Distinct Clonotypes of Anti-DNA Antibodies in Mice with Lupus Nephritis

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Abstract

Clonotypes of IgG anti-DNA antibodies were studied by isoelectric focusing in various autoimmune mice with or without lethal lupus nephritis. MRL/MpJ-*lpr/lpr* mice exhibited the most heterogeneous spectrotypes of anti-DNA antibodies in the pH range from 6.5 to 8.5, with marked variation in individual mice. Female (NZB × NZW)F1 mice expressed rather uniform DNA-binding bands composed of at least five to six distinct subgroups, having isoelectric points from 6.5 to 8.0. Male BXSB mice showed major characteristic bands confined to alkaline pH range from 7.8 to 8.5, similar to C57BL/6J-*lpr/lpr* mice, which showed markedly restricted bands in this region. Both AKR/J-*lpr/lpr* and C3H/HeJ-*lpr/lpr* mice expressed DNA-binding bands mostly focused between pH 6.5 and 8.2. The aging study indicated that three autoimmune mice (MRL/MpJ-*lpr/lpr*, [NZB × NZW]F1, and male BXSB) that developed fatal glomerulonephritis showed clonal expansion of anti-DNA antibodies throughout their life. In contrast, such age-dependent expansion of anti-DNA clonotypes was not evident in three *lpr* cogenic mice (C57BL/6J-*lpr/lpr*, AKR/J-*lpr/lpr*, and C3H/HeJ-*lpr/lpr*) that developed only mild glomerulonephritis; rather, their expression of anti-DNA spectrotypes diminished as they aged. Anti-DNA activities in renal eluates from nephritic autoimmune mice were mostly distributed in the pH range from 6.5 to 8.0, without significant concentrations in the high alkaline range of more than pH 8.0. These results suggest that there exist distinct anti-DNA clonotypes in each mouse strain and that the development of lupus nephritis does not appear to be associated with particular spectrotypes of anti-DNA antibodies. Rather, the age-dependent expansion of anti-DNA clonotypes may be a feature more characteristic of mice developing lethal lupus nephritis.

Introduction

The occurrence of circulating anti-DNA antibodies is one of the abnormalities closely associated with the pathological manifestations of human and murine systemic lupus erythematosus (SLE).[1] The involvement of DNA-anti-DNA immune complexes (IC) in the pathogenesis of lupus nephritis was demonstrated by the significant concentration of anti-DNA antibodies found in eluates from the diseased kidneys (1, 2). The induction of tolerance to DNA antigens in (NZB × NZW)F1 mice prolonged their survival and decreased lupus nephritis, further supporting the pathogenic significance of anti-DNA antibodies (3, 4).

It has been suggested that the qualities of anti-DNA antibodies such as specificity or avidity or isotypes may be as important as their quantities in provoking renal disease (5–8). In addition, isoelectric focusing analysis (IEF) of serum anti-DNA isoelectric spectrum (spectrotype), which is a reflection of the repertoire of anti-DNA-producing B cell clones (9), has demonstrated the development of new clonotypes of anti-DNA antibodies that appeared to be coincident with the onset of renal disease in SLE-prone mice (10, 11). However, it is still unclear whether particular spectrotypes of anti-DNA antibodies are indeed associated with the development of lupus nephritis (12).

In the present study, we performed, by IEF, an extensive analysis and comparison of the anti-DNA spectrotypes in sera and renal eluates from autoimmune mice with or without lethal lupus nephritis. The first group consisted of three different lupus-prone mice (MRL/MpJ-*lpr/lpr* [MRL-*lpr/lpr*], [NZB × NZW]F1, and male BXSB) that develop severe autoimmune disease characteristic of SLE and die of glomerulonephritis (13). The second group consisted of three lymphoproliferation (*lpr*) congenic strains (C57BL/6J, AKR/J, and C3H/HeJ) without apparent SLE backgrounds. The latter three strains of mice bearing the *lpr* gene develop only mild chronic glomerulonephritis late in life, despite the production of various autoantibodies, including anti-DNA antibodies (14, 15). Therefore, we thought it beneficial to compare the spectrotypes of anti-DNA antibodies and their age-related changes in these strains to determine whether the particular anti-DNA clonotypes are involved in the pathogenesis of lupus nephritis.

Methods

Mice. MRL-*lpr/lpr* and BXSB mice were purchased from The Jackson Laboratory, Bar Harbor, ME. (NZB × NZW)F1 female mice were bred in our colonies from the breeding pairs purchased from Olac 1976 Limited, Oxon, England. Three different congenic strains bearing the *lpr* gene in nonautoimmune background, C57BL/6J-*lpr/lpr* (B6-*lpr/lpr*), AKR/J-*lpr/lpr* (AKR-*lpr/lpr*), and C3H/HeJ-*lpr/lpr* (C3H-*lpr/lpr*), were originally developed by E. D. Murphy and J. B. Roths (The Jackson Laboratories) and have been bred in our colonies since 1981. With the exception of BXSB mice, only female mice were used in the present study. Mice were bled by retroorbital puncture and the sera were stored at −20°C until use.

Preparation of DNA. Heat-denatured calf thymus DNA (Type V; Sigma Chemical Co., St. Louis, MO) was used as single-stranded DNA (ssDNA). Double-stranded DNA (dsDNA) was purified by digestion with S1 nuclease (16) and by the subsequent fractionation with benzoyl-naphthoyl-DEAE cellulose column chromatography (17). Hydroxylapatite column chromatography (17) has shown no detectable

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1. *Abbreviations used in this paper:* AKR-*lpr/lpr*, AKR/J-*lpr/lpr*; B6-*lpr/lpr*, C57BL/6J-*lpr/lpr*; C3H-*lpr/lpr*, C3H/HeJ-*lpr/lpr*; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; GBM, glomerular basement membrane; IC, immune complexes; IEF, isoelectric focusing; *lpr*, lymphoproliferation; mBSA, methylated BSA; MHC, major histocompatibility complex; MRL-*lpr/lpr*, MRL/MpJ-*lpr/lpr*; pI, isoelectric points; SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA.

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amounts of ssDNA in this dsDNA preparation. Both ssDNA and dsDNA were labeled with ^{125}I according to the methods of Commerford (18). The purity of ^{125}I-dsDNA was documented by hydroxylapatite column chromatography. The specific activity of each labeled DNA was 3–4 × 10^6 cpm/μg of ssDNA and 2–3 × 10^6 cpm/μg of dsDNA.

Solid-phase anti–DNA assay. To measure the activities of anti–ssDNA or anti–dsDNA antibodies solid-phase radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) was employed. For anti–ssDNA solid-phase RIA, polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) were coated with 50 μl of ssDNA (10 μg/ml) in phosphate-buffered saline (PBS) for 3 h at 37°C, followed by washing with PBS. After incubation with 0.5% bovine serum albumin (BSA) in PBS for 1 h at room temperature, 50 μl of samples diluted with 2% BSA in PBS containing 0.05% Tween 20 was added to the wells for an overnight incubation at 4°C. After washing, wells were incubated with 50 μl of ^{125}I-labeled goat anti–mouse γ-chain specific antibody (Cappel Laboratories, Cochranville, PA) at 20–50 × 10^4 cpm/well for 5 h at 4°C, followed by washing with PBS, and the bound radioactivity was counted.

