0031-3998/91/2910-0024$03.00/0
PEDIATRIC RESEARCH

Vol. 29, No. 1, 1991
Printed in U.S.A.

Inherited Selective Intestinal Cobalamin Malabsorption and Cobalamin Deficiency in Dogs

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ABSTRACT. Inherited selective intestinal malabsorption of cobalamin (Cbl) was observed in a family of giant schnauzer dogs. Family studies and breeding experiments demonstrated simple autosomal recessive inheritance of this disease. Affected puppies exhibited chronic inappetence and failure to thrive beginning between 6 and 12 wk of age. Neutropenia with hypersegmentation, anemia with anisocytosis and poikilocytosis, and megaloblastic changes of the bone marrow were present. Serum Cbl concentrations were low, and methylmalonic aciduria and homocysteinemia were present. Parenteral, but not oral, cyanocobalamin administration rapidly eliminated all signs of Cbl deficiency except for low serum Cbl concentrations. Cbl malabsorption in affected dogs was documented by oral administration of [^{57}Co]cyanocobalamin with or without simultaneous oral administration of intrinsic factor or normal dog gastric juice. Quantitation and function studies of intrinsic factor and transcobalamin-II from affected dogs revealed no abnormality. Other gastrointestinal functions and ileal morphology were normal, indicating a selective defect of Cbl absorption at the level of the ileal enterocyte. Immunoelectron microscopy of ileal biopsies showed that the receptor for intrinsic factor-Cbl complex was absent from the apical brush border microvillus pits of affected dogs. This canine disorder resembles inherited selective intestinal Cbl malabsorption (Imerslund-Gräsbeck syndrome) in humans, and is a spontaneously occurring animal model of early onset Cbl deficiency. (*Pediatr Res* 29: 24–31, 1991)

Abbreviations

Cbl, cobalamin
IF, intrinsic factor
IF-Cbl, intrinsic factor-cobalamin complex
TC-II, transcobalamin-II
CN-Cbl, cyanocobalamin
[^{57}Co]CN-Cbl, radioisotopically labeled cyanocobalamin
UCBC, unsaturated cobalamin binding capacity
MMA, methylmalonic acid
THCys, total homocysteine

Received June 1, 1990; accepted August 13, 1990.
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Supported by the NIH University of Pennsylvania Human Genetics Center (NIH Grant GM 32592), the National Referral Center for Animal Models of Human Genetic Disease (NIH Grant RR 02512), the Mrs. Cheever Porter Foundation, and the Lucille B. Markey Charitable Trust.

Dietary Cbl absorption is a complex and highly specific process (1). Cbl is freed from foodstuffs and binds to IF, a glycoprotein produced by the gastric mucosa and, in dogs, also by the pancreatic duct epithelium (2, 3). IF-Cbl complex binds specifically to a receptor in the microvillus pits of enterocytes lining the ileum (4). Transcytosis of Cbl subsequent to IF-Cbl receptor binding is a poorly understood process, but newly absorbed Cbl is found in portal circulation bound to TC-II for transport to tissues. Several inherited defects causing selective Cbl malabsorption and life-threatening Cbl deficiency in the first few years of life have been described in humans. These include secretion of insufficient or abnormal IF (5–7) and selective intestinal malabsorption of Cbl (5, 8–10). TC-II deficiency is also associated with Cbl malabsorption, but the greater severity and earlier onset of clinical signs of this disease are related primarily to impaired transport of Cbl between tissues (11). The molecular or cellular defects causing selective intestinal Cbl malabsorption are unknown.

In our report, we describe clinical, metabolic, pathologic, and genetic features of inherited selective intestinal Cbl malabsorption in a large family of dogs. We also provide immunohistochemical evidence that absence of the receptor for IF-Cbl complex from the ileal apical brush border is the cause of Cbl malabsorption in this family of dogs.

MATERIALS AND METHODS

Animals. Seventeen affected dogs were studied. Detailed clinical and laboratory descriptions of the proposita and a related purebred giant schnauzer have been published (12). Eight offspring of a mating of those two dogs and seven mixbred offspring of F_1-backcross and F_2 matings were produced. Clinically normal littermates (obligate heterozygotes from F_1-backcross matings) served as controls in metabolic and hematologic studies. In Cbl absorption tests, five normal dogs (two standard poodles, one beagle, and two keeshonds) that were 15–26 mo old, weighing 11–31 kg, served as controls. The dogs were maintained in the facilities of the University of Pennsylvania Unit of Laboratory Animal Resources, where they were allowed water *ad libitum* and fed a complete and balanced, commercial canine maintenance diet (Lab Canine Diet no. 5006, Ralston-Purina Co., St. Louis, MO). Puppies were weaned at 1 mo of age, and lactating bitches and puppies under 6 mo of age were fed a commercial puppy diet (Purina Puppy Chow, Ralston-Purina Co.) supplemented with canned meat (Big Bet, Big Bet Pet Foods, Dublin, PA). Affected dogs exhibiting clinical, hematologic, and metabolic signs of Cbl deficiency received 370–740 nmol (0.5–1.0 mg) of CN-Cbl intramuscularly as needed to maintain remission of clinical and laboratory abnormalities. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Reagents. [^{57}Co]CN-Cbl (300 μCi/nmol) was obtained from Amersham Corp., Inc., Arlington Heights, IL. Crystalline CN-Cbl, cyanocobinamide, Sepharose-bound Cbl, and practical grade porcine secretin were obtained from Sigma Chemical Co., St. Louis, MO. Reagents used in Cbl absorption studies *in vivo* were gelatin capsules each containing 0.56 μCi of [^{57}Co]CN-Cbl (1.14 μCi/nmol), gelatin capsules each containing one unit (National Formulary-XI) of porcine IF, a reference solution of [^{57}Co]Cl_2, and unlabeled CN-Cbl for injection (Rubratope-57 Diagnostic Kit). These and cholecystekinin (Kinevac) were obtained from Squibb Diagnostics, New Brunswick, NJ. Rabbit anti-dog ileal IF-Cbl receptor serum was the generous gift of Dr. Bellur Seetharam, Medical College of Wisconsin, Milwaukee.

Hematologic and metabolic studies. Complete blood counts were done by routine methods. Bone marrow aspirates and core biopsy specimens were collected from the iliac crest under ultrashortacting barbiturate anesthesia. Serum Ig concentrations were measured by radial immunodiffusion using canine-specific reagents. Serum Cbl and folate concentrations were measured by a commercial radiobinding assay (Quantaphase B_{12}/Folate Radioassay, Bio-Rad, Hercules, CA).

