

ASSOCIATION OF CIRCULATING RETROVIRAL
gp70-ANTI-gp70 IMMUNE COMPLEXES WITH
MURINE SYSTEMIC LUPUS ERYTHEMATOSUS*

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New Zealand black (NZB) mice and their F₁ hybrids (NZB x W) spontaneously develop a disease that closely resembles human systemic lupus erythematosus (SLE)¹ (1, 2). The immunologic abnormalities of this disorder are characterized by the formation of several types of autoantibodies and immune complex (IC) glomerulonephritis (3-5). Endogenous retroviruses and their gene products have been implicated in the pathogenesis of murine SLE (6, 7), partially because of the remarkably high concentrations of the retroviral envelope glycoprotein, gp70, found in sera of NZ mice (6, 8, 9), and partly because gp70 is deposited in diseased glomeruli along with host immunoglobulins (Ig) and complement and nuclear antigens (4, 6, 7). Like NZB and NZB x W mice, the newly developed murine strains, MRL/1 and BXSB, also spontaneously develop an SLE-like disease and although their serum levels of gp70 are not as high as those of NZ mice, gp70 is deposited in diseased glomeruli (10). Considering that serum gp70 may form gp70-anti-gp70 ICs and, subsequently, deposit in glomerular lesions, we compared the four strains of SLE-prone mice to several immunologically normal murine strains with comparable levels of serum gp70 for the presence of circulating ICs. For this purpose, the molecular size of serum gp70 was determined by sucrose density gradient analysis, and the amount of rapidly sedimenting gp70 which was found bound to Ig was quantitated throughout the course of disease.

We found that sera from all SLE mice contained a heavy form of gp70 complexed to Ig, and that these complexes appeared with the onset of renal disease and persisted throughout its course. By contrast, the normal strains of mice with comparable serum gp70 levels did not have heavy gp70.

Materials and Methods

Mice. Of the SLE-prone strains, NZB and NZW mice were originally obtained from the

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; CP, cyclophosphamide; FeLV, Feline leukemia virus; IC, immune complex; Ig, immunoglobulin; *lpr*, lymphoproliferation; MuLV, murine leukemia virus; NRS, normal rabbit serum; PBS, phosphate-buffered saline; SLE, systemic lupus erythematosus; Staph A, *Staphylococcus aureus*; ssDNA, single-stranded DNA.

Laboratory Animals Centre, Medical Research Council, Surrey, England, and since 1965 have been maintained by brother-sister matings at Scripps Clinic and Research Foundation. Mating of NZB females with NZW males produced the NZB x W hybrids. The inbred strains BXSB, MRL/1, and MRL/n were obtained from the research colony of Dr. E. D. Murphy of The Jackson Laboratory, Bar Harbor, Maine. BXSB is a recombinant inbred strain derived from crossing a C57BL/6 female and an SB/Le male. Nearly 100% of the BXSB develop a spontaneous progressive lethal SLE-like disease affecting males much earlier than females. The MRL substrains were derived mainly from strain LG/J with contributions from AKR/J, C3H/Di, and C57BL/6. A spontaneous autosomal recessive mutation, *lpr* (lymphoproliferation) gene, producing massive T-cell proliferation and an early onset SLE-like syndrome was first observed at the 12th generation of inbreeding (11). Two inbred substrains that shared $\cong 89\%$ of their genomes were developed: MRL/1 (*lpr/lpr*), with massive lymphoproliferation, and MRL/n (+/+), without *lpr* and with late-life SLE. The immunologically normal strains, C57BL/6, BALB/c, and C3H mice were purchased from Leonell C. Strong Research Foundation, San Diego, Calif. AKR, DBA/2, LG/J, and 129 mice came from The Jackson Laboratories. Blood samples were collected by orbital sinus puncture, and the sera were stored at -20°C until use.

Retroviral Envelope Glycoprotein, gp70. gp70 of Rauscher murine leukemia virus (MuLV) and NZB xenotrope virus prepared as described by Strand and August (12) were kindly provided by Dr. J. Elder (the Scripps Clinic and Research Foundation). gp70 from AKR ecotrope virus and major structural protein p30 of Rauscher murine leukemia virus (MuLV) were a gift from Dr. A. F. Esser and Dr. R. M. Bartholomew (the Scripps Clinic and Research Foundation).

Antisera. Goat anti-feline leukemia virus (FeLV) antisera were obtained from the National Cancer Institute. Anti-NZB xenotrope virus antibodies were raised in goats by repeated injections of virus in Freund's adjuvant. Anti-mouse IgG and anti-goat IgG antibodies came from rabbits immunized by repeated injections of each respective IgG (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.) in Freund's adjuvant. Monospecific rabbit anti-mouse IgG antiserum was purchased from Litton Bionetics, Kensington, Md.

Radiolabeling Procedures. gp70 from Rauscher MuLV, NZB xenotropic virus and AKR ecotrope virus were labeled with radioactive iodine (^{125}I) by the chloramine T method (13). Single-stranded DNA (ssDNA) was labeled with ^{125}I by the method of Commerford (14).

Radioimmunoassay for gp70 and p30. The concentrations of gp70 in serum samples, gradient fractions, or eluates from kidneys were determined by inhibiting the binding of goat anti-FeLV antibody to ^{125}I -labeled gp70 from Rauscher MuLV. For the radioimmunoassay, 0.05 ml of test samples diluted in 0.1 M borate buffer (pH 8.0) containing 10% normal rabbit serum (NRS), 1% normal goat serum and 0.01% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) was added to 0.025 ml of goat anti-FeLV antiserum diluted in the same buffer. 0.025 ml ^{125}I -labeled Rauscher MuLV gp70 (1 ng) in borate buffer containing 0.2% bovine serum albumin (BSA) was added to these mixtures. After an overnight incubation at room temperature, 0.1 ml of rabbit anti-goat IgG antiserum which was diluted in borate buffer (sufficient to precipitate $> 95\%$ of IgG present in the reaction mixtures) was added, and incubation continued at 37°C for 30 min, followed by an additional 30-min incubation at 4°C . In the absence of the competing viral antigen, $\cong 40\text{--}50\%$ specific precipitation of ^{125}I -Rauscher MuLV gp70 was obtained. A standard inhibition curve was established with the buffer containing known amounts of gp70 from Scripps leukemia virus (7) and the concentration of gp70 present in tested samples was estimated by reference to the standard curve. In some experiments, the concentration of gp70 in samples was determined by a similar radioimmunoassay with ^{125}I -labeled NZB xenotropic gp70 and goat anti-NZB xenotropic virus antibodies. gp70 of NZB xenotropic virus was used as reference antigen for the latter radioimmunoassay.

