IMMUNOPATHOLOGY OF NZB/BL MICE

VI. Virus Separable from Spleen and Pathogenic for Swiss Mice

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Plates 5 and 6

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An ultrastructurally typical virus-like filtrable agent has been recovered from NZB/Bl mice (1); at the same time, preliminary observations have been reported on the activity of the agent in Swiss mice. The present study, an extension of this work, points to two (perhaps related) circumstances which may be requisites for the pathogenic action of this agent within and outside the strain NZB: infection of newborn, or infant, mice; persistent, possibly tolerant, infection of adult mice.

Methods and Materials

Animals.—The care and the methods of study of our colony of NZB/Bl mice, derived from breeding stock provided by Dr. Marianne Bielschowsky and now in the 70th generation of brother-sister matings, are described elsewhere (1, 2).

A male CBA/T6 mouse, in a breeding nucleus obtained from Jackson Laboratories (Bar Harbor, Maine) by Dr. Eugene Lance, was crossbred with an NZB/Bl female to obtain the CBA ♂ × NZB ♀ F_1 hybrids.

Adult Webster-Swiss mice (Carworth Farms, New City, N. Y.) were used as short-term random breeders to obtain newborn mice. In addition, six pregnant female gnotobiotic CFW mice were obtained from Carworth Farms to provide newborn mice through the courtesy of Mr. Dennis Baker. The digestive microflora (3) associated with the gnotobiotic colony included lactobacilli, streptococci (Group N), and enterococci. A panel of eight serological tests (to be discussed below) for murine virus antibodies had been performed on 18 of the 72 gnotobiotic CFW mice in the Carworth colony, in each instance with negative results.

Adult Swiss mice were obtained from a colony (Charles River Farms, North Wilmington, Mass.) known to be free from infection with lymphocytic choriomeningitis (LCM) virus and were used as provisional intermediate hosts in experiments described below.

Murine Virus Antibody Determinations.—These serological tests were performed by Dr. John C. Parker of Microbiological Associates, Inc. (Washington, D.C.) on serums obtained from 10 aged NZB/Bl mice, long residents in our nonbreeding NZB/Bl mouse rooms. The serological procedures (see reference 8) are indicated in Table IV: hemagglutination-inhibition method for antibodies against pneumonitis virus of mice (PVM), reovirus 3, K, Sendai, Theiler GD VII, and polyoma virus; complement-fixation method for antibodies against mouse adenovirus and mouse hepatitis virus.

An indirect method was used to determine whether NZB/Bl mice harbored a latent (viremic) infection with LCM virus (4): 0.2 ml of fresh whole blood was collected from each of 10 young (2–3 months old) NZB/Bl mice and was injected intraperitoneally into an equal number of Swiss mice obtained from a colony known to be free from infection with this virus. At 28 days, the recipient Swiss mice were bled and the serums were tested for the presence of complement-fixing antibody against LCM virus (Table IV), normally absent from these mice but inducible in the event of infection.

Cell-Free Filtrates.—Using standardized methods described elsewhere (1), cell-free filtrates were prepared mainly from individual spleens obtained from 17–20 months old NZB/Bl mice which presented the characteristic findings of autoimmune hemolytic disease (5, 6), including positive antiglobulin (Coombs') tests, anemia, reticulocytosis, and splenomegaly. In some instances, the spleen filtrates were carried a step beyond the customary purification procedure by further centrifugation for 60 min at 23,000 rpm (30,000 *g*) in a Spinco model L preparative ultracentrifuge. The pellets so formed were then reconstituted in pH 7.2 buffered saline so that 1 ml was equivalent to 0.2 g weight of spleen (as with all filtrates). These "purified" cell-free filtrates were, as all others, passed through sterile Millipore filters with rated porosities of 0.45 μ or 0.22 μ and shown to be capable of retaining marker bacteria (*Serratia marcescens*). The standard filtrate contained about 1 mg protein (or organic material) per milliliter, the purified filtrate about 0.08 mg protein per milliliter. Newborn and infant mice received an intraperitoneal injection of 0.25 ml of filtrate, older mice 0.5 ml of filtrate.