In ELISA, polystylene microtiter plates (Flow Laboratories, Inc., McLean, VA) were coated with ssDNA and incubated with samples in the same way as in RIA. Wells were then incubated with alkaline phosphatase-conjugated goat anti–mouse γ-chain specific antibody for 5 h at 4°C at 1 μg/ml of antibody concentration. After washing five times, 1 mg/ml of enzyme substrate (paranitrophenyl phosphate; Sigma Chemical Co.) was added in 0.01 M diethanolamine solution, pH 9.8. The color intensity was measured at OD 405 nm with Titertek Multiskan (Flow Laboratories, Inc.). To determine anti–dsDNA antibody activities by ELISA, wells were coated with 0.1% protamine sulfate before 50 μl of dsDNA (10 μg/ml) was added to the wells, according to the method of Klotz et al. (19). Samples and antibody conjugates were diluted with 10% normal goat serum in 2% BSA in PBS containing 0.05% Tween 20 to reduce the nonspecific binding of immunoglobulins to protamine sulfate. The results of IEF fractions are directly expressed as the OD 405 nm after background values are subtracted.

Farr anti–DNA assay. IgG anti–ssDNA antibodies in serially diluted sera from *lpr* congenic mice were measured with a modified Farr DNA-binding RIA in the presence of 0.1 M 2-mercaptoethanol to inactivate specifically IgM antibodies (20).

Solid-phase assay for IgG. The concentration of IgG was quantitated by a solid-phase RIA or ELISA using goat anti–mouse γ-chain specific antibody similar to that described for the determination of IgM (21). Levels of IgG were computed by referring to the standard curve obtained by mouse IgG (Miles Ames Div., Miles Laboratories, Inc., Elkhart, IN).

Analytical IEF. Analytical IEF was performed in a vertical slab gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) with a modification of the procedure by Baumann and Chrambach (22) and Bionda et al. (23). The gel solution was composed of 4.25% acrylamide, 0.75% *N,N'*-diallyltartardiamide, 8 M urea, 2% Nonidet P-40 (NP-40), and 2% ampholites (Ampholines; LKB Produkter, Bromma, Sweden), pH 3.5–5, 5–7, 6–8, 7–9, and 9–11. After the solution was degassed, 0.1% *N,N,N',N'*-tetramethylethylenediamine, 6.8 μg/ml riboflavin 5'-phosphate, and 150 μg/ml ammonium persulfate were added to polymerize the solution. The gel (14 × 16 × 0.15 cm) was formed under fluorescent light for 4–5 h. The pore size of the gel excluded macromolecules such as IgM and C1q. The upper electrode chamber contained 0.05 M phosphoric acid and 0.01 M taurine; the lower electrode chamber contained 0.1 N NaOH and 0.01 M L-lysine. After the gel was prefocused for 1 h, 20 μl of heat-inactivated serum or renal eluate containing 1–40 μg of IgG, diluted with 80 μl of 6.7 M urea solution containing 5% Ampholine (pH 3.5–10) and 2% NP-40, was loaded to a sample well and focused for 14–15 h at a constant voltage of 500. Using high concentrations of urea and NP-40 permitted solubilization of proteins even in the form of IC in samples and prevented precipitation of proteins during focusing (23). Separated immunoglobulins were precipitated in 20% sodium sulfate solution for 4 h at room temperature, cross-linked with 4 mg/ml dimethylsuberimidate in 0.05 M phosphate buffer for 3 h at 4°C, and washed with PBS overnight.

To reveal DNA-binding bands, gels were pretreated with 0.005% sodium dodecyl sulfate (SDS) in PBS at room temperature for 1 h. Then, gels were incubated with sonicated ^{125}I-DNA (1–4 × 10^5 cpm/ml) in PBS containing 0.005% SDS or in PBS for 16–20 h at room temperature. Unbound radioactivity was removed by extensive washing with PBS for 3 d. After gels were dried, autoradiography was performed with Kodak X-Omat films. After 5–30 d exposure, the film was developed. In general, the number of DNA-binding bands did not change after 3 wk of exposure.

Densitometry of autoradiographic films was performed with a high-resolution laser beam densitometer built in the laboratory of Prof. Gabbiani, Department of Pathology, University of Geneva. The scanning profile was analyzed by a computer (Microcomputer based on the S-100 bus running under CP/M operating system; Digital Research Inc., Pacific Grove, CA) programmed for scanning, which corrects base line and minimizes the background noise, as described by Kocher et al. (24).

Elution of separated immunoglobulins from analytical IEF gel. Immunoglobulins were eluted from the IEF gel by the following procedure. After the gel was focused, it was rapidly cooled at −20°C for 2 h to precipitate urea on the surface of the gel. After the gel was washed three times for 1 h with PBS to remove urea and to adjust pH, it was cut in 2- or 3-mm slices. Each gel slice was homogenized in 500 μl of 1% BSA in PBS containing 0.05% Tween 20. After an overnight incubation at 4°C, proteins eluted from the gel were obtained by centrifugation. IgG concentrations and IgG anti–DNA antibody activities in eluates from each IEF fraction were measured by solid-phase RIA or ELISA. Using ^{125}I-labeled mouse IgG, ~50% of IgG were shown to be recovered from every IEF fraction by this procedure.

Elution of immunoglobulins from kidneys of autoimmune mice. The method similar to Dang and Harbeck (25) was used. Each of two pools of 8–10 kidneys from 4–5-mo-old MRL-*lpr/lpr*, 8–10-mo-old (NZB × NZW)F1 female, and 6–9-mo-old BXSB male mice was weighed, minced, and pressed through No. 200 stainless steel mesh (Bellco Glass, Inc., Vineland, NJ). Washed homogenates were incubated with DNase I (Worthington Biochemical Corp., Freehold, NJ) in 3 × 10^{-3} M $MgCl_2$ in PBS at 37°C for 2 h at 4°C overnight. After centrifugation, the first DNase eluate was separated. Then, the homogenates were further treated with 0.1 M citrate buffer, pH 3.2, at 37°C for 2 h to obtain the acid eluate. Both eluates were dialyzed against 0.01 M phosphate buffer, pH 7.8, pooled, and concentrated.

Results

Specific detection of DNA-binding bands in analytical IEF. To minimize the nonspecific binding to ^{125}I-ssDNA by positively charged proteins or highly concentrated immunoglobulins in analytical IEF gels, the effect of SDS during the incubation of IEF gels with ^{125}I-ssDNA was tested. In the absence of SDS, several significant ssDNA binding bands were obtained in the range of pH 6.8–8.0 by nonheated sera from 2-mo-old C57BL/6 and MRL-*lpr/lpr* mice (Fig. 1 *A*). Some of these bands were diminished or removed by heating of sera at 56°C for 30 min, but significant bindings were still observed, especially in the serum from 2-mo-old MRL-*lpr/lpr* mouse. 40 μg of separated IgG_{2a} myeloma protein (LPC-1), which does not have any anti–DNA activity, developed the ssDNA-binding bands under this condition. Methylated BSA (mBSA), a basic protein, has also shown a strong binding to ^{125}I-ssDNA, particularly in the range of pH 8.2–8.5. On the other hand, when the gel was pretreated with 0.005% SDS and incubated with ^{125}I-ssDNA in the presence of 0.005% SDS (Fig. 1 *B*), ssDNA-binding bands by sera from 2-mo-old C57BL/6, and MRL-*lpr/lpr* mice, LPC-1 myeloma protein and mBSA were markedly or com-

