WILD-TYPE GROSS LEUKEMIA VIRUS AND THE PATHOGENESIS OF THE GLOMERULONEPHRITIS OF NEW ZEALAND MICE*

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Renal glomerular disease (membranous glomerulonephritis) is the main cause of death of NZB mice, especially of females, starting at about 10 months of age, and is almost certainly an immunologically induced disorder separate from, but not wholly unrelated to, the autoimmune hemolytic disease which typifies this inbred strain (1–3). The renal disorder is characterized by: an insidious onset beginning in some mice at about 4 months of age; a progressive, chronic, usually fatal course which sometimes is acutely accelerated and associated with positive lupus erythematosus (LE)[1] cell tests such as occur in human systemic LE and typify (NZB × NZW) F_1 hybrid mice (1, 4); significant proteinuria noted commonly in the female in the later stage of the disease; and histopathological changes involving the capillary basement membrane and the mesangium of the glomeruli (5, 6).

NZB mice carry wild-type Gross leukemia virus apparently throughout life (9) but, unlike other G (Gross) antigen–positive inbred strains (7, 15), NZB mice break tolerance to G antigens and produce G natural antibody in later life (9). Proteinuria, a functional manifestation of severe renal glomerular

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[1] *Abbreviations used in this paper:* CF, complement-fixation; EBSS, Earle's balanced salt solution; G, Gross; GSA, Gross soluble antigen; HA, hemagglutination-inhibition; IF, immunofluorescence; LE, lupus erythematosus; MuLV, murine leukemia virus; PBS, phosphate-buffered saline.

disease, becomes increasingly prevalent as G soluble antigen, a type-specific soluble antigen specified by wild-type Gross leukemia virus (7, 8), undergoes immune elimination from the plasma (9). The cumulative mortality of NZB mice, mainly attributable to renal glomerular disease, increases in phase with the production of free G antibody (9). Murine leukemia virus (MuLV)–specified antigens and host immunoglobulins, presumably antibodies, are localized in vivo in the glomerular lesions (9, 10). These findings implicate Gross leukemia virus in the etiology of glomerulonephritis of NZB mice and suggest that the host immune response to antigens of the G system (7, 15, 16), and hypersensitivity mediated by the deposition of G soluble antigen–antibody complexes in the glomeruli, are important contributory factors in the pathogenesis of the renal disease (9). The present paper provides further supporting evidence for the hypersensitivity pathogenesis of the glomerulonephritis of NZB mice and also presents new and parallel findings which similarly implicate wild-type Gross leukemia virus and associated hypersensitivity mechanisms in the pathogenesis of the accelerated renal disease (lupus glomerulonephritis) that typifies (NZB $\times$ NZW) F_1 hybrid mice.

Methods and Materials

Mice.—The following mice were used: NZB mice from our colony (10); NZB, NZW, and (NZB $\male$ $\times$ NZW $\female$) F_1 hybrid mice produced from a breeding nucleus of NZB and NZW mice obtained from the Texas Inbred Mice Co., Houston, Tex., through the courtesy of Mr. Samuel M. Poiley, National Cancer Institute, National Institutes of Health, Bethesda, Md.; C57BL/6 and AKR mice from our colony and from Jackson Memorial Laboratory, Bar Harbor, Maine; and CFW mice from Carworth Farms, New City, N. Y. The general methods of care and study were described elsewhere (9, 10).

Urinalysis.—A fresh drop of urine was collected and tested for proteinuria by the use of reagent strips (Albustix or Labstix, Ames Co., Elkhart, Ind.) as previously described (10). Reactions of 2+ or greater on repeated tests at regular, including daily, intervals were graded as significant proteinuria.

Kidney Homogenate.—The kidneys of anesthesized mice were perfused *in situ* with cold (4°C) Earle's balanced salt solution (EBSS) until grossly free of blood. A block of kidney tissue was frozen at −70°C for immunofluorescence study (as described subsequently) and the remainder was placed in an equal volume of EBSS, homogenized in a glass hand homogenizer, and centrifuged at 4°C and 1200 g, followed by collection of supernatant fluid (homogenate in Table I).

Frozen sections of the perfused kidneys were stained by the direct immunofluorescence method for mouse (host) immunoglobulins, confirming the adequacy of the perfusion in removing unbound host immunoglobulins and the almost exclusive location of bound immunoglobulin in the renal glomerular lesions of NZB and (NZB $\times$ NZW) F_1 hybrid mice (10, 11).

Kidney Eluate.—The kidneys were perfused and homogenized as previously described. The pH of the homogenate was lowered to 3.2 by the addition of 2.5 M citric acid, followed by incubation at 4°C for 90 min with constant stirring, centrifugation at 4°C and 1200 g, collection of the supernatant fluid, restoration of its pH to 7.4 by the addition of 2 N NaOH, further centrifugation, and collection of the supernatant fluid (acid-buffer eluate in Tables I and II).

In order to obtain a purified eluate in sufficient amount for multiple tests, the acid-buffer eluate in Table III was prepared from the many times washed, saline-insoluble sediment of

pooled kidney homogenate, as follows. Approximately 0.5 g of kidney tissue from each mouse was minced with scissors and washed three times with six volumes of cold (4°C) EBSS, followed each time by centrifugation at 4°C and 1935 g for 15 min. The washed tissue was frozen and stored overnight at −70°C. The tissue was thawed, pooled, placed in six volumes of EBSS, homogenized in a glass hand homogenizer, and centrifuged at 4°C and 12,100 g for 45 min. The supernatant fluid was removed. The remaining sediment was washed three times with pH 7.6, 0.01 M phosphate-buffered isotonic saline (PBS), followed each time by centrifugation at 4°C and 3020 g for 15 min. The washed sediment was suspended in several volumes (20 ml per g of starting tissue) of pH 3.2, 0.02 M citrate:phosphate-buffered isotonic saline, and incubated at 37°C for 90 min with constant stirring. The suspension was then centrifuged at 4°C and 12,100 g for 30 min. The supernatant fluid (240 ml) was removed, dialyzed against PBS at 4°C for 48 hr, and concentrated 4-, 8-, 16-, 32-, and 64-fold in a Diaflo Ultrafiltration Cell (Model 50, Amicon Corp., Lexington, Mass.). The eluate was shown by immunoelectrophoretic analysis to contain mouse immunoglobulins but not other mouse serum proteins. Mouse 7S immunoglobulin concentration and antibody activity in the eluate were determined and compared with similar analyses of the serums of the donor mice.

An acid-buffer eluate of the pooled kidneys of 14 (NZB × NZW) F_1 hybrid mice, 10 females and 4 males with an average age of 8 months (SD 1.5), was made by the preparation of the saline-insoluble sediment of kidney homogenate and overnight treatment with deoxyribonuclease (obtained from Worthington Biochemical Corp., Freehold, N. J.), 0.2 mg per ml at pH 6.9 and 4°C, according to the procedure of Koffler et al. (12), followed by elution with isotonic 0.05 M glycine-HCl buffer at pH 2.4.

