IMMUNOPATHOLOGY OF NZB/BL MICE

V. Viruslike (Filtrable) Agent Separable from Lymphoma Cells and Identifiable by Electron Microscopy

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Plates 97 to 99

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Autoimmune hemolytic anemia (1–3), membranous glomerulonephritis (4–9), systemic connective-tissue disease (7–10), and malignant lymphoma (11, 12) develop in aging NZB/Bl mice. The hemolytic (13) and the renal (8) diseases can be induced (within a few weeks and independently) in young NZB/Bl mice by the transplantation of syngeneic adult spleen cells. The malignant lymphomas, which develop in older NZB/Bl mice with already established hemolytic and renal diseases (12), are of two histological types, reticulum cell sarcoma and pleomorphic malignant lymphoma (12). These lymphomas are maintained in our laboratory by serial transplantation within the strain NZB/Bl (9).

In recent years, murine leukemias (and lymphomas) of several varieties have been shown to be caused by viruses (14–18), a circumstance which has led us to ask whether some, or all, of the immunopathological disorders arising in NZB/Bl mice might also be initiated by viruses or other filtrable agents (9). Accordingly, relevant studies were undertaken of the biological activity of cell-free filtrates prepared from NZB/Bl malignant lymphomas; in the course of this investigation viruslike particles were identified by electron microscopy in malignant lymphoid cells and in nonneoplastic cells and tissues of NZB/Bl mice. A brief account of this work follows.

Methods and Materials

Animals.—The care and the methods of study of our colony of NZB/Bl mice, which was derived from breeding stock provided by Dr. Marianne Bielschowsky, are described elsewhere (6) as are also the cervical lymph node origin and the initial transplantation of reticulum cell sarcoma (hereinafter also called malignant lymphoma, 93-line) within this strain (12). We also maintain the CBA/T6 strain of mice and Swiss-Webster albino mice.

Lymphoma (Cell-Free) Filtrates.—These were prepared from subcutaneous tumors obtained from NZB/Bl mice bearing transplants of malignant lymphoma (12), 93-line. In the finally standardized procedure, the tumor tissue was obtained and handled under sterile conditions, minced, and ground in a chilled mortar with pestle and sand to provide a 10 to 20% suspension in pH 7.2 buffered isotonic saline. The suspension was centrifuged in a (Servall Super-

speed) refrigerated centrifuge successively at 3900 RPM (1800 *g*) and 4200 RPM (2300 *g*) for 20 min each and at 9200 RPM (10,000 *g*) for 2 min to obtain a "clear" supernatant fluid. The supernatant fluid was then passed through sterile Millipore filters with rated porosities of 0.45 μ and on occasion 0.22 μ, and shown to be capable of retaining marker bacteria (*Serratia marcescens*). Aliquots of the bacteriologically sterile cell-free filtrate were injected subcutaneously (0.15 to 0.3 ml) and intraperitoneally (0.25 to 0.5 ml) into preweanling (2 to 3 wk-old) NZB/Bl mice on the day of preparation and were also frozen and stored at −70°C. Similar injections were given to preweanling CBA/T6 mice and to young adult Swiss-Webster mice.

Electron Microscopy.—Tissues were fixed either with 1% osmium tetroxide in phosphate buffer at pH 7.4 or with 4% glutaraldehyde in phosphate buffer followed by postfixation with 1% osmium tetroxide in phosphate buffer. Tissues were dehydrated in ethyl alcohol and propylene oxide and embedded in epoxy mixture containing Epon 812. Sections were cut with diamond knives using the Porter-Blum model I microtome and were picked up on bare copper grids. All sections were double-stained with uranyl acetate and lead acetate. Electron micrographs were made with a Siemens Elmiskop I at an initial magnification of 20,000 or 40,000. These plates were enlarged 3 times in the preparation of illustrations.