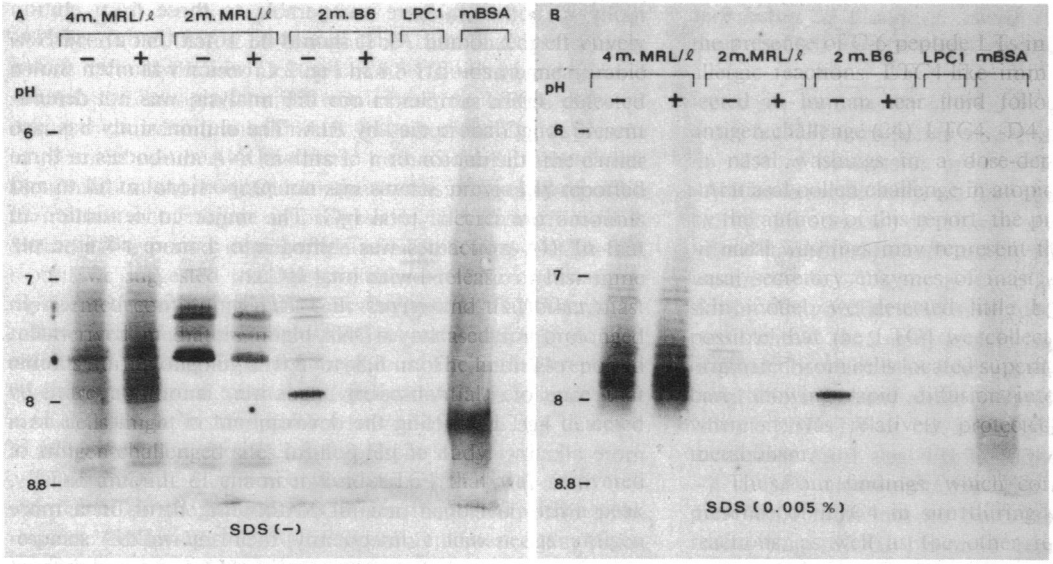

Figure 1. 20 μl of sera from 4-mo-old MRL-*lpr/lpr* (4 m. MRL/*1*), 2-mo-old MRL-*lpr/lpr* (2 m. MRL/*1*), and 2-mo-old B6 (2 m. B6) mice were subjected to IEF with 40 μg of IgG_{2a} myeloma protein (LPC-1) and 25 μg of mBSA in duplicated gels. After focusing, autoradiographs were obtained by incubating the gel with ^{125}I-ssDNA without SDS (*A*) or in the presence of 0.005% SDS (*B*). (−), Non-heated serum; (+), heat-inactivated serum.

pletely diminished. However, even under this condition, many of the ssDNA-binding bands obtained from serum from a 4-mo-old MRL-*lpr/lpr* mouse remained in the pH range of 6.5–8.2, almost the same intensity as that obtained without SDS. Therefore, throughout the study, the IEF gel was pretreated with 0.005% SDS and incubated with ^{125}I-ssDNA in the

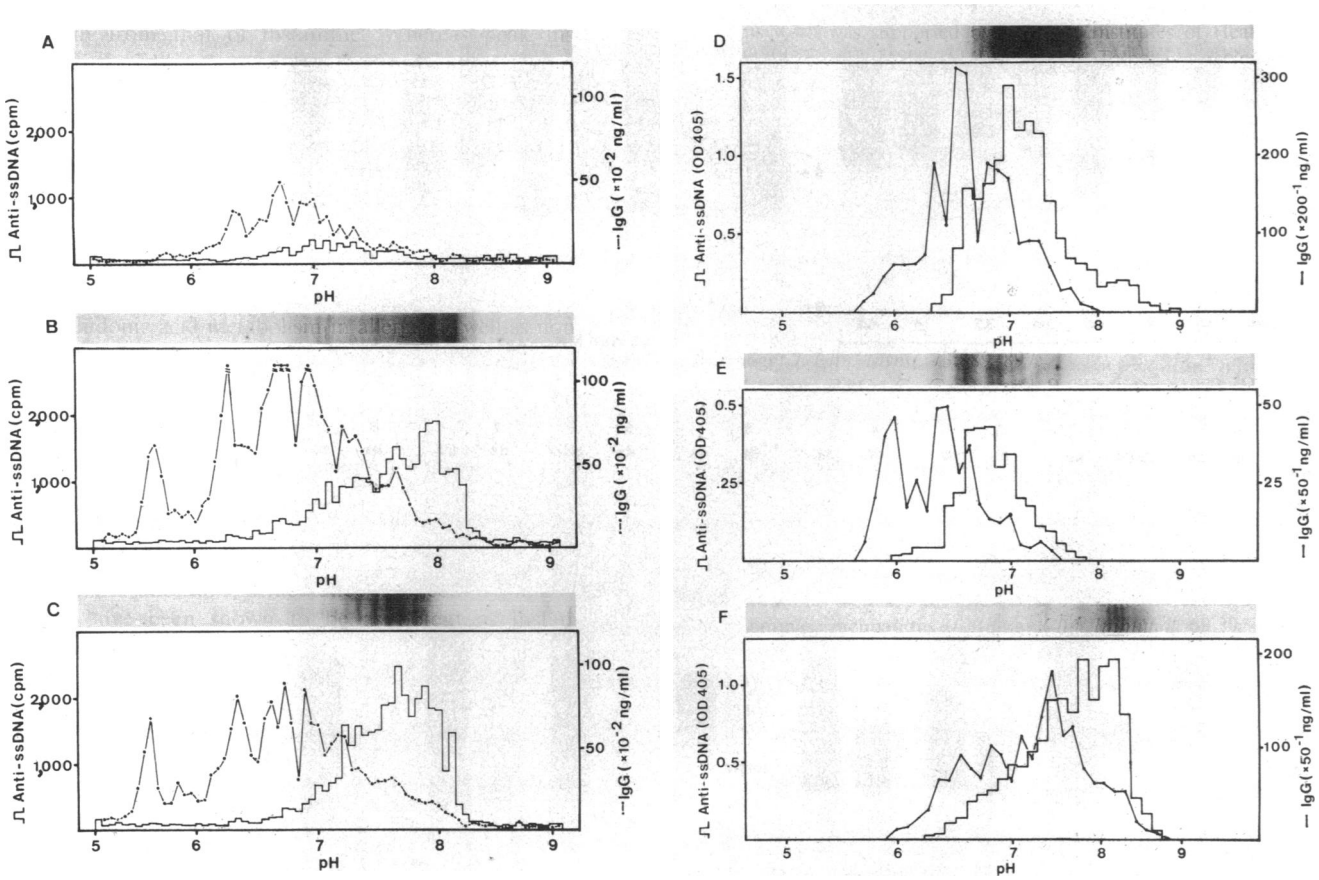

Figure 2. Serum proteins from 2-mo-old MRL-*lpr/lpr* (*A*), three 5.5-mo-old MRL-*lpr/lpr* (*B, C,* and *D*), 10-mo-old female (NZB × NZW)F1 (*E*), and 6-mo-old male BXSB (*F*) mice were separated by IEF in duplicate. Spectrotypes of ssDNA-binding bands were compared with the elution profiles from the gel slices of anti-ssDNA activities and IgG levels detected by RIA (*A, B,* and *C*) or ELISA (*D, E,* and *F*).

presence of 0.005% SDS. However, it should be noted that nonspecific bands were sometimes observed after the long time exposure of the film.

