IMMUNOLOGICAL STUDIES CONCERNING THE NEPHRITIS OF SYSTEMIC LUPUS ERYTHEMATOSUS*

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Patients with systemic lupus erythematosus (SLE) form a spectrum of antibodies which react in various test systems with native or altered autologous antigens (1–4). The stimuli eliciting antibody production and the pathogenetic role of these antibodies in SLE have been the subject of intensive investigation and debate. Earlier studies stressed the importance of such antibodies as direct cytotoxic agents (5–7). More recently, however, evidence has been accrued suggesting that circulating antigen-antibody complexes are formed during the course of SLE and are deposited in the kidneys of patients with the nephritis of SLE. Serum complement is depressed during active stages of nephritis (8, 9) and the clinical manifestations are similar to the chronic serum sickness syndrome elicited in animals by antigen-antibody complexes formed in vivo (10). Floccular and granular deposits of γ-globulin and complement (11–13) have been localized in glomeruli utilizing the fluorescent antibody technique. These deposits resemble those found in experimental immune complex–induced nephritis of Dixon and associates (14). Antinuclear antibodies have been eluted from the glomeruli of kidneys from patients with SLE nephritis (15–18). Recently, one potential antigen, deoxyribonucleic acid (DNA), has been observed in sera from certain patients with active nephritis (19). These data suggest that antigen-antibody complexes composed of nuclear antigen and antinuclear antibodies might well be involved in the pathogenesis of the nephritis.

The present studies were undertaken to obtain further support for the antigen-antibody complex hypothesis. In particular, efforts were made not only to demonstrate antinuclear antibodies in glomeruli but to show by quantitative immunochemical techniques that they are concentrated at this site in association with nuclear antigens.

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Material and Methods

(1) Tissues and Sera

Whole kidneys were obtained at autopsy within 12 to 24 hr of death and frozen at −20° to −50°C. Specimens were obtained from patients with clinical symptoms of SLE (10), subacute glomerulonephritis (1), ovarian carcinoma (1), cerebral hemorrhage (1), pulmonary infarction (1), and carcinoma of lung (1). Renal biopsies were also obtained from five patients with SLE. Small blocks of tissue were frozen at −70°C in dry ice and isopentane when fresh tissue was available for immunofluorescent examination.

Histological examination of kidneys from patients with SLE revealed typical features of subacute nephritis. Glomeruli showed irregular thickening of capillary tufts by dense eosinophilic deposits, focal areas of necrosis, occasional crescent formation, and varying degrees of hyalinization. The kidneys from a patient with subacute glomerulonephritis showed microscopic changes similar to those with SLE nephritis. Sections of kidney from the remaining patients were essentially normal.

(2) Fluorescent Antibody Technique

Antisera to human γ-globulin, γG-globulin, γM-globulin, fibrinogen, and β_{1C}-globulin (C'3 component of complement) were prepared, conjugated with fluorescein isothiocyanate, and used with direct and indirect incubations as previously described (13). The final molar fluorescein-protein ratios of conjugates (20) were 2 to 3. The conjugates were used in a dilution which gave agar precipitin reactions at 1:8 to 1:32 dilutions with 1 mg/ml of protein in the antigen well. Human serum was used for the assay of anti-C'3 sera. In vitro complement fixation using human serum was carried out following the method of Lachmann et al. (21).

Cryostat sections were fixed routinely in acetone for 10 min prior to incubation with a fluoresceinated antiserum or a test serum and eluate in the indirect fluorescent antibody studies. Selected cryostat sections were treated with 0.02 M, pH 3.2, citrate buffer for 2 hr at 37°C; with 1 M or 2 M NaCl for periods of 5 min to 3 hr at 37°C; with deoxyribonuclease (DNase, 2 × crystallized pancreatic deoxyribonuclease; Worthington Biochemical Corp., Freehold, N.J.), 100–200 μg for 30 min to 2 hr; and with DNase followed by 0.02 M, pH 3.2, citrate buffer.