Immunodiffusion and Immunoelectrophoresis Analysis.—Mouse 7S (γG) immunoglobulin concentrations in the kidney eluate and sera were determined by quantitative radial immunodiffusion analysis according to the method of Mancini et al. (13). Immunoelectrophoretic analysis of mouse serum proteins was performed by the micromethod of Scheidegger (14).

Anti-Nuclear Antibody.—Cryostat sections of normal guinea pig liver were air dried, fixed in 95% ethyl alcohol for 30 min at room temperature, washed with PBS, and used as nuclear antigen for the detection of anti-nuclear antibody in mouse serums (or kidney eluate). The indirect method was used in the usual manner, as follows: incubation of sections at room temperature for 30 min with 0.1 ml of serum was followed by PBS wash and treatment with fluorescein-labeled goat antibody to mouse immunoglobulins. Reactions were graded as definite positive (specific nuclear fluorescence) or negative, and positive serums were titrated by doubling dilutions to an end point. Specificity of the reaction was verified by the absorption of the serum with calf thymus deoxyribonucleoprotein, and by the treatment of the sections with deoxyribonuclease. Positive control serum was obtained from NZB mice, negative control serum from CFW mice.

Anti-Erythrocyte Antibody.—A 6–8% suspension of ficin-treated normal mouse erythrocytes obtained from CFW mice or Coombs' test–negative young NZB mice was prepared and used in the usual manner for the serological detection of incomplete warm antibody to mouse erythrocytes. 2 drops of mouse serum (or kidney eluate) were mixed in a test tube with 1 drop of the erythrocyte suspension, incubated for 3 min at room temperature, centrifuged for 30 sec in a Sero-Fuge (Clay-Adams, Inc., Parsippany, N. J.), and observed for macroscopic agglutination. Tubes showing no, or doubtful, agglutination at this time were incubated further at 37°C for 30 min, centrifuged, and reexamined. Reactions were graded as definite positive (macroscopic agglutination) or negative, and positive serums were titrated by doubling dilutions to an end point. Positive control serum was obtained from NZB mice, negative control serum from CFW mice.

G (Gross) Typing Serum.—This serum was prepared by immunization of C57BL/6 female mice with the G+ AKR ♀ spontaneous leukemia K36. This serum did not contain hemag-

glutination-inhibition (HI) polyoma antibody. A complete description of the G typing system is given elsewhere (15).

Determination of G Antibody.—The serums, homogenates, and eluates were tested for G antibody by the indirect immunofluorescence method with suspensions of viable G+ E♂G2 indicator cells. Reactions were graded according to the proportion of cells showing characteristic membrane fluorescence: $\leq 20\%$, negative; 21–50%, weak positive; 51–100%, strong positive. A complete description of this method is given elsewhere (16).

Demonstration of G Soluble Antigen (GSA) Adsorbed on Viable Indicator Cells.—The test was performed on plasma, homogenates, and eluates, and is based on the capacity of GSA to adsorb to cell membranes where it is demonstrable by immunofluorescence with G typing serum (7). Ascites leukemia EL4 cells served as the indicator cells; EL4 does not carry G or FMR cellular antigens (15). The plasma of AKR mice served as positive control. A detailed description of this method is given elsewhere (7).

MuLV CF Antigen.—The complement-fixation (CF) method for the detection of CF antigens with murine leukemia virus (MuLV) group and type specificities is described elsewhere (17, 18). The rat antisera used in the CF test were transplanted lymphomas or sarcomas induced respectively by murine leukemia viruses or the Moloney strain of murine sarcoma virus (19), and were selected for antibody specificity with respect to MuLV antigens and for absence of nonspecific reactions with extracts of normal tissues of various mouse strains. CF tests were made on 10% extracts of tissues at a starting dilution of 1:2 or 1:4, and CF-positive extracts were titrated at doubling dilutions to an end point. Positive 3+ and 4+ reactions in the absence of anticomplimentary effects and in the absence of positive reactions with negative control extracts were scored as positive.

MuLV IF Antigens.—The indirect immunofluorescence (IF) method for the detection in unfixed frozen tissue sections of IF antigens with MuLV group and G type specificities is described elsewhere (9). The rat antiserum used in the IF test was prepared in the laboratory of Dr. Lloyd Old by the method of Geering et al. (20), and kindly provided by these authors for this study. This antiserum was obtained from (W/Fu × BN) F_1 rats bearing subcutaneous transplants of the Gross virus–induced leukemia W/Fu (C58NT)D. The antiserum did not contain detectable anti-nuclear antibody by the indirect IF method, nor HI polyoma antibody, and did not react in immunodiffusion tests with mouse serum proteins. The fluorescein-labeled goat antibody against rat γ-globulins gave a single line of precipitation of rat γ-globulins in immunodiffusion tests with rat serum, and was thoroughly absorbed with lyophilized normal mouse serum to remove cross-reacting antibody against mouse γ-globulins. This absorbed reagent was negative in direct IF reactions with mouse kidneys, whether normal or glomerulonephritic. Negative control reactions in the indirect IF method included the use of normal (nonimmune) W/Fu rat serum and partial inhibition of (W/Fu × BN) F_1 rat antiserum by absorption with soluble extract of E♂G2 leukemia cells prepared by the method of Geering et al. (20), or by absorption with the plasma of AKR mice carrying leukemia K36, whereas the plasma of C57BL mice failed to do so. The normal kidneys of C57BL mice served as additional negative controls. All mouse tissues were unfixed, frozen at −70°C, and sectioned at 1–2 μ. Mouse immunoglobulins were located by the direct IF method with fluorescein-labeled antibody against mouse γ-globulins, as previously described (10).

Histopathology.—The autopsy procedures and histological methods were described elsewhere (10).

Electron Microscopy.—The methods of procedure were described elsewhere (21).

RESULTS

NZB Mice

G Soluble Antigen (GSA).—Tests for GSA were performed on the plasma and on the homogenate and the acid-buffer eluate of the perfused kidneys of female

NZB mice 2–14 months of age (Table I). The results obtained on the kidneys of the individual mice are illustrated in Fig. 1. The mice were also tested for proteinuria.

Significant proteinuria (2+ reaction or greater) occurred in NZB mice 10 and 14 months of age and slightly in advance of this, in keeping with previous findings (9). GSA was detectable in the plasma at 10 and 14 months, although it is known to appear in NZB mice somewhat earlier (9).

The tests for GSA were negative in each of 16 kidney homogenates. In significant contrast, the tests for GSA were positive in 5 of 12 kidney eluates, with positive results occurring at 6–14 months.