To assess the specificity of binding revealed by the autoradiography, immunoglobulins were eluted from IEF gel slices and their anti-ssDNA activities were determined by RIA or ELISA. Results were compared with the spectrotypes of ssDNA-binding bands of the duplicate samples in the same gel. Serum from a 2-mo-old MRL-*lpr/lpr* mouse showed a group of barely visible, weak ssDNA-binding bands in pH 6.7–7.2. Minimum bindings (<400 cpm) to ssDNA were detectable by RIA in the same pH range from IEF gel eluates (Fig. 2 *A*). When three sera from 5.5-mo-old MRL-*lpr/lpr* mice were analyzed, multiple but quite different ssDNA-binding bands were obtained from each sample in the pH range from 6.5 to 8.5. The elution study on anti-ssDNA activities of IEF gels from these sera gave results comparable to their autoradiographs (Fig. 2, *B–D*). Similar studies were performed on sera from 10-mo-old (NZB × NZW)F1 female and 6-mo-old BXSB male mice. Results from ssDNA-binding bands obtained by autoradiography in both sera were comparable to those from elution studies (Fig. 2, *E* and *F*). It should be noted that an ssDNA-binding band at ~ pH 8.8 in Fig. 2 *C*, which was often shown by other serum samples in our IEF analysis, was not demonstrated as IgG antibodies by RIA. The elution study has also shown that the distribution of anti-ssDNA antibodies in three different SLE-prone strains was not proportional at all to and distinct from that of total IgG. The major concentration of anti-ssDNA antibodies was shifted into a more alkaline pH range when compared with total IgG.

Anti-DNA spectrotypes in MRL-lpr/lpr female mice. In MRL-*lpr/lpr* mice, many ssDNA-binding bands became evident in the pH range from 6.8 to 8.0 at 3 mo of age. Some subgroups of ssDNA-binding bands were more intensified by 5 mo of age, paralleling the development of new bands in a more alkaline range of pH 8.0–8.5. Representative results of two MRL-*lpr/lpr* mice are shown in Fig. 3 *A*. Occasionally, some mice developed new ssDNA-binding bands in a more acidic range as well. Consequently, their anti-ssDNA spectrotypes were extremely heterogeneous in pH 6.5–8.5 and differed

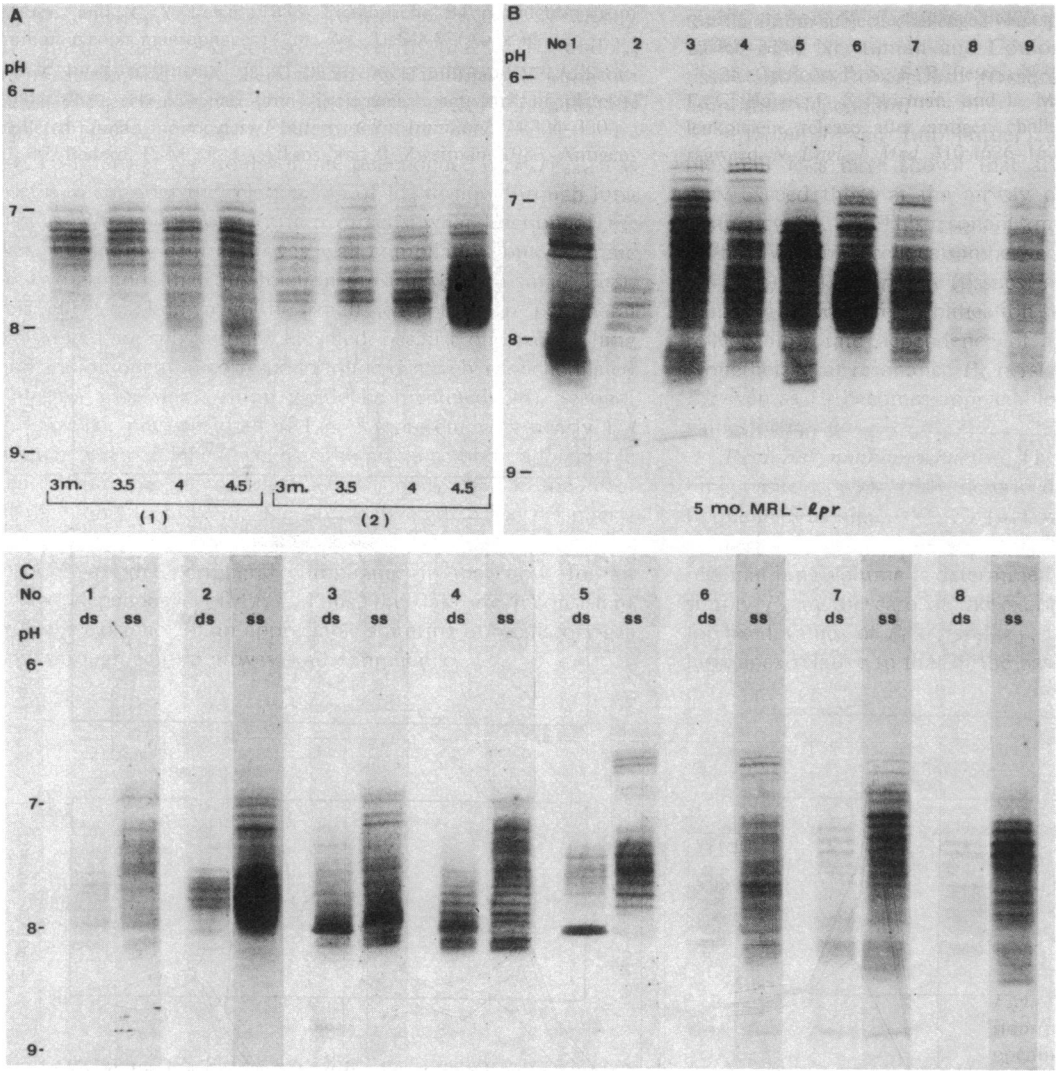

Figure 3. (*A*) Age-dependent changes of ssDNA-binding bands in two MRL-*lpr/lpr* mice from 3 (3 m.) to 4.5 mo of age. (*B*) ssDNA-binding bands in individual MRL-*lpr/lpr* mice of 5 mo of age. (*C*) Comparison between dsDNA-binding bands (ds) and ssDNA-binding bands (ss) in eight individual MRL-*lpr/lpr* mice of 4.5–5 mo of age.

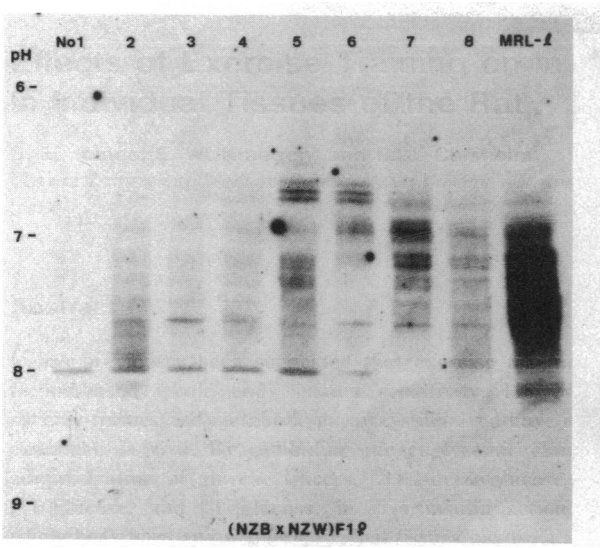

Figure 4. Anti-DNA spectrotypes in eight female (NZB × NZW)F1 mice of 8–10 mo of age. For comparison, a serum from 5-mo-old MRL-*lpr/lpr* mouse (MRL-*l*) is included.

greatly among individual mice at 5 mo of age (Fig. 3 *B*). When anti–dsDNA spectrotypes were compared with those of anti–ssDNA in these mice, dsDNA-binding bands shared dominant bands reacting with ssDNA (Fig. 3 *C*). Again, anti–dsDNA spectrotypes varied greatly among individual mice.