Urinary organic acids were analyzed by gas-liquid chromatography and mass spectrometry after extraction and conversion to trimethylsilyl derivatives, with hexadecandioic acid as internal standard. Free serum and urinary amino acids were separated and quantified by ion-exchange chromatography using a Beckman model 7300 amino acid analyzer (Beckman Instruments, Palo Alto, CA) with lithium citrate buffers. Total serum homocysteine (THCys=free + disulfide bound + protein bound) was measured by a previously described method of capillary gas chromatography-mass spectrometry with selective ion monitoring and deuterated homocysteine as an internal standard (13). Endogenous creatine clearance (14) and 24-h protein excretion (15) were determined by reported methods.

Cbl absorption studies. Three Cbl-treated affected dogs, one heterozygous dog, and five normal control dogs underwent radiolabeled-Cbl absorption tests. The proposita was 51 mo old and weighed 32 kg. A heterozygous normal and two affected F_1-backcross littermates were 9 mo old and weighed 32–38 kg. Dogs were housed separately in cages with grated floors, and received 740 nmol (1 mg) of CN-Cbl intramuscularly 4 and 2 d before starting the study. They were fasted for 12 h before each phase of study.

Intestinal absorption of Cbl was assessed by oral administration of 0.49 nmol (0.66 μg) [^{57}Co]CN-Cbl alone (phase I) or with simultaneous oral administration of IF or normal dog gastric juice (phase II). In phase I, a single capsule of [^{57}Co]CN-Cbl was administered orally to each dog, and 2 h later, 740 nmol (1 mg) of unlabeled CN-Cbl was administered intramuscularly. The dogs were fed at that time and every 12 h thereafter. Blood samples were collected in heparinized tubes at the time of [^{57}Co]CN-Cbl administration and repeatedly thereafter for 24 h. For 6 d, all feces were collected immediately when passed to prevent contamination with urine. Phase II tests in the three affected dogs and the heterozygous dog began 10 d after the beginning of the phase I. Phase II was done identically to phase I except that each dog received two units of IF orally with a capsule of [^{57}Co]CN-Cbl. Ten d after the beginning of phase II, phase II was repeated in two of the affected dogs and the heterozygous dog as before, except that 130 mL of normal dog gastric juice was administered with the [^{57}Co]CN-Cbl.

The radioactivity of 2.4-mL aliquots of each plasma sample was determined in a gamma counter and expressed as the fraction of the administered dose in the calculated total plasma volume (0.05 of total body weight). For determination of fecal excretion of radioactivity, stools were compressed into even layers in the bottom of cylindrical cardboard containers. The height of each fecal layer was recorded, and the radioactivity of each container of feces was determined in a deep-well gamma counter. A linear correction factor was calculated to correct for variable fecal sample geometry by counting 10 mL of [^{57}Co]Cl_2 reference solution diluted with varying volumes of tap water. Fecal excretion of [^{57}Co] was expressed as the fraction of the administered dose.

IF and TC-II characterization. Gastric juice was collected through a fiberoptic endoscope from anesthetized dogs with or without pentagastrin administration (6 μg/kg body weight). Aliquots were taken for gastric acid titration and the remaining gastric juice was immediately chilled and depepsinized by raising the pH to 10.0 for 20 min with 2 M NaOH. After neutralization to pH 7.2 with 2 M HCl, gastric juice was stored at −20°C. Pancreatic juice was collected essentially as described (16). We modified the procedure by collecting pancreatic secretions for 6 h under continuous i.v. infusion of 1 unit secretin/(kg·h) and 0.1 μg cholecystekinin/(kg·hr). Two mM phenylmethylsulfonylfluoride (final concentration) was added to collection tubes before sample collection.

Serum, gastric juice, and pancreatic juice UCBC were determined by modification of the method of Gottlieb *et al.* (17). IF determinations were done by the cyanocobinamide-blocking assay of Begley and Trachtenberg (18). IF was purified from gastric and pancreatic juice samples essentially by the method of Allen and Mehlman (19), except that before application of clarified and buffered samples to the affinity column, non-IF Cbl-binding proteins were blocked with 1000-fold excess of cyanocobinamide. Binding assays of purified IF to Triton X-100 extracts of tissue homogenates were performed by the method of Seetharam *et al.* (20). *In vivo* function of the affected dog's purified pancreatic IF was tested in normal dogs. At laparotomy, the lumen of a 30-cm segment of ileum (30 cm proximal to the ileocecal valve) was washed with 500 mL of warmed buffered electrolyte solution (Normosol-R, Abbott Laboratories, North Chicago, IL) with 10 mM Ca-gluconate added. The segment was isolated with Doyen's intestinal clamps, and a cannula was placed at the confluence of mesenteric veins draining the segment. A sample of 3.7 pmol of affected dog IF bound to [^{57}Co]CN-Cbl (0.75 μCi) in 20 mL of the lavage solution was put into the lumen of the isolated segment through a 26-gauge needle. Seven hundred forty nmol (1 mg) of unlabeled CN-Cbl were given i.v. at the same time and intramuscularly 90 min later. Blood samples were taken from the mesenteric vein cannula at 30-min intervals for 4 h. Plasma radioactivity was determined in a gamma counter. In another dog, the same procedure was done except that 3.7 pmol (0.75 μCi) of free [^{57}Co]CN-Cbl was placed in the isolated ileal segment.

Sephadex-G200 gel filtration of Cbl-binding proteins was performed by previously described methods (21). Serum samples for TC-II analysis were separated from blood cells by centrifugation immediately upon collection without anticoagulant. Cultured fibroblasts were derived from skin biopsies taken under local anesthesia. Fibroblast culture and TC-II function studies were performed as previously described (22). In brief, serum samples were labeled to excess with [^{57}Co]CN-Cbl, and free label was removed with albumin-coated charcoal to measure binding capacity. Flasks containing 3 mL minimal essential medium without FCS were seeded with 5×10^5 cells/flask of normal dog fibroblasts. Serum, with TC-II labeled to capacity, was added to each flask at 185 fmol TC-II-[^{57}Co]CN-Cbl/mL of medium. Cells were cultured for 24 h at 37°C, harvested with trypsin, suspended, washed, sonicated, and centrifuged. Radioactivity of each fraction was determined at each step. Cell-associated label resisting trypsin and washing and remaining in the soluble fraction after sonication and centrifugation was interpreted to have been bound and internalized. Cbl-binding proteins elaborated by cultured fibroblasts into the culture medium were identified by gel filtration.