TABLE I

G Soluble Antigen and G Natural Antibody in Plasma and Serum and in Homogenate and Acid-Buffer Eluate of Perfused Kidneys of NZB Mice at Various Ages, with and without Proteinuria

Age	Proteinuria No. positive/ No. tested	G soluble antigen* (No. positive /No. tested)			G natural antibody‡ (No. positive/No. tested)		
		Plasma	Homogenate	Eluate	Serum	Homogenate	Eluate
Months							
2–4	0/4	0/2	0/4	0/2	0/4		1/8
6	0/4	0/2	0/2	1/2	0/4	0/2	3/4
8	1/4	0/2	0/4	2/2	0/4	0/4	3/4
10	6/6	3/3	0/4	1/3	0/6	0/4	5/6
14	6/6	2/3	0/2	1/3	2/6	0/2	5/6
Summation			0/16	5/12		0/12	17/28
P (chi-square test)			P < 0.01			P < 0.001	

* Indicator cells, EL4 cells.

‡ Indicator cells, E♂G2 cells.

GSA in the kidney eluate was shown to react with G antibody from several sources (Table II), including the individual serums of 4 NZB mice 11–15 months of age, a serum pool obtained from retired breeder C57BL/6 mice, and the G typing serum *C57BL/6 anti-AKR spontaneous leukemia K36*. As indicated, not only by the per cent of fluorescent indicator cells (Table II), but also by the intensity and magnitude of membrane fluorescence (Fig. 2), the serum of some NZB mice (for example, No. 4667) reacted more strongly with the antigen in kidney eluate than did the G typing serum.

G Natural Antibody.—Tests for G natural antibody were performed on the serum and on the homogenate and the acid-buffer eluate of the perfused kidneys of female NZB mice 2–14 months of age (Table I). The results obtained on the kidneys of the individual mice are shown in Fig. 1.

Positive tests for G natural antibody in the serum were observed in some mice by 14 months of age, in keeping with previous findings (9).
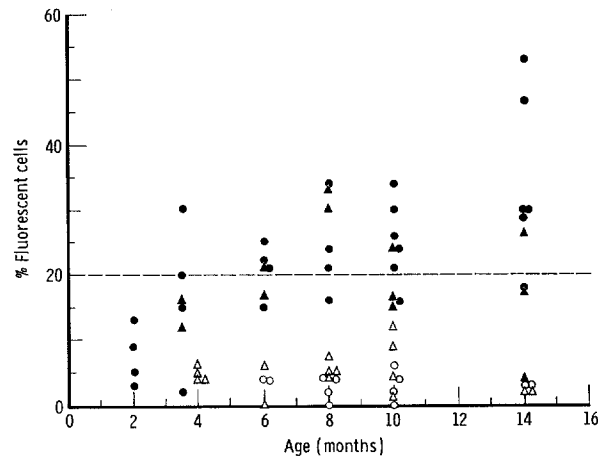
FIG. 1. Tests for G soluble antigen (triangles) and G natural antibody (circles) in homogenates (open symbols) and acid-buffer eluates (closed symbols) prepared from the kidneys of NZB mice at various ages. Reactions are graded by proportion of indicator cells showing membrane immunofluorescence: negative ($\leqq 20\%$); weak positive ($21-50\%$); strong positive ($51-100\%$).

TABLE II

Reactivity of G Soluble Antigen in Acid-Buffer Eluate of Kidneys of 6 NZB Mice with Proteinuria (Mean Age, 13.6 Months, SD 0.9) with G Antibody in Serums of NZB and C57BL/6 Mice

G soluble antigen Source	G antibody Source	Fluorescent cells*
		%
Kidney eluate, NZB mice	11 month old ♀ NZB mouse No. 5183 serum	80
" " " "	12 " " " " " No. 5060 "	55
" " " "	14 " " " " " No. 4667 "	96
" " " "	15 " " " " " No. 5230 "	37
" " " "	Retired breeder C57BL/6 mouse serum pool	61
" " " "	C57BL/6 anti K36 mouse serum pool	86
Plasma, AKR mouse	" " " " " "	86
" " "	10 month old ♂ NZB mouse No. 5311 serum	57

* EL4 cells, positive reaction, $>20\%$.

The tests for G natural antibody were negative in 12 kidney homogenates. Contrasting very significantly with these results, the tests for G natural antibody were positive in 17 of 28 kidney eluates. The first positive test for G natural antibody in the kidney eluate was observed at 4 months of age (Fig. 1), and the majority of tests were positive at 6–14 months.

Autoantibodies.—Tests for anti-nuclear antibody, anti-erythrocyte antibody, and G natural antibody were performed on the acid-buffer eluate prepared from the saline-insoluble sediment of the kidneys of NZB mice and on representative serums obtained at the same time from these mice. As described under Methods and Materials, the antibody tests on the eluate were made on samples taken
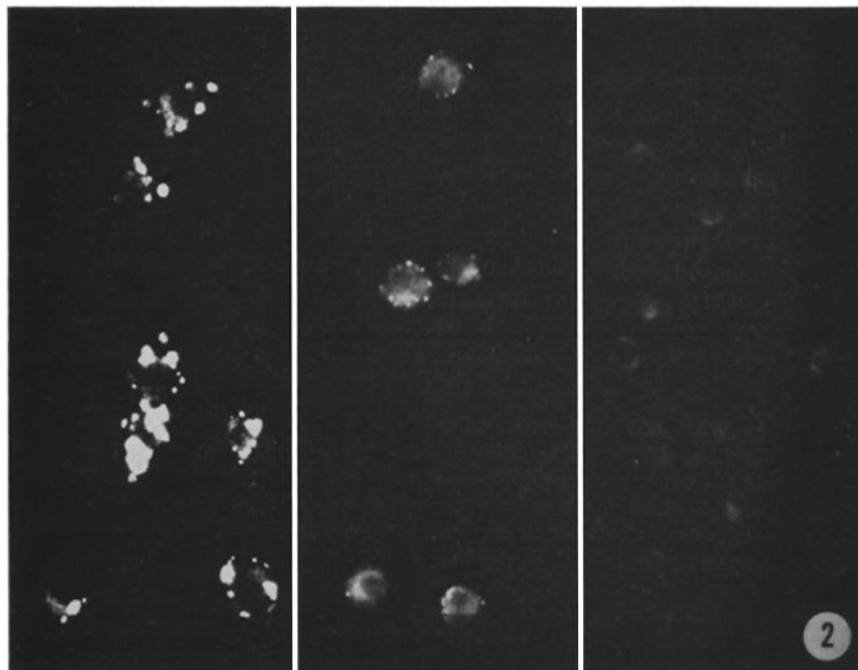

Fig. 2. GSA in NZB kidney eluate adsorbed to surface of EL4 indicator cells and reacted with NZB mouse serum No. 4667 (*Left*) and with G typing serum *C57BL/6 anti-AKR spontaneous leukemia K36* (*Middle*); EL4 cells alone, negative control (*Right*). Membrane immunofluorescence, indirect method, using fluorescein-labeled goat antibody to mouse immunoglobulins. $\times$ 500.

during the concentration procedure. The concentration of mouse 7S immunoglobulins (Ig) in the eluate and the serums was also measured. The antibody titer in the serums was expressed as the reciprocal of the highest serum dilution giving a positive end point. The arithmetic mean titer was calculated and normalized to a 7S Ig concentration of 10 mg/ml for the comparison of the antibody activities in the eluate and the serums.