Anti–DNA spectrotypes in (NZB × NZW)F1 female mice. The ssDNA-binding bands in (NZB × NZW)F1 females began to be detectable at 6 mo of age. At 8–10 mo of age, at least five to six relatively distinct subgroups of ssDNA-binding bands became apparent in pH 6.5–8.0. Seven of eight sera tested exhibited these common bands, although intensity of each band varied among individual animals (Fig. 4). In contrast to the wide distribution of anti–ssDNA spectrotypes in MRL-*lpr/lpr* mice, (NZB × NZW)F1 females did not develop significant alkaline bands of more than pH 8.0 even later in their life. The faint but significant dsDNA-binding bands that corresponded to heavy ssDNA-binding bands were observed in these mice (data not shown).

Anti–DNA spectrotypes in BXSB male mice. The ssDNA-binding bands in male BXSB mice were relatively confined to the alkaline range of pH 7.8–8.5. By 6 mo of age, these alkaline bands became more intensified with the development of relatively weak additional bands between pH 7–8 (Fig. 5 *A*). In fact, 11 of 13 mice tested showed major alkaline ssDNA-binding bands characteristic of BXSB male mice (Fig. 5 *B*). Similar alkaline bands of lesser intensity were observed with ^{125}I-dsDNA (data not shown).

Anti–DNA spectrotypes in B6-lpr/lpr, AKR-lpr/lpr, and C3H-lpr/lpr female mice. Three strains of mice (B6, AKR, and C3H) bearing the *lpr* gene spontaneously produced significant amounts of anti–DNA antibodies (14, 15). However, their levels reached maximum at ~6–8 mo of age and decreased gradually. Most sera from 6–8-mo-old B6-*lpr/lpr* mice exhibited markedly restricted ssDNA-binding bands in alkaline pH from 7.8 to 8.4, similar to those obtained by male BXSB mice (Fig. 6 *A*). Only 1 of 20 mice examined developed additional bands in pH 7–7.5. In contrast, both AKR-*lpr/lpr* and C3H-*lpr/lpr* mice at 6–8 mo of age showed a variety of anti–ssDNA spectrotypes differing in individual mice in the pH range from 6.5 to 8.2 (Figs. 7 *A* and 8 *A*). The number of anti–ssDNA bands was relatively limited in these three *lpr* mice as compared with MRL-*lpr/lpr* mice. Further, both the number and intensity of anti–ssDNA bands in these *lpr* mice decreased as they aged (Figs. 6 *B*, 7 *B*, and 8 *B*). At 12 mo of age, ssDNA-binding bands were hardly detectable in the majority of mice.

To examine whether the difference in the number of ssDNA-binding bands between MRL-*lpr/lpr* and the other three *lpr* strains was related to their anti–ssDNA antibody levels, various amounts of sera (2.5–40 μl) from three MRL-*lpr/lpr* (4.5-mo-old) and three B6-*lpr/lpr* (6–8-mo-old) mice, and one C3H-*lpr/lpr* (6-mo-old) and one AKR-*lpr/lpr* (6-mo-old) mouse were analyzed for their anti–ssDNA spectrotypes in the same experiment (Fig. 9). The measurement of IgG anti–ssDNA antibody levels by Farr assay of these samples showed comparable amounts (±60% bindings) in sample Nos. 1, 7, 9 (B6-*lpr/lpr*), 5, 13, 18 (MRL-*lpr/lpr*), 14 (C3H-*lpr/lpr*), and 16 (ARK-*lpr/lpr*). In sera from three MRL-*lpr/lpr* mice, the individual anti–DNA spectrotypes were essentially identical, independent of the amounts of sera applied to the IEF gels

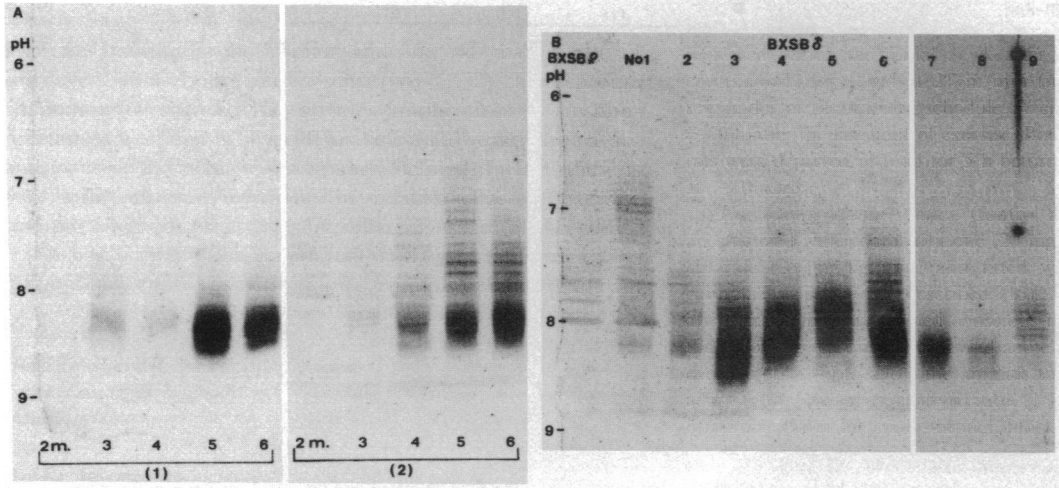

Figure 5. (*A*) Age-related changes of anti-DNA spectrotypes in two male BXSB mice from 2 (2 m.) to 6 mo of age. (*B*) Anti-DNA spectrotypes in nine male BXSB mice of 6 mo of age. A serum from 2-mo-old female BXSB mouse is included at the left end as a control.

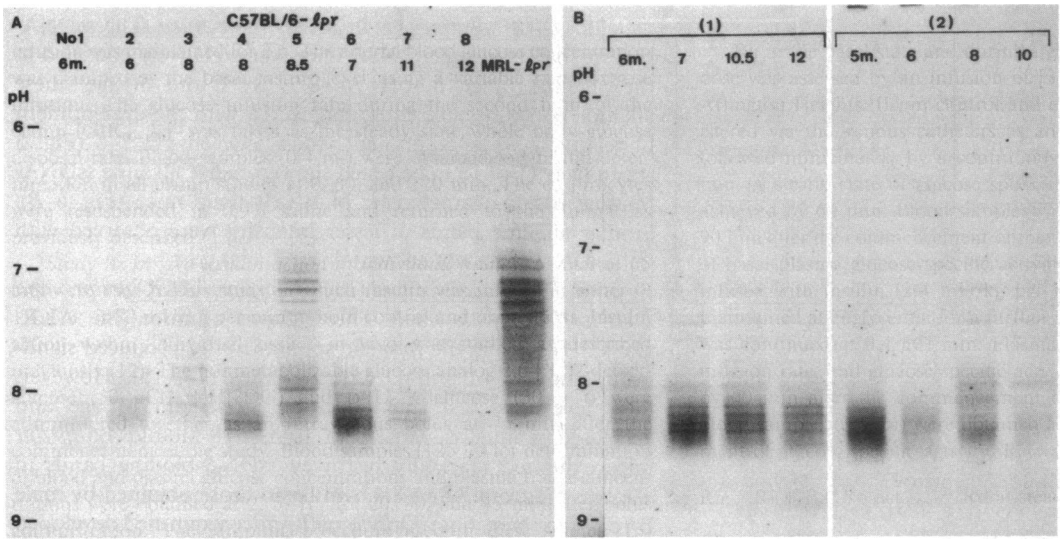

Figure 6. (*A*) Anti-DNA spectrotypes in individual B6-*lpr/lpr* mice of 6(6 m.)–12 mo of age. A serum from 5-mo-old MRL-*lpr/lpr* (MRL-*lpr*) is included at the right end. (*B*). Age-related changes of anti-DNA bands in two B6-*lpr/lpr* mice from 6–12 and 5–10 mo of age.