RESULTS

Malignant Lymphoma, 93-Line.—Transplants of this reticulum cell sarcoma grew locally in the subcutaneous trochar-inoculation site, reaching a dimension of 3 to 4 cm within 3 to 4 wk after each of the early passages. The degree of lymphoid hyperplasia and the number of plasma cells, including periodic acid–Schiff positive Russell-body types, in spleen, lymph nodes, and thymus of tumor-bearing mice appeared to exceed even that which commonly occurs in NZB/Bl mice of comparable age (4 to 7 months) as they, and similarly the tumor–bearing mice, undergo antiglobulin (Coombs') test conversion and develop other manifestations of autoimmune hemolytic anemia. Surprisingly, the glomerular lesions of focal membranous glomerulonephritis (6, 8) developed in a 2 month-old NZB/Bl mouse which had carried a tumor transplant for 4 wk and, in addition, diffuse membranous glomerulonephritis occurred in a tumor-bearing mouse when only 4 months of age. The structural manifestation of membranous glomerulonephritis in older NZB/Bl mice bearing transplanted tumors was in general severe and similar to that occurring in NZB/Bl mice of comparable age but not bearing tumor grafts (6, 8).

At the eighth serial transplantation, the malignant lymphoma displayed more rapid local growth and widespread dissemination, involving spleen, lymph nodes (of axillary, inguinal, cervical, brachial, intrathoracic, and mesenteric regions), thymus, liver, kidney, and even bone marrow at this and subsequent passages, which were in fact then made at 2 to 3 wk intervals rather than at 3 to 4 wk as previously. The blood leukocyte count (Wbc/mm^3) was 5300, normal, as in other recipients. The histological and cytological appearance of this reticulum cell sarcoma, presently in the eighteenth transplant generation within the strain NZB/Bl, has not changed from that of the original primary tumor (12), except perhaps for more active mitosis. Tumor transplantation to

normal mice of the strain CBA/T6 and to Swiss-Webster mice has thus far been unsuccessful.

Lymphoma (Cell-Free) Filtrates.—These were prepared in the initial experiment from subcutaneous lymphomas obtained from NZB/Bl mice bearing the eleventh serial transplant of malignant lymphoma, 93-line. Aliquots of the cell-free filtrate were injected subcutaneously (0.15 to 0.3 ml) and intraperitoneally (0.25 to 0.5 ml) into five young (3-wk-old) male NZB/Bl mice, all litter-mates. Tumors did not develop in the subcutaneous inoculation sites

TABLE I

Laboratory Examinations of Young NZB/Bl Mice at 5 to 7 wk After Receiving Inoculations of Lymphoma Cell-Free Filtrate

Recipient No.	1	2	3	4	5
Proteinuria	++	++	++	+++	++
Direct antiglobulin test	−	−	−	−	−
Hematocrit, %	38*	48	49	48	49
Reticulocytes, %	5	23*	17*	12*	2
Platelets/mm^3	1,100,000	2,500,000	2,560,000	1,860,000	2,360,000
Wbc/mm^3	7,150	8,500	6,500	7,500	3,500
Serum urea nitrogen, *mg/100 ml*	19	21	25	23	42*
Serum cholesterol, *mg/100 ml*	159	149	158	169	252*
Serum proteins, *g/100 ml*	5.2	5.1	5.3	5.1	5.7
Albumin	1.8*	1.7*	1.6*	1.5*	1.8*
α_1-globulins	0.5	0.3	0.4	0.5	0.4
α_2-globulins	1.0	1.1	1.3*	1.1	0.3
β-globulins	0.9	1.1	0.9	1.0	1.0
γ-globulins	1.0*	0.9*	1.0*	1.0*	1.2*

* More than 2 standard deviations from normal mean.

during the period of observation (5 to 7 wk). Weekly urinalyses showed no departure from the normal until the third week following inoculation, when significant proteinuria (++) occurred in three of the five mice. Tests for hematuria were negative throughout the period of observation.

Complete laboratory examinations were made at 5 to 7 wk after inoculation of the cell-free filtrate. Results are given in Table I where significant changes in quantitative determinations, that is, values more than two standard deviations from the normal mean for healthy NZB/Bl mice of similar age, 8 to 12 wk old (8), are indicated by asterisks. Significant proteinuria (++ to +++) occurred in each recipient within 3 to 5 wk after inoculation of cell-free filtrate.