The following results were obtained (Tables III and IV). The normalized titer of G natural antibody in the kidney eluate was 25, in the serum, 12; the

ratio (eluate/serum) was 2.1. The normalized titer of antinuclear antibody in the kidney eluate was 25, in the serum 32; the ratio (eluate/serum) was 0.8. The normalized titer of anti-erythrocyte antibody in the kidney eluate was 25, in the serum 56; the ratio (eluate/serum) was 0.5. Thus, of the three serum antibodies, all were detectable in the kidney eluate but only G natural antibody

TABLE III

Antibody Titers in Acid-Buffer Eluate Prepared from Pooled Kidneys of 28 NZB Mice with Proteinuria (Mean Age, 11.6 Months, SD 2.3), Contrasted with Serum Antibody Titers*

Antibody	Antibody in eluate					Normalized antibody titer (7S Ig concn., 10 mg/ml)	Normalized antibody titer ratio
	7S Ig conc. (mg/ml) and result						Eluate/Serum* (7S Ig Concn., 10 mg/ml)
	<0.2	0.2	0.4	0.8	3.0		
G natural antibody‡	−(9%)	−(19%)	+(42%)	+(29%)	+(28%)	25	2.1
Anti-nuclear antibody	−	−	+	+	+	25	0.8
Anti-erythrocyte antibody	−	−	+	+	+	25	0.5

* Normalized serum antibody titers for mice in this experiment are given in Table IV.
‡ (%), % fluorescent cells (E♂G2 cells), positive reaction, >20%.

TABLE IV

Antibody Titers in Serums Obtained from 9 NZB Mice with Proteinuria (Mean Age, 11.1 Months, SD 1.5)

Antibody	Number of tests	Serum dilution					Antibody titer	
		Number of positive tests					Arithmetic mean‡	Normalized§ 7S Ig concn., 10 mg/ml
		1:8*	1:16*	1:32*	1:64	1:128		
G natural antibody*	9	3	3	1			11	12
Anti-nuclear antibody	9	2	4	2		1	30	32
Anti-erythrocyte antibody	9	2		4	1	2	52	56

* In the test for G natural antibody, serum dilutions were actually 1:7, 1:15, and 1:30; two of nine serums were negative and graded 0. Indicator cells, E♂G2 cells.
‡ Calculated from reciprocal of dilutions.
§ Measured 7S Ig concn. was 9.4 mg/ml.

was more concentrated in the eluate than in the serum. However, it is to be noted that if only the G antibody-positive serums in Table IV were used, the normalized titer of G antibody in the serum would be 15 and the ratio (eluate/serum) would be somewhat less, namely 1.7.

(*NZB* × *NZW*) *F₁ Hybrid Mice (and Parental Strains)*

MuLV CF Antigens.—The complement-fixation (CF) test for murine leukemia virus (MuLV) CF antigens was performed on tissue extracts of the spleen,

liver, and kidneys of 13 (NZB × NZW) F₁ hybrid mice 6–13 months of age (Table V). All of the tissue extracts tested were CF antigen–positive at a dilution of 1:8, and a large majority was positive at a dilution of 1:16.

The test for MuLV CF antigens was performed on tissue extracts of the spleen, liver, and kidneys of 7 NZW mice of 18 months of age. All of the extracts tested were CF antigen–positive at a dilution of 1:8, and a large majority was positive at a dilution of 1:16.

Comparable positive results were obtained on extracts of the spleen, liver, and kidneys of NZB mice at 8 months, in keeping with previous findings (9).

MuLV IF Antigens.—Unfixed frozen sections of the perfused kidneys of 6 (NZB × NZW) F₁ hybrid mice were studied by the indirect immunofluorescence (IF) method, using a broadly reactive rat antiserum (see Methods and Materials), for the location of murine leukemia virus IF antigens. The hybrid mice were females. One was 6 months, three were 7 months, and two were 8 months

TABLE V

MuLV CF Antigens in Tissue Extracts of (NZB × NZW) F₁ Hybrid, NZW, and NZB Mice

Mice	No.	Age	Tissue extract (dilution)					
			Spleen		Liver		Kidney	
			1:8	1:16	1:8	1:16	1:8	1:16
		Months	*No. antigen positive/No. tested*					
(NZB × NZW) F₁	20	6–13	19/19	14/19	20/20	20/20	8/8	7/8
NZW	7	18	6/6	5/6	7/7	7/7	7/7	6/7
NZB	5	8	5/5	3/4	5/5	4/4	3/3	2/3

of age, and all had highly significant (3+) proteinuria. MuLV IF antigens were located in the renal glomeruli in the four younger mice, almost exclusively in the wall of the peripheral capillary loops and in the mesangium of the glomeruli; in two of these mice, MuLV IF antigens were detectable in virtually all glomeruli in the frozen sections (Fig. 3). The deposits of MuLV IF antigens were granular or homogeneous; their distribution within a glomerulus was segmental or diffuse. The negative controls in the indirect method for the detection of MuLV IF antigens included the use of normal rat serum (Fig. 4) and the study of frozen sections of the kidneys of normal C57BL/6 mice (Fig. 5). These controls and the direct stain gave negative reactions. The indirect reaction for MuLV IF antigens (Fig. 6) was partially inhibited by prior absorption of the rat antiserum with the plasma of AKR mice carrying the G+ AKR spontaneous leukemia K36 (Fig. 7), or with a soluble extract of E♂G2 leukemia cells (Fig. 8).