Experimental Design.—To date, 50 filtrates of NZB/Bl mouse tissues, including spleen, kidney, liver, lung, and lymphoma have been prepared; 15 of these preparations have been inoculated into more than 150 Swiss and CBA/T6 mice, in many instances for the purpose of long-term studies which are in progress. The present report is based upon initial studies with four NZB/Bl spleen filtrates which were found to be biologically active when injected intraperitoneally into newborn or very young (less than 1 wk old) Swiss mice, including the 1 day old progeny of gnotobiotic CFW females, but not when injected into Swiss mice at 6 wk of age. NZB/Bl lymphoma filtrate had previously been found inactive with respect to induction of hemolytic and renal disease when injected into 2–3 wk old Swiss mice (1).

RESULTS

Swiss Mice

Control Observations.—Normal healthy adult Swiss mice were found by urinanalysis to have (−) or at most (+) proteinuria. The normal average and the 95% confidence interval, based upon 10–20 individual determinations, of those blood constituents immediately relevant to the present study are given in Table I. The laboratory findings on numerous Swiss mice under observation in our mouse colony during the period of this study were, with rare exception, normal by the criteria just mentioned, as were the data obtained on a group of Swiss mice injected at 3 wk with an NZB/Bl lymphoma filtrate. 81 direct and 41 indirect antiglobulin (Coombs') tests at 37°C were performed on 81 adult Swiss mice, and all tests were negative. Having no evidence of either hemolytic or renal disease, these animals served as controls for the observations on inoculated mice presented here.

Hemolytic Disease.—Some of the indications that hemolytic disease was induced in Swiss mice by the intraperitoneal inoculation of NZB/Bl spleen cell-

free filtrate into newborn and infant mice are given in Table II. The changes occurred in 2–5 months, frequently near the middle of this time interval, and often persisted through a major segment of the period of observation, which is being continued. The indirect antiglobulin (Coombs') test performed for in-

TABLE I

Laboratory Examinations of Healthy Adult Swiss Mice

Determination	Average	Average ± 2 SD (95% confidence interval)
Hematocrit, %	52	44–60
Reticulocytes, %	2	0–3
Platelets/mm^3	1,590,000	1,250,000–1,950,000
WBC/mm^3	10,750	5,500–18,100
Serum urea nitrogen, *mg/100 ml*	25	17–33
Serum cholesterol, *mg/100 ml*	133	73–193
Serum proteins, *g/100 ml*		
Total	5.7	5.1–6.3
Albumin	2.7	2.0–3.4
α_1-globulins	0.5	0.2–0.8
α_2- "	0.8	0.6–1.0
β- "	1.0	0.6–1.4
γ- "	0.7	0.5–0.9

TABLE II

Evidence of Hemolytic Disease in Swiss Mice at 2–5 Months After Inoculation of Cell-Free Filtrate

Abnormality	Criterion*	Mice		Range of abnormality
		Per cent	Fraction	
				per cent
Indirect Coombs' test‡	Positive	17	(5/29)	
Reticulocytosis	Ret. > 3%	28	(10/36)	4–20
Anemia	Hct. < 44%	8	(3/40)	41–43

* Values outside 2 SD interval for the normal (see Table I).

‡ Papain method, using erythrocytes of Swiss mice.

complete antibodies active at 37°C became positive in 17% (5 of 29) of the inoculated mice, at low serum titers (approximately 1:2) comparable to the early findings on young NZB/Bl mice prior to positive conversion in the direct test. Papain-treated Swiss mouse erythrocytes of either homologous or autologous source served equally well as test antigens. The direct antiglobulin (Coombs') tests have remained negative to date. Reticulocytosis developed in 28% (10 of 36) of the inoculated mice, and low grade anemia occurred in 8%

(3 of 40). It must be emphasized that the method of study always utilized the collection of minimal samples of blood by a single bleeding at *monthly or longer intervals*, a procedure which in our experience does not factitiously induce reticulocytosis and anemia in normal Swiss mice.

Autopsies and histological examinations of major organs were performed on five of the inoculated mice. The presence in four mice of both vigorous (erythroid and myeloid) hematopoiesis and prominent hemosiderosis in spleen and to a certain extent liver, in our experience unusual findings in untreated Swiss mice of comparable age, provided substantiating evidence of hemolytic disease.