Immunoelectron microscopy. Ileal biopsies were preserved and prepared for electron microscopic examination as previously described (4). Sections were coded, and the examiner did not know which were from affected and which were from normal dogs.

Statistical analysis. Results of breeding studies were assessed by the χ^2 test. Serum Cbl concentrations of affected dogs and clinically normal littermates were compared by analysis of variance. Fecal [^{57}Co] excretion data were compared using the Wilcoxon rank-sum test, and plasma radioactivity data were compared by analysis of variance for repeated measures.

RESULTS

Disease Manifestations. Clinical features. Detailed clinical and laboratory information about the proposita and another affected purebred giant schnauzer has been published previously (12). In those and all affected puppies subsequently studied, onset of clinical signs occurred between 6 and 12 wk. Most apparent were chronic inappetence and failure to thrive. Linear skeletal growth was normal or near normal, but affected puppies did not gain weight normally and were weak. Muscle mass was poorly developed and, by 4 mo of age, the puppies were cachectic. During intercurrent febrile episodes, they became anorectic and were markedly depressed.

Laboratory features. Absolute neutropenia (0.66–3.8 × 10^9/L, littermate controls 5.2–11.6 × 10^9/L) developed in each affected puppy between 7 and 16 wk of age and was followed by development of nonregenerative anemia (packed cell volume 0.21–0.33; littermate controls 0.38–0.45) that was evident by 20 wk of age. Although erythrocyte Wintrobe indices were normal, blood smears revealed moderate to severe anisocytosis and poikilocytosis. Large ovalocytes, hypersegmented neutrophils, giant platelets, and occasional macrocytic and fully hemoglobinized normoblasts with immature nuclei were seen. Platelet numbers were normal. On bone marrow examinations, megaloblastic changes were particularly evident in the myeloid series. Giant metamyelocytes and band forms (Fig. 1) were present. Erythroid precursors were reduced in number, and cellularity of the marrow was normal or decreased. Bone marrow iron stores appeared normal, and serum iron concentrations and total iron-binding capacities were normal. Serum IgG concentrations were low (1.1–4.2 g/L, age-matched normal controls 10–25 g/L) but serum IgA and IgM concentrations were normal.

Before weaning at 1 mo of age, serum Cbl concentrations of less than 75 pmol/L were noted in all puppies, three affected and seven obligate heterozygotes, born in F_1-backcross litters when the bitch, the proposita, had not been supplemented with parenteral CN-Cbl during the last two trimesters of gestation or during lactation. After weaning, serum Cbl concentrations of the heterozygous puppies rapidly increased to above 150 pmol/L, but those of the affected puppies remained <75 pmol/L. In the F_2 litter raised by an obligate heterozygote bitch, only the affected puppy had a low serum Cbl concentration before or after weaning. At 8 wk of age (3 wk postweaning), affected puppies of all litters had significantly lower serum Cbl concentrations (all <75 pmol/L, $n = 7$) than clinically normal littermates (203 ± 11 pmol/L, $n = 11$, $F_{1,17} = 49.66_1$, $p < 0.0001$). Clinically normal, obligate heterozygote puppies had serum Cbl concentrations that were not significantly different from those of unrelated normal dogs (150–375 pmol/L). Serum folate concentrations were normal in all puppies throughout these studies. Low serum Cbl concentrations reported here are expressed as <75 pmol/L because that was the Cbl concentration of the lowest standard in the radiobinding assay used. Serum Cbl concentrations were undetectable in samples from untreated affected puppies when checked by the more sensitive microbiologic assay using *Euglena gracilus* (data not shown).

Urinary organic acid analysis revealed metabolites of alternative pathways of propionyl-CoA catabolism in Cbl-deficient puppies. Large amounts of MMA and smaller amounts of methylcitrate, 3-OH propionic acid, and propionylglycine were present. Methylmalonic aciduria in Cbl-deficient puppies ranged from 4.0 to 46 mol MMA/mol creatinine. Clinically normal littermates and unrelated age-matched control puppies excreted less than 0.034 mol MMA/mol creatinine. Methylmalonic aciduria was noted before weaning in all puppies that were raised by a Cbl-deficient affected bitch, the proposita. After weaning, significant methylmalonic aciduria was present only in affected puppies (Fig. 2). In an F_2 litter whelped and raised by a clinically normal, obligate heterozygote bitch, on the affected puppy had methylmalonic aciduria before or after weaning.

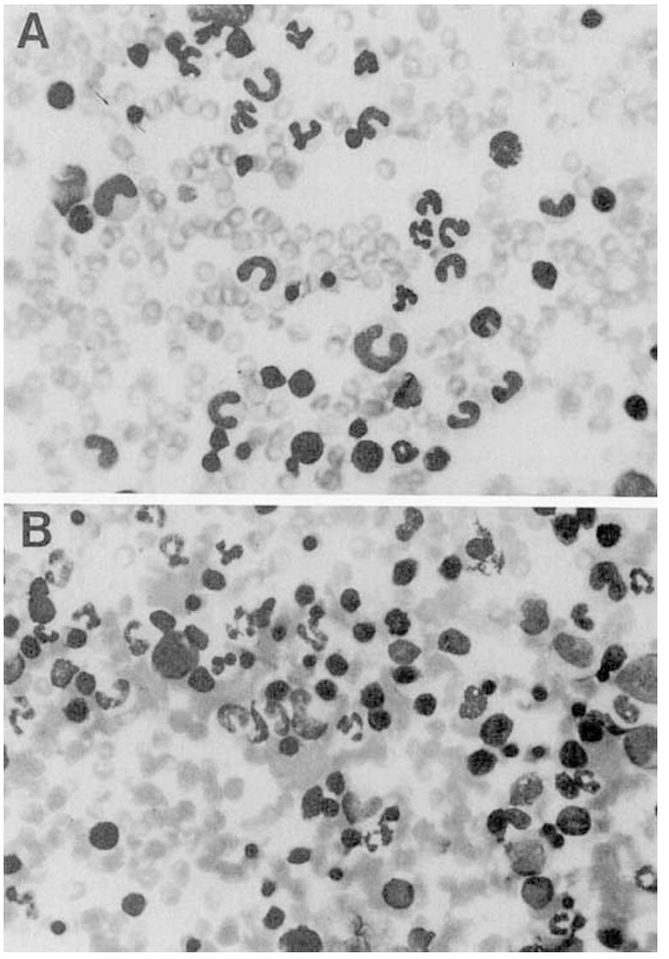

Fig. 1. Bone marrow aspirate from a Cbl-deficient and an age-matched normal puppy. *A*, a typical aspirate from a 20-wk-old, untreated, affected puppy exhibiting clinical and laboratory signs of Cbl deficiency. Megaloblastic changes are visible in some of the myeloid cells shown. Giant metamyelocytes and band neutrophils are present. Erythroid precursors are lacking and cellularity of the marrow is reduced from normal. *B*, a normal bone marrow aspirate is presented for comparison.