Autologous (host) immunoglobulins were bound to the glomeruli of (NZB × NZW) F₁ mice (11), as shown by the parallel study of adjacent sections with fluorescent antibody against mouse immunoglobulins. The bound immuno-
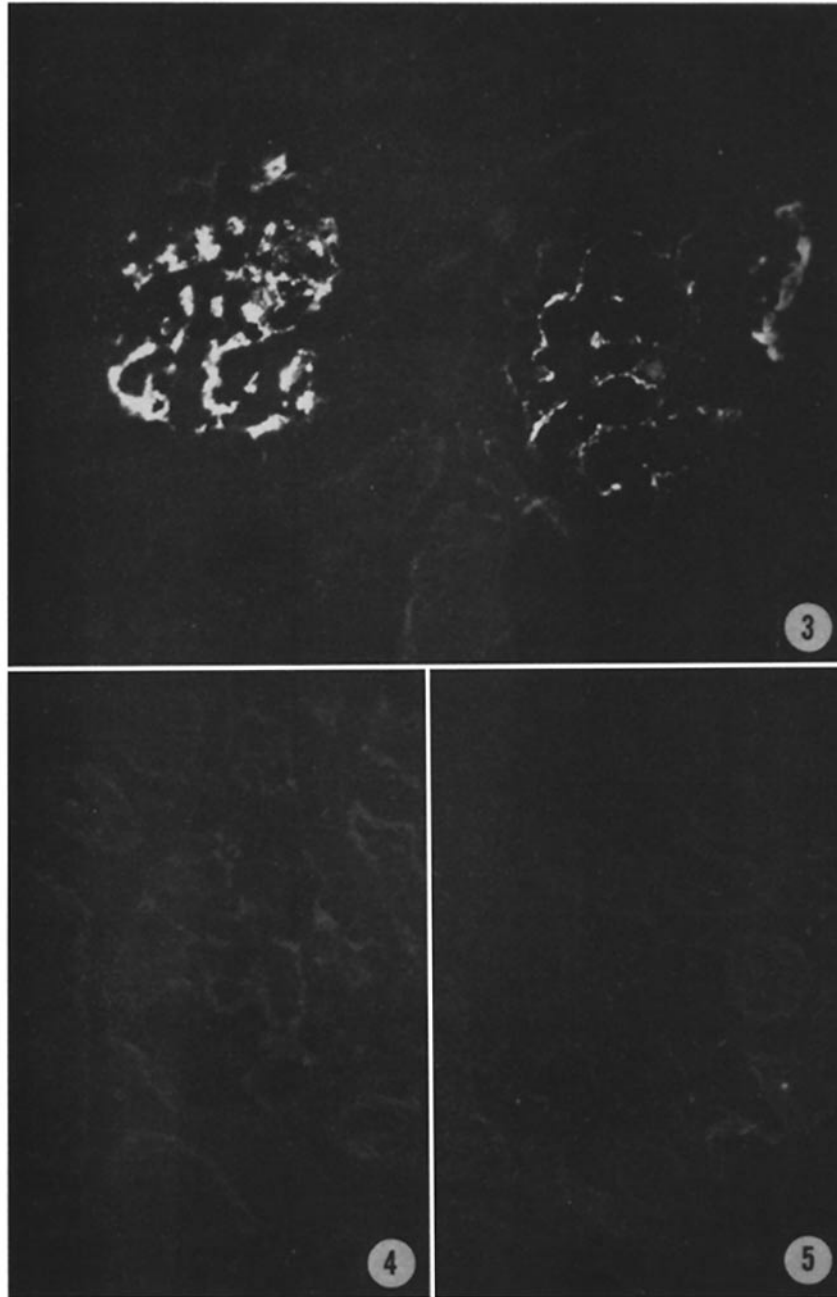
FIG. 3. Frozen section of perfused kidney of 7 month old female (NZB × NZW) F₁ hybrid mouse. MuLV IF antigens (white areas) located in the mesangium (left) and in the wall of the peripheral capillary loops (right) of two glomeruli. Indirect IF procedure using rat antiserum against MuLV IF antigens followed by fluorescent anti-rat γ-globulins, neither of which reagents reacted with mouse serum proteins. (see Methods and Materials). × 418.

FIG. 4. Negative control for Fig. 3. Frozen section of same kidney. Indirect IF procedure using normal rat serum followed by fluorescent anti-rat γ-globulins. × 418.

FIG. 5. Negative control for Fig. 3. Frozen section of perfused kidney of 2 month old C57BL/6 mouse. Indirect IF procedure using rat antiserum against MuLV IF antigens followed by fluorescent anti-rat γ-globulins. × 418.
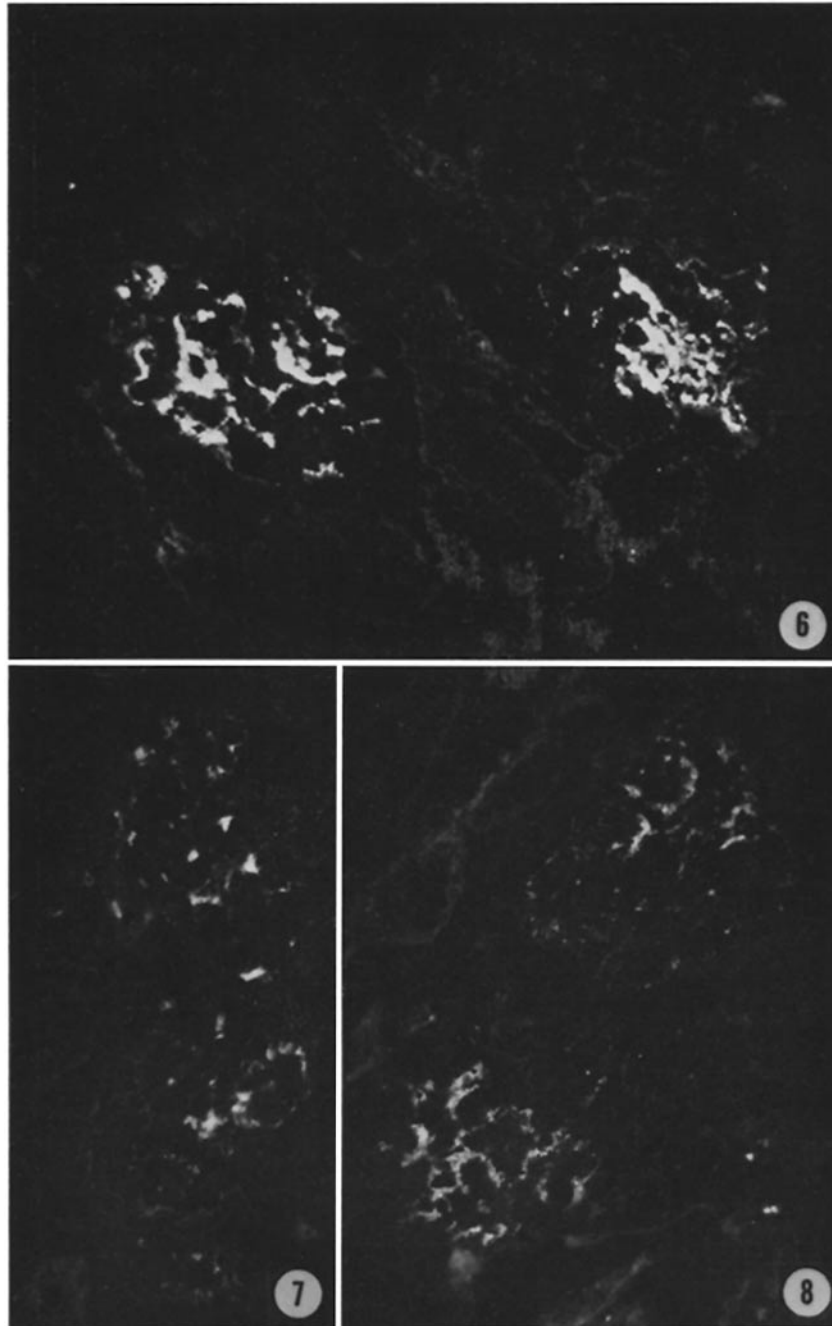
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FIG. 6. Frozen section of perfused kidney of 7 month old female (NZB × NZW) F₁ hybrid mouse. MuLV IF antigens (white areas) located in the mesangium and the wall of the peripheral capillary loops of two glomeruli. Indirect IF procedure using rat antiserum against MuLV IF antigens followed by fluorescent anti-rat γ-globulins. × 418.