Renal Disease.—Some of the indications that renal disease was induced in Swiss mice by the intraperitoneal inoculation of NZB/Bl spleen cell-free filtrate into newborn and infant mice is presented in Table III. These changes occurred

TABLE III

Evidence of Renal Disease in Swiss Mice at 2–5 Months after Inoculation of Cell-free Filtrate

Abnormality	Criterion*	Mice		Range of abnormality
		Per cent	Fraction	
Proteinuria	>+	27	(11/41)	++ to +++
Hypoalbuminemia	<2 g/100 ml	21	(6/29)	0.9–1.9 g/100 ml
Elevated α_2-globulins	>1 g/100 ml	24	(7/29)	1.1–1.2 g/100 ml
Hypergammaglobulinemia	>0.9 g/100 ml	76	(22/29)	1.0–2.3 g/100 ml
Hypercholesterolemia	>193 mg/100 ml	41	(17/42)	196–250 mg/100 ml

* Values outside 2 SD interval for the normal (see Table I).

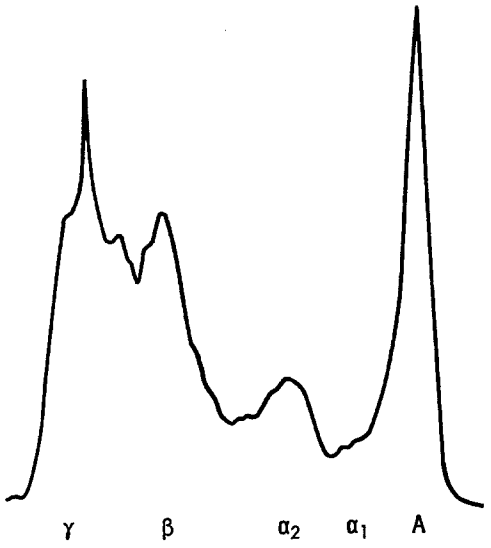
in 2–5 months and, once developed, tended to persist. Significant proteinuria (++ to +++) occurred in 27% (11 of 41) of the inoculated mice, hypoalbuminemia in 21% (6 of 29), elevated α_2-globulins in 24% (7 of 29), hypergammaglobulinemia in 76% (22 of 29), and hypercholesterolemia in 41% (17 of 42). An impressive example of induced hypergammaglobulinemia is shown in Text-fig. 1.

Histological examinations of major organs of five of the inoculated mice revealed the following data: in all, intense lymphoid cell and plasma cell hyperplasia of spleen and lymph nodes; in three, plasma cell hyperplasia (infiltration) of thymus with relative depletion of lymphocytes; in two, atrophy of cortex and medulla of thymus; and in three, renal glomerular lesions. The renal changes consisted of an homogeneous thickening and alteration of the glomerular capillary basement membrane (Fig. 1) and an accumulation of basement membrane-like materials in the axis of the glomerular tuft. These changes were usually focal, that is, they involved some but not all of the glomeruli in the kidneys,

and segmental, that is, they affected a part of an individual glomerulus and were similar to those seen in the early phases of "spontaneous" (9, 10) and experimentally accelerated (9) renal disease of NZB/Bl mice. Lesions of this type were not seen in the kidneys of control Swiss mice of comparable age (Fig. 2).

Murine Virology

Antibody Determinations.—Serological tests for evidence of prior immunizing nfection of old NZB/Bl mice with pneumonitis virus of mice (PVM), reovirus 3,



TEXT-FIG. 1. Paper electrophoresis pattern for serum proteins of a Swiss mouse inoculated with NZB/Bl cell-free filtrate. Albumin (A) migration to the right. Total protein, 8.0 g/100 ml; A, 1.8; α_1-globulins, 0.5; α_2, 1.1; β, 2.2; γ, 2.4.

mouse adenovirus, and mouse hepatitis virus as well as for evidence of latent (viremic) infection of young NZB/Bl mice with lymphocytic choriomeningitis (LCM) virus were negative in each of 10 determinations (Table IV). Antibodies against K virus and against Sendai virus were present in serum specimens 4 and 5 respectively. Serological tests for antibodies against GD VII virus and against polyoma virus were positive in each instance.

