**IMMUNOHISTOPATHOLOGICAL ANALYSIS OF IMMUNE-
COMPLEXES RELATED TO ANTI-DNA ANTIBODIES
IN THE SERUM AND ON THE GLOMERULUS
IN MRL/1 MICE**

ATSUSHI KOHNO, KEN-ICHI SEKITA AND YOSHIHIRO HAMASHIMA

*Department of Pathology, Faculty of Medicine,
Kyoto University, Kyoto 606*

Received for publication December 27, 1982

To analyze the characteristics of the immune deposits related to anti-DNA antibodies on the glomeruli of MRL/MP-1pr/1pr (MRL/1) mice, an elution study and an immunofluorescent study using antiultraviolet-irradiated (uv) DNA antisera (rabbit) were carried out. MRL/1 mice developed a high concentration of immunoglobulin G (IgG), anti-DNA antibodies, rheumatoid factors (RF), and circulating immune complexes (CIC) in the sera. In contrast, RF and IC were not significantly detected in the renal eluates in MRL/1 mice, although anti-DNA antibodies revealed high titers, while the titers of anti-dsDNA antibodies in the sera and eluates after DNase digestion did not increase significantly. Furthermore, DNA were not detected as the antigens (Ag) on the glomeruli by the immunofluorescent study using anti-uvDNA antisera which are specific to DNA Ag, though we detected anti-DNA antibodies in the eluates in MRL/1 mice. These results suggest either that other antigen-antibody systems constitute the major IC component in lupus nephritis of MRL/1 mice or that DNA Ag fixed to the glomeruli was digested with endogeneous DNase into oligonucleotide which is resistant to treatment with DNase, and does not produce conformational changes after uv-irradiation.

MRL/Mp-1pr/1pr (MRL/1) mice are characterized by a spontaneous occurrence of early and severe immune complex (IC) glomerulonephritis, which is associated with elevations of serum immunoglobulin G (IgG) and a variety of autoantibodies, including anti-DNA antibodies and rheumatoid factors (RF) (2, 12). Lambert and Dixon (14) demonstrated a significant concentration of anti-DNA antibodies in the renal eluates in New Zealand black mice [NZB] × New Zealand white mice [NZW] F₁ (B/W F₁) hybrid mice, thereby suggesting the pathogenic importance of DNA-anti-DNA immune complexes (DNA-IC) for the glomerular lesions. This hypothesis was in part supported by the findings that the induction of tolerance to DNA antigens prolonged the survival associated with decreased anti-DNA antibody production in these B/W F₁ hybrid mice (3, 19). The accumulating evidence has suggested that IgG-anti-dsDNA antibodies in the sera contribute to lupus nephritis (18, 21) and that IgG-RF have a significant

association with vasculitis (24) and glomerular lesion (12). Based on this data, we tried to determine the IgG-class autoantibodies which contribute to lupus nephritis in the sera and renal eluates in MRL/l mice and other autoimmune mice.

Harbeck *et al.* (8) using DNase digestion technique have shown that the presence of DNA-IC in sera was correlated with active renal disease, but this result has not always been confirmed by other authors (5, 11). In recent years, Izui *et al.* reported that at first DNA antigens bind to glomeruli, the anti-DNA antibodies bind to DNA antigens *in situ* on the glomeruli (10), and suggested a hypothesis that IgG-RF bind with IgG-anti-DNA antibodies in the serum, then circulate as IC, and act as an immunosorbent on the glomeruli after binding to DNA antigens on the glomeruli in MRL/l mice. However, whether DNA-IC circulate in the serum or DNA-IC are deposited *in situ* is still controversial. In the present studies, we attempted to clarify these points by an elution study and an immunofluorescent study using anti-uvDNA antisera.

MATERIALS AND METHODS

MICE AND SERA: MRL/l mice were obtained from Jackson Laboratories (Bar Harbor, Maine, U.S.A.). NZB, (NZB $\times$ NZW) F₁-hybrids (B/W F₁) and BALB/c mice were obtained from our own colonies. To avoid the influence of sex hormones, only female mice which were about 4 months of age were used in this study. Sera were bled from preorbital sinus, and stocked in -70°C until use.