Free amino acid concentrations in serum and urine from untreated, affected dogs were within normal limits. However, serum THCys concentration was elevated (23.0 μmol/L; normal adult dogs 6.3 ± 1.4 μmol/L, mean ± SD, $n = 10$) in the proposita at 3 y of age. At that time, the dog had not had parenteral CN-Cbl treatment for 3 mo and her serum Cbl concentration was <75 pmol/mL, but she was showing no clinical signs of Cbl deficiency. One mo subsequent to parenteral CN-Cbl treatment (740 nmol CN-Cbl/d for 7 d), her serum THCys concentration was 6.1 μmol/L.

In urine samples of sp gr 1.035–1.050 from CN-Cbl treated and untreated affected dogs, 2^+ protein was consistently found by a qualitative colorimetric dipstick technique. This, however, was not considered abnormal for dogs with concentrated urine. In more quantitative studies, protein to creatinine ratios were consistently less than 0.4 in affected dogs (normal 0.08–0.54), and per kg body weight 24-h protein excretion determinations [7–12 mg/(kg·d)] using a sensitive quantitative method were

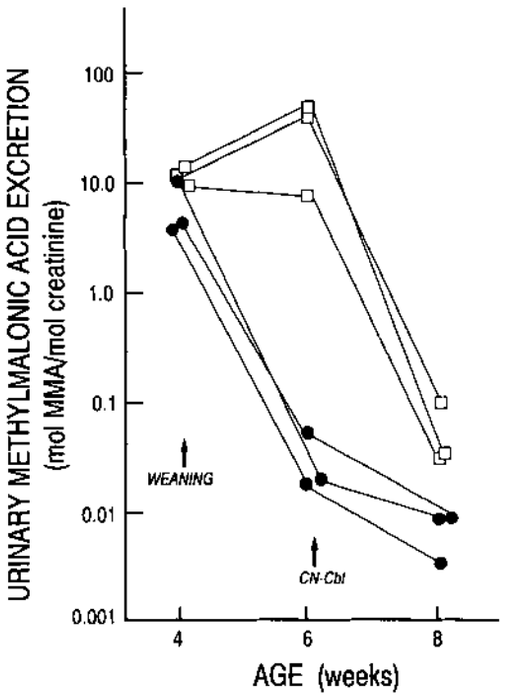

Fig. 2. Urinary MMA excretion. MMA concentrations of urine samples were measured by gas-liquid chromatography, and creatinine concentrations were measured by the picric acid method of Jaffe. The values shown are from urine samples obtained 1 d before weaning, 2 wk postweaning, and 2 wk after parenteral CN-Cbl administration [740 nmol (1 mg), intramuscularly, once]. Three puppies (□) were affected with selective intestinal Cbl malabsorption, and three (●) were normal littermates. All six puppies were the offspring of an F_1-backcross mating, and the dam, the proposita of this study, was Cbl deficient during the latter part of gestation and during lactation.

repeatedly within normal limits for dogs [normal 13.9 ± 7.7 mg/(kg·d)].

Response to treatment. As reported previously (12), no response was seen in a male affected puppy after 7 d of simultaneous parenteral folic acid administration 11 μmol/d (5 mg/d) and oral CN-Cbl administration 7.4 nmol/d (10 μg/d). However, affected puppies that were treated with a single intramuscular injection of 740 nmol (1 mg) of CN-Cbl had a rapid and complete resolution of all clinical, hematologic, and metabolic abnormalities except for persistent subnormal serum Cbl concentrations. Urine collected 10–60 min postinjection was pink (negative for blood or Hb), suggesting that some of the administered CN-Cbl was lost rapidly in the urine. Appetite and weight gain returned in 12 to 48 h. All hematologic abnormalities resolved within 2 to 4 wk. Reticulocytosis (0.024–0.106) began 3–4 d after parenteral CN-Cbl administration and lasted for 10–14 d. MMA excretion decreased to 0.45–0.57 mol MMA/mol creatinine within 4 d of Cbl treatment and ranged between 0.014 and 0.113 mol MMA/mol creatinine for 8 wk thereafter. Serum Cbl concentrations of these puppies were 150–375 pmol/L at 1 wk after parenteral CN-Cbl administration, but dropped below 75 pmol/L by 2 wk posttreatment. A single 740-nmol (1 mg) injection of CN-Cbl in 8-wk-old affected puppies showing signs of Cbl deficiency was sufficient to support normal growth and prevent a relapse of clinical or laboratory abnormalities for 8 wk despite subnormal serum Cbl concentrations. Eight to 10 wk after treatment, the puppies relapsed with methylmalonic aciduria and growth retardation. In six affected puppies 11–17 wk of age, growth retardation and methylmalonic aciduria were rapidly reversed by daily s.c. administration of 1.9–3.7 nmol (2.5–5 μg) of CN-Cbl. Such treatment for 10 d prevented relapse for up to 4 wk. Affected dogs treated with 740 nmol (1 mg) CN-Cbl monthly remained healthy and grew to normal size and weight. Sperm production, ovarian cycling, and fertility were apparently normal in treated animals.