Electron Microscopy.—Virus-like particles, with close ultrastructural resemblance to the type "C" murine oncogenic virus particles and indistinguishable from those previously described (1), were identified in lymphoid and renal tissues by electron microscopy. The virus-like particles were present in distinctive intra- and extracellular locations, notably in the latter respect in the basal

foldings of renal tubules, of an embryonic (18 day stage) NZB/Bl mouse in-utero, a newborn NZB/Bl mouse (Fig. 3), 15–17 month old NZB/Bl mice, a 3 wk old hybrid mouse produced by the cross CBA ♂ × NZB ♀ (Fig. 4), and a Swiss mouse (the progeny of a gnotobiotic CFW female) at 4 wk after receiving an intraperitoneal injection of a purified cell-free filtrate of NZB/Bl mouse spleen (Fig 5). Similar particles were not found on equivalent examination of the tissues of untreated Swiss and CBA mice.

TABLE IV
Murine Virus Antibody Determinations on 10 NZB/Bl Mice (21–23 Months Old)

Specimen	Serum end point titrations								
	Hemagglutination-inhibition test						Complement fixation test		
	PVM	Reo 3	K	Sendai	GD VII	Polyoma	Mouse adeno-virus	Mouse hepatitis virus	LCM*
1	–	–	–	–	1:80	1:320	–‡	–‡	–
2	–	–	–	–	1:80	1:160	–‡	–‡	–
3	–	–	–	–	1:80	1:320	–‡	–‡	–
4	–	–	≦1:40	–	≦1:20	≦1:40	–	–	–
5	–	–	–	1:40	1:40	1:320	–	–‡	–
6	–	–	–	–	1:40	1:160	–	–	–
7	–	–	–	–	1:20	1:160	–‡	–‡	–
8	–	–	–	–	1:320	1:320	–	–	–
9	–	–	–	–	1:160	1:320	–	–	–
10	–	–	–	–	1:40	1:320	–	–	–
Initial test dilution	1:20	1:20	1:10	1:10	1:20	1:20	1:10	1:10	1:10

* These serological tests were performed on Swiss mice which 28 days previously had received intraperitoneal injections of 0.2 ml fresh whole blood obtained from young (2–3 months old) NZB/Bl mice.

‡ Negative at 1:20, anticomplementary activity at 1:10 dilution.

DISCUSSION

With respect to both ultrastructure and pathogenic effects, assuming these to be attributes of one and the same agent, the virus of NZB/Bl mice appears to be an entity not represented by the viruses listed in Table IV. For example K virus of mice (11) shares many properties of the papovaviruses (12); Sendai virus is a murine parainfluenza virus (13); GD VII virus (14) is a murine picornavirus and causes encephalomyelitis of mice; polyoma virus is a murine papovavirus capable of causing tumors of diverse histogenesis, including those of salivary gland origin, in mice (15). Neutralization studies which require

specific antiserums and, preferably, a more convenient bioassay system should determine the antigenic relation, if any, to known murine oncogenic viruses (16–19) with typical type "C" ultrastructure.

According to the four postulates of Koch, reaffirmed for virology by Rivers, proof that a microorganism causes a disease is established by (*a*) demonstrating the occurrence of the microorganism in association with the disease, (*b*) isolating this microorganism in pure culture (or tissue culture), (*c*) reproducing the disease in susceptible animals by inoculation of the microorganism in pure culture, (*d*) finding the microorganism in the susceptible animals. Our studies have consistently demonstrated that typical virus-like particles are present in the nephrons of NZB/Bl mice from birth until advanced age. The virus-like particles have been identified in the urinary space and the epithelial cells (podocytes) of the glomeruli, in the lumens and the basal foldings of the renal convoluted tubules, and in lymphoid cells of spleen and thymus. Renal and hemolytic diseases, with early functional and structural changes similar to those occurring in NZB/Bl mice at comparable age (10), were induced in Swiss mice by inoculating them, as newborns or infants, with cell-free filtrates prepared from the spleens of adult NZB/Bl mice. Typical virus-like particles were identified in the nephrons of an inoculated Swiss mouse. In vitro studies utilizing tissue cultures for the isolation of the virus are next to be undertaken.