To determine the amounts of gp70 bound to Ig, sera were depleted of Ig by immune precipitation with rabbit anti-mouse IgG antisera, in which anti-goat IgG activity was preabsorbed by goat IgG-coated immunoabsorbent columns because anti-goat IgG activity interferes with the radioimmunoassay for gp70. After the removal of Ig precipitates by centrifugation, the amount of gp70 in the supernate was determined. As a control, sera were treated with NRS. From the concentrations of gp70 in both samples, the amounts of gp70 bound to Ig were calculated.

The absorption of serum gp70 by *Staphylococcus aureus* protein A (Staph A, provided by Dr.

J. Elder) was carried out as follows. 0.2 ml of sera diluted 1/10 in 2 mg/ml BSA in borate buffer was incubated with 0.05 ml washed, packed Staph A pretreated with formaldehyde for 30 min at room temperature.

Concentrations of p30 in gradient fractions were determined in a blocking assay using ^{125}I -Rauscher MuLV p30 and goat anti-MuLV p30 antibodies (provided by Dr. R. V. Gilden of Flow Laboratories, Inc., Rockville, Md.) as described by Parks and Scolnik (15).

Radioimmunologic Detection of Anti-gp70 and Anti-DNA Antibodies. The presence of antibodies to AKR ecotropic gp70, Rauscher MuLV gp70, or NZB xenotropic gp70 was determined by using a radioimmunoassay in which the primary binding to each of these retroviral gp70s labeled with ^{125}I was followed by precipitation with rabbit anti-mouse IgG antisera. 0.005 ml of test serum, diluted 1/10 in borate buffer, was incubated with 0.025 ml of ^{125}I -gp70 (1 ng) overnight at room temperature. Then, 0.1 ml of rabbit anti-mouse IgG antisera (sufficient to precipitate > 95% of the IgG) was added, and mixtures were incubated at 37°C for 30 min and at 4°C for 30 min. The results are expressed as the percentage of ^{125}I -gp70 specifically precipitated after correction for the nonspecific precipitation which was obtained from a pooled serum of NZB mice that did not contain any anti-gp70 activity (8, 16).

Serum levels of anti-ssDNA antibodies were determined by using a modification of the Farr DNA-binding radioimmunoassay as described previously (17). The results are expressed as a percentage of 20 ng of ^{125}I -ssDNA precipitated by 0.01 ml serum at 50% saturated ammonium sulfate. The normal mean value of ^{125}I -ssDNA precipitation, assessed by testing sera from normal mice of five different strains (AKR, BALB/c, C3H, C57BL/6, and DBA/2), was $8.5 \pm 2.8\%$ (1 SD).

Sucrose Density Gradient Ultracentrifugation. The sedimentation characteristics of gp70 from serum or renal eluates was determined by using sucrose density ultracentrifugation. Samples of 0.05–0.15 ml were layered on 5–20% (wt/vol) linear sucrose gradients in 0.01 M phosphate-buffered saline, pH 7.0 (PBS) and centrifuged at 36,000 rpm for 15 h at 4°C with a SW60 rotor in a Beckman L-75 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The positions of IgM, IgG, or gp70 were established by radioactive markers, then the gradients were divided into 12 or 15 fractions. Each fraction was radioimmunoassayed for the presence of gp70. In some experiments, sera were layered on a 5–20% sucrose gradient in PBS containing 0.5 M KSCN and centrifuged at 36,000 rpm for 15 h at 4°C. The 8 or 12 fractions obtained from KSCN-containing gradients were immediately dialyzed against PBS before the determination of the gp70 content by the radioimmunoassay. IgG was detected in gradient fractions by radial double immunodiffusion with monospecific rabbit anti-mouse 7S IgG antisera.

Partial Purification of Serum gp70. Serum gp70 was partially purified by affinity column chromatography. Pooled sera from NZB, NZB x W, MRL/1, or BXSB mice were applied to a column containing concanavalin A-coupled Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). Bound gp70 was eluted by 0.1 M α -methyl-D-mannoside in PBS, concentrated, and dialyzed against PBS.

Elution of Kidney-Fixed gp70 and Ig. Renal eluates were prepared as described previously (18). Briefly, kidneys from each of the SLE strains (NZB, NZB x W, MRL/1, and BXSB) were pooled, cut into small pieces, and washed extensively with PBS overnight at 4°C. The tissues were homogenized and washed five times by centrifugation in PBS. The precipitates were resuspended in PBS containing Mg^{++} , digested with DNase, and eluted with 2.5 M KSCN in PBS. Obtained eluates were concentrated with a Diaflo ultrafiltration membrane (PM10, Amicon Corp., Scientific Sys. Div., Lexington, Mass.) in a microfiltration system. The IgG concentration was determined by radial immunodiffusion in agar (19).

Results

Retroviral gp70 and Anti-gp70 Antibodies in Sera. Levels of gp70 were determined in sera from four strains of mice which were considered immunologically abnormal because of their predisposition toward SLE (NZB, NZB x W, MRL/1, and male BXSB) and nine immunologically normal strains. In a radioimmunoassay with anti-FeLV antiserum and ^{125}I -Rauscher MuLV gp70, all the murine strains with SLE-like

TABLE I
Serum Retroviral gp70

Mice	Sex	Age		
		1-3 Mo	4-6 Mo	7-9 Mo
NZB	Female	42* (11)‡	32 (20)	46 (24)
	Male	75 (14)	53 (22)	56 (27)
NZB x W	Female	32 (28)	26 (38)	35 (16)
	Male	49 (31)	42 (59)	40 (27)
MRL/1	Female	18 (2)	18 (23)	7 (12)
	Male	25 (44)	25 (48)	23 (12)
BXSB	Female	11 (33)	7 (48)	6 (29)
	Male	17 (44)	20 (46)	11 (11)
NZW	Female	41 (14)	37 (34)	31 (25)
	Male	69 (18)	50 (35)	56 (24)
MRL/n	Female	8 (8)	6 (54)	5 (20)
	Male	21 (25)	13 (57)	12 (32)
DBA/2	Female	17 (16)	20 (20)	15 (12)
	Male	28 (14)	34 (13)	37 (12)
AKR	Female	8 (13)	22 (9)	12 (5)
	Male	11 (17)	14 (9)	14 (7)
LG/J	Male	58 (13)	NT§	NT
129	Male	21 (17)	NT	NT
C57BL/6	Female	1 (9)	NT	4 (8)
	Male	3 (20)	NT	4 (7)
C3H	Female	2 (7)	NT	3 (7)
	Male	5 (8)	NT	4 (3)
BALB/c	Female	2 (6)	NT	1 (7)
	Male	2 (5)	NT	4 (5)

* μg gp70/ml determined by a radioimmunoassay with ^{125}I -Rauscher MuLV gp70 and anti-FeLV antibodies.

‡ Number of mice tested.