ELUTION OF IMMUNOGLOBULINS FROM RENAL TISSUE: Elutions of renal tissues were carried out according to the method of Woodroffe and Wilson with certain modifications (7, 26). Kidneys of mice were perfused completely with physiological saline. The cortex was cut and weighed in each mouse, then washed with PBS containing 1% BSA (pH 7.4) at 4°C , then pressed gently through a No. 100 mesh stainless steel sieve, and pressed through a No. 100 mesh again. Then these glomerulus-rich homogenates were washed with 30 ml of PBS containing 1% BSA at 4°C , and centrifuged 8 times at 1,500 rpm for 5 min at 4°C . Incubation was then carried out with 2.5 ml of 0.02 M citrate buffer containing 1% BSA (pH 3.2) at 23°C for 1 hr with continuous shaking. The mixtures were centrifuged at 3,500 rpm at 4°C for 15 min. Eluates were rapidly neutralized to pH 7.0 with 1N-NaOH in order to prevent denaturation of antibody activity, then dialyzed against PBS (pH 7.4), and concentrated to 1 ml, and stocked in -70°C until use. In the assay of the eluates, results were expressed as follows: μg or cpm/g kidney.

IMMUNOLOGIC REAGENTS: Goat anti-mouse IgG antisera [F(ab')₂ fragment] was obtained from Cappel Laboratories, Springfield, Va, U.S.A. Highly polymerized calf thymus DNA (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) was digested with S1 nuclease (Seikagaku Kogyo, Tokyo, Japan), dialyzed, and fractionated on a methylated albumin kieselguhr column (22). Hydroxyapatite analysis (17) revealed less than 5% of contaminating single-stranded DNA (ssDNA). This was used as the double-stranded DNA (dsDNA). Single-stranded DNA was obtained by heating dsDNA at 100°C for 10 min, then immediately cooling it in an ice bath. Deoxyribonuclease 1 (DNase 1) was obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Fresh C₁q solution was

isolated by the method of Yonemasu and Stroud (27). Fc fragments of mouse gamma globulin (MGG) were prepared according to the method of Izui and Eisenberg (12) from pooled BALB/c mice sera. Anti-mouse κ chain antisera (rabbit) and mouse κ light chains (mouse urine-MOPC 41) were obtained from Litton Bionetics, Inc. Kensington, Md., U.S.A. Anti-ultraviolet irradiated (uv) DNA antisera (rabbit) was prepared in our laboratory according to the method of Tan *et al.* (23). Anti-uvDNA antibodies are known to recognize the thymine dimer in ss or dsDNA after uv light irradiation (15, 20). In the Ouchterlony test, the anti-uvDNA antisera formed the precipitation lines with both uvDNA and ssDNA. This observation indicates that the anti-uvDNA antibodies recognize the antigenic determinant in uvDNA which are composed with pyrimidine dimers and the altered conformational structure associated with these lesions. This was used after absorption with mouse liver powder in order to inhibit non-specific binding. FITC-labeled goat-anti-rabbit antisera prepared in our laboratory was used after absorption with BALB/c sera.

RADIOLABELING PROCEDURES: Protein A (Pharmacia, Sweden) was labeled using Na- ^{125}I (Radiochemical Centre, Amersham, U.S.A.) and chloramine-T (16). Free iodide was removed by chromatography on Sephadex G-25. Anti-mouse κ chains were affinity-labeled with mouse κ light chains coupled Sepharose 4B, using Na ^{125}I and chloramine T.

DETERMINATION OF CONCENTRATIONS OF IgG: Solid phase radioimmunoassay (RIA) was performed to assess the levels of IgG in the sera and eluates. One hundred μl of 250 μg of antibody protein/ml anti-IgG [F(ab')_2] in PBS (pH 7.4) was used to coat the wells of flexible microtiter plates (Dynatech Laboratories Inc., Alexandria, Va, U.S.A.) for 2 hr at room temperature (RT). After being washed three times with PBS, the wells were further coated with 1% solution of BSA before 100 μl of appropriately diluted test samples in PBS containing 1% BSA were added. The plates were then incubated for 1 hr at RT and washed five times with PBS, after which 5×10^4 cpm of ^{125}I -labeled protein A in PBS containing 1% BSA was added. These plates were incubated another 30 min at RT and washed, after which individual wells were cut out for counting. A standard curve was established by using the standard sample (prepared in our laboratory from pooled BALB/c mice sera). In order to correctly evaluate the effect of elution, we examined a relative index in each mouse as the elution index, which represents the eluate IgG proportion at the same serum IgG concentration as follows;

$$\text{Elution index} = \frac{\text{IgG concentration of the eluate}}{\text{IgG concentration of the serum}} \times 100$$

ASSAYS OF IgG ANTI-DNA ANTIBODIES: IgG-anti-DNA antibodies were measured by a modified solid-phase RIA according to the method of Koike *et al.* (13). with the following exceptions: (a) methylated bovine serum albumin (M-BSA) was not used in the anti-ssDNA assay, (b) 100 μl of 1 : 10 diluted sera or 1 : 2 diluted eluates were tested, (c) 100 μl of ^{125}I -labeled protein A (1×10^4 cpm in serum or 5×10^4 cpm in eluate) was added.