These facts point to two circumstances which may be requisites for the pathogenic action of this viral agent within and outside the strain NZB: infection of newborn, or infant, mice or of embryos in utero; persistent infection of adult mice. While viruses are known to be protected by fixation to the living cells of susceptible animals (23), which in itself can account for persistent infection, the combined features of congenital (or neonatal) infection and abundance of virus-like particles in extracellular sites resemble those observed in mice with persistent *tolerant* infection with lymphocytic choriomeningitis virus (20). In fact, renal disease, with glomerular lesions and perivascular accumulations of lymphocytes and plasma cells similar to those occurring in NZB/Bl mice, is a prominent aspect of the "late onset disease" which develops in these tolerant mice (20). Hotchin and Collins have suggested (20) that this late onset disease may occur as a result of the gradual waning of immunological tolerance formerly exhibited by the mouse towards an otherwise harmless virus infection or, alternatively, may arise from a slow cytopathic effect of the virus on target organs such as the kidneys. An early study (21) in which neonatal thymus of NZB/Bl mice was transplanted to neonatally thymectomized CBA/T6 mice, resulting in the induction of autoimmune hemolytic and renal disease in the latter, might find eventual explanation in virus, as well as lymphoid cell, transfer.

The findings in viral plasmacytosis (Aleutian disease) of mink (22) bear similarity to those which prevail in NZB/Bl mice (10): hypergammaglobuli-

nemia, overproliferation of plasma cells, glomerular and vascular lesions, and genetic predisposition (for Aleutian and related color types). Recently this viral infection has been experimentally transmitted to standard dark (not Aleutian) mink and has been shown to induce positive antiglobulin (Coombs') test conversion in these animals at about 8 months (22).

SUMMARY AND CONCLUSIONS

Hemolytic disease characterized by slight anemia, reticulocytosis, hemosiderosis, extramedullary hematopoiesis, and positive indirect antiglobulin (Coombs') tests and renal disease with proteinuria, hypoalbuminemia, and glomerular lesions were produced in Swiss mice by neonatal intraperitoneal inoculation of cell-free filtrates prepared from the spleens of old NZB/Bl mice. Lymphoid cell and plasma cell hyperplasia as well as hypergammaglobulinemia occurred in some of these inoculated mice.

Type "C" murine oncogenic virus-like particles, indistinguishable from those previously described (1), were shown by electron microscopic study to be present in distinctive locations, notably in the basal foldings of convoluted tubules in the kidneys of a newborn NZB/Bl mouse, old NZB/Bl mice, a CBA $\times$ NZB F_1 hybrid mouse, and a Swiss mouse inoculated with NZB/Bl spleen cell-free filtrate.

These observations point to two (perhaps related) circumstances which may be requisites for the pathogenic action of this newly discovered virus within and outside the strain NZB: infection of newborn, or infant, mice; persistent, possibly tolerant, infection of adult mice.

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Addendum.—In a continuation of this work, 3 of 28 Swiss mice were found at 7 months after inoculation with NZB/Bl cell-free filtrate to have developed malignant lymphoma with similar organ distribution and leukemic blood picture, and in addition to have hemolytic disease and progressive renal disease (diffuse membranous glomerulonephritis). The incidence of induced lymphoma (~10%) was about 10 times the incidence of spontaneous lymphoma (~1%) observed in 100 control Swiss mice.