Controls included incubation of sections with unfluoresceinated antiserum prior to incubation with fluoresceinated antiserum and treatment of sections with normal fluoresceinated globulin fractions. Inhibition studies were performed by incubating aliquots of an eluate or antiserum with an equal volume of antigen solution (100–500 μg/ml) or saline for 60 min at 37°C and 60 min at 4°C. 0.5 cc aliquots of eluates were incubated with strands obtained from 0.5 cc of nucleoprotein (discussed below) for studies on the quantitation of antinuclear antibody.

(3) Preparation of Fluoresceinated Antibodies to Nuclear Antigens

Anti-DNA antibody was isolated from plasma of a patient (B.R.) with SLE. The equivalence zone of a quantitative precipitin test with calf thymus DNA was determined (22). A mixture of plasma and DNA at the early equivalence zone was incubated at 37°C for 1 hr, placed at 4°C for 2 hr, centrifuged at 2000 rpm for 20 min and washed three times in cold pH 7.4 phosphate-buffered saline. After the last wash, approximately 1.0 cc of precipitate was suspended in 10 cc of pH 6.9 buffer with 0.003 M $MgCl_2$. 1.0 mg of DNase was added to the suspension and incubated at 37°C for 1 hr (23). The suspension was then dialyzed overnight against 0.001 M EDTA; 0.2 cc of residual precipitate remained after DNase treatment. The supernatant was conjugated with fluorescein at a protein concentration of 0.5 mg/ml. The conjugated anti-DNA antibody produced bright fluorescence of nuclei when incubated with human liver in a 1:8 dilution without evidence of background fluorescence. The staining

was completely inhibited by incubating equal volumes of DNA (1 mg/ml) with an equal volume of anti-DNA conjugate.

Antinucleoprotein antibody was prepared from the supernatant of plasma (B.R.) remaining after absorption with DNA at equivalence. The supernatant was incubated with nucleoprotein strands (3 cc of packed strands per 10 cc of plasma) followed by DNase treatment as above. Agar gel diffusion studies of this supernatant revealed a precipitin line with nucleoprotein extract but not DNA. Immunofluorescence staining of human liver nuclei with the fluorescein-conjugated isolated antinucleoprotein antibody was inhibited by incubation with nucleoprotein strands but not by incubation with DNA.

A globulin fraction containing antibodies to Sm antigen and other phosphate buffer extractable nuclear antigens was prepared from a pool of 10 serums from patients with SLE and conjugated with fluorescein. The serums exhibited precipitin reactions only with phosphate buffer extract of nuclei. Globulin fractions were conjugated at protein concentrations of 10 mg/ml. Fluorescence staining of hepatic nuclei was completely inhibited by phosphate buffer extract of nuclei.

(4) Preparation of Antigens

Nuclei were prepared from calf thymus following the method of Mirsky and Pollister (24). The purity of the preparations was checked by staining smears with hematoxylin and eosin. Less than 10% contamination by whole cells was noted.

(*a*) Nuclear extract was prepared by stirring a 10% suspension of thymus nuclei in pH 7.2 phosphate-buffered 0.15 M saline for 4–6 hr at room temperature. The supernatant was centrifuged at 2500 rpm for 30 min and diluted to a final protein concentration of 3 mg/ml determined by the Folin technique (25). This extract was used for the eluate inhibition studies.

(*b*) Nuclear extract containing Sm antigen was prepared following the method of Tan and Kunkel (26) and used for the immunodiffusion studies.

(*c*) Nucleoprotein was prepared following the method of Mirsky and Pollister (24). A viscous solution was stored at 5°C in 1 M NaCl. Prior to use, strands were precipitated by addition of 6 volumes of distilled water, washed three times in saline, sonicated for 3–4 min at full strength in a Branson sonicator, and used at a protein concentration of 3 mg/ml.

(*d*) Native DNA (Calf thymus deoxyribonucleic acid, Worthington Biochemical Corp.) was used at 1 mg/ml for agar gel diffusion and inhibition studies.