DNASE DIGESTION: DNase digestion was carried out according to the method of Harbeck *et al.* (8) with the following exceptions: (a) in the dilution of eluates,

PBS containing 1% BSA was used as a diluent, (b) the final dilution of sera and eluates were 1 : 10 and 1 : 5, respectively. As a positive control, 50 μ l of a MRL/1 mouse serum which had the original titer of 3,200 cpm in 1 : 10 dilution was incubated with the same volume of dsDNA solution (10 μ g/ml) for 2 hr at 37°C before these treatments.

IgG-RF RADIOIMMUNOASSAY: IgG-RF were measured by a solid-phase RIA according to the method of Izui and Eisenberg (12) with the following exceptions: (a) 100 μ l of 1 : 10 diluted sera or 1 : 2 diluted eluates were tested, (b) in the dilution of the eluates, borate buffer containing 3.6% BSA, 0.09% Tween 80 and 0.27 M 2-ME was used as a diluent, (c) 100 μ l of 125 I-labeled anti-mouse κ chain antisera (1×10^4 cpm) was added. Every sample tested was controlled for binding to microtiter plate wells lacking the Fc fragment coating, as well as for binding the radiolabeled antibody to the wells coated, but not exposed, to test sera.

SOLID PHASE C_{1q} BINDING ASSAY (SP- C_{1q} BA): This assay was performed as a modification of the method of Hay, Nineham and Roitt (9) with the following exceptions: (a) microtiter wells (Dynatech) were used instead of polystyrene tubes, (b) the final dilution of sera and eluates were 1 : 30 and 1 : 5, respectively, (c) 100 μ l of 125 I-labeled protein A (2×10^4 cpm) was added, (d) the results were expressed as cpm.

IMMUNOFLUORESCENCE: Tissues were rapidly frozen in dry ice acetone. Cryostat sections at 2 μ m were air dried and washed 3 times in PBS (pH 7.4) for 30 min without fixation. And the sections in PBS were irradiated by uv-light (Toshiba Electric Co. Ltd., 15 W) from a distance of 10 cm for 15 min. The control sections were not irradiated by uv-light. All sections were stained with 1 : 20 diluted anti-uvDNA antisera (rabbit) for 30 min at RT. After being washed 3 times in PBS, the sections were stained with 1 : 10 diluted FITC-labeled anti-rabbit antisera (Goat). Specificity of the reaction to uvDNA on the tissue was ascertained as follows: The mouse fibroblasts on the slide (ANA-TEST, MELOY) were incubated with 1 : 10 diluted human SLE serum (reciprocal titer of ANA: 640) for 30 min at RT, and were washed with PBS. After irradiation by uv-light, the fibroblasts were stained with 1 : 20 diluted anti-uvDNA antisera, and then the slide was washed with PBS, and the fibroblasts were stained with 1 : 10 diluted FITC-labeled anti-Rabbit antisera (goat). In this experiment, uv-irradiated fibroblasts gave positive nuclear staining, although no nucleolus of the fibroblasts was stained without uv-irradiation. This observation indicated that uv-irradiation made a conformational change forming uvDNA *in situ* as new antigens, and that these new antigens reacted with anti-uvDNA antisera in spite of overlaying anti-DNA antibodies on DNA antigens.

STATISTICAL ANALYSIS: The results were analyzed with Student's *t*-test, Wilcoxon's signed rank test, and Spearman rank correlation analysis. Probability values of more than 0.05 were considered to be insignificant.

RESULTS

EFFECT OF ELUTION FROM RENAL GLOMERULI: Table 1 shows the mean of IgG concentration in the sera and eluates in 15 MRL/1, 4 B/W F₁, 5 NZB, and 18 BALB/c mice of 4 months of age. The mean of IgG concentration in MRL/1 and

TABLE 1. *Effect of elution from renal glomeruli of mice*

Mice (no.)	IgG concentration		Elution index ^a
	Sera (mg/ml)	Eluates (μ g/g kidney)	
MRL/1 (15)	16.3 $\pm$ 3.2 ^b $p < 0.005^c$	52.8 $\pm$ 23.7 $p < 0.01$	1.46 $\pm$ 0.920 $p < 0.05$
B/W F ₁ (4)	7.5 $\pm$ 2.7 $p < 0.01$	34.3 $\pm$ 15.3 $p < 0.005$	0.41 $\pm$ 0.080 $p < 0.005$
NZB (5)	1.2 $\pm$ 0.2 NS	0.5 $\pm$ 0.1 $p < 0.025$	0.04 $\pm$ 0.015 $p < 0.005$
BALB/c (18)	2.7 $\pm$ 0.6	0.1 $\pm$ 0.05	0.02 $\pm$ 0.004

^a: Elution index = $\frac{\text{IgG concentration of the eluate}}{\text{IgG concentration of the serum}} \times 100$

^b: Mean $\pm$ SEM.

