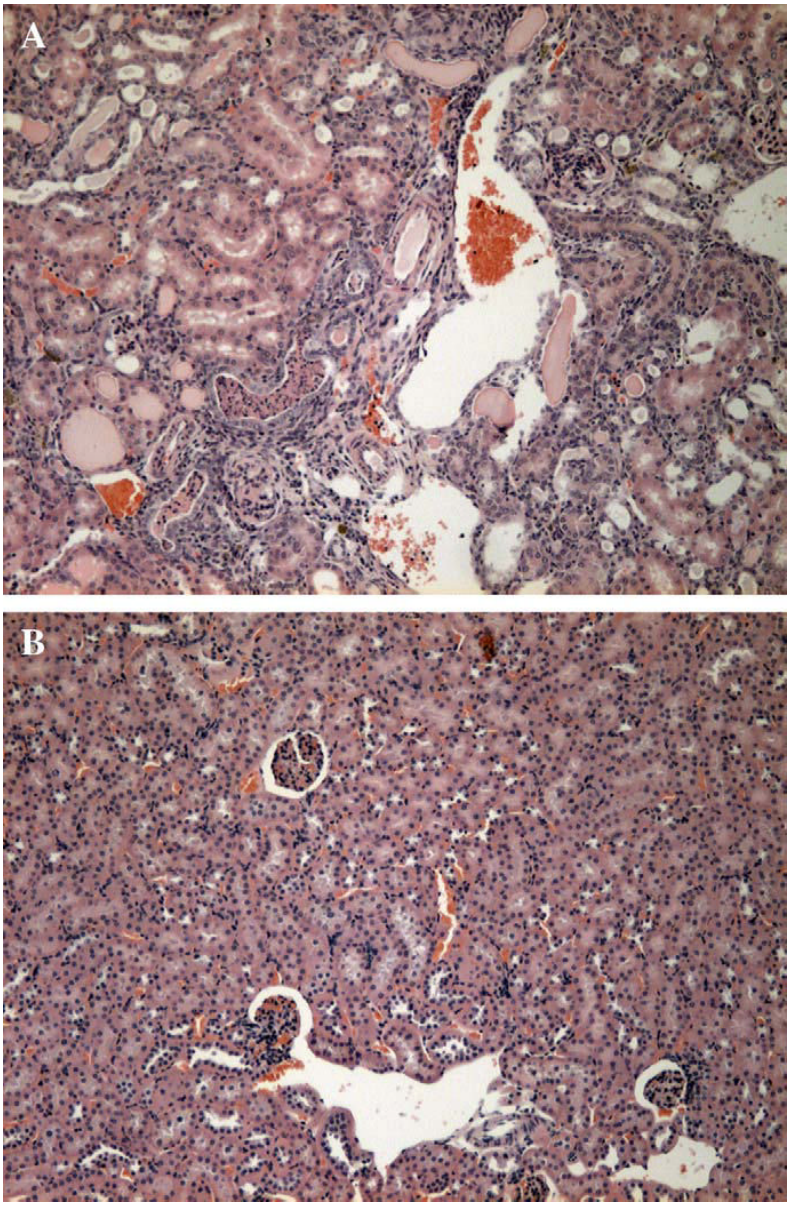

Fig. 1.

H&E-stained sections of kidneys of B6.*kd/kd* SPF and B6.*kd/kd* GF mice. (A) Severe interstitial with mild tubular dilatation in a 153-day-old SPF mouse. (B) No disease in a 226-day-old GF mouse. Magnification is 100×.