(*e*) Denatured DNA was prepared by boiling native DNA at 0.5 mg/ml for 10 min and immediately plunging the solution into an ice bath.

(*f*) Purified ribosomes were prepared from rabbit liver (27) and suspended in pH 7.4 phosphate-buffered saline for use in agar diffusion studies.

(5) Immunoprecipitation

Immunoprecipitation was carried out by a modification of the Ouchterlony technique (26) with Petri dishes containing 25 ml of 0.5% agar (Baltimore Biological Laboratories, Baltimore, Md.) in 0.01 M, pH 7.4, phosphate-buffered saline with sodium azide (0.1%). Holes were cut, 8 mm in diameter and 4 mm apart. The plates were incubated at room temperature.

Quantitative radial immunodiffusion plates were prepared (28) and used for determination of immunoglobulin concentration in serums and eluates. Several early studies utilized immunodiffusion plates purchased from Hyland Laboratories, Los Angeles, Calif.

(6) Hemagglutination

Anti-γ-globulin antibodies were assayed following the method of Osterland and associates (29), and titers of blood group antibodies were determined by incubating serial dilutions of serum and eluates with a 1% suspension of erythrocytes of blood groups A, B, and O. Hemag-

glutination titer was recorded as the highest dilution with agglutination after centrifugation. Rheumatoid factor was assayed following the method of Waller (30) using Ripley coated cells.

(7) Glomerular Elution Procedures

The glomerular isolation procedure was performed as described by Krakower and Greenspan (31) with certain modifications. Procedures were carried out at 4°C, unless otherwise stated. After allowing one-half of a kidney to thaw for 12 hr, the cortex was separated and cut into small blocks, weighed (60–80 g/kidney), suspended in cold 0.85% saline, and homogenized in an ice-jacketed Waring Blendor for 1–2 min at low speed. In three cases (2, 3, 11) liver was subjected to a similar procedure. The particulate matter was then pressed through a 100-mesh stainless steel sieve with a spatula. The sediment was suspended in approximately 400 cc of cold 0.85% saline, divided into aliquots, and washed until the supernatant was clear (6–7 washings). The superficial layers of sediment containing mainly tubular cells were removed. Centrifugation was performed for 3 min at 250 *g* in a refrigerated centrifuge.

Aliquots of selected washes were examined for the presence of antinuclear antibody by the indirect fluorescent technique. The first two or three washes contained antinuclear antibody in low titer, but the final two or three washes were uniformly negative for antibody. The final sediment contained mainly glomeruli and a moderate amount of cellular debris. Counts revealed that approximately 250,000–350,000 glomeruli were isolated from one-half of each kidney. Hematoxylin and eosin stains revealed that about 10% of the intact glomeruli were encapsulated. In addition, scattered glomerular fragments and tubular cells were observed.

The material obtained from 11 kidneys was suspended in 75 cc of 0.02 M citrate buffer at pH 3.2, pH 2.4 glycine buffer, or pH 7.2 phosphate buffer and stirred at room temperature for 2 hr. γ-globulin (fraction II) was added to eluates from two normal kidneys to a final concentration of 1.0 mg/ml of eluate. Eluates were then dialyzed against pH 7.2, 0.02 M phosphate-buffered saline, and concentrated to $\frac{1}{5}$ of their volume by ultrafiltration and stored at −20°C.

DNase treatment of glomeruli obtained from five kidneys was performed in a manner analogous to that used for the isolation of anti-DNA antibody. Approximately 5 cc of glomerular suspension was diluted to 20 cc with pH 6.9 phosphate-buffered saline. 0.003 M $MgCl_2$ was added and the pH adjusted to 6.9 with 1 N NaOH. 0.2 mg of DNase was added to the suspension which was incubated for 1–2 hr at 37°C or overnight at 4°C, and then centrifuged for 1 hr at 4°C (2000 rpm). The supernatant was then dialyzed for 24 hr against pH 7.2 phosphate-buffered saline containing 0.001 M EDTA and concentrated.