§ Not tested.

disease had relatively high concentrations of gp70 in their sera, but several normal strains had equally high levels (Table I). Among the SLE-prone strains, NZB mice were the highest in concentration of gp70, an amount that was relatively large at only 1 wk after birth (mean of seven mice \pm 1 SD: $24 \pm 3 \mu\text{g}/\text{ml}$). The circulating gp70 reached maximum concentrations when the animals were 1 mo old and did not change greatly throughout life, in contrast to the continuous increase in serum levels of several autoantibodies and in the frequency of glomerulonephritis as the animals aged (3). Similar results were obtained in NZB x W, MRL/1, and male BXSB mice.

Because free antibodies to AKR ecotrope gp70 have been detected in mice carrying AKR ecotrope viral genome (16), we used a primary binding assay and three different sources of retroviral protein—AKR ecotrope gp70, Rauscher MuLV gp70, and NZB xenotrope gp70—to test the presence of serum antibodies to retroviral gp70.

When 2 mo old, binding to ^{125}I -AKR ecotrope gp70 was greater in the sera of two SLE-prone strains (NZB x W and MRL/1), as well as in several normal strains (NZW, MRL/n, LG/J, and C3H), than in the remaining strains tested (Table II). The activity was highest in NZB x W mice and generally became obvious when the mice were 4 wk old. After 2 mo, there was no age-dependent increase in the levels of anti-AKR ecotrope gp70 antibodies in contrast to the increase in anti-DNA antibodies.

TABLE II
Serum Anti-Retroviral gp70 and Anti-DNA Activity

Mice	Age	No.	Anti-AKR	Anti-Rauscher	Anti-Xeno	Anti-DNA
NZB × NZW female	1 wk	(5)	2.4 ± 0.6*	0.4 ± 0.4	NT‡	NT
	2 wk	(7)	1.8 ± 0.3	-0.1 ± 0.1	NT	NT
	3 wk	(7)	2.6 ± 0.4	0.7 ± 0.5	NT	NT
	4 wk	(19)	20.5 ± 9.9	3.4 ± 6.2	NT	10.0 ± 3.4§
	2 mo	(21)	32.2 ± 13.4	13.2 ± 13.5	0.4 ± 0.9	33.5 ± 12.7
	5 mo	(20)	34.7 ± 4.0	19.3 ± 13.8	NT	45.3 ± 20.6
MRL/1 female + male	8-10 mo	(63)	24.5 ± 8.3	4.6 ± 6.2	0.5 ± 1.3	69.0 ± 17.1
	2 wk	(8)	1.1 ± 1.4	-0.5 ± 0.8	1.9 ± 1.1	10.2 ± 3.9
	3 wk	(5)	3.0 ± 4.2	-0.6 ± 0.5	1.3 ± 0.7	NT
	4 wk	(9)	9.1 ± 4.5	8.6 ± 14.1	0.9 ± 0.8	11.5 ± 7.1
	2 mo	(22)	13.9 ± 10.3	2.8 ± 2.4	0.2 ± 0.7	30.9 ± 10.6
	4-5 mo	(23)	17.4 ± 10.3	6.2 ± 2.5	NT	81.2 ± 11.8
NZB female	2 wk	(7)	2.6 ± 0.5	0.1 ± 0.5	NT	NT
	4 wk	(8)	2.2 ± 0.3	-0.1 ± 0.2	NT	12.7 ± 5.3
	2 mo	(11)	2.2 ± 0.9	0.3 ± 0.5	NT	28.8 ± 8.8
	10 mo	(12)	3.3 ± 1.7	0.1 ± 1.1	0.9 ± 0.8	54.3 ± 9.4
BXSB male	2-3 mo	(18)	0.5 ± 1.9	0.2 ± 0.5	0.4 ± 0.7	16.6 ± 9.3
	6-8 mo	(28)	1.2 ± 1.9	0.5 ± 1.6	-0.3 ± 0.4	29.3 ± 16.6
MRL/n female + male	2 mo	(10)	12.8 ± 9.7	1.2 ± 1.7	1.5 ± 1.7	20.2 ± 9.3
NZW female + male	2 mo	(7)	10.4 ± 7.5	0.4 ± 1.0	0.5 ± 0.6	10.2 ± 4.7
LG/J	2 mo	(10)	24.1 ± 8.9	9.3 ± 5.0	NT	4.2 ± 1.2
129	2 mo	(12)	0.8 ± 1.2	-0.1 ± 0.4	-0.1 ± 0.9	8.2 ± 1.3
AKR	2 mo	(10)	-0.2 ± 0.6	0.1 ± 1.2	NT	7.5 ± 2.8
BALB/c	2 mo	(12)	0.1 ± 0.9	-0.5 ± 0.9	NT	9.1 ± 2.2
C3H	2 mo	(15)	4.7 ± 5.0	0.1 ± 0.9	NT	11.4 ± 2.5
C57BL/6	2 mo	(12)	-0.9 ± 1.0	0.3 ± 1.0	NT	11.0 ± 2.8
DBA/2	2 mo	(7)	-0.3 ± 1.0	2.3 ± 1.4	NT	4.9 ± 2.3

* Percent binding of ¹²⁵I-gp70 by 5 μl of serum. Mean ± 1 SD.

‡ Not tested.

§ Percent binding of ¹²⁵I-ssDNA by 10 μl of serum. Mean ± 1 SD.

In fact, the binding activity to AKR ecotrope gp70 was comparatively higher in sera of 2-mo-old mice than in 8-mo-old mice. As might be expected, the sera from NZB or BXSB mice which have no AKR ecotrope genome and therefore no exposure to AKR antigens, exhibited no significant binding to AKR ecotrope gp70.

Some sera from NZB × W, MRL/1, and LG/J mice also bound significant quantities of Rauscher MuLV gp70 (Table II). Increased anti-Rauscher MuLV gp70 activity was found more frequently in sera from 2- and 5-mo-old NZB × W mice than those from 8-mo-old mice.

In contrast to AKR ecotrope gp70 and Rauscher MuLV gp70 activities, none of the sera bound significant amounts of ¹²⁵I-NZB xenotrope gp70 (Table II). These data clearly indicate that significant levels of anti-AKR ecotrope gp70 and anti-Rauscher MuLV gp70 antibodies are not necessarily associated with murine SLE syndrome.

Sedimentation Rate of Serum gp70. Sera from SLE-prone mice and normal mice of various ages were next compared in terms of the sedimentation of gp70 molecules. After ultracentrifugation in sucrose density gradients, the gp70 was measured in each

of 15 serial gradient fractions by the radioimmunoassay.