Pathology. A 19- and 28-wk-old affected puppy and a clinically normal, sex-matched littermate of each were euthanized. The older puppies had received 740 nmol (1 mg) of CN-Cbl parenterally at 8 wk of age, but the younger puppies had had no Cbl treatment. A third untreated, affected puppy died suddenly at 24 wk of age. All affected puppies were inappetent and had been losing weight daily at the time of death. Gross and histopathologic findings in the affected dogs were emaciation and moderate to severe lymphoid depletion or hypoplasia of the thymus, lymph nodes, and gastrointestinal lymphoid follicles. One had moderate diffuse atrophy of the gastric mucosa, and all affected puppies had mild to moderate diffuse atrophy of the duodenal and proximal jejunal mucosa and mild to moderate mucosal edema and lymphangiectasia of the entire intestinal tract. Many lymph node macrophages contained phagocytized erythrocytes and hemosiderin, and bone marrow contained mild to moderate amounts of hemosiderin. The bone marrow was hypocellular with scattered aggregates of megaloblastic erythroid and myeloid cells. Small numbers of normally maturing erythrocytes and myelocytes and normal numbers of megakaryocytes were present. No abnormalities were noted in the central or peripheral nervous systems. No significant gross or histopathologic lesions were found in the clinically normal littermates. Examination of biopsy specimens from 28-wk-old affected dogs that were in hematologic and metabolic remission after parenteral CN-Cbl administration revealed no histologic abnormalities of the gastrointestinal tract, lymph nodes, or bone marrow.

Genetic Studies. A pedigree of litters containing affected animals is shown in Figure 3. The proposita (no. 100), a purebred giant schnauzer, was the result of a father-daughter mating. Two male littermates (no. 105 and no. 106) had identical clinical and laboratory signs and were undoubtedly affected but were destroyed before studies of Cbl metabolism could be made. A related male giant schnauzer (no. 70) with a proven defect in Cbl absorption was the only affected puppy in a litter of six. In all, these two litters contained 16 puppies, four of which were affected (three of nine males and one of seven females). Both litters resulted from consanguineous matings between clinically normal parents, and all four parents shared a common ancestor (no. 30). From clinical descriptions by the breeder and the attending veterinarian, another male giant schnauzer (no. 51) sharing the same familial criteria was suspected to have had the same disease, but died before it could be studied. In a mating between two affected dogs (no. 100 and no. 70), all eight puppies (three male and five female) surviving the neonatal period were affected.

These findings strongly suggested autosomal recessive inheritance, and this hypothesis was tested by a series of experimental matings. Offspring were determined as affected when they exhibited growth failure, subnormal postweaning serum Cbl concentrations, hematologic abnormalities, methylmalonic aciduria, and rapid positive response to parenteral CN-Cbl administration. The proposita was outcrossed to a normal male mongrel (no. 90) and produced an F_1 generation of nine puppies, eight of which survived the neonatal period, all clinically normal. Two of the F_1 offspring were mated and produced a litter of 10 F_2 puppies; six survived the neonatal period, and one of these was affected. Normal F_1 males backcrossed to their affected dam produced three litters containing 17 surviving puppies, six of which (three male and three female) were affected. These results were consistent with inheritance of the disease as a simple autosomal recessive trait ($\chi^2 = 1.47$, $df = 1$, $p > 0.25$).

Laboratory Investigations. In all of the investigations described below, affected dogs studied or from which samples were derived had been treated with weekly injections of 740 nmol (1 mg) CN-Cbl and were in clinical, hematologic, and metabolic remission.

Cobalamin absorption tests. To test the hypothesis that affected dogs malabsorbed Cbl, three affected dogs, a clinically normal

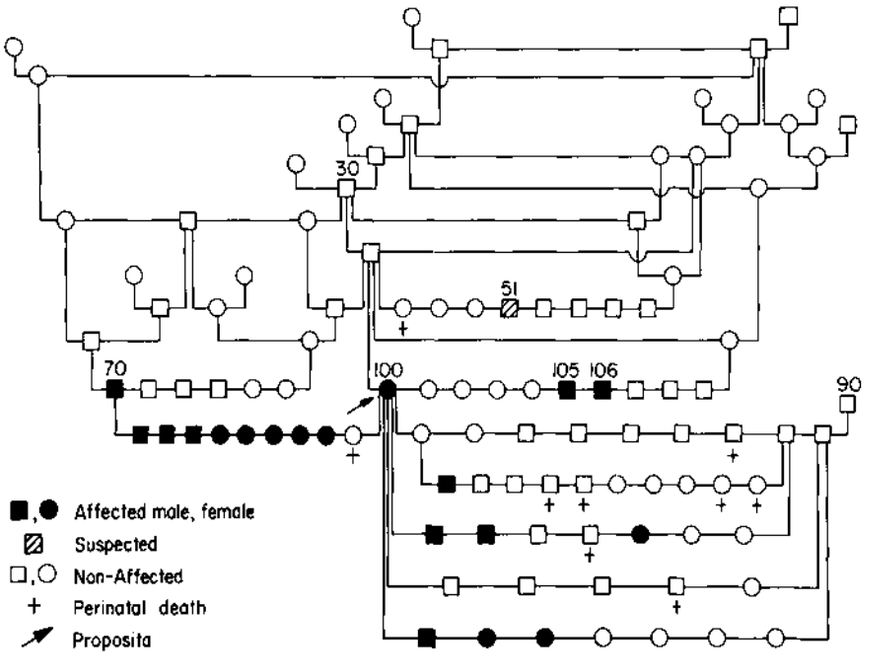

Fig. 3. Pedigree of a family of dogs with inherited selective intestinal Cbl malabsorption. A mating of two dogs is indicated by the symbols for offspring arranged on a horizontal line joining a vertical line from the bottom of the symbol for each parent. The proposita (*arrow*) and dog no. 70 were purebred giant schnauzers. Dog no. 90 was an unrelated, normal mongrel. Note that affected dogs were born to normal parents, that both sexes were affected, and that dog no. 30 is a common ancestor of every parent of an affected dog. These features and the numerical results of experimental outcross and F_1-backcross matings are consistent with simple autosomal recessive inheritance.

heterozygous dog, and five normal control dogs underwent [^{57}Co]CN-Cbl absorption tests. In phase I tests (Fig. 4), significant radioactivity was first detected in the plasma of control dogs 3–4 h after oral administration of [^{57}Co]CN-Cbl and was maximal at 6–7 h (0.013 ± 0.003 of administered dose, mean ± SD). Between 10 and 24 h postadministration, the $t_{1/2}$ of plasma radioactivity was 10.3 h. In contrast, no radioactivity was detected in the plasma of affected dogs at any time point ($p < 0.0002$). The same results were obtained in phase II tests; radioactivity was not detected in the plasma of affected dogs after oral administration of IF or normal dog gastric juice concurrent with the oral administration of [^{57}Co]CN-Cbl. The temporal pattern and magnitude of [^{57}Co]CN-Cbl absorption during phase I in the clinically normal, obligate heterozygote were similar to those during phase I tests in the normal control dogs. Oral administration of IF or gastric juice did not improve or suppress [^{57}Co]CN-Cbl absorption during subsequent phase II tests in this dog.