(Fig. 9 *A*). In fact, all three MRL-*lpr/lpr* mice exhibited heterogeneous anti–DNA spectrotypes in pH 6.6–8.4 with the lowest amount of serum (Nos. 5, 13, and 18). However, samples from B6-*lpr/lpr* (Nos. 1, 7, and 9), C3H-*lpr/lpr* (No. 14), and AKR-*lpr/lpr* (No. 16), whose anti–DNA contents were comparable to those of sample Nos. 5, 13, and 18 from MRL-*lpr/lpr*, showed restricted spectrotypes in pH 7.8–8.4 for B6-*lpr/lpr* and in pH 7.0–8.0 for C3H-*lpr/lpr* and ARK-*lpr/lpr* mice. Notably, no new anti–DNA clonotypes were revealed in sera from B6-*lpr/lpr* and ARK-*lpr/lpr* mice, even when twice as many sera (40 μl) were used or when the IEF gels were exposed for longer periods.

Densitometric analysis on the same autoradiographic films confirmed the distinct spectrotypes of anti–DNA antibodies in these *lpr* mouse strains. Representative results are shown in Fig. 9 *B*. It should be noted that increasing amounts of a B6-*lpr/lpr* serum from 20 μl (No. 7) to 40 μl (No. 6) did not result in the appearance of new spectrotypes. Likewise, when IEF gels of Nos. 2–5 (20–2.5 μl of serum) and Nos. 10–13 (20–2.5 μl of serum) from MRL-*lpr/lpr* mice were similarly analyzed, the number of anti–DNA spectrotypes in the lowest amount of sera (Nos. 5 and 13) was identical to that observed with the highest amount of sera (Nos. 2 and 10) (data not shown). These findings suggest that the difference in anti–DNA spectrotypes among these *lpr* strains was not a reflection of serum antibody concentrations but represented unique qualitative characteristics of each mouse strain.

Anti–DNA antibodies in renal eluates. Significant activities of anti–ssDNA and anti–dsDNA antibodies were recovered from renal eluates of MRL-*lpr/lpr*, (NZB × NZW)F1, and male BXSB mice (Table I). Concentrations of anti–ssDNA antibodies in renal eluates were 10–40 times greater than those of anti–dsDNA antibodies. Relative concentrations of anti–ssDNA antibodies in IgG eluted from the kidneys of three SLE-prone strains were from 3 to 18 times higher than those of anti–ssDNA antibodies in serum IgG. Anti–dsDNA anti-

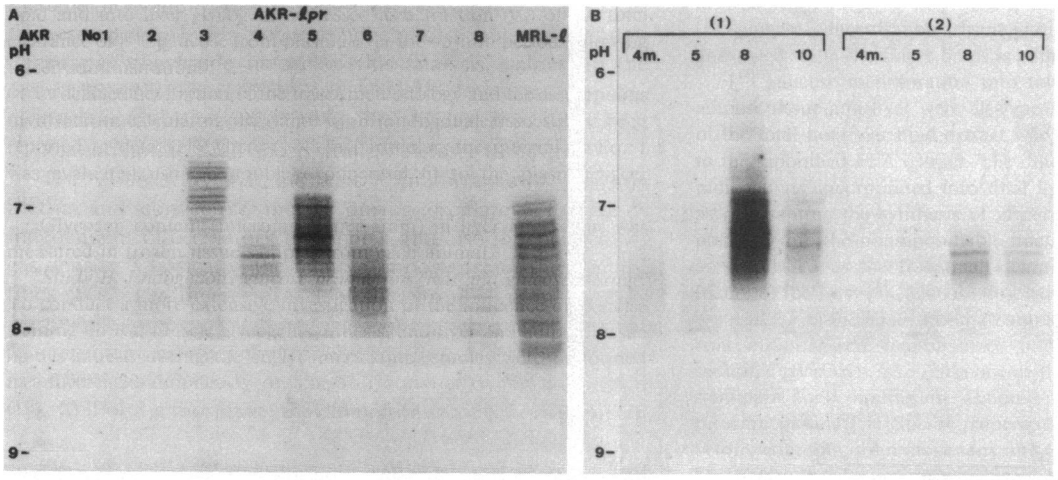

Figure 7. (*A*). Anti-DNA spectrotypes in individual AKR-*lpr/lpr* mice of 6–8 mo of age. For comparison, sera from 2-mo-old AKR (left end) and 5-mo-old MRL-*lpr/lpr* (right end) are included. (*B*) Age-related changes of DNA-binding bands in two AKR-*lpr/lpr* mice from 4 (4 m.) to 10 mo of age.

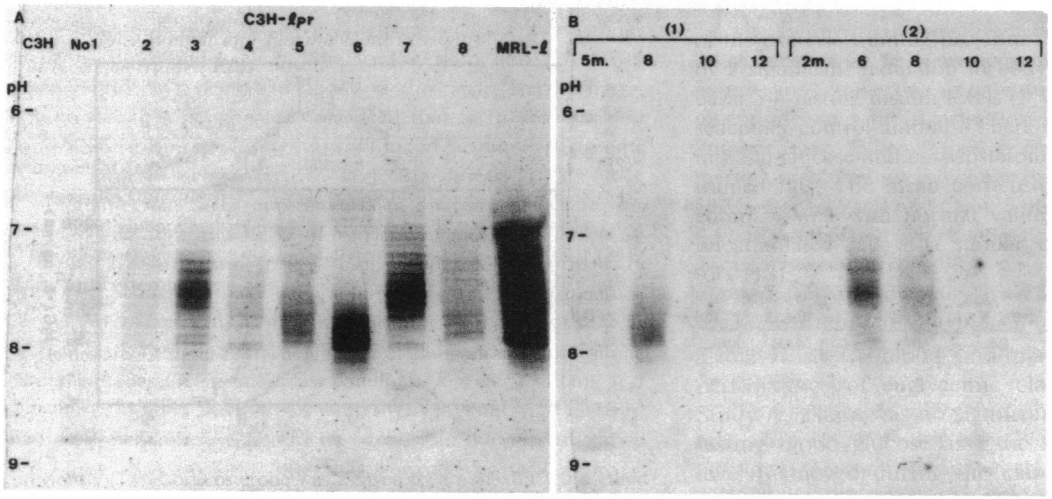

Figure 8. (*A*) Anti-DNA spectrotypes in individual C3H-*lpr/lpr* mice of 6–8 mo of age. Sera from 2-mo-old C3H (left end) and 5-mo-old MRL-*lpr/lpr* (right end) mice are compared. (*B*) Age-related changes of DNA-binding bands in two C3H-*lpr/lpr* mice from 5(5 m.)–12 and 2–12 mo of age.

bodies were also concentrated in renal eluates from these mice, being four to six times greater than in serum.