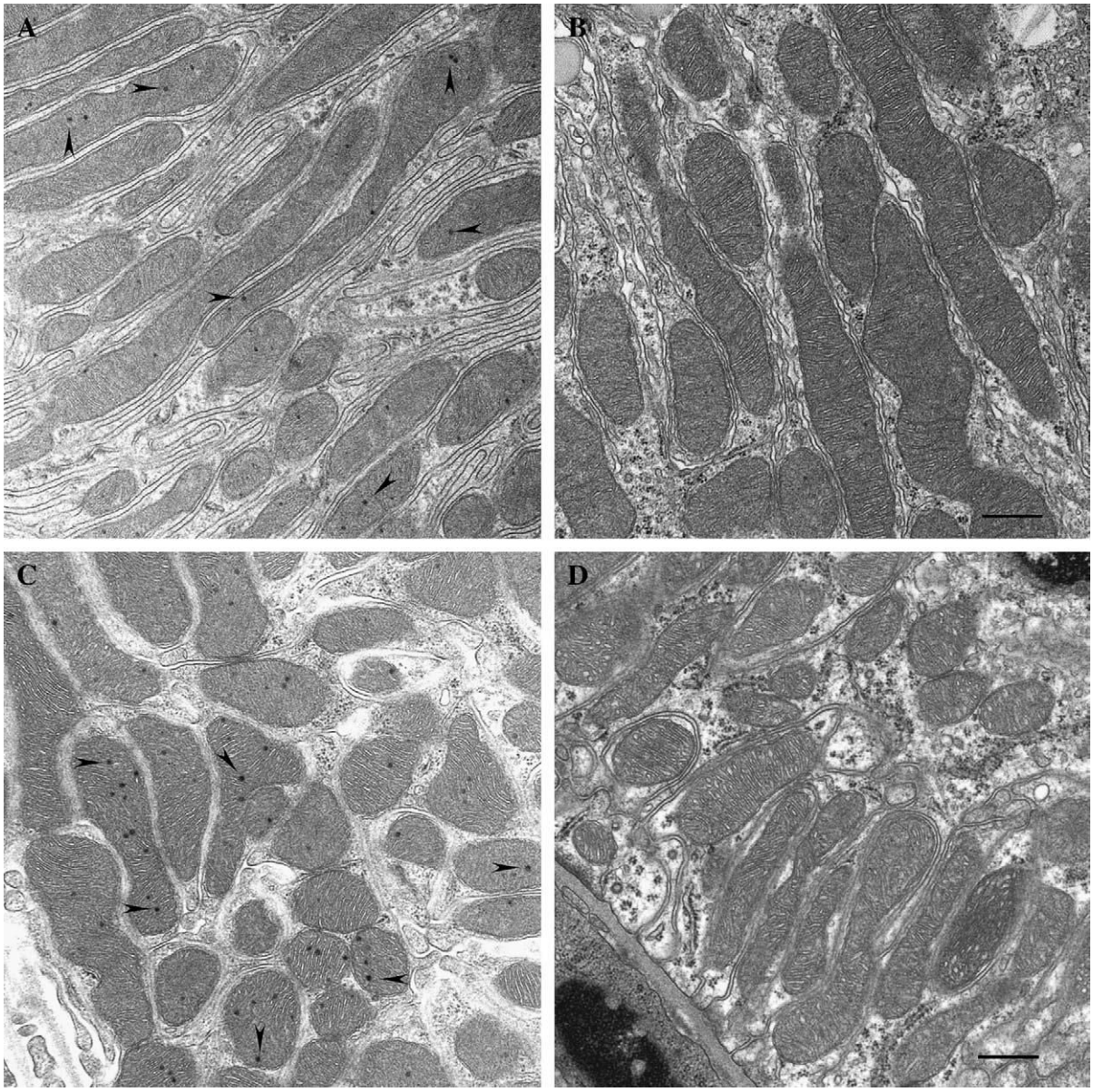

Fig. 2.

Electron micrographs of kidney mitochondria from mice maintained under SPF or GF conditions. (A) Kidney mitochondria from an SPF B6.*kd*/+ mouse at age 42 days. (B) Kidney mitochondria from an SPF B6.*kd/kd* mouse at age 42 days. (C) Kidney mitochondria from a B6.*kd/kd* mouse at 155 days of age maintained under GF conditions. (D) Kidney mitochondria from an SPF B6.*kd/kd* mouse at 102 days of age. Matrix granules are indicated by arrows in (A) and (C). Magnification is 30,000×; scale bar, 500 nm.