The direct antiglobulin (Coombs') tests remained negative. Blood studies revealed low hematocrit per cent in recipient 1 and elevated reticulocytes per cent in recipients 2, 3, and 4. A decrease of serum albumin and an increase of serum γ-globulins occurred in each recipient. A decrease in total serum proteins and increases in serum urea nitrogen and cholesterol occurred in recipient 5. The urinary and the serum changes in the inoculated mice were similar in kind (proteinuria, hypoalbuminemia, hypergammaglobulinemia, and exceptionally hypercholesterolemia) to those which occur in adult NZB/Bl mice with severe renal disease and in young NZB/Bl mice with glomerular lesions induced by transplants of syngeneic adult spleen cells (8). Blood leukocyte counts (Wbc/mm^3) in the inoculated mice were normal (3500 to 8500) as were the differential counts (lymphocytes, 76 to 82 per cent; mononuclears, 1 to 3 per cent; polymorphonuclears, 13 to 20 per cent; eosinophils, 0 to 7 per cent).

Autopsies were performed on these five NZB/Bl mice which, apparently in good health, were sacrified at 8 to 12 wk of age, having received injections of cell-free filtrate 5 to 9 wk previously. Total body weights were 33 to 38 g; spleen, 0.09 to 0.12 g; thymus, 0.04 to 0.09 g; and kidneys (both together), 0.45 to 0.54 g. There was neither gross nor microscopic evidence of tumor at the inoculation site or elsewhere. The lymph nodes (cervical, axillary, inguinal, and mesenteric) were grossly hyperplastic in each animal, and microscopically the cellular increase was mainly of lymphoid, including reticular and plasma cell, variety. Renal lesions, characterized in 1 μ sections fixed in Carnoy's solution and stained with the periodic acid–methenamine silver technique (19) by membranous thickening and multiple nodule formation along the outer (subepithelial) aspect of the glomerular capillary basement membrane, occurred in the kidneys of each recipient of cell-free filtrate. This structural change is the earliest, and a practically pathognomonic, lesion of membranous glomerulonephritis of NZB/Bl mice (8); it does not *in association with significant proteinuria and hypoalbuminemia* develop spontaneously at less than 4 months of age; and, becoming diffuse (involving every glomerulus), it is characteristically associated with the functional manifestations of severe renal disease in older NZB/Bl mice (8, 9).

Similar findings were obtained on a comparable group of NZB/Bl mice injected as preweanlings with another preparation of NZB/Bl lymphoma filtrate. On the other hand, significant proteinuria, hypoalbuminemia, and glomerulonephritis were not observed on examination of either six healthy, *untreated*, 2 month-old NZB/Bl mice or nine young Swiss-Webster mice and eight young CBA/T6 mice *injected* as preweanlings with NZB/Bl lymphoma filtrate. In all these experiments, the direct antiglobulin (Coombs') tests remained negative.

Viruslike Particles.—Viruslike particles with close resemblance to the typical "C" type murine oncogenic virus particles (25) were identified by means of electron microscopy in malignant lymphoma cells, not only in the primary tumor but in transplants of this lymphoma at the first and seventh consecutive

passage, Fig. 1. Similar viruslike particles were identified in nonneoplastic cells of the kidneys, Fig. 2, thymus, Fig. 3, and spleen, Fig. 4, of NZB/Bl mice bearing this transplantable lymphoma. In the kidneys, viruslike particles were most numerous near the basal foldings of the distal convoluted tubules and were also present in the epithelial cells (podocytes) of the glomeruli and in the tubular lumens as well. In the thymus and the spleen, viruslike particles were most abundant in cytoplasmic vesicles within lymphoid and reticular cells and were also present in extracellular spaces.

Structurally similar viruslike particles were present in comparable sites in the kidneys, Fig. 5, spleen, and thymus of nontumor bearing NZB/Bl mice at 11 and 15 months of age, the thymus (but not the kidneys) of a healthy 6 wk-old NZB/Bl mouse, and the kidneys and spleen, Fig. 6, of 2- to 3-month-old NZB/Bl mice which had been injected with NZB/Bl lymphoma filtrate or had received transplants of NZB/Bl adult spleen cells (8). The typical mature viruslike particle in NZB/Bl mouse tissues, Fig. 8, had a centrally located electron-opaque nucleoid measuring approximately 55 mμ in diameter and a double-limiting membrane with round or oval shape and over-all diameter of about 100 mμ.