^c: Significance of difference from BALB/c mice by the Student's *t*-test; NS = not significant.

TABLE 2. *Detection of IgG-anti-DNA antibodies in the sera and eluates*

Mice (no.)	IgG-anti-dsDNA antibodies		IgG-anti-ssDNA antibodies	
	Sera (cpm)	Eluates (cpm/g kidney)	Sera (cpm)	Eluates (cpm/g kidney)
MRL/1 (16)	2565.3 $\pm$ 187.0 ^a $p < 0.005^b$	655.6 $\pm$ 211.9 $p < 0.005$	2848.4 $\pm$ 95.4 $p < 0.005$	618.6 $\pm$ 254.8 $p < 0.01$
B/W F ₁ (4)	2523.0 $\pm$ 325.0 $p < 0.005$	132.0 $\pm$ 47.0 $p < 0.005$	3052.0 $\pm$ 315.5 $p < 0.005$	28.0 $\pm$ 15.0 NS
NZB (5)	1364.0 $\pm$ 456.3 NS	70.0 $\pm$ 10.3 $p < 0.025$	1839.0 $\pm$ 469.6 $p < 0.005$	83.4 $\pm$ 36.6 NS
BALB/c (18)	881.5 $\pm$ 104.2	34.8 $\pm$ 6.9	1122.3 $\pm$ 111.8	8.3 $\pm$ 3.4

^a: Mean $\pm$ SEM.

^b: Significance as compared with BALB/c mice; NS = not significant (Student's *t*-test).

B/W F₁ mice was significantly higher than that in BALB/c mice in both serum and eluate ($t=4.11$, $p < 0.005$, and $t=2.54$, $p < 0.01$ in the serum, and $t=2.44$, $p < 0.01$ and $t=9.30$, $p < 0.005$ in the eluate, respectively). In contrast, the mean of IgG concentration in NZB was not higher than that in BALB/c in the serum, but that in NZB was slightly higher than that in BALB/c in the eluate ($t=2.00$, $p < 0.025$). In addition, in MRL/1 and B/W F₁ mice, the elution index was significantly higher than that in BALB/c ($t=1.72$, $p < 0.05$ and $t=20.49$, $p < 0.005$, respectively). Although NZB did not reveal a high IgG concentration in the serum and eluate, the elution index in NZB was slightly higher than that in BALB/c ($t=41.41$, $p < 0.005$). These data suggest that MRL/1 and B/W F₁ mice severely develop IgG deposits on the glomeruli of the kidney.

DETECTION OF ANTI-DNA ANTIBODIES: Table 2 shows the mean of IgG-class anti-DNA antibodies in the sera and eluates in 16 MRL/1, 4 B/W F₁, 5 NZB, and 18 BALB/c mice of 4 months of age. The mean of IgG-anti-dsDNA

antibodies in MRL/1 and B/W F₁ mice was significantly higher than that in BALB/c mice in both serum and eluate ($t=8.09$, $p<0.005$ and $t=7.27$, $p<0.005$, in the serum, and $t=3.11$, $p<0.005$, and $t=3.89$, $p<0.005$ in the eluate, respectively). In contrast, the mean of IgG-anti-dsDNA antibodies in NZB was slightly higher than that in BALB/c in the serum ($t=1.55$, $p>0.05$, not significant), but that in NZB was significantly higher than that in BALB/c in the eluate ($t=2.47$, $p<0.025$).

The mean of IgG-anti-ssDNA antibodies in the serum in MRL/1, B/W F₁, and NZB mice, was significantly higher than that in BALB/c mice ($t=11.58$, $p<0.005$ and $t=7.97$, $p<0.005$, and $t=2.96$, $p<0.005$, respectively). In contrast the mean of IgG-anti-ssDNA antibodies in the eluate in MRL/1 mice was significantly higher than that in BALB/c mice ($t=2.51$, $p<0.01$), but in B/W F₁ and NZB mice that was not significantly higher than that in BALB/c mice ($t=0.27$, $p>0.05$ and $t=1.09$, $p>0.05$, respectively). These data suggest that IgG-anti-dsDNA antibodies are a more important component of immune depositions on glomeruli of murine lupus nephritis than IgG-anti-ssDNA antibodies, since IgG-anti-dsDNA antibodies in the eluate were commonly observed in MRL/1 and B/W F₁ mice, although IgG-anti-ssDNA antibodies in the eluate were observed in MRL/1 mice only. These findings are compatible with our previous study using B/W F₁ × NZW backcross progeny (28).