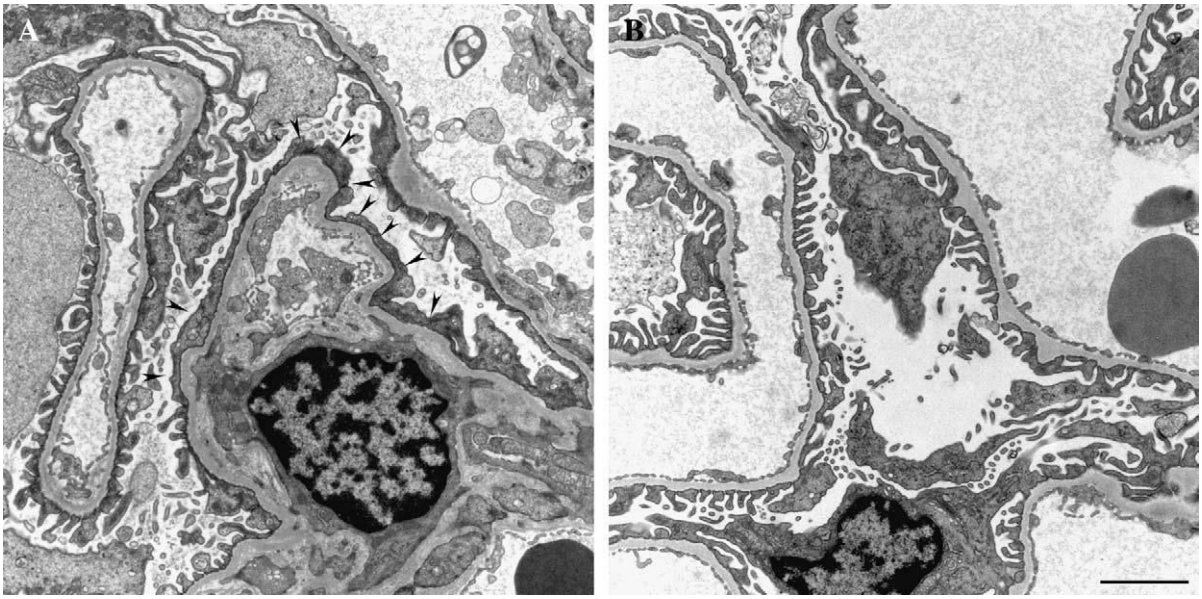

Fig. 3.

Comparison of electron micrographs of glomeruli from B6.*kd/kd* mice raised under SPF and GF conditions. (A) 173-day-old SPF mouse; podocyte effacement indicated by arrows. (B) 218-day-old GF mouse. Magnification in both is 10,000×; scale bar, 500 nm.

Table 1

Occurrence of interstitial nephritis in B6.*kd/kd* mice at various ages under SPF or GF conditions

Age at dissection (days)	Number positive/total dissected		χ^2	*p*
	SPF	GF		
100–120	13/19	1/20	17.03	<0.00001
121–140	8/9	1/5	6.64	<0.05
Over 140	26/27	3/15	26.26	<0.0001

Table 2

Matrix granules in mitochondria of SPF and GF mice

Mice	Conditions	Age (days)	Mutant phenotype[a]
B6	SPF	50–100	0/5
B6	SPF	>100	0/4
B6.*kd/kd*	SPF	50–100	5/7
B6.*kd/kd*	SPF	>100	3/4
B6.*kd/kd*	GF	>100	0/8

[a]The mutant phenotype is defined as having essentially no matrix granules in the mitochondria.

Table 3

Effect of caloric restriction on frequency of interstitial nephritis in B6.*kd/kd* mice[a]

Number positive/total dissected		χ^2	*p*
Normal diet	**Caloric restriction**		
50/67	0/9	19.6	<0.0001

[a] All of the mice in this experiment were between 100 and 180 days of age.