It should be emphasized that the typical viruslike particles were found in the tissues of NZB/Bl mice obtained from two sources, namely, mice received from Australia in 1963, reared elsewhere (6), and kept in our animal quarters for only 18 days prior to sacrifice, Fig. 7, and those received from New Zealand in 1964 as a breeding nucleus and maintained for 6 months in our NZB/Bl mouse breeding room in isolation from all other laboratory animals. At all times, personnel in this breeding room wore caps, gowns, disposable masks, and gloves, and exercised precautions against cross-infections. Comparable viruslike particles were not found in the kidneys and spleen of Swiss-Webster and CBA/T6 mice on equivalent search by electron microscopy.

Further studies of fine structure will be presented at a later date.

DISCUSSION

The present study indicated that a filtrable agent separable from NZB/Bl malignant lymphomas was capable, upon inoculation into preweanling NZB/Bl mice, of inducing lymphoid cell hyperplasia, hypergammaglobulinemia, proteinuria, hypoalbuminemia, and pathological changes (focal membranous glomerulonephritis) of kidneys—functional and structural changes qualitatively similar to those of "spontaneous" occurrence in older NZB/Bl mice (4–10); and, moreover, that typical viruslike particles, identifiable by electron microscopy, were present in NZB/Bl mouse tissues and cells, including malignant and benign lymphoid cells, epithelial cells of renal glomeruli and tubules, and extracellular sites. To our knowledge, these observations are the first of their kind dealing with NZB/Bl mice.

While the pathogenesis of the induced renal disease and its relation to the

"spontaneous" disease are not yet fully elucidated, the following possible sequence of events can be suggested. Once a viral infection is established, the virus or its products might: have a directly injurious effect on renal glomeruli; provide antigenic stimulation of lymphoid cells; induce renal disease by hypersensitivity mechanisms, as by the injurious meeting of circulating antibody with viral antigens in glomeruli or by the glomerular localization of circulating viral antigen-antibody complexes; and might further expand the population of lymphoid cells and of cell types susceptible to viral action and transform some of these into lymphoid cells with autoaggressive (autoimmune) and neoplastic behavior. Conceivably, the virus might also be only a passenger virus persisting in a tolerant or an immune host.

Studies are in progress to determine whether the filtrable agent has oncogenic properties and to investigate its role in the hemolytic disease (1–3) which characterizes NZB/Bl mice. Circulating and in vivo–localized antibodies with specificity for the structurally altered glomeruli in "spontaneous" renal disease of NZB/Bl mice have already been described (6), and, conceivably, these immunoglobulins may prove to be at least in part antiviral antibodies. A recent paper has described the occurrence of glomerulonephritis in AKR mice with leukemia produced by the inoculation of Friend or Rauscher virus (22).

While uniquely successful in their positive aspects, the present experiments are not the first reported attempts to uncover a transmissible agent in NZB/Bl mice. The inoculation of twice frozen and thawed NZB/Bl spleen cell suspensions failed to induce Coombs' test conversion in young NZB/Bl mice (20); and the intraperitoneal injection of cell-free filtrates prepared from the pooled lymphoid organs of 24- to 27-wk-old NZB/Bl mice had no effect on hybrid (C57BL × C3H/Bi)F_1 and C3H/Bi mice (21).

Genetic studies of the offspring of reciprocal crosses between NZB/Bl and other strains of mice and of back crosses to the parent strain have been made in an effort to distinguish between the possible action of a vertically transmitted virus and chromosomal factors in the causation of autoimmune hemolytic anemia. While some of the findings were more in conformity with inheritance by a dominant autosomal gene (23), the results in other studies (24) were equally in accord with the hypothesis that a virus is transmitted vertically by sperm as well as ovum of NZB/Bl mice and demonstrably affects only animals of a strictly limited range of genotypes.