EFFECT OF DNASE DIGESTION ON ANTI-DNA IN THE SERUM AND ELUATE: In order to quantitatively estimate DNA-anti-DNA complexes, levels of IgG-anti-dsDNA antibodies in serum and eluate after DNase treatment of samples were compared with those in undigested samples (Table 3). The control sample showed a significant effect from DNase digestion, since there was a notable increase in IgG-anti-dsDNA antibody activity. However, when samples were digested with DNase, all samples did not show an increase in IgG-anti-dsDNA antibody activity after DNase digestion. Using Wilcoxon's signed rank test as a statistical analysis, there was no significant difference between undigested samples and DNase digested

TABLE 3. *Effect of DNase digestion on IgG-anti-dsDNA antibodies in the sera and eluates*

Mice (no.)	IgG-anti-dsDNA in the sera (cpm)		IgG-anti-dsDNA in the eluates (cpm/g kidney)	
	Undigested	DNase digested	Undigested	DNase digested
MRL/1 (9)	2632.2 ± 271.4 ^a	2681.2 ± 256.1	1028.9 ± 302.1	785.6 ± 247.5
	NS (T=18, n=9, $p>0.05$) ^b		NS (T=6, n=7, $p>0.05$)	
B/W F ₁ (5)	2382.0 ± 288.4	2204.0 ± 547.0	134.0 ± 36.2	90.0 ± 40.5
	NS (T=3, n=5, $p>0.05$)		NS (T=2, n=5, $p>0.05$)	
NZB (5)	1366.0 ± 456.3	1272.0 ± 442.1	70.0 ± 9.5	78.0 ± 38.6
	NS (T=7, n=5, $p>0.05$)		NS (T=7.5, n=5, $p>0.05$)	
Control ^c	150	3,400		

^a: Mean ± SEM.

^b: Significance of difference between undigested sample and DNase digested sample by the Wilcoxon's signed rank test; NS=not significant.

^c: Control sample was prepared by incubating 50 μ l of a MRL/1 mouse serum which had the original titer of 3,200 cpm in 1:10 dilution with the same volume of dsDNA solution (10 μ g/ml) for 2 hr at 37°C.

TABLE 4. *IgG-SPC1q and Ig-RF in the sera and eluates*

Mice (no.)	IgG-SPC1q		IgG-RF	
	Sera (cpm)	Eluates (cpm/g kidney)	Sera (cpm)	Eluates (cpm/g kidney)
MRL/l (14)	8415.3 $\pm$ 1660.9 ^a $p < 0.005^b$	880.8 $\pm$ 56.5 NS	306.6 $\pm$ 43.8 $p < 0.005$	171.4 $\pm$ 9.5 NS
B/W F ₁ (5)	1973.8 $\pm$ 300.8 $p < 0.005$	1104.8 $\pm$ 32.9 $p < 0.01$	194.8 $\pm$ 18.7 NS	180.2 $\pm$ 17.1 NS
NZB (5)	1132.0 $\pm$ 89.7 NS	995.0 $\pm$ 32.8 NS	170.0 $\pm$ 22.3 NS	189.6 $\pm$ 44.1 NS
BALB/c (18)	1105.8 $\pm$ 35.5	816.3 $\pm$ 57.6	179.0 $\pm$ 20.5	172.9 $\pm$ 21.8

^a: Mean $\pm$ SEM.^b: Significance as compared with BALB/c mice; NS = not significant (Student's *t*-test).

samples in the serum and eluate in 9 MRL/l, 5 B/W F₁, and 5 NZB mice of 4 months of age (Ts=18 [NS], Ts=3 [NS] and Ts=7 [NS] in the serum, and Ts=6 [NS], Ts=2 [NS] and Ts=7.5 [NS] in the eluate, respectively). These findings are compatible with Izui *et al.* (11) and Yoshida *et al.* (29). These results suggest either that DNA-IC systems might not constitute the major IC component in murine lupus nephritis or that the DNA antigens which constitute IC are resistant to treatment with DNase.

FIG. 1. The relationship between the amount of IgG-anti-dsDNA antibodies and IgG-RF. (A); Serum IgG-anti-dsDNA activity is plotted against IgG-RF for individual sera. There was a significant correlation between the 2 measures ($r_s = 0.53$, $p < 0.05$). (B); Eluate IgG-anti-dsDNA activity is plotted against IgG-RF for individual eluates. There was no significant correlation between the 2 measures ($r_s = -0.08$, NS).