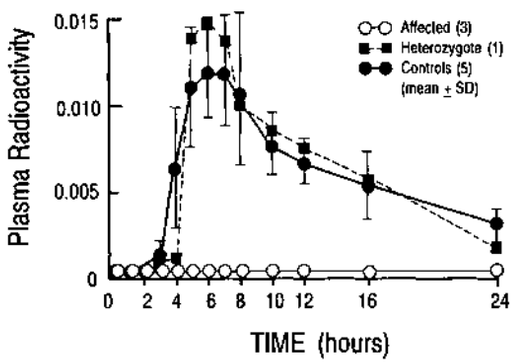

Fig. 4. Plasma radioactivity during phase I oral [^{57}Co]CN-Cbl absorption tests. At time zero, 0.49 nmol (0.67 μg) of [^{57}Co]CN-Cbl (1.14 μCi/nmol) was administered orally to each dog, and 740 nmol (1 mg) of unlabeled CN-Cbl was administered intramuscularly to each dog 2 h later. Values are expressed as the fraction of the administered dose in the total calculated plasma volume (0.05 of body weight). *Solid circles* and *error bars* indicate mean ± 1 SD of the values obtained from five normal control dogs. In the affected dogs, plasma radioactivity was not detected at any time point. The heterozygote was not significantly different from control dogs. The same results were obtained when IF or normal dog gastric juice and [^{57}Co]CN-Cbl were administered concurrently (phase II), suggesting an ileal enterocyte defect of Cbl absorption.

In phase I tests, fecal recovery of radiolabeled Cbl in five control dogs was 0.65 ± 0.13 of the administered dose after 6 d, indicating that at least 0.35 of the administered dose was absorbed. Most of the fecal radioactivity was recovered on d 1 and 2, but 0.04 ± 0.02 of the administered dose was recovered during d 4–6. In contrast, the three affected dogs excreted 0.88 ± 0.05 ($p < 0.074$) of the administered radioactivity within 2 d, and only 0.002 was recovered during d 4–6 ($p < 0.036$).

IF studies. Gastric and pancreatic juices were collected from an affected and a normal dog. In unstimulated gastric secretions, the affected dog produced 11.1 pmol IF/(h·kg body weight), and the normal dog produced 11.6 pmol IF/(h·kg). In both, IF represented about 0.1 of the UCBC. Pentagastrin-stimulated gastric acid secretion was normal in both dogs (23). In the 30-min fraction, 16.4 mL of 0.1 M NaOH were required to neutralize 10 mL of the affected dog's gastric juice to pH 7.0, whereas 17.2 mL were required for the normal dog's. In pancreatic juice, the affected dog produced 77 pmol IF/(h·kg body weight), and the normal dog produced 88 pmol/(h·kg). IF represented 0.5–0.6 of pancreatic juice UCBC in these two dogs.

IF was purified from gastric and pancreatic juice of both dogs by affinity chromatography on Cbl-Sepharose after blocking of non-IF Cbl-binding proteins with 1000-fold excess of cyanocobinamide. Sephadex G-200 gel filtration of the prepared proteins from both dogs gave identical elution profiles, a single symmetrical peak with retention coefficient (V_e/V_o) of 1.58 corresponding to an apparent M_r of 66 000.

The purified pancreatic IF of each dog was assayed for IF-Cb binding to Triton X-100 extracts of homogenates of jejunal and ileal mucosal biopsies from a normal dog. There was no IF-Cb binding activity of either in jejunal extracts. In ileal extracts both affected and normal dog IF exhibited specific IF-Cbl binding

that was Ca^{2+} and pH dependent, binding being inhibited by 10 mM EDTA, pH below 6.0 or above 9.0, or by 1000-fold excess of unlabeled IF-Cbl. One thousand-fold excess of free Cbl, IF devoid of Cbl, or the Cbl-binding protein of dog bile did not inhibit IF-Cbl binding of either normal or affected dog IF to ileal extracts. Affected dog serum did not inhibit either IF binding of Cbl or binding of the IF-Cbl complex to ileal extracts. Affected dog IF bound ileal extracts at 130 fmol/mg ileal protein, and normal dog IF bound at 147 fmol/mg protein. Dissociation constants were 0.46 and 0.53 nM, respectively.

Purified pancreatic IF of the affected dog was bound to [^{57}Co]CN-Cbl and put into an isolated ileal segment of an anesthetized normal dog. Radioactivity was detected in the portal plasma 90 min later and increased to 0.0092 of the administered dose in the total plasma volume at 4 h postadministration. No radioactivity was detected in the portal plasma for 4 h after free radiolabeled Cbl was placed in an isolated ileal segment in a control dog.

TC-II studies. No difference in serum Cbl-binding proteins was detected between affected and control dogs. The UCBC of three affected dogs' serum ranged from 1.39 to 1.98 nmol/L, whereas those of two control dogs were 1.56 and 1.46 nmol/L. In both affected and normal, more than 0.9 of protein-bound Cbl was eluted from a Sephadex-G200 gel filtration column in a single symmetrical peak with retention coefficient (V_e/V_o) of 2.00–2.05. These results are similar to those previously reported for canine serum Cbl-binding proteins, the major peak being TC-II (21). In assays using [^{57}Co]CN-Cbl-labeled serum of an affected and a normal dog, no differences were seen in TC-II-Cbl internalization and incorporation into intracellular soluble material by normal dog fibroblasts. The serum of the affected dog mediated uptake by normal dog fibroblasts of 39.8 fmol [^{57}Co]CN-Cbl/10^6 cells. The normal dog serum mediated uptake by the same normal dog fibroblasts of 41.5 fmol/10^6 cells. Cultured fibroblasts of the affected and normal dogs released TC-II into the culture medium. There were no differences in hepatic uptake and biliary excretion of radiolabel after injection of [^{57}Co]CN-Cbl bound to TC-II into the portal circulation of anesthetized affected and normal dogs (Fyfe JC, Simpson KW, unpublished observations).