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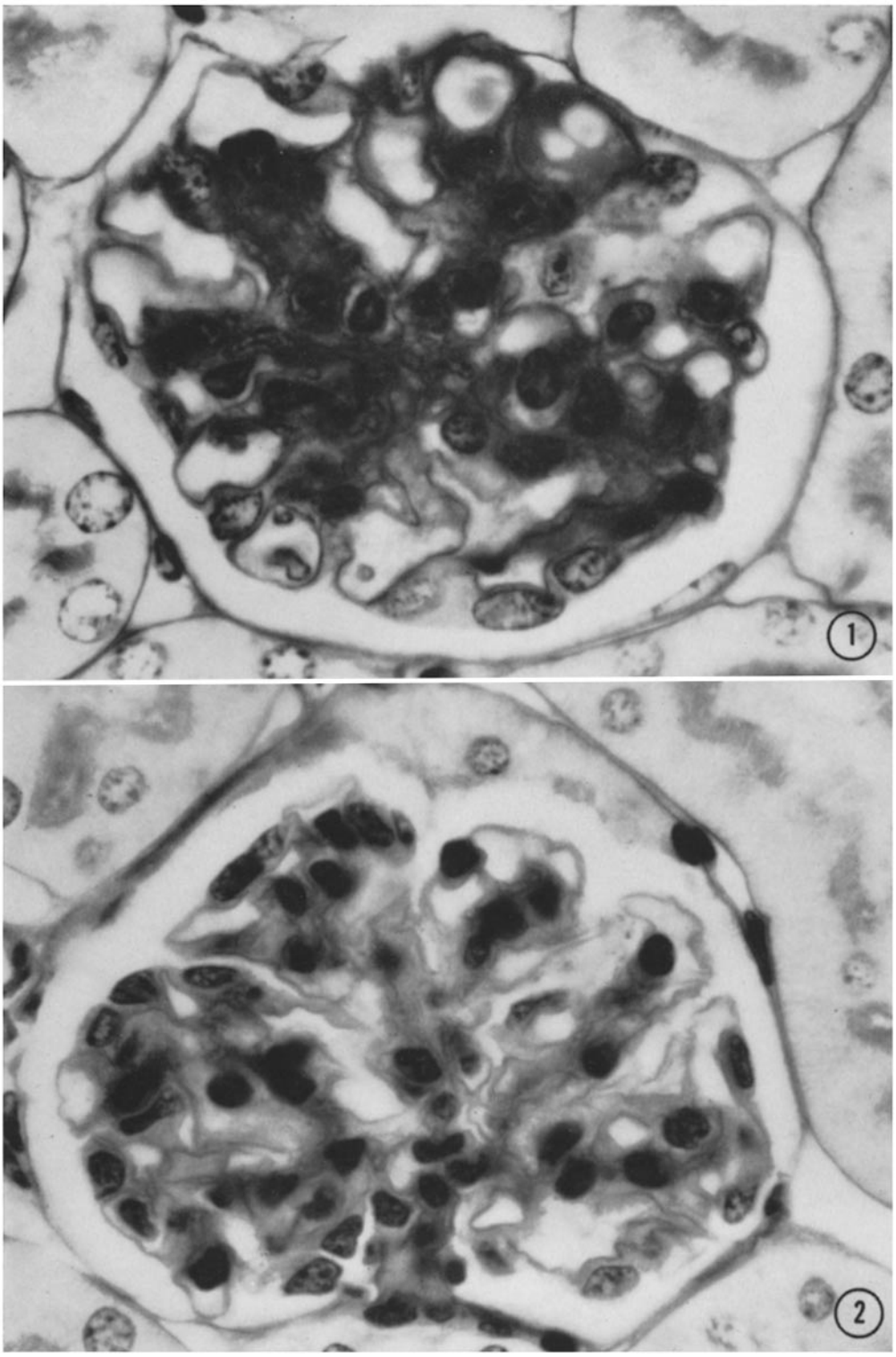
EXPLANATION OF PLATES

PLATE 5

FIG. 1. Kidney (cortex) of Swiss mouse inoculated with NZB/Bl spleen cell-free filtrate. Membranous and mesangial lesions involving a segment of a glomerulus. 2 μ section. Periodic acid-Schiff stain, × 1800.

FIG. 2. Kidney (cortex) of a control Swiss mouse. A normal glomerulus. 2 μ section. Periodic acid-Schiff stain, × 1500.