DETECTION OF SPC1q-CIC AND IgG-RF: Table 4 shows the mean of IgG-SPC1q and IgG-RF in the serum and eluate in 14 MRL/l, 5 B/W F₁, 5 NZB, and 18 BALB/c mice of 4 months of age. The mean of IgG-SPC1q in MRL/l and B/W F₁ mice was significantly higher than that in BALB/c mice in the serum ($t=5.02$, $p<0.005$, and $t=5.39$, $p<0.005$, respectively). In contrast, the mean of IgG-SPC1q in MRL/l mice was not significantly higher than that in BALB/c mice in the eluate ($t=0.80$, NS), but that in B/W F₁ mice was slightly higher than that in BALB/c mice in the eluate ($t=2.61$, $p<0.01$). On the other hand, the mean of IgG-RF in the serum in MRL/l mice was significantly higher than that in BALB/c mice ($t=2.84$, $p<0.005$), but the mean of IgG-RF in the serum in B/W F₁ and NZB mice was not significantly higher than that in BALB/c mice ($t=0.39$, NS, and $t=0.16$, NS, respectively). In contrast, the mean of IgG-RF in the eluate in MRL/l mice had no significant difference as compared with BALB/c mice ($t=0.10$, NS), the same as in B/W F₁ and NZB mice ($t=0.17$, NS and $t=0.35$, NS, respectively).

RELATIONSHIP BETWEEN IgG-ANTI-ds-DNA AND IgG-RF: To determine the correlation between the quantities of IgG-anti-dsDNA and IgG-RF in the serum and eluate, the sera from 14 individual MRL/l mice and the eluates from 11 individual MRL/l mice at 4 months of age were analyzed for the 2 measures. As shown in Fig. 1A, the Spearman rank correlation analysis revealed a significant

FIG. 2. The immunofluorescent findings of MRL/l mouse kidney. The MRL/l mouse kidney section on which PBS was mounted was irradiated by uv light, then stained with anti-uvDNA antisera. After washing in PBS, the section was stained with FITC-labeled anti-rabbit rantisera. The kidney section demonstrated only nuclear staining, and there was no clear fluorescence at the capillary wall or mesangial area. $\times 200$ For details, see text.

correlation in the serum ($r_s=0.53$, $p<0.05$), however, there is no correlation between IgG-anti-dsDNA and IgG-RF in the eluate ($r_s=-0.08$, NS) (Fig. 1B).

IMMUNOFLUORESCENT STUDIES: The frozen section of kidney from 16 MRL/1, 4 B/W F₁, and 5 NZB mice of 4 months of age were tested by the indirect immunofluorescent method using anti-uvDNA antisera (rabbit) and FITC-labeled anti-rabbit-antisera (goat) absorbed with BALB/c serum. After pretreatment of uv-irradiation, these kidney sections demonstrated only nuclear staining on the glomeruli and tubulus, and there was no clear fluorescence at the capillary wall or mesangial area of glomeruli (Fig. 2). In contrast, these kidney sections showed no positive staining without pretreatment of uv-irradiation. This observation suggests that DNA antigens might not exist on the glomerulus in the form of polynucleotide chains which produce thymine dimers after uv-irradiation.

DISCUSSION

Our present studies have dealt with the DNA-IC in the serum and on the glomerulus in MRL/1 and other autoimmune mice. There have been several reports documenting the existence of DNA-IC in the sera in SLE (8); however these results are somewhat controversial (5, 11). We applied the elution study to detect DNA-IC in the eluate and serum in autoimmune mice. We detected anti-dsDNA antibodies in the eluates and sera in MRL/1 mice, and IC in the serum, but very little in the elute in MRL/1 mice. Taking into account the results from DNase digestion in the serum and eluate, it would appear that DNA-IC do not predominate in IC. This assumption is given support by the study of Izui *et al.* (11) who did not find significant levels of this type of IC in SLE patient's sera by four different RIA. However, in recent years, Bruneau *et al.* (4) suggested that DNA-IC might be small cyclic complexes constituting small strands of helical DNA and of one or two IgG antibody molecules, which are resistant to treatment with DNase. Considering their data, our observations suggest either that DNA-IC are not a major component or that DNA-IC are composed by small-sized DNA which are resistant to DNase digestion and anti-DNA antibodies, since DNA-IC which are formed *in vitro*, and composed by polynucleotide chains and anti-DNA antibodies are very sensitive to treatment with DNase. In order to extend our observation, we applied the indirect immunofluorescent method using the anti-uvDNA antisera to detect DNA antigens on the glomeruli of autoimmune mice. In this assay, we did not detect DNA antigens at the capillary walls or mesangial area on glomeruli in spite of our detection of anti-DNA antibodies in eluates. If the thymine dimers are produced after uv-irradiation, these new antigens could be recognized by anti-uvDNA antisera, since these antisera react with uvDNA in spite of overlaying anti-DNA antibodies on DNA antigens. In order to increase the sensitivity of this method, we applied an immunoenzymatic electron microscopic method using peroxidase-labeled anti-uvDNA antisera, but the same findings were observed (manuscript in preparation; Kanemura *et al.*). These observations seem to be not compatible with Andres *et al.* (1). They demonstrated that FITC-labeled antisera reactive with thymine or cytosine were bound in the glomerular capillary walls of the patient with active SLE glomerular nephritis after treating with 0.02 M citrate buffer (pH 3.2) or 0.2 M NaCl. Considering their observation, our data