FIG. 7. Frozen section of same kidney as in Fig. 6. Indirect IF procedure using rat antiserum against MuLV IF antigens absorbed with the plasma of AKR mice carrying the G+ AKR spontaneous leukemia K36. Glomerular immunofluorescence is decreased relative to Fig. 6. × 418.

FIG. 8. Frozen section of the same kidney as in Fig. 6. Indirect IF procedure using rat antiserum against MuLV IF antigens absorbed with soluble extract of E♂G2 leukemia cells. Glomerular immunofluorescence is decreased relative to Fig. 6.
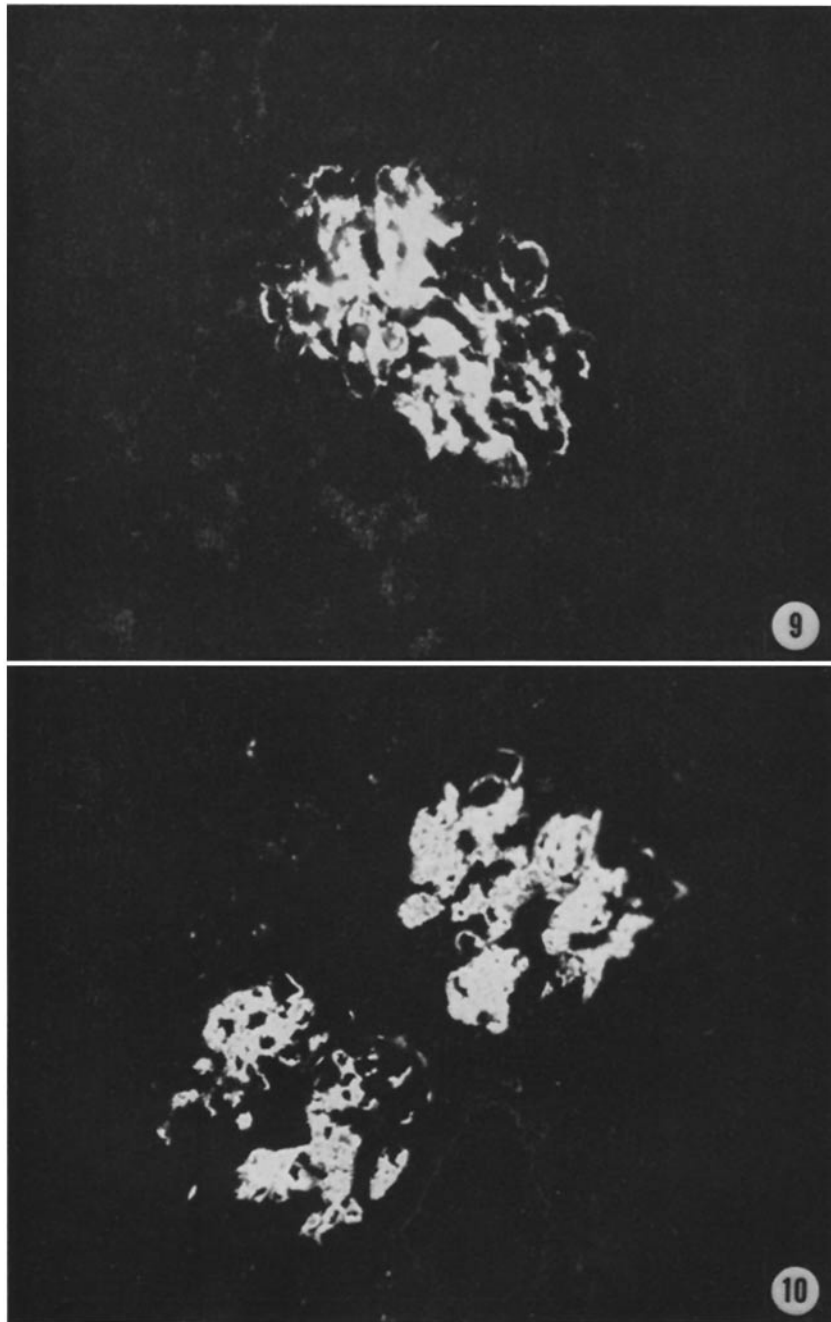
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FIG. 9. Frozen section of perfused kidney of 7 month old female (NZB × NZW) F₁ hybrid mouse. Autologous bound immunoglobulins located in the wall of the peripheral capillary loops and in the mesangium of the glomerulus. Direct IF procedure using fluorescent anti-mouse γ-globulins. × 418.

FIG. 10. Frozen section of perfused kidney of 11 month old female NZB mouse. Autologous bound immunoglobulins located mainly in the mesangium of two glomeruli. Direct IF procedure using fluorescent anti-mouse γ-globulins. × 418.

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globulins were located in the wall of the peripheral capillary loops and in the mesangium of the glomeruli (Fig. 9); their glomerular distribution was similar to but somewhat more widespread than the MuLV IF antigens.