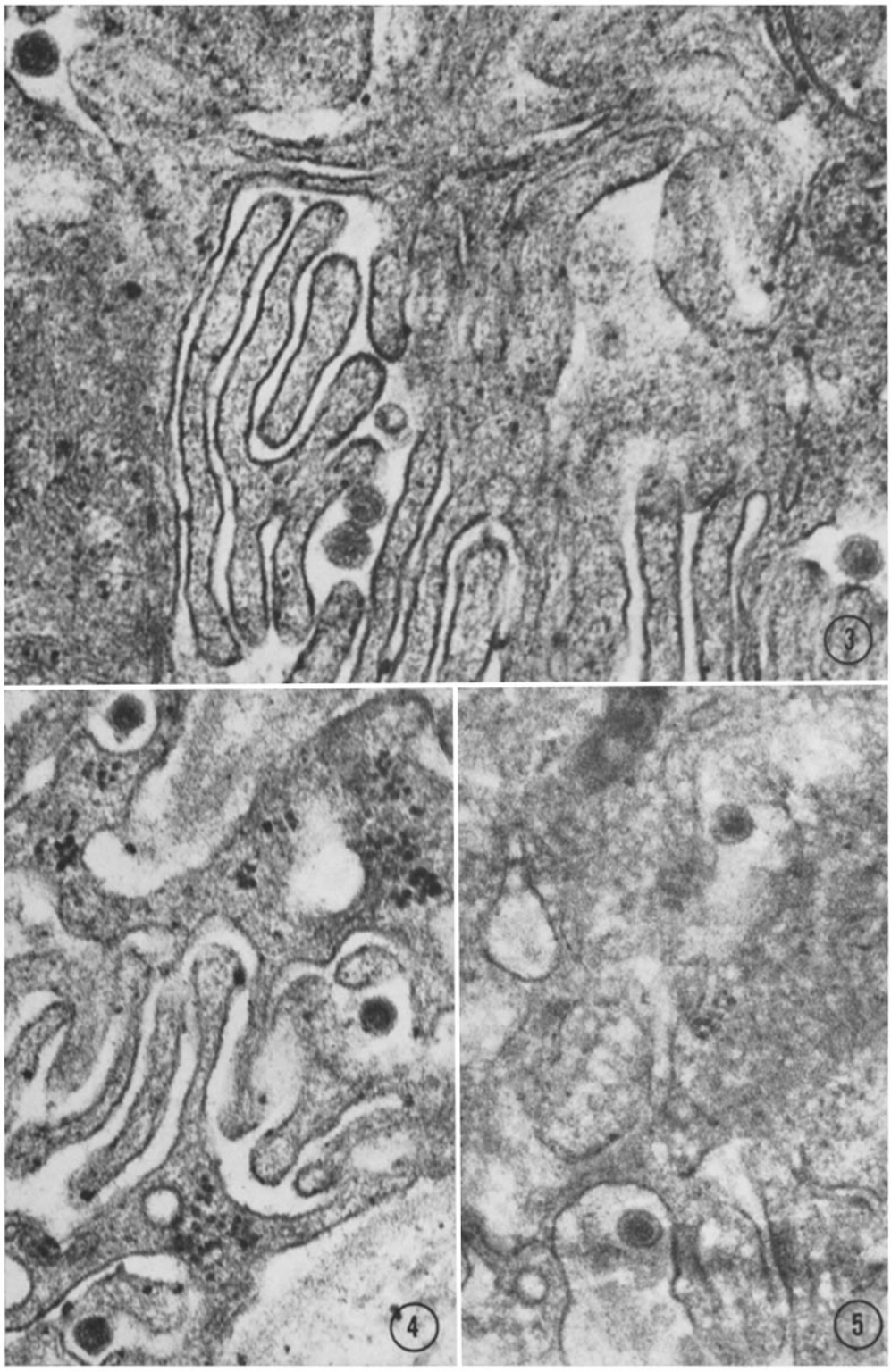


(Mellors and Huang: Immunopathology of NZB/Bl mice)

PLATE 6

FIGS. 3–5. Electron micrographs of thin sections fixed with glutaraldehyde and osmium tetroxide and double-stained with uranyl acetate and lead acetate. × 60,000.

Ultrastructurally similar virus-like particles are present within the spaces of basal foldings of proximal convoluted tubules in the kidneys of newborn NZB/Bl mouse (Fig. 3), a CBA × NZB F_1 hybrid mouse (Fig. 4), and a Swiss mouse inoculated with NZB/Bl spleen cell-free filtrate (Fig. 5).



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