When anti–DNA spectrotypes of renal eluates from these lupus-prone mice were analyzed by analytical IEF, no well-defined DNA-binding bands were seen, although less sharp, dull bands were observed in high alkaline pH range (pH 8.2–9.2). Therefore, immunoglobulins were eluted from IEF gel slices and their anti–ssDNA and anti–dsDNA antibodies were determined by ELISA in comparison with IgG levels. Representative results from renal eluates of MRL-*lpr/lpr*, (NZB × NZW)F1, and male BXSB mice are shown in Fig. 10. All three renal eluates showed almost identical spectrotypes of anti–ssDNA and anti–dsDNA antibodies. Most of both anti–ssDNA and anti–dsDNA activities were distributed within pH 6.5–8.0. However, it should be noted that there was no significant anti–DNA activity detectable by ELISA in high alkaline pH range of more than pH 8.0 in any eluates. Spectrotypes of IgG from renal eluates were mostly obtained in pH 6–8, peaking at around pH 7.0. Again, there were no detectable amounts of IgG in alkaline range of more than pH 8.0 in any renal eluates.

Discussion

In the present study, we analyzed and compared the spectrotypes of IgG anti–DNA antibodies in various autoimmune mice by IEF in relation to the pathogenesis of lupus nephritis. Our results demonstrated that each murine strain bears distinct spectrotypes of anti–DNA antibodies and that the development of lupus nephritis does not appear to be associated with particular spectrotypes of anti–DNA antibodies. Rather, the age-dependent expansion of anti–DNA clonotypes appears to be more characteristic in mice developing lethal lupus nephritis.

It is clear that the use of low concentration SDS (0.005%) during the incubation of the focused gel with ^{125}I-DNA enhanced the specificity of DNA-binding bands because of the elimination of the binding with basic proteins. Such concentration of SDS almost completely eliminated nonspecific bands

Table I. Comparison of Concentrations of IgG, Anti–ssDNA, and Anti–dsDNA Antibodies between Renal Eluates and Corresponding Sera from Three Strains of Lupus-prone Autoimmune Mice

Samples of	IgG*	Anti-ssDNA‡	Anti-ssDNA/IgG	Concentration index of anti-ssDNA§	Anti-dsDNA‖	Anti-dsDNA/IgG	Concentration index of anti-dsDNA¶
			%			%	
MRL-*lpr/lpr*							
Renal eluate	39	1.26	3.23	2.8	0.12	0.31	4.4
Serum	41,500	486	1.17		29	0.07	
(NZB × NZW)F1							
Renal eluate	57	4.52	7.93	17.6	0.12	0.21	4.2
Serum	13,100	59	0.45		6	0.05	
BXSB male							
Renal eluate	66	0.56	0.85	12.1	0.04	0.06	6.0
Serum	9,500	7	0.07		1	0.01	

*,‡,‖ The concentrations of IgG, anti–ssDNA, and anti–dsDNA antibodies are expressed as micrograms per gram of kidneys for renal eluate and micrograms per milliliter for serum. §,¶ Concentration index of anti–ssDNA or anti–dsDNA antibodies is expressed by the ratio of anti-ss(or ds)DNA/IgG in renal eluate to anti-ss(or ds)DNA/IgG in serum.

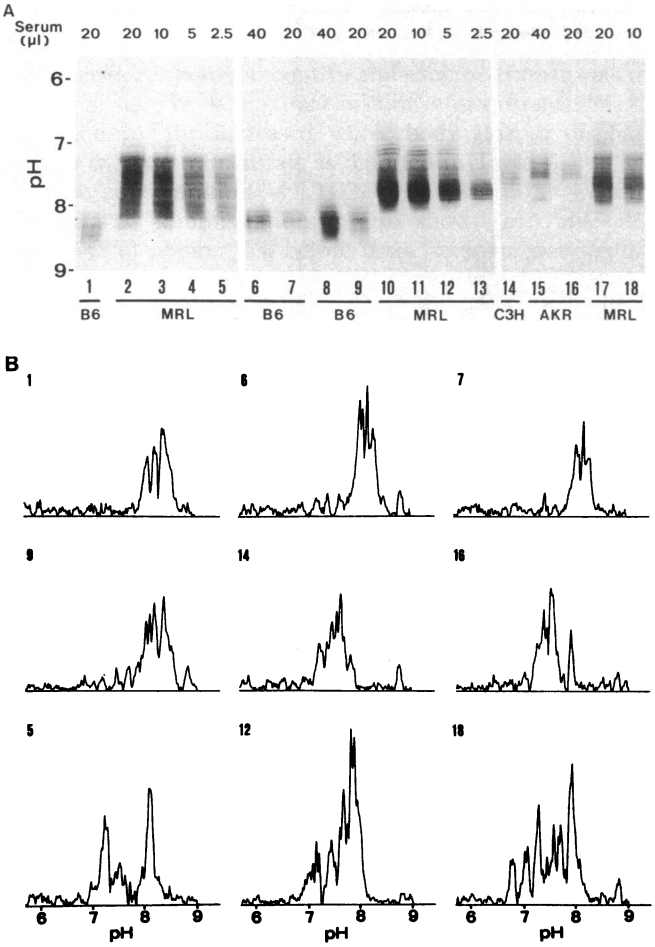

Figure 9. (*A*) Anti-ssDNA spectrotypes in three different MRL-*lpr/lpr* (MRL) mice (4.5-mo-old) were compared with those in three B6-*lpr/lpr* (B6) mice, one C3H-*lpr/lpr* (C3H) mouse, and one AKR-*lpr/lpr* (AKR) mouse (6–8-mo-old) in the same experiment. Various amounts (2.5–40 µl) of sera were analyzed for comparison. For example, spectrotypes of samples 2–5 were obtained from the same MRL-*lpr/lpr* mouse using four different amounts of serum as indicated. Note that sample Nos. 1, 7, 9, 14, and 16, which exhibited ssDNA-binding activities comparable to those of sample Nos. 5, 13, and 18, showed limited numbers of ssDNA-binding bands. (*B*) Densitometric analysis with programmed background reduction and baseline correction of the DNA-binding bands of nine samples in Fig. 9 *A*. The sample numbers coincide with those in Fig. 9 *A*. Intensity of the bands is expressed in the same vertical scale. The results were comparable to the spectrotypes shown in Fig. 9 *A* with high resolution.