The glomeruli from four kidneys were treated with 0.02 M pH 3.2 citrate buffer for 4 hr at room temperature after DNase treatment.

Ribonuclease (bovine pancreatic ribonuclease 3 × crystallized, Worthington Biochemical Corp.) treatment was performed by washing glomeruli in 0.01 M Tris-HCl, pH 7.4 buffer, and suspending 5 cc of glomeruli in 20 cc of 0.01 M Tris-HCl, pH 7.4, with 0.001 M $MgCl_2$. Ribonuclease was added to a final concentration of 50 μg/ml and the suspension was incubated at 37°C for 1 hr. After centrifuging to remove glomeruli, the supernatant ribonuclease was inactivated by addition of polyvinyl sulfate, 400 μg/ml, and dialyzed against Tris buffer and then pH 7.2 phosphate-buffered saline for 48 hr.

RESULTS

(1) Tissue Studies

(a) Localization of Immunoglobulins and Complement (Table I).—Cryostat sections from each kidney were examined by the fluorescent antibody technique prior to the elution procedures in order to evaluate the amount of γ-globulin and complement present and the effect of procedures designed to dissociate

antigen-antibody complexes on the γ-globulin deposits in glomeruli. Kidneys obtained at autopsy from patients with the subacute nephritis of SLE exhibited deposits of γG-globulin and the C'3 component of complement in all cases studied. Lesser amounts of γM-globulin were demonstrated. These proteins were deposited along glomerular capillary loops in a diffuse or focal lumpy pattern (Fig. 1). Aggregates of immunoglobulins and complement were also found focally distributed in the walls of small and medium sized arterioles.

Biopsy specimens from five patients with earlier stages of nephritis revealed lesser amounts of immunoglobulins and complement. In these sections a finely granular deposit was visualized along the basement membrane (Fig. 2) in contrast to the more advanced lesions which demonstrated a homogeneous pattern of fluorescence.

Treatment of cryostat sections of kidney with pH 3.2, 0.02 M citrate buffer for 2 hr, which has been shown to elute γ-globulin (32), caused a moderate reduction in the amount of immunoglobulins demonstrable in glomeruli. In contrast, immunoglobulins were not eluted from glomeruli of sections treated with pH 7.2 phosphate-buffered saline under similar conditions. Sections from two kidneys (cases 7 and 9) were treated with DNase in an attempt to elute antibody bound to DNA or nucleoprotein. No appreciable decrease in immunoglobulins was observed, although when DNase treatment of sections was followed by acid buffer incubation a marked decrease in glomerular bound immunoglobulins was noted. Concentrated salt solution, utilized to dissociate antigen-antibody complexes (33), caused a moderate to marked reduction in immunoglobulins after incubation of sections from cases 7 and 9.

(*b*) *Attempts of Direct Localization of Specific Antibody.*—Fluoresceinated phosphate buffer extract of nuclei and fluoresceinated histone were incubated with cryostat sections of kidneys (cases 7 and 9) in an attempt to demonstrate the presence of antinuclear antibodies in γ-globulin deposits. Staining of glomerular capillary tufts was not observed, although fluoresceinated histone was found to react weakly with nuclei.

(*c*) *Localization of Antigen.*—Highly purified anti-DNA antibody labeled with fluorescein was utilized in a search for DNA antigen. Deposits of DNA were found in glomeruli (Figs. 3 *a* and 3 *b*) and vessel walls in multiple sections of kidney studied (case 7). The pattern of localization was similar to that of γ-globulin. Glomeruli had intense focal fluorescence in lumpy deposits or within intravascular thrombi. The fluorescent staining of the anti-DNA conjugate was completely inhibited by prior incubation of the conjugate with DNA (Fig. 3 *c*). Optimal localization of DNA was achieved in cryostat sections treated for 30–90 min at 37°C with 2 M NaCl prior to incubation with the fluoresceinated antiserum. Incubation periods of 2–3 hr resulted in markedly decreased