We have now to study further the natural mode of transmission of the filtrable agent described in the present work, its strain specificities, and relation to known murine viruses.

SUMMARY AND CONCLUSIONS

A filtrable agent separable from transplantable malignant lymphomas of NZB/Bl mice was capable, upon inoculation into preweanling NZB/Bl mice, of inducing lymphoid cell hyperplasia, hypergammaglobulinemia, proteinuria, hypoalbuminemia, and pathological changes (focal membranous glomerulone-

phritis) of kidneys—renal functional and structural changes qualitatively similar to those of spontaneous occurrence in older NZB/Bl mice.

Viruslike particles with close resemblance to the typical "C" type murine oncogenic virus particles were identified by means of electron microscopy in NZB/Bl mouse tissues and cells, including malignant lymphoma cells, benign lymphoid cells of thymus and spleen, epithelial cells of renal tubules, and extracellular sites.

The relevance of these observations, the first of their kind dealing with NZB/Bl mice, is discussed in relation to the several immunopathological disorders which characterize this strain of mice.

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Addendum.—In a continuation of this work, we have been able to induce in Swiss mice features of renal and hemolytic disease, including positive antiglobulin (Coombs' tests, by neonatal intraperitoneal inoculation of ultracentrifugally purified cell-free filtrates of splenic tissue obtained from old NZB/Bl mice not having malignant lymphoma.

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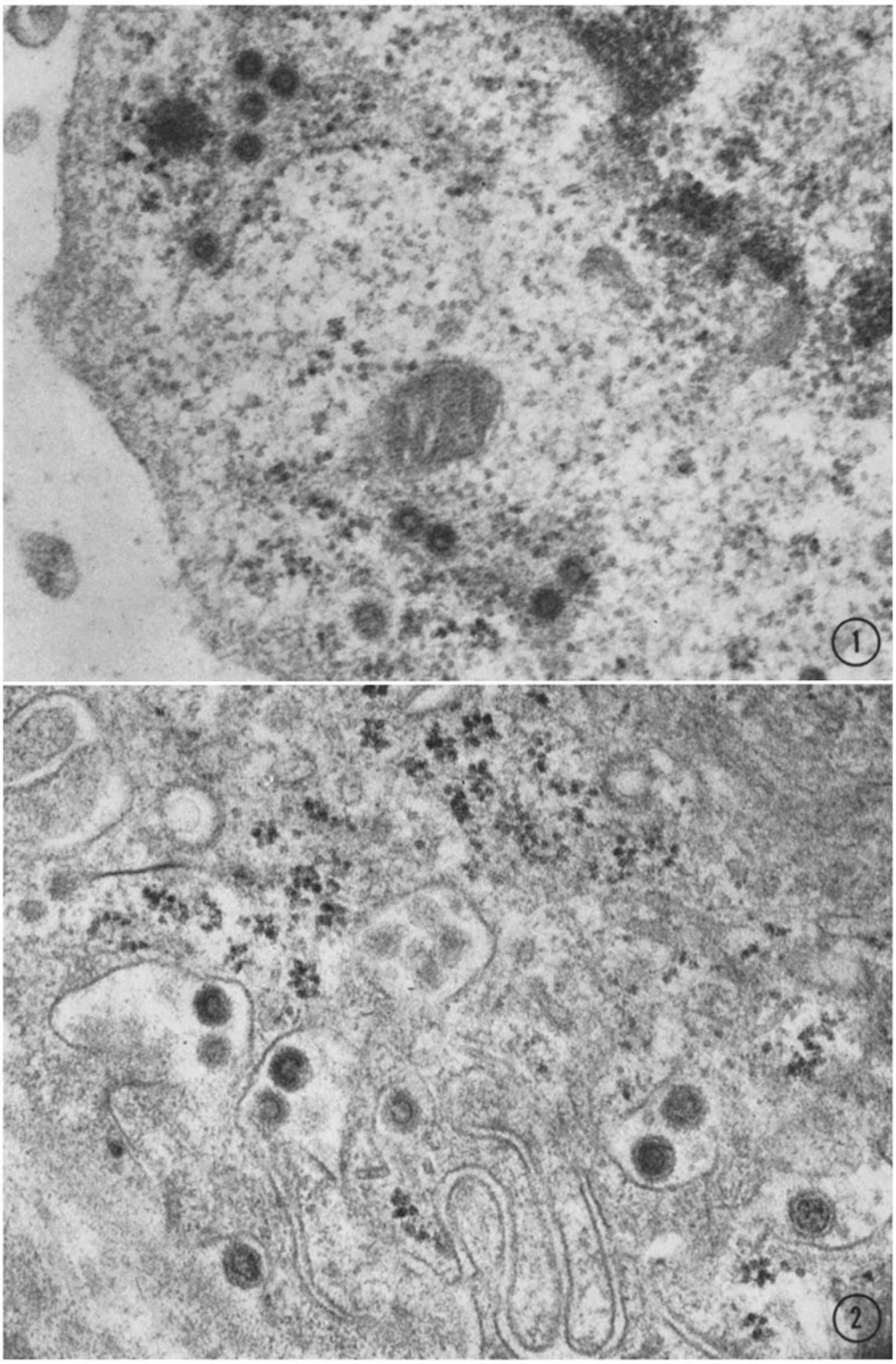
EXPLANATION OF PLATES