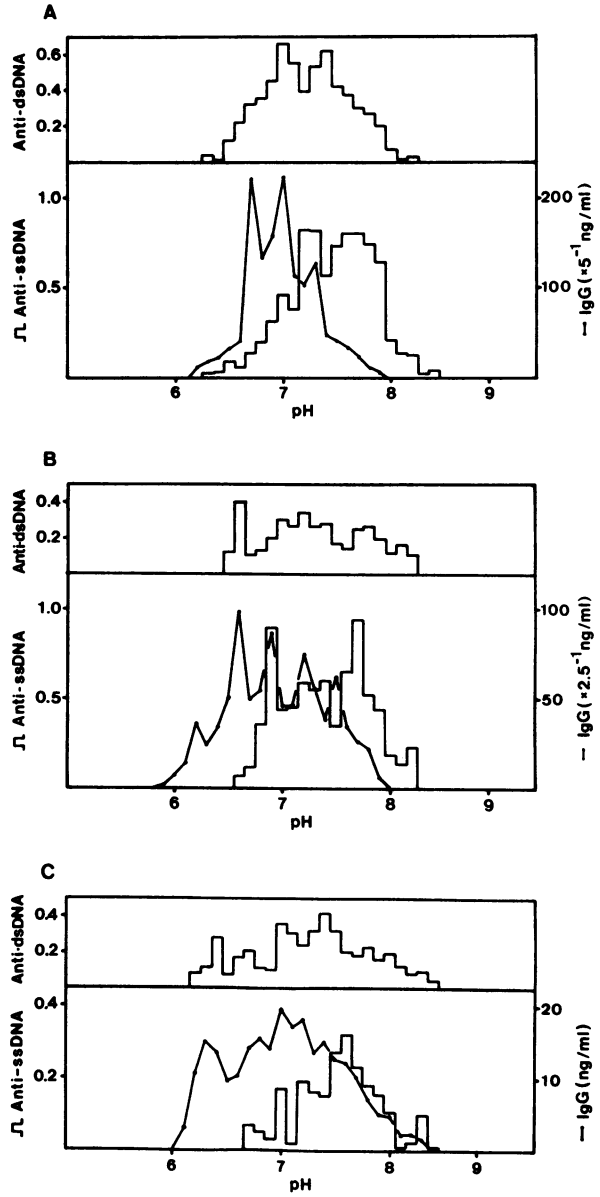

Figure 10. Spectrotypes of anti-dsDNA and anti-ssDNA activities and IgG levels in IEF fractions from renal eluates. (*A*) 4–5-mo-old MRL-*lpr/lpr* mice; (*B*) 8–10-mo-old (NZB × NZW)F1 mice; (*C*) 6–9-mo-old BXSB male mice.

obtained by non-DNA binding immunoglobulins such as myeloma protein, LPC-1, or mBSA. It also reduced the DNA-binding bands seen in 2-mo-old nonautoimmune B6 mice without affecting the intensity and number of DNA-binding bands of sera from adult SLE mice. Such an effect of SDS has been demonstrated for the specific determination of anti–DNA antibodies by the Farr-DNA binding RIA (26). Although the use of SDS may affect the detection of low avidity anti–DNA antibodies, the elution study has demonstrated the correlation of DNA-binding bands obtained in the presence of SDS with the anti–DNA binding activities determined by solid-phase RIA or ELISA, which can detect low as well as high avidity antibodies (27). It should also be noted that anti–DNA binding activity eluted from the IEF gel was not correlated with the concentration of immunoglobulins, an observation supporting the specific detection of DNA-binding bands in our assay. However, one should be aware that even in the presence of SDS several samples, particularly renal eluates, showed dull DNA-bindings, occasionally in high alkaline range, which did not appear to be IgG anti–DNA antibodies.

When the dsDNA-binding bands were compared with ssDNA-binding bands in sera from autoimmune mice, almost all of anti–dsDNA bands shared strong anti–ssDNA bands in spite of the high purity of ^{125}I-dsDNA preparation. Since it has been shown that most of anti–dsDNA antibodies bind to both dsDNA and ssDNA (28–31), it is likely that our ssDNA-

binding bands reflect the total expression of both anti-ssDNA and anti-dsDNA antibodies.

Analysis of anti-DNA spectrotypes in SLE-prone mice developing lethal glomerulonephritis has demonstrated that each strain has distinct and characteristic spectrotypes of anti-DNA antibodies. MRL-*lpr/lpr* mice exhibited the most heterogeneous spectrotypes of anti-DNA antibodies, with marked variation in individual animals. In contrast, in female (NZB × NZW)F1 and male BXSB mice anti-DNA spectrotypes were relatively uniform. Female (NZB × NZW)F1 mice appeared to bear five to six common clonotypes in pH 6.5–8.0, and the major anti-DNA spectrotype of male BXSB mice was confined to a pH range from 7.8 to 8.5. This limited size of anti-DNA spectrotypes in BXSB mice would explain a relatively low concentration of anti-DNA antibodies in this strain as shown in the present study and others (13, 20). Our results are contrasted with the recent observation that the most frequent anti-DNA antibodies common in these three autoimmune mice were focused in the alkaline range from pH 8.0 to 8.5 (10). The difference between our results and theirs may be partly related to the use of SDS, which efficiently eliminated the binding of DNA by cationic nonimmunoglobulin proteins.

It has been recently suggested that the anti-DNA antibodies with isoelectric points (pI) from 8 to 9 may be more pathogenic because of the appearance of such alkaline anti-DNA antibodies coincident with the onset of renal disease and the concentration of these antibodies in renal eluates (10). This hypothesis was further supported by the findings that highly positively charged immunoglobulins (32) or immune complexes (33) could localize in glomerular basement membrane (GBM) through electrostatic interaction with the anionic sites in GBM (34, 35) compared with those having neutral or acidic pI. However, our results have demonstrated that the development of lupus nephritis does not appear to be associated with particular spectrotypes of anti-DNA antibodies. First, the appearance of alkaline anti-DNA clonotype with high pI was not a common feature of mice developing severe glomerulonephritis. Second, B6-*lpr/lpr* mice produced relatively high concentration of alkaline anti-DNA antibodies but failed to develop severe renal disease. Third, not only anti-DNA antibodies but also IgG was eluted from diseased kidneys, distributed mostly in the range of pH 6.5–8.0 without any concentration of particular clonotypes of alkaline pI >8.0. Autoradiographs of DNA bindings by renal eluates from nephritic autoimmune mice have shown dull DNA bindings in high pH range (pH 8.2–9.2) similar to those described previously (10). However, such bands did not appear to be IgG anti-DNA antibodies because highly sensitive and specific ELISA failed to demonstrate anti-DNA antibodies or IgG in this high alkaline range. In fact, our results are compatible with the recent studies which have demonstrated no significant correlation between the alkaline anti-DNA spectrotype of serum and renal disease in human SLE (12). It should be noted that cationic IC composed of unmodified focused fractions of antigens and antibodies (pI 8–10) did not bind significantly to GBM compared with the high binding activity of IC composed of chemically modified cationic antigens and antibodies (33).

Instead, our results suggest that the age-dependent expansion of anti-DNA clonotypes may be more characteristic of mice that develop fatal glomerulonephritis. In fact, only mice developing fatal glomerulonephritis exhibited clonal expansion of anti-DNA antibodies throughout their lives. New spectrotypes can be developed not only in the alkaline range but also in the acidic range. However, such expansion was not apparent in three *lpr* congenic mice that failed to develop severe glomerulonephritis. Their expression of anti-DNA spectrotypes rather decreased when they became old (>10 mo).

It is significant that spectrotypes of anti-DNA antibodies differed in three different congenic inbred strains bearing the *lpr* mutation, although their serum levels of anti-ssDNA antibodies were comparable (14). Apparently, the expression of anti-DNA spectrotypes was greatly influenced by the background genome of each strain. Of interest, B6-*lpr/lpr* mice have the major anti-DNA spectrotype in alkaline range similar to that observed in BXSB mice. Since both strains have the same immunoglobulin allotype (Igh-1^b) and major histocompatibility complex (MHC) haplotype (H-2^b) (13, 36), such a limited spectrotype in these mouse strains may be related to the immunoglobulin allotype gene or MHC-linked Ir gene for the specific immune response to DNA antigen. In fact, MRL-*lpr/lpr*, C3H-*lpr/lpr*, and AKR-*lpr/lpr* mice that bear H-2^k haplotype express different anti-DNA spectrotypes from B6-*lpr/lpr* and BXSB mice. Experiments using B6-*lpr/lpr* mice congenic for the immunoglobulin allotype gene or MHC gene would help elucidate the understanding of the differences in anti-DNA spectrotypes in various strains of mice.

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