Immunoelectron microscopy. Immunohistochemical examination of normal and affected dog ileal biopsies was done by electron microscopy using polyclonal rabbit antibody against IF-Cbl receptor purified from dog ileum (4, 20). In the normal dog biopsy, immunoreactive material was seen on many intracellular membranes, in tubular vesicles, and in particular, in many microvillus pits of the apical brush border of villus tip enterocytes in all of the sections examined (Fig. 5). These findings were exactly as previously reported for dog ileum (4). In contrast, in the affected dog ileal biopsy, although immunostaining was present on the same intracellular structures, IF-Cbl receptor staining was never found on the apical microvillus surface membrane of villus tip enterocytes. These results suggest that the inherited trait causing selective Cbl malabsorption in this family of dogs is absence of IF-Cbl receptor from the apical brush border of the ileum.

DISCUSSION

This family of dogs exhibits inherited selective intestinal Cbl malabsorption that causes life-threatening Cbl deficiency early in life. Family studies and breeding experiments demonstrated simple autosomal recessive inheritance of this trait. Clinical signs, serum Cbl concentrations, urinary MMA concentrations, and [^{57}Co]CN-Cbl absorption of obligate heterozygotes were not different from those of normal controls.

Malabsorption of Cbl in affected dogs was documented by [^{57}Co]CN-Cbl absorption tests in which plasma radioactivity in the control dogs was similar to that observed in dogs previously 21) and was comparable to levels in humans studied in a similar way (24). The observation that fecal excretion of small amounts of orally administered [^{57}Co]CN-Cbl lasted significantly longer in control dogs than in affected dogs suggests that enterohepatic recirculation of Cbl occurring in normal dogs was interrupted in the affected dogs. The reported biologic $t_{1/2}$ of placentally derived (25) and parenterally administered (26) Cbl in dogs is 6–16 wk, much shorter than the $t_{1/2}$ of 11–14 mo reported for humans (27). Thus, in contrast to humans, onset of clinical and laboratory signs of Cbl deficiency in the 2nd and 3rd mo of life and rapid relapse after treatment are compatible with congenital Cbl malabsorption in dogs.

The complete and rapid response of affected dogs to parenteral CN-Cbl administration as the sole treatment suggested that the absorptive defect was selective for Cbl. Gastrointestinal histology by light and electron microscopy was normal in CN-Cbl-treated affected dogs. Furthermore, gastrointestinal function studies in the proposita revealed no abnormalities beyond selective Cbl malabsorption (12). Clinical signs suggestive of more generalized malabsorption were not observed in any of the dogs reported here, nor was there laboratory evidence of achlorhydria or exocrine pancreatic insufficiency.

Although the molecular or cellular nature of the inherited defect in this canine family was not defined, the defect was localized to the ileal enterocyte because no abnormality of IF or TC-II was detected. Moreover, the immunohistochemical findings strongly implicate a defect of the ileal receptor for IF-Cbl complex, either of expression or function. Further studies are needed to confirm this conclusion.

The disease in this family of dogs closely resembles inherited selective intestinal malabsorption of Cbl in humans (Imerslund-Gräsbeck syndrome; McKusick catalogue no. 26110) (28). In both, Cbl deficiency is caused by failure of the ileal enterocyte to mediate transcytosis of Cbl. It has been described in about 200 human patients worldwide and is inherited as a simple autosomal recessive trait (8, 8a, 10). Severe Cbl deficiency usually develops between 1 and 4 y of age. Patients in most families also exhibit proteinuria and/or amino aciduria that persists despite parenteral Cbl treatment and remission of all other abnormalities (8, 8a, 29, 30). Although high amounts of IF-Cbl receptor have been found in renal proximal tubular epithelium of humans, dogs, and rats (31), it is not yet clear why selective intestinal Cbl malabsorption and proteinuria are inherited together.

Reported amounts of proteinuria in Imerslund-Gräsbeck patients range from 0.013–1.46 g/d (normal is <0.2 g/d), most of which is albumin (29, 30). Normal values of urinary protein excretion for 35-kg dogs, as were studied here, range up to 0.75 g/d (15). For this reason, it was not clear from our studies whether the proteinuria seen in Imerslund-Gräsbeck syndrome patients was occurring in these dogs. Because the definition of this disease is only operational at this time, it is likely that the syndrome in humans may encompass many defects along the pathway of ileal enterocyte Cbl transcytosis, only some of which also cause proteinuria or amino aciduria. An obvious advantage of this canine model is that it is genetically defined at the disease locus, all of the affected animals having the same mutation. Notably, a study of proteinuric Imerslund-Gräsbeck patients in one family indicated an absence or dysfunction of the IF-Cbl receptor (32). These dogs may represent at least a subset of human families with selective intestinal Cbl malabsorption.

The dogs are also a spontaneously occurring, nonhuman model of Cbl deficiency. The metabolic abnormalities found in the affected dogs were similar to those found in Cbl-deficient humans and other species. Cbl deficiency in the inadequately treated proposita caused low serum Cbl concentrations and methylmalonic aciduria in her otherwise normal, heterozygous puppies until weaning, as has been reported in human infants nursed by strict vegetarians or mothers with untreated pernicious anemia (33, 34). In subsequent gestations, the proposita received more aggressive Cbl therapy, and only her affected puppies became Cbl deficient.