Figs. 1 to 8 are electron micrographs of thin sections of NZB/Bl mouse tissues double-stained with uranyl acetate and lead acetate.

PLATE 97

FIG. 1. Portion of malignant lymphoma cell containing ten viruslike particles in the cytoplasm. Portion of nucleus is shown in upper right corner. Glutaraldehyde-osmium fixation. × 60,000.

FIG. 2. Portion of kidney of lymphoma-bearing mouse. Viruslike particles in basal portion of distal convoluted tubule. Glutaraldehyde-osmium fixation. × 60,000.

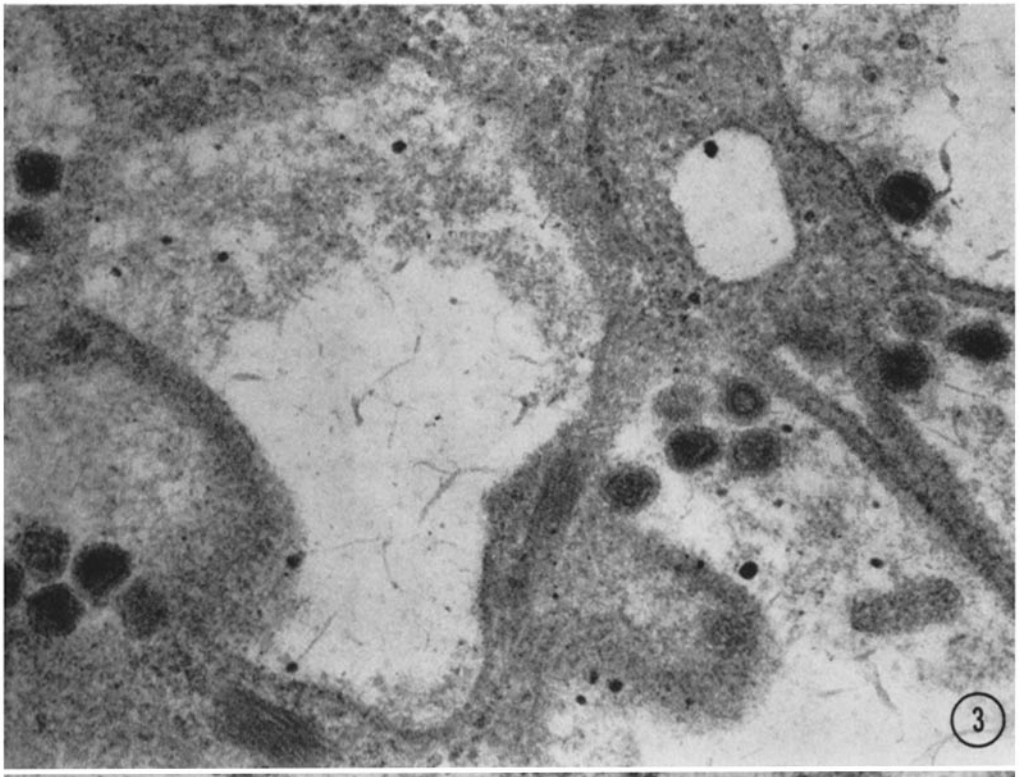


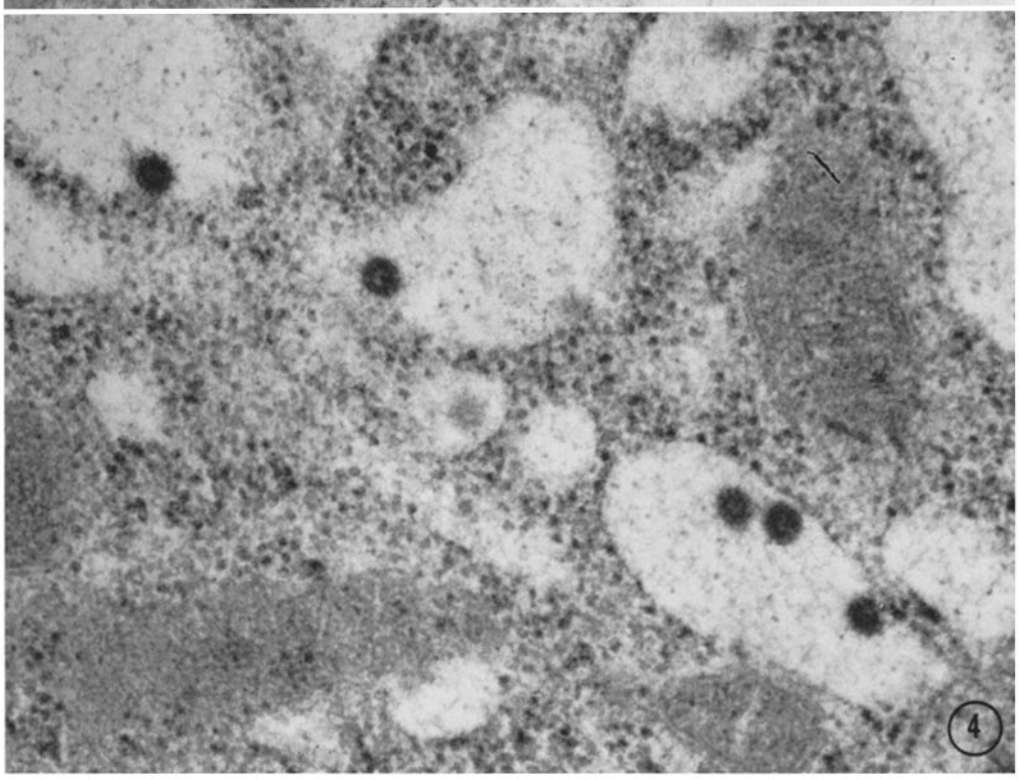
(Mellors and Huang: Immunopathology of NZB/Bl mice)

PLATE 98

FIG. 3. Portion of thymus of lymphoma-bearing mouse. Viruslike particles in cytoplasm and vesicles of a reticular cell. Glutaraldehyde-osmium fixation. × 60,000.

FIG. 4. Portion of spleen of lymphoma-bearing mouse. Viruslike particles in vesicles of a lymphoid cell. Glutaraldehyde-osmium fixation. × 60,000.





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

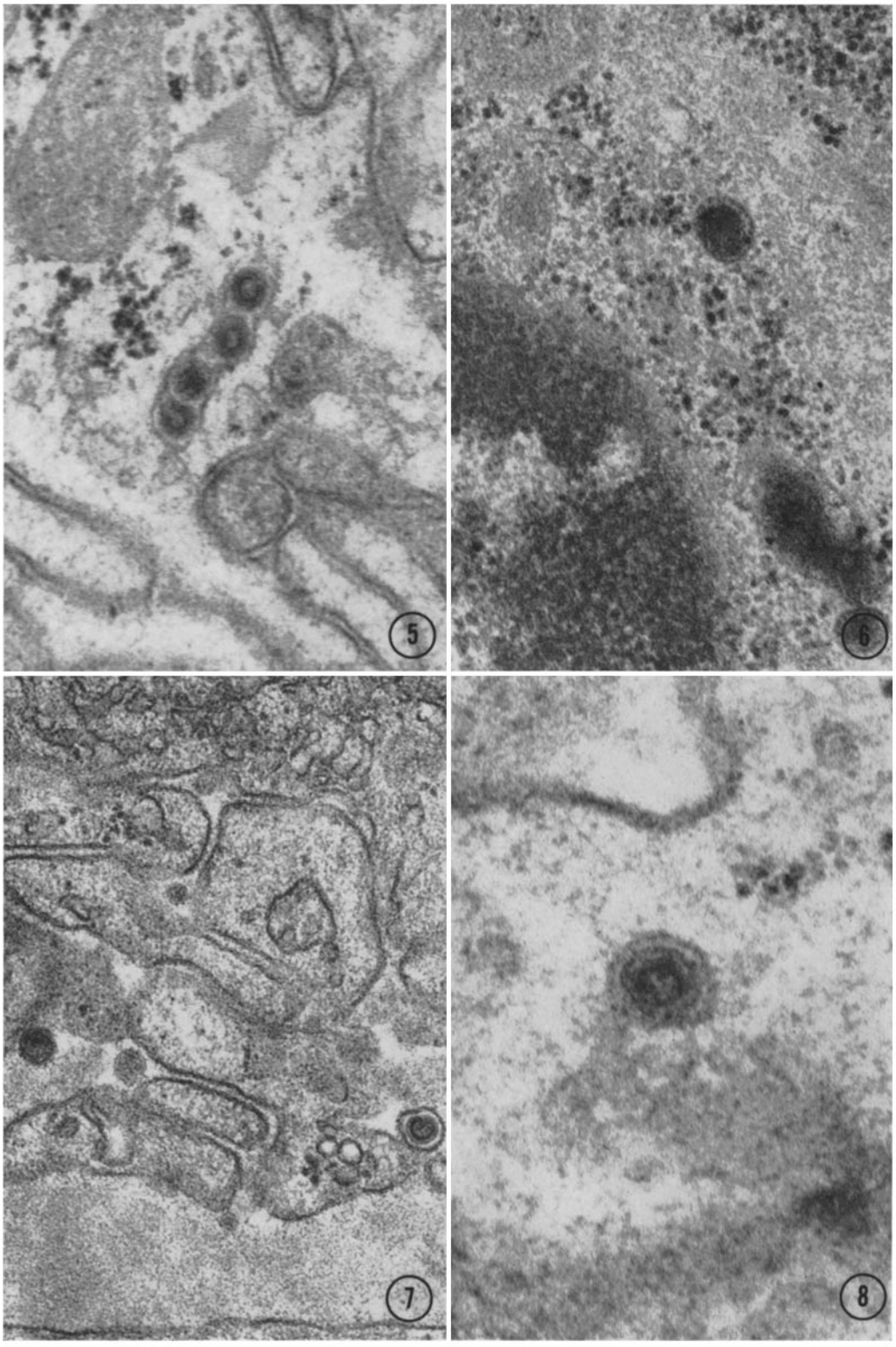
PLATE 99

FIG. 5. Portion of kidney of 11 month-old mouse. Four viruslike particles in vesicle close to basal foldings of distal convoluted tubule. Osmium tetroxide fixation. × 60,000.

FIG. 6. Portion of spleen of 3 month-old mouse which had been injected with lymphoma filtrate. Viruslike particle in cytoplasm of lymphoid cell. Glutaralde-hyde-osmium fixation. × 60,000.

FIG. 7. Portion of kidney of 5 month-old mouse obtained from Australia. Two viruslike particles in basement membrane of proximal convoluted tubule. Osmium tetroxide fixation. × 60,000.

FIG. 8. Portion of collecting tubule of kidney. Viruslike particle (in small vesicle) with a centrally located electron-opaque nucleoid (~55 mμ in diameter) and a limiting membrane (~100 mμ in diameter). Osmium tetroxide fixation. × 120,000.



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