Frozen sections of the perfused kidneys of 6 NZB mice 2–10 months of age were similarly studied for MuLV IF antigens. The reactions for MuLV IF antigens were negative at 2–4 months, weakly positive in glomerular mesangial
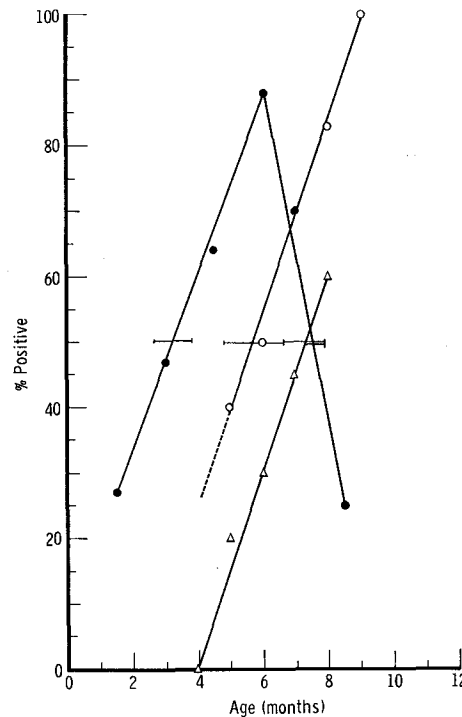

FIG. 11. Tests (% positive) for GSA (●), G natural antibody (○), and significant proteinuria (△) performed on (NZB × NZW) F_1 hybrid mice at various ages. Horizontal bar at 50% positive, age mean ±SE.

foci at 6–8 months, and more strongly positive at 9–10 months. Autologous (host) immunoglobulins were bound to the glomeruli at 4–12 months, increasing in amount with age (Fig. 10). These results on NZB mice corresponded to previous findings (9, 10).

The reactions for MuLV IF antigens were weakly positive in some glomeruli in the perfused kidneys of a female AKR mouse 8 months of age.

GSA.—81 tests for GSA were performed on the plasmas of 16 (NZB × NZW) F_1 hybrid mice equally divided as to sex and studied at 1–9 months of age. The results are shown in Figs. 11 and 12 and in Table VI. The first positive reactions

for GSA in the hybrid mice occurred at 2 months; 50% of the reactions were positive at 3.3 ± 0.6 months, and nearly 90% were positive at 6 months. Beyond the 6th month, GSA underwent elimination from the plasma; positive reactions were reduced to 50% at 7.6 ± 0.3 months and continued to fall thereafter.

The first positive reactions for GSA in NZB mice occurred at 3 months, and
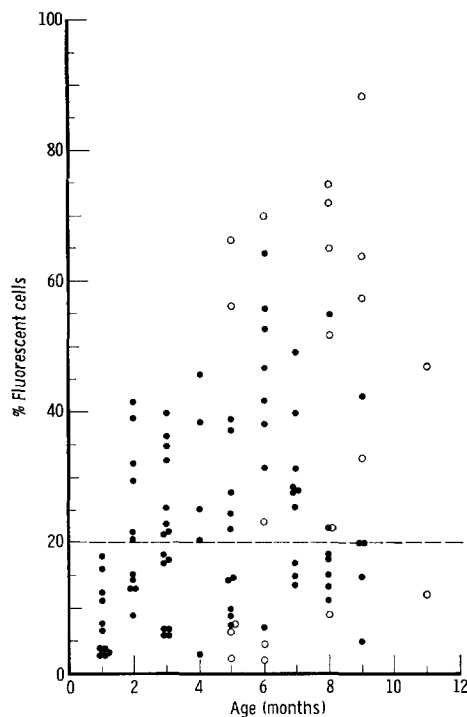

FIG. 12. Tests for GSA (●) and G natural antibody (○) performed on (NZB × NZW) F₁ hybrid mice at various ages. Reactions are graded by per cent of indicator cells showing membrane immunofluorescence: negative ($\leq 20\%$); weak positive (21–50%); strong positive (51–100%).

the 50% response time for GSA production and elimination was 5.3 ± 0.4 and 13.3 ± 0.6 months, respectively, as previously reported (9). This occurrence is significantly later than that observed in (NZB × NZW) F₁ hybrid mice.

74 tests for GSA were performed on the plasmas of 10 NZW mice 1–11 months of age. The reactions were graded by the per cent of indicator cells showing membrane immunofluorescence (see Methods and Materials) as follows: negative ($\leq 20\%$), weak positive (21–50%), and strong positive (51–100%). The following results were obtained: 1–6 months, 1 weak positive (at 2 months)

and 40 negatives; 7–10 months, 12 weak positives and 7 negatives; 11 months, 4 strong positives, 5 weak positives, and 5 negatives. Except for one early, weak positive reaction, GSA production in NZW mice was delayed in time and amount in comparison with NZB mice and (NZB × NZW) F_1 hybrid mice.

G Natural Antibody.—21 tests for G natural antibody were performed on the serums of 10 (NZB × NZW) F_1 hybrid mice equally divided as to sex and studied at 5–11 months of age. The results are shown in Figs. 11 and 12 and in Table VI. The first positive reactions for G natural antibody in the hybrid mice occurred at 5 months of age; 50% of reactions were positive at 5.8 ± 0.9 months, and a large majority was positive thereafter.

The first positive reactions for G natural antibody in NZB mice occurred at 10 months, and the 50% response time was 13.3 ± 0.4 months, as previously

TABLE VI

50% Response Time (T_{50}) for GSA Production and Elimination and G Antibody Production in (NZB × NZW) F_1 Hybrid Mice

Response	T_{50}*	SE	SD	N'
	Months	*Months*	*Months*	
GSA production	3.3	0.6	3.1	51
G natural antibody	5.8	0.9	2.4	15
GSA elimination	7.6	0.3	1.3	15

* Estimated by logarithmic-probit procedure and based upon N' determinations in the 6.7–93.3% range of response. Standard error (SE) equals SD $\div \sqrt{\dfrac{N'}{2}}$.

reported (9). This occurrence is significantly later than observed in (NZB × NZW) F_1 hybrid mice.

31 tests for G natural antibody were performed on the serums of 10 NZW mice 8–18 months of age. The results were as follows: 8–9 months, 14 negatives; 11 months, 2 positives and 8 negatives; 18 months, 6 positives and 1 negative. The detectability of G natural antibody in NZW mice was substantially delayed in comparison with (NZB × NZW) F_1 mice, but not appreciably delayed in comparison with NZB mice.

Proteinuria.—46 tests for proteinuria were performed on 10 (NZB ×NZW) F_1 hybrid mice of both sexes, and the mice were studied at 4–8 months of age. The results are shown in Fig. 11 and in Table VII. Significant proteinuria (positive reactions of 2+ or greater) occurred in some of the hybrid mice at 5 months of age and in 50% at 7.3 ± 0.6 months.

Cumulative Mortality.—Mortality statistics were tabulated at monthly intervals on 40 (NZB × NZW) F_1 hybrid mice equally divided as to sex. The results are shown in Table VII. 50% of the hybrid mice were dead at 8.0 ± 0.2

months. The major cause of death was renal glomerular disease (membranous and lupus glomerulonephritis) as verified by autopsy.

DISCUSSION

The present paper deals with the pathogenesis of "spontaneous" glomerulonephritis of NZB and (NZB × NZW) F_1 hybrid mice as studied from an immunological point of view, and as related to the antigens associated with wild-type Gross leukemia virus infection.