In pooled sera from 1- to 3-mo-old NZB x W female mice (50% mortality 8–9 mo old) gp70 always appeared in or near gradient fractions containing the labeled 5S marker (Fig. 1). In comparison, when sera from 5-mo-old mice were ultracentrifuged, gp70 banded in a broader peak. Although the peak of gp70 was found in the 5S position, significant amounts of gp70 were detected in bands heavier than 7S IgG. In sera from 8- to 10-mo-old mice, larger amounts of gp70 appeared in the heavy fractions. When 15 individual sera of 8- to 10-mo-old mice were analyzed, all had the heavy form of gp70, but quantities of gp70 present in the heavy position varied in each sample. Although the sedimentation patterns were not uniform, many of sera contained two peaks of gp70: a wide peak of 9–19S region and another peak in the 5S position. In some sera, only a 9–19S peak of gp70 was observed. One should note that the amounts of heavy gp70 increased as the animals aged and as amounts of the light form of gp70 decreased. This heavy gp70 appeared 2–3 mo later in the sera of male NZB x W mice than in their female counterparts.

NZB mice live longer than NZB x W females (50% mortality in NZB males and females at 16–17 mo old), and the heavy form of gp70 was detectable in NZB sera only after the mice reached 8–10 mo of age (Fig. 1). Even then, less heavy gp70 was apparent than in NZB x W females of the same age. Heavy gp70 appeared at the same times in NZB males and females.

The newly developed SLE-prone murine strain, MRL/1, that is characterized by an early fatal glomerulonephritis (50% mortality: 5–6 mo old for both sexes) manifested the heavy form of gp70 earlier than NZB x W female mice. Sera from 1.5-mo-old MRL/1 mice exhibited only a single peak of gp70 at about the 5S region, but by 3 mo of age, both sexes had gp70 of the heavier variety (Fig. 1). The amounts of gp70 present in the heavy fraction increased as the MRL/1 mice aged, similarly to the NZB x W and NZB mice. On the other hand, MRL/n mice, which are a substrain of MRL mice and develop glomerulonephritis much later in life (50% mortality: female, 17 mo old; male, 23 mo old), had no detectable heavy gp70 during the first 8–10 mo of life. However, when 1 yr old, female, but not male MRL/n mice, expressed considerable heavy gp70 in their sera.

Another newly developed SLE-prone strain, BXSB, has a male dominant disease characterized by early fatal glomerulonephritis despite low levels of anti-DNA antibodies (50% mortality: male, 6 mo old; female, 15 mo old), only males formed rapidly sedimenting gp70 beginning at 3 mo of age (Fig. 1). As the animals aged, an increased proportion of the gp70 sedimented toward the bottom of the gradient. However, female BXSB mice, which are immunologically normal throughout the first 9 mo of life, developed no heavy gp70 until 8–10 mo old.

A possibility exists that anti-Rauscher MuLV gp70 antibodies present in sera from 2- to 3-mo-old NZB x W mice and 8-mo-old LG/J mice might interfere with detection of heavy gp70, because ¹²⁵I-Rauscher MuLV gp70 was used as the labeled antigen in the radioimmunoassay. Therefore, selected serum samples from 3- and 8-mo-old NZB x W mice and 8-mo-old LG/J mice which contained anti-Rauscher MuLV gp70 antibodies were analyzed in a different radioimmunoassay using ¹²⁵I-NZB xenotrope gp70 and anti-NZB xenotrope virus antibodies. In a serum from an 8-mo-old NZB x W mouse, considerable amounts of heavy gp70 were demonstrated with this assay,

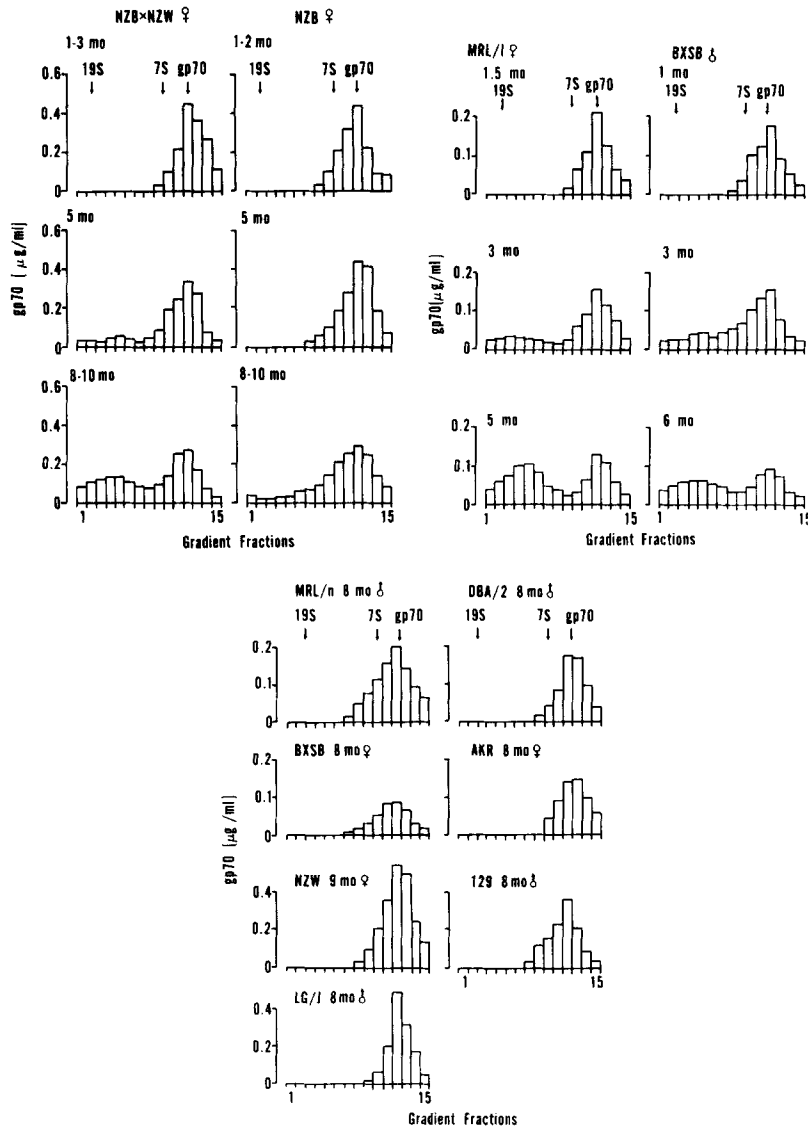


FIG. 1. Sedimentation rate of serum gp70 from SLE-prone and normal mice at various ages analyzed by sucrose density gradient ultracentrifugation. The concentration of gp70 was determined by the radioimmunoassay with anti-FelV antiserum and ^{125}I -Rauscher MuLV gp70. The position of markers is indicated by the arrows.

whereas none of the sera from 3-mo-old NZB x W or 8-mo-old LG/J mice had the heavy gp70 (data not shown).

The sedimentation pattern of serum p30 of a pooled serum from 8-mo-old NZB x W female mice was compared to that of gp70. In contrast to the wide range of sizes found for gp70, all the p30 molecules sedimented in the 3-5S gradient fractions, indicating an absence of complexes containing this major viral protein.