suggests that DNA antigens might not exist on the glomeruli in the form of polynucleotide chains which produce thymine dimers by uv-irradiation, but exist in the form of small strands of helical DNA which do not produce conformational changes after uv-irradiation. These assumptions from the elution study and the immunofluorescent study are compatible with each other. These assumptions which oligonucleotide DNA-IC exist in the serum, and are deposited on the glomeruli, might be given support by the study of Chused *et al.* (6) who demonstrated that B/W F₁ mice do not differ from normal mice in their clearance of nucleic acid, and by the study of Wakizaka and Okuhara (25) who reported that the minimum chain length required for an antigenic determinant of DNA is either tetra- or pentanucleotide which is resistant to treatment with DNase.

On the other hand, we detected IgG-RF in the sera in MRL/l mice, but not in those in other autoimmune strains. The amount of IgG-RF correlates with the amount of IgG-anti-dsDNA antibodies in the serum. These observations are based in part on a common pathogenetic mechanism such as genetic predisposition, however in the eluate, there is no correlation between IgG-RF and IgG-dsDNA antibodies. Therefore it does not seem likely that the IgG-RF which bind to IgG-anti-DNA antibodies act as an immunosorbent on the glomeruli.

In conclusion, our studies suggest either that DNA-IC which are composed by long polynucleotide chains are not a major component of IC or that small-sized DNA-IC which are composed by oligonucleotide derived from endogeneous DNase digestion are circulating in the sera and are deposited on the glomeruli.

ACKNOWLEDGEMENTS

This work was supported by the grant from the Ministry of Health and Welfare, Japan.

We are grateful for the advice of Dr. H. Yoshida, WHO Immunology Research and Training Centre, Geneva, Switzerland, for the expert technical assistance of Mr. K. Tsuchida, and for the secretarial assistance of Ms. K. Shimizu.

SLE=systemic lupus erythematosus, CIC=circulating immune complexes, SPCLqBA=solid phase Clq binding assay, dsDNA=double-stranded DNA, ssDNA=single-stranded DNA, uvDNA=ultraviolet-irradiated DNA, RF=rheumatoid factors, PBS=phosphate-buffered-saline, BSA=bovine serum albumin, FITC=fluorochrome isothiocyanates.

REFERENCES

1. Andres, G. A., Accinni, L., Beiser, S. M., Christian, C. L., Cinotti, G. A., Erlanger, B. F., Hsu, K. C. and Seegal, B. C.: Localization of fluorescein-labeled antinucleoside antibodies in glomeruli of patients with active systemic lupus erythematosus nephritis. *J. Clin. Invest.* 49: 2106-2118, 1970.
2. Andrews, B. S., Eisenberg, R. A., Theofilopoulos, A. N., Izui, S., Wilson, C. B., McConahey, R. J., Murphy, E. D., Roths, J. B. and Dixon, F. J.: Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestation in several strains. *J. Exp. Med.* 148: 1198-1215, 1978.
3. Borel, Y., Lewis, R. M. and Stollar, B. D.: Prevention of murine lupus nephritis by carrier dependent induction of immunologic tolerance to denatured DNA. *Science* 182: 76-78, 1973.
4. Bruneau, C. D., Edmonds, J. P., Hughes, G. R. V., Aarden, L.: Detection and characterization