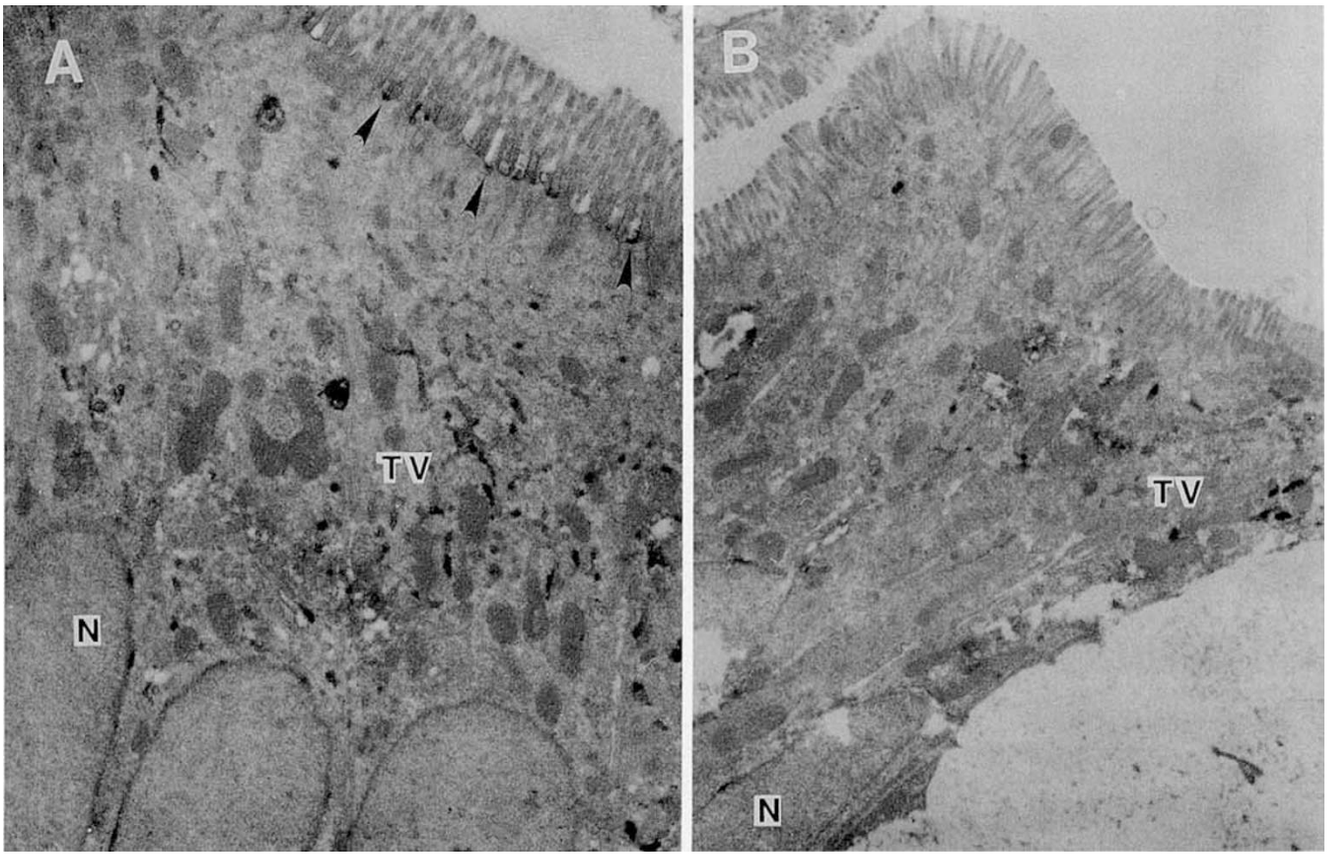

Fig. 5. Immunoelectron microscopy of ileal biopsy specimens from affected and normal dogs. The sections were stained by incubation with anti-dog IF-Cbl receptor antiserum and horseradish peroxidase. Villus tip enterocytes are shown. *A*, In the normal dog, immunocross-reactive receptor is present on many tubulovesicular membranes (*TV*), the nuclear membrane (*N*), and in many microvillus pits of the apical brush border (*arrows*) ×11 000. *B*, In sections from dogs that could not absorb Cbl, immunocross-reactive receptor was present on the same intracellular membranes and in tubulovesicles as seen in normal dogs. However, receptor was never found on the apical microvillus surface membrane or in microvillus pits (×8000). Receptor was not present in mucus-secreting cells of normal or affected dogs.

In the Cbl-deficient dogs, the proximal metabolites of Cbl-dependent enzymes accumulated and were metabolized by alternative pathways, producing abnormal organic acids and glycine conjugates that were excreted in urine. Untreated affected dogs excreted large amounts of MMA and smaller amounts of methyl citrate, 3-OH propionate, and propionylglycine. After parenteral CN-Cbl treatment, affected puppies excreted only normal or slightly elevated amounts of MMA and no other abnormal organic acid metabolites. Stabler *et al.* (13) have discussed the difficulties caused by *in vivo* and *in vitro* disulfide formation of measuring serum homocysteine by routine ion exchange methods. In this study, elevated serum or urine concentrations of free homocysteine were not found in any of the untreated, affected dogs when measured by routine automated ion exchange techniques. However, in the affected dog studied by capillary gas chromatography-mass spectrometry with selected ion monitoring, serum THCys was elevated almost 4-fold above normal dog values and returned to normal after parenteral Cbl treatment.

The hematologic effects of Cbl deficiency in these dogs were similar to those in humans. Megaloblastic bone marrow is a hematologic hallmark of Cbl deficiency in humans (35) and was present in these dogs. Their bone marrow had more megaloblastic changes in myeloid cells than erythroid cells, and this was reflected in the peripheral blood. In humans, macrocytic anemia is a commonly recognized hematologic feature of Cbl deficiency. In the dogs, the presence of small numbers of macrocytic erythrocytes seen in peripheral blood smears was masked by the presence of numerous microcytic cells when assessed by Wintrobe indices.

Inappetence causing prolonged protein-calorie malnutrition or elevated concentrations of abnormal metabolites may have produced nonspecific abnormalities in blood cell production, morphology, and/or survival in these dogs. Additionally, gastrointestinal mucosal atrophy, lymphoid depletion or hypoplasia, and low IgG concentrations noted in the untreated, affected dogs were changes probably caused or exacerbated by protein-calorie malnutrition. We did not observe neurologic changes in Cbl-deficient dogs analogous to subacute combined degeneration of the CNS in Cbl-deficient humans, monkeys, or fruit bats (35–37). This may have been the result of species differences of Cbl-dependent metabolism or of insufficient duration of Cbl deficiency in the dogs.

This family of dogs represents a unique opportunity to address several central issues regarding intestinal Cbl absorption and Cbl-dependent metabolism in a genetically and phenotypically defined animal model.

Acknowledgments. The authors thank John R. Hansell, M.D., Veterans Administration Medical Center, Philadelphia, PA, for advice and technical support in the determination of fecal [^{57}Co] excretion and serum Cbl concentrations; members of the Division of Biochemical Development and Molecular Diseases, The Children's Hospital of Philadelphia, for analyses of free serum and urinary amino acid concentrations and urinary organic acid quantitation; Robert H. Allen, M.D., and Sally P. Stabler, M.D., University of Colorado Health Sciences Center, Denver, CO, for serum THCys determinations; Kenneth W. Simpson B.V.M.&S., Ph.D., School of Veterinary Medicine, University of Pennsylvania, Philadelphia, for surgical preparations; Frances Shofer, Ph.D., University of Pennsylvania, Philadelphia, for sta-

tistical data analysis; and James A. Begley, M.S., Veterans Administration Medical Center, Albany, NY, and Bellur Seetharam, Ph.D., Medical College of Wisconsin, Milwaukee, for valuable discussion and review of the manuscript.

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