Characterization of Heavy Form of Serum gp70. Because the presence of heavy gp70 in

sera correlates well with the progression of murine SLE, the heavy gp70 may represent ICs. To examine this possibility, we incubated the sera with a Staph A bacterial absorbent, which effectively removes both IC and IgG from serum (20, 21). When pooled sera from 8-mo-old NZB x W female mice were absorbed with Staph A, then subjected to sucrose density ultracentrifugation, the heavy form of gp70 was no longer detectable (Fig. 2). However, amounts of gp70 in the 5S fractions were similar to those in unabsorbed sera, indicating that Staph A selectively absorbed heavy gp70. We then quantitated gp70 before and after absorbing the sera with Staph A and found that absorption removed \cong 50% of the circulating gp70 of 8-mo-old NZB x W females (Table III). Considerable amounts of gp70 were also absorbed from the sera of 5-mo-old MRL/1, 6-mo-old male BXSB, and 10-mo-old NZB mice with Staph A. Most of this gp70 was recovered by treating the absorbent with 2.5 M KSCN for 30 min at room temperature. However, Staph A did not significantly absorb gp70 from the sera of 1-mo-old mice of lupus strains or of 8- to 10-mo-old mice of normal strains tested (NZW and DBA/2).

To further characterize the heavy gp70, immunoabsorptions were performed. For this purpose, gp70 was partially purified from sera by passage through concanavalin A-coupled Sepharose 4B columns and concentrated. This procedure led to about a fivefold increase in the concentration of gp70, whereas the concentration of IgG decreased > 10 times. Then, 0.05 ml of concentrated serum gp70 preparations from 8-mo-old NZB x W female mice were treated with 0.1 ml of rabbit anti-mouse IgG antibodies, which precipitated most of the IgG in the samples leaving the supernates to be analyzed by sucrose density gradient ultracentrifugation. Control samples were treated with NRS in a similar manner. In addition, comparable samples absorbed with Staph A were centrifuged in parallel runs on sucrose density gradients. A representative experiment is shown in Fig. 3. Samples treated with anti-IgG antibodies did not have the heavy form of gp70 compared to those treated with NRS, although the amounts of gp70 in 5S regions were similar in both gradients. The distribution of gp70 in the gradient fractions after absorption with anti-IgG antibodies was essentially identical to that in samples absorbed with Staph A. Similar results were obtained in concentrated gp70 samples from MRL/1, BXSB, and NZB mice, although absorption with Staph A was generally more efficient in removing the heavy form of gp70 than absorption with anti-IgG antibodies.

Our final means of establishing that the heavy gp70s were ICs was to investigate the effect of KSCN, an IC-dissociating agent, on heavy gp70. A serum pool from 8-mo-old NZB x W female mice was subjected to ultracentrifugation on sucrose density gradients in the presence or absence of 0.5 M KSCN. Treatment of sera with KSCN led to a disappearance of heavy gp70 but increased concentrations of gp70 in the 5S position compared to sera not exposed to KSCN (Fig. 4 a). Similar experiments were performed with partially purified, concentrated serum gp70 from 8-mo-old NZB x W females. All the gp70 shifted to the 5S regions in gradients containing KSCN in contrast to the diffuse distribution of gp70 when KSCN was absent (Fig. 4 b). IgG always appeared in fractions heavier than 7S as well as in 7S position of gradients without KSCN, whereas IgG was located only in the 7S region of gradients with KSCN. When this experiment was repeated on the 10-19S components containing heavy gp70 obtained from these gradients without KSCN, the addition of KSCN

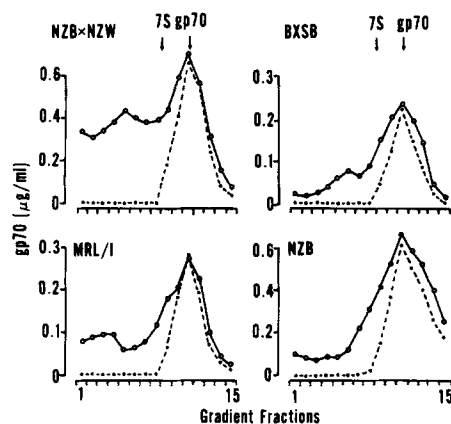


FIG. 2. Ultracentrifugation analysis of molecular size of serum gp70 before (○) and after (●) treatment of sera with Staph A. 0.2 ml of sera diluted one-half in 2 mg/ml BSA were incubated with 0.25 ml of washed, packed Staph A for 30 min at room temperature. The position of markers is indicated by the arrows.

TABLE III
Absorption of Serum gp70 with Staph A

Mice	Age	gp70 (μg/ml)			
		Unabsorbed	Absorbed*	(Percent absorption)	Eluate from Staph A‡
NZB × NZW female	8 mo	30.4	16.2	(46.7)	12.3
	1 mo	35.0	35.8	(0)	NT§
MRL/1 female	5 mo	12.4	7.9	(36.3)	4.3
	1 mo	15.6	15.5	(0)	NT
BXSb male	6 mo	17.5	11.3	(35.4)	6.0
	1 mo	15.8	15.4	(2.5)	NT
NZB female	10 mo	34.1	26.5	(22.3)	5.6
	1 mo	36.2	37.8	(0)	NT
NZW female	10 mo	47.9	47.2	(1.5)	NT
	1 mo	44.4	42.5	(4.3)	NT
DBA/2 female	8 mo	10.5	10.8	(0)	NT
	1 mo	16.8	16.1	(3.6)	NT

* 200 μl of pooled sera diluted 1/10 in 2 mg/ml BSA in PBS were incubated with 50 μl packed Staph A for 30 min at room temperature.

‡ To elute gp70 bound to Staph A, Staph A was first washed five times with 1 ml of PBS and treated with 200 μl of 2.5 M KSCN in PBS for 30 min at room temperature. Eluates were immediately dialyzed against PBS.

§ Not tested.

caused almost all the gp70 to sediment in the 5S regions (Fig. 4 C). In contrast, without KSCN, most of gp70 again sedimented in 10–19S regions.

To examine whether KSCN-dissociated gp70 and Ig could reassociate and form heavy gp70, 7S and 5S fractions were obtained by ultracentrifuging concentrated gp70 in the presence of KSCN. Both fractions were extensively dialyzed against PBS, mixed and incubated overnight at room temperature, then ultracentrifuged again. In the mixed fractions, considerable amounts of gp70 shifted toward the bottom of the

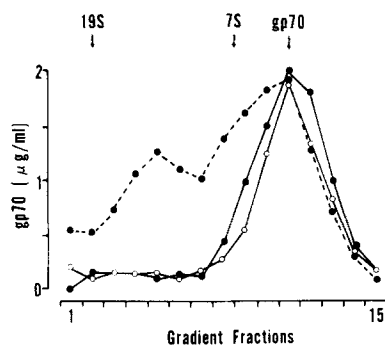


FIG. 3. Absorption of the heavy gp70 with anti-IgG antibodies or Staph A. Partially purified and concentrated gp70 from 8-mo-old NZB x W female mice was treated with anti-IgG antibodies (●—●), Staph A (○—○) or NRS (●—●) before the ultracentrifugation analysis. The position of markers is indicated by the arrows.

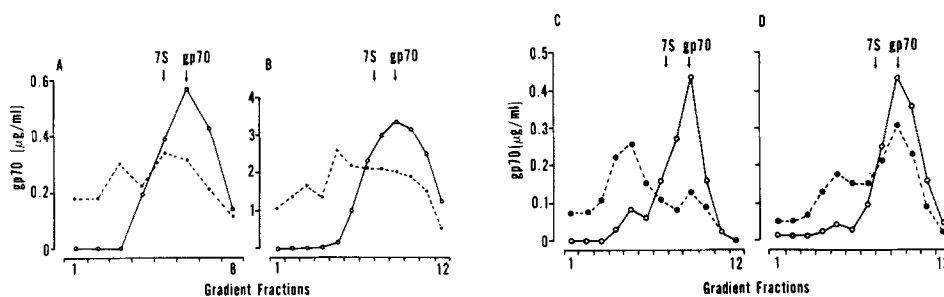


FIG. 4. (A) Effect of KSCN on the molecular size of serum gp70. A pooled serum from 8-mo-old NZB x W female mice was subjected to sucrose density gradient ultracentrifugation in the presence (○) or absence (●) of 0.5 M KSCN. Fractions obtained from KSCN-containing gradient were immediately dialyzed against PBS before the determination of gp70 content by the radioimmunoassay. (B) Effect of KSCN on the molecular size of serum gp70 partially purified by passage through concanavalin A-coupled Sepharose 4B. gp70 was partially purified from sera of 8-mo-old NZB x W female mice and subjected to ultracentrifugation in the presence (○) or absence (●) of 0.5 M KSCN. (C) Dissociation of the heavy gp70 by KSCN. The partially purified serum gp70 from 8-mo-old NZB x W female mice was subjected to ultracentrifugation without KSCN. Then, 10-19S components containing heavy gp70 were pooled and ultracentrifuged again in the presence (○) or absence (●) of 0.5 M KSCN. (D) Reassociation of KSCN-dissociated gp70 and Ig. The partially purified serum gp70 from 8-mo-old NZB x W female mice was subjected to ultracentrifugation in the presence of 0.5 M KSCN. 7S and 5S fractions were extensively dialyzed against PBS, mixed and incubated overnight at room temperature, then ultracentrifuged again without KSCN (●). 5S fractions alone were ultracentrifuged in a parallel run (○). The position of markers is indicated by the arrows.

gradient, indicating reassociation of recovered reactants (Fig. 4 d). However, such a reassociation was completely inhibited when Ig, which were present in the mixtures, were removed by precipitation with anti-IgG antibodies. When only 5S fractions were tested, most of the gp70 remained in the 5S regions.

Quantitation of Free and Ig-Bound Serum gp70. Concentrations of gp70 were measured after treatment of individual test sera with rabbit anti-mouse IgG antibodies, or as a control with NRS, to quantitate free and Ig-bound gp70. In 1- to 3-mo-old NZB x W female mice, no significant amounts of gp70 were removed with anti-IgG antibodies (Fig. 5). However, \cong 30% of sera from 5-mo-old mice and almost all the sera from 8- to 10-mo-old mice lost one-fifth to one-half of their content of gp70 through absorption

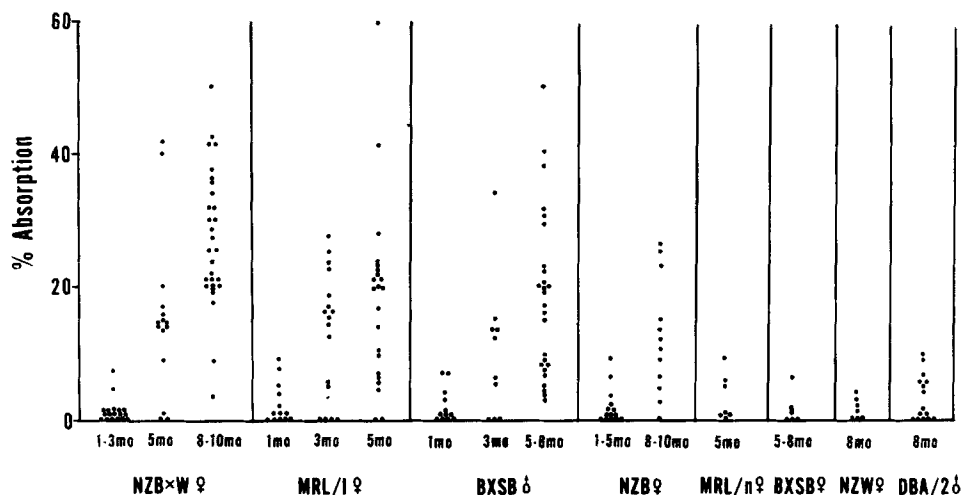


FIG. 5. Quantitation of IgG-bound serum gp70 from SLE-prone and normal mice at various ages. Concentrations of gp70 were determined before and after removal of serum Ig by rabbit anti-mouse IgG antibodies. Results are expressed as the percentage of serum gp70 absorbed with anti-IgG antibodies.

by anti-Ig. Compared to female NZB x W mice, the males developed Ig-bound gp70 2–3 mo later. MRL/1 and male BXS B mice had Ig-bound gp70 earlier than NZB x W mice. About 70% of 5- to 8-mo-old mice had significant amounts ($> 1/10$) of gp70 complexed with Ig (Fig. 5). However, no gp70 was absorbable from sera of MRL/n or female BXS B mice at 5–8 mo old. In NZB mice, the complexing of gp70 to Ig appeared first at 8–10 mo old, and the incidences and amounts were lower than in other SLE-prone strains (Fig. 5). In contrast, none of the sera from normal strains contained significant amounts of Ig-bound gp70 during the first 8–10 mo of age.

Effect of Cyclophosphamide-Treatment on the Formation of Heavy gp70. The development of the Ig-bound, heavy form of gp70 was examined in NZB x W females which had received the immunosuppressive drug, cyclophosphamide (CP) from 4 mo of age. CP had a dramatic effect because this treatment prolonged the animals survival time (at 14 mo of age: CP-treated, 100% survival; untreated, 20% survival) and suppressed the spontaneous development of anti-DNA antibodies (percent binding of ^{125}I -ssDNA at 8 mo of age. CP-treated, 12.8 ± 3.9 ; untreated, $70.1 \pm 8.8\%$). However, there was no significant difference in serum levels of gp70 between CP-treated ($21.5 \pm 6.3 \mu\text{g/ml}$) and untreated mice ($21.9 \pm 8.3 \mu\text{g/ml}$). The details of this experiment will be described elsewhere.² We then compared the development of heavy gp70 in three 13-mo-old NZB x W females treated with CP to that in three 8-mo-old untreated mice of the same strain and sex. As analyzed after ultracentrifugation, none of the CP-treated mice had the heavy form of gp70 in their sera (Fig. 6); in fact, the sedimentation pattern of their serum gp70 was essentially identical to that of 2-mo-old mice. On the other hand, all three serum samples from untreated mice had relatively large amounts of gp70 in the gradient fractions heavier than 7S IgG. When the two groups of sera were compared after absorption, considerable amounts of gp70 from untreated

²Theofilopoulos, A. N., R. A. Eisenberg, S. Izui, P. S. McConehey, and F. J. Dixon. Manuscript in preparation.

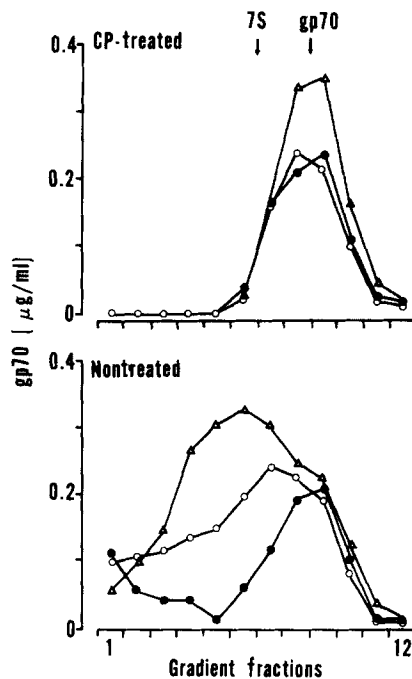


FIG. 6. Failure to develop the heavy gp70 in NZB x W female mice treated with CP. Three sera from CP-treated mice at 13 mo of age and three from untreated mice at 8 mo of age were subjected to sucrose density gradient analysis for the presence of the heavy gp70. The position of markers is indicated by the arrows. CP-treated: Δ , No. 7; \circ , No. 8; \bullet , No. 9. Nontreated: \circ , No. 13; \bullet , No. 14; Δ , No. 15.

mice were absorbed with anti-IgG antibodies or Staph A, whereas no significant amounts of gp70 from sera of CP-treated mice were absorbed with anti-IgG antibodies or Staph A (Table IV).

Sedimentation Rate of gp70 in Renal Eluates. Considerable amounts of retroviral gp70 were demonstrated in renal eluates from 2-mo-old ($5 \mu\text{g/g}$ of tissue) and 8- to 10-mo-old NZB x W female ($16 \mu\text{g/g}$ of tissue) mice, whereas IgG was demonstrable only in eluates from 10-mo-old mice ($590 \mu\text{g/g}$ of tissue). The distribution of gp70 as seen by immunofluorescence was limited to the tubules, particularly the apical portions of the convoluted tubules, in the 2-mo-old mice but involved glomeruli as well as tubules in the older mice (6, 9, 10). When we analyzed the molecular size of this gp70, renal eluates from old NZB x W mice contained some heavy gp70, although $>50\%$ of gp70 sedimented near the 5S regions (Fig. 7). By contrast, gp70 in renal eluates obtained from 2-mo-old mice sedimented only in the 5S position. This heavy gp70 in renal eluates was selectively absorbed by Staph A or anti-mouse IgG antibodies. A similar heavy gp70 was also found in renal eluates from 10-mo-old NZB, 6-mo-old BXSB, and 5-mo-old MRL/1 mice.

Discussion

The major envelope glycoprotein, gp70, of murine retroviruses is present in the serum of virtually all mice (6, 8, 9), but its expression does not correlate with the

TABLE IV
Absorption of Serum gp70 with Staph A or Anti-IgG Antibodies in CP-Treated and Nontreated (NZB × NZW) F₁ Female Mice

Treatment	No.	Staph A*			Anti-IgG‡		
		Unab-sorbed	Ab-sorbed	Absorp-tion %	Unab-sorbed	Ab-sorbed	Absorp-tion %
CP	7	32.4§	31.9	1.5	30.9	32.1	0
	8	22.9	21.0	8.3	22.4	21.6	3.6
	9	20.6	19.6	4.9	21.7	20.6	5.1
Control	13	31.4	19.4	38.2	32.2	25.4	21.1
	14	20.0	14.3	28.5	19.4	15.8	18.6
	15	32.8	12.8	61.0	32.1	20.7	35.5

* 200 μ l sera diluted $\frac{1}{10}$ in 2 mg/ml BSA in PBS were incubated with 50 μ l packed Staph A for 30 min at room temperature.

‡ 100 μ l sera diluted $\frac{1}{10}$ in 2 mg/ml BSA in PBS were treated with 100 μ l rabbit anti-mouse IgG antibodies or NRS for 30 min at 37°C and for 60 min at 4°C.

§ Retroviral gp70 μ g/ml determined by radioimmunoassay with ¹²⁵I-Rauscher gp70 and anti-FeLV antibodies.

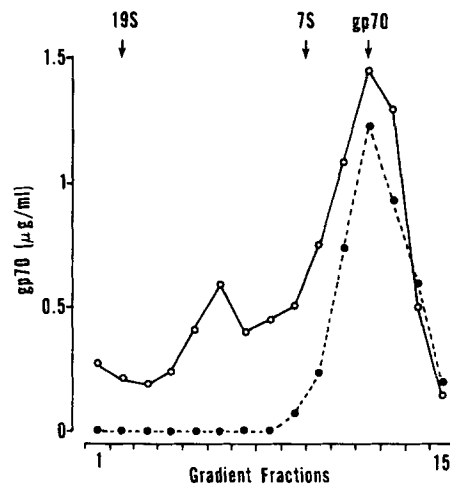


FIG. 7. Ultracentrifugation analysis of molecular size of gp70 in renal eluates obtained from 2-mo-old (●) and 8- to 10-mo-old (○) NZB × W female mice. The position of markers is indicated by the arrows.

progression of nephritis nor of autoimmune disease in mice that characteristically succumb to SLE-like symptoms (10, 16, 22, 23). However, our investigation of gp70-anti-gp70 ICs present in murine sera demonstrated that all the SLE-prone strains contain considerably more complexed, heavy form of gp70 than SLE-free mice. This complexed gp70 varies in molecular size from 9S to 19S position on sucrose density gradient ultracentrifugation. Unlike the 5S gp70, the heavy gp70 increases not only in incidence, but also in amounts with the progression of disease in all the SLE strains, although the total levels of serum gp70 do not change after the animals reach 1 mo

of age. The appearance of heavy gp70 varies with age among the SLE susceptible strains, but parallels the onset of renal disease and persists throughout the course of disease. In fact, MRL/1 and BXSB mice, which have a 50% mortality at 5–6 mo, develop the heavy gp70 in their sera earlier than other SLE mice do. Similarly, heavy gp70 appears in sera of female NZB x W mice earlier than in the males. However, a substrain of MRL mice, MRL/n, and female BXSB mice, which develop glomerulonephritis in the 2nd yr of life with a 50% mortality at >15 mo, fail to develop the heavy gp70 during the first 8–10 mo of age. In contrast, sera from other immunologically normal strains with high concentrations of gp70, such as NZW, LG/J, and DBA/2 mice, have no heavy gp70, at least during the first 8–10 mo of life.

Several findings suggest that this heavy form of gp70 is a complex with antibodies to the viral protein. First, the heavy form of gp70 is selectively absorbed with anti-IgG antibodies as well as Staph A. The appearance of Ig-bound gp70 complexes in sera is concomitant with that of heavy gp70, and the degree of gp70 sedimented in the heavy position of gradients correlates with the amounts of gp70 absorbable with anti-IgG antibodies or Staph A. Second, these complexes are dissociated by a chaotropic ion, KSCN, and when the reactants recovered from 7S regions and 5S regions of KSCN gradients are mixed under associating conditions, they recombine and form the heavy gp70. The facts that the reassociation of recovered reactants is completely inhibited when IgG is removed from the reactants and that components obtained from 5S regions do not form the heavy gp70 by themselves, indicate that IgG is required to form the heavy form of gp70. Finally, the finding that NZB x W females treated with an immunosuppressive drug, CP, have no heavy gp70 even at 1 yr of age, but still have levels of gp70 as high as untreated mice supports an immunologic mechanism for the development of heavy gp70.

Because antibodies to viral envelope components of MuLV were previously observed in sera from NZB and NZB x W mice (16, 24), in the present study, serum levels of antibodies against three antigenically different gp70 were measured. It was clear that all strains of mice with AKR ecotrope genome spontaneously develop antibodies against AKR ecotrope gp70 (16). Among these strains, NZB x W mice formed the greatest amount of antibody. In addition, some NZB x W, MRL/1, and LG/J mice produced antibodies reacting with anti-Rauscher MuLV gp70. However, in view of the facts that (a) the serum titers of these antibodies do not correlate with the progression of disease; (b) NZB mice and BXSB males, which develop the Ig-bound, heavy gp70, do not have detectable amounts of anti-AKR ecotrope or anti-Rauscher MuLV gp70 antibodies; (c) several immunologically normal murine strains spontaneously develop these antibodies; and (d) these antibodies were not concentrated in renal eluates from any of SLE strains (10), it appears that anti-AKR ecotrope or anti-Rauscher MuLV gp70 antibodies are not involved in the formation of gp70 ICs.

In view of the lack of correlation between anti-AKR and Rauscher gp70 antibodies and the formation of gp70 ICs, it is most likely that the antibody involved is directed to xenotropic gp70 which is the usual kind of gp70 in the serum of virtually all mice (36). If this is the case, the mice which develop SLE would not have a unique kind or amount of gp70 but instead, they would have the unique ability to make an antibody response to the ubiquitous molecule perhaps as part of their general immunologic hyperresponsiveness. Possible bases for such a hyperresponsiveness of SLE-prone mice

have been suggested (25–30), for example, loss of suppressor T cells (31, 32) and/or increased B cell activity (22, 33, 34). Alternatively, SLE mice might produce a unique and as yet undiscovered form of retroviral gp70 during the course of disease, possibly as a result of an abnormality in genes that regulate expression of retroviral gp70 or a recombination event, e.g., between ecotrope virus and xenotrope virus as observed in mink cell focus-inducing viruses (35).

The presence of retroviral gp70 ICs in sera and tissue lesions does not imply that retroviral production is involved in the disease of SLE mice, because the expression of retroviral gp70 may occur independently of complete virus production as in the case of serum gp70 (8, 36). Indeed, no consistent pattern has been found in the virologic phenotypes of four different SLE strains (16, 37). Recently, Datta et al. (38) demonstrated that the phenotypic expression of xenotropic viruses was unrelated to glomerulonephritis in crosses between NZB and SWR mice. However, one cannot exclude the possibility that the production of other as yet unrecognized kinds of retroviruses might be involved in murine SLE.

Our results strongly suggest that circulating gp70 ICs are a potential source of tissue injury in all mice with SLE. The presence of the complexed form of gp70 in renal eluates from the diseased kidneys of adult mice but not in those from 2-mo-old mice also supports the involvement of these gp70 ICs in the renal lesions. It is also noteworthy that in the sera, we did not find a heavy form of p30, the major structural protein of retroviruses. This is compatible with the previous observations of relatively infrequent deposits of p30 in glomerular lesions (6, 10).

Our observation, however, does not imply that gp70 ICs are the primary or major cause of glomerulonephritis in SLE strains. As described previously, lupus mice produce a variety of antibodies reactive with native or altered autologous antigens (3–5, 10, 39). A number of these antibodies seem to be involved in the pathogenesis of murine SLE through the combination with soluble tissue antigens released in circulating blood or in extravascular spaces to form ICs resulting in tissue damage. In fact, unidentified circulating IC-like materials have been found in sera from SLE mice by a Raji-cell assay (10) and conglutinin-binding assay (40), and significant concentrations of anti-DNA antibodies in the renal eluates were demonstrated in these mice (4, 10). The induction of tolerance to DNA antigen in NZB x W mice was shown to prolong survival and decrease nephritis as well as anti-DNA production (41, 42), supporting the pathological importance of DNA-anti-DNA complexes in SLE mice. Therefore, it will be necessary to quantitate the amounts of gp70 ICs in the serum and tissue lesions in relation to other ICs to determine the magnitude of the role of gp70 ICs. However, the present study clearly shows that the Ig-complexed, heavy form of gp70 is a new useful marker of murine SLE.

Summary

Endogenous retroviral gp70 was investigated as a participant in the pathogenesis of a lupus-like disease that spontaneously develops in four kinds of mice (NZB, NZB x W, MRL/1, and male BXSB). Sera from these strains contain a heavy form of gp70 that varies in sedimentation rates from 9S to 19S in sucrose density gradient analysis and appears with the onset of disease and persists throughout its course. Immunologically normal strains of mice do not develop rapidly sedimenting gp70 by 8–10 mo of life. The fact that the heavy gp70 is selectively absorbed with anti-IgG antibodies or

with *Staphylococcus aureus* protein A suggests that it is complexed with antibodies. The incidence and quantities of these gp70 ICs rise with the progression of disease in all strains with lupus. These findings suggest that Ig-complexed heavy gp70 may be involved in the pathogenesis of glomerulonephritis of mice with SLE.

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