- of DNA-anti-DNA complexes in a patient with systemic erythematosus. *Clin. Exp. Immunol.* 28: 433-436, 1977.
5. Cano, P. D., Jerry, L. M., Sladowski, J. P. and Osterland, C. K.: Circulating immune complexes in systemic lupus erythematosus. *Clin. Exp. Immunol.* 29: 197-204, 1977.
 6. Chused, T. M., Steinberg, A. D. and Talar, N.: The clearance and localization of nucleic acid by New Zealand and normal mice. *Clin. Exp. Immunol.* 12: 465-476, 1972.
 7. Ebling, F. and Hahn, B. H.: Restricted subpopulations of DNA antibodies in kidneys of mice with systemic lupus.—Comparison of antibodies in serum and renal eluates. *Arthritis Rheum.* 23: 392-403, 1980.
 8. Herback, R. J., Bardana, E. J., Kohler, P. F. and Carr, R. I.: DNA-anti-DNA complexes—Their detection in systemic lupus erythematosus area. *J. Clin. Invest.* 52: 789-795, 1973.
 9. Hay, E. C., Nineham, L. J. and Roitt, I. M.: Routine assay for the detection of immune complexes of known immunoglobulin class using solid phase Clq. *Clin. Exp. Immunol.* 24: 396-400, 1976.
 10. Izui, S., Lambert, P. H. and Miescher, P. A.: In vitro demonstration of a particular affinity of glomerular basement membrane and collagen for DNA—A possible bases for local formation of DNA-anti-DNA complexes in systemic lupus erythematosus. *J. Exp. Med.* 144: 428-443, 1976.
 11. Izui, S., Lambert, P. H. and Miescher, P. A.: Failure to detect circulating DNA-anti-DNA complexes by four radioimmunological methods in patient with systemic lupus erythematosus. *Clin. Exp. Immunol.* 30: 384-392, 1977.
 12. Izui, S. and Eisenberg, R. A.: Circulating anti-DNA-rheumatoid factor complexes in MRL/1 mice. *Clin. Immunol. Immunopathol.* 15: 536-551, 1980.
 13. Koike, T., Nagasawa, R., Nagata, N. and Shirai, T.: Specificity of mouse hybridoma antibodies to DNA. *Immunol. Lett.* 4: 93-97, 1982.
 14. Lambert, P. H. and Dixon, F. J.: Pathogenesis of the glomerulonephritis of NZB/W mice. *J. Exp. Med.* 127: 507-521, 1968.
 15. Levine, L., Seaman, E., Hammerschlag, E. and Van Vanakis, H.: Antibodies to photoproducts of deoxyribonucleic acids irradiated with ultraviolet light. *Science* 153: 1666-1667, 1966.
 16. McConahey, P. J. and Dixon, F. J.: A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* 29: 185-189, 1966.
 17. Paetkau, V. and Langman, L.: A quantitative, batch hydroxyapatite method for analyzing native and denatured DNA at room temperature. *Anal. Biochem.* 65: 525-532, 1975.
 18. Papoian, R., Pillarisetty, R. and Talal, N.: Immunological regulation of spontaneous antibodies to DNA and RNA. II. Sequential switch from IgM to IgG in NZB/NZW F₁ mice. *Immunology* 32: 75-79, 1977.
 19. Parker, L. P., Hahn, B. H. and Osterland, C. K.: Modification of NZB/NZW F₁ autoimmune disease by development of tolerance to DNA. *J. Immunol.* 113: 292-297, 1974.
 20. Seaman, E., Levine, L. and Van Vanakis, H.: Immunological evidence for the existence of thymine dimers in ultraviolet irradiated DNA. In *Nucleic Acid in Immunology*, ed. by O. J. Plescia and W. Braum, Springer-Verlag, New York, 1968, pp. 157-164.
 21. Steward, M. W. and Hay, F. C.: Changes in immunoglobulin class and subclass of anti-DNA antibodies with increasing age in NZB/NZW F₁ hybrid mice. *Clin. Exp. Immunol.* 26: 363-370, 1976.
 22. Sueoka, N. and Cheng, T. Y.: Fractionation of nucleic acid with the methylated albumin column. *J. Mol. Biol.* 4: 161-172, 1962.
 23. Tan, E. M. and Stoughton, R. B.: Ultraviolet light alteration of cellular deoxyribonucleic acid *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 62: 708-714, 1969.
 24. Theofilopoulos, A. N., Burtonboy, G., LoSpalluto, J. J., Ziff, M.: IgM rheumatoid factor and low molecular weight IgM.—An association with vasculitis. *Arthritis Rheum.* 17: 272-284, 1974.
 25. Wakizaka, A. and Okuhara, E.: Immunological studies on nucleic acids—An investigation

- of the antigenic determinations of denatured deoxyribonucleic acid (DNA) reactive with rabbit anti-DNA antisera by a radioimmunoassay technique. *Immunochemistry* 12: 843-845, 1975.
26. Woodroffe, A. J., Wilson, C. B.: An evaluation of elution techniques in the study of immune complex glomerulonephritis. *J. Immunol.* 118: 1788-1794, 1977.
 27. Yonemasu, K. and Stroud, R. M.: Clq-Rapid purification method for preparation of mono-specific antisera and for biochemical studies. *J. Immunol.* 106: 304-313, 1971.
 28. Yoshida, H., Kohno, A., Ohta, K., Hirose, S., Maruyama, N. and Shirai, T.: Genetic studies of autoimmunity in New Zealand mice. III. Associations among anti-DNA antibodies, NTA, and renal disease in (NZB $\times$ NZW) F₁ $\times$ NZW backcross mice. *J. Immunol.* 127: 433-437, 1981.
 29. Yoshida, H., Muso, E., Inoue, H., Furukawa, F., Sekita, K., Kawai, C. and Hamashima, Y.: Evaluation of solid phase Clq and conglutinin binding assay in systemic lupus erythematosus in relation to anti-DNA antibodies and nephritis. In preparation.