This study revealed the presence of G (Gross) soluble antigen (GSA) and the early and selective concentration of G natural antibody in the kidneys of NZB mice. These two reactants were apparently present in the kidneys as antigen-antibody complexes but not as free forms, for they were detected only in the eluates and never in the homogenates of kidneys. While the quantity of G antibody detected in the kidney eluate did not necessarily represent the precise

TABLE VII

50% Response Time (T_{50}) for Significant Proteinuria and Cumulative Mortality in (NZB × NZW) F_1 Hybrid Mice

Response	T_{50}*	SE	SD	N'
	Months	*Months*	*Months*	
Significant proteinuria	7.3	0.6	2.7	41
Cumulative mortality	8.0	0.2	1.4	160

* See footnote, Table VI.

amount present, owing to the possibility of antibody recombination with co-existing GSA in the eluate during the measurement, the quantity of G antibody actually measured in this study can be taken as an indication of the relative amount. G antibody was detectable in the kidney eluates of mice approximately 4–6 months of age, whereas it first appeared in the serum of NZB mice at about 10 months of age (9). The detection of G antibody in the kidney eluates, even before the manifestation of proteinuria, is in accord with the fact that glomerular lesions and glomerular-bound immunoglobulins are demonstrable in NZB mice in advance of proteinuria (10). The interpretation of these findings is that G antibody is produced by NZB mice at a young age and is combined with GSA to form antigen-antibody complexes which deposit in the kidneys (mainly in the glomeruli) and contribute to the development of glomerulonephritis, and that, in the later life of NZB mice, G antibody becomes detectable in the serum in free form only when its formation exceeds its neutralization by GSA. The finding that the activity of G antibody (per milligram 7S immunoglobulin) in the acid-buffer eluate of the kidneys of NZB mice with proteinuria was somewhat greater than that found in the serum supports this interpretation.

The observations were extended to the study of the pathogenesis of the fulminating renal glomerular disease (lupus glomerulonephritis) that typifies (NZB × NZW) F_1 hybrid mice (1, 4, 5). This renal glomerular disease is associated with the development of positive LE cell test; (1, 4) and anti-nuclear antibodies in high titer (5). DNA-anti-DNA immune complexes are implicated in the pathogenesis of the renal disease (22). The present study revealed the presence of murine leukemia virus (MuLV) CF antigens in extracts of the spleen, liver, and kidneys of (NZB × NZW) F_1 hybrid mice, as well as NZW and NZB mice, as shown earlier for NZB mice (9). C type murine leukemia virus-like particles have also been found in the corresponding tissues of (NZB × NZW) F_1 hybrid mice and NZW mice by electron microscopic study (23; Mellors and Huang, unpublished work), as reported previously for NZB mice (21, 23-27). The present study further revealed that both GSA and G natural antibody were detectable in the serum of (NZB × NZW) F_1 hybrid mice earlier than in NZB mice, and that the 50% response time for GSA elimination and for proteinuria manifestation of renal glomerular disease occurred in the hybrid mice at 7.3-7.6 months of age. In addition, murine leukemia virus IF antigens, together with host immunoglobulins, were demonstrated in the renal glomerular lesions of (NZB × NZW) F_1 hybrid mice, as shown previously for NZB mice by the immunofluorescence method (9) using a broadly reactive rat antiserum (20) with specificities for MuLV group- and viral and cellular G type-specific antigens. The acid-buffer eluate of the kidneys of (NZB × NZW) F_1 mice has been prepared but not yet analyzed for GSA, G antibody, and anti-nuclear antibody.

In the mouse system, the antibody against MuLV group–specific antigens has not yet been produced in any strains of mice. On the other hand, the antibodies against viral or cellular G type–specific antigens can be formed separately in different mouse strains. The G typing serum *C57BL anti-AKR spontaneous leukemia K36* contains only the antibody reactive with cellular G antigen(s) (8); the serum of some NZB mice contains the antibody against viral envelope G antigen(s) (8). Although as yet not proved it seems very likely that individual NZB mice may react immunologically with both viral and cellular G antigens. The illustrations of GSA in the acid-buffer eluate of the kidneys of NZB mice, as demonstrated by the immunofluorescence method (Fig. 2), suggested that the NZB mouse serum reacted with either a broader range or a greater quantity of G antigens, resulting in more intense and larger areas of positive staining than shown by the G typing serum *C57BL/6 anti-K36*. Thus, in terms of G type specificities, the serum of NZB mice may have a broad reactivity against G antigens similar to that of the rat antiserum against G antigens.

The implication of murine leukemia and sarcoma viruses in the pathogenesis of glomerulonephritis in mice is supported by other observations. AKR mice are, similar to NZB mice, congenitally and persistently infected with wild-

type Gross leukemia virus; glomerulonephritis occurs in some AKR mice in association with lymphatic leukemia (28). Mice experimentally infected with Rauscher, Friend, and Passage A Gross leukemia viruses (28, 29) develop renal glomerular lesions. BALB/c mice neonatally infected with Molony leukemia and sarcoma viruses (MLV and MSV) have MLV- or MSV-antibody complexes in the circulation and develop glomerular lesions, with viral antigen and antiviral antibody detectable in the kidneys (30).

As a general phenomenon, wild-type murine leukemia virus or virogene is virtually ubiquitous in mice (31). Its emerging role in the etiology and pathogenesis of spontaneous murine glomerulonephritis may be more widespread than is presently recognized.

NZB and (NZB × NZW) F_1 hybrid mice are a widely studied animal model of several human diseases of unknown etiology for which better understanding is urgently sought. These diseases include idiopathic glomerulonephritis, systemic lupus erythematosus, and related connective tissue and autoimmune diseases. In the association of autoimmunity, connective tissue disease, and lymphoid neoplasia (2, 32, 33, 6), NZB mice provide a uniquely challenging model of human diseases, because a similar constellation of disorders, or clinical transitions among them, are known to occur sometimes in man (34–37).

SUMMARY

The pathogenesis of the spontaneous glomerulonephritis of NZB and (NZB × NZW) F_1 hybrid mice is related at least in part to the formation of natural antibody against antigens of the G (Gross) system, and apparently to the deposition in the glomeruli of immune complexes of G natural antibody with G soluble antigen (GSA), type-specific antigen specified by wild-type Gross leukemia virus. G natural antibody and GSA are detectable in the acid-buffer eluate of the kidneys of NZB mice during the course of the glomerulonephritis. (NZB × NZW) F_1 hybrid mice develop glomerulonephritis and produce GSA and free G natural antibody earlier in life than do NZB mice. The proteinuria manifestation of the gomerulonephritis of (NZB × NZW) F_1 hybrid mice becomes increasingly prevalent as GSA undergoes immune elimination from the circulation. Gross leukemia virus–specified antigens together with bound immunoglobulins are located in the glomerular lesions of (NZB × NZW) F_1 hybrid mice, both in the mesangium as observed in NZB mice and also in the wall of the peripheral capillary loops of the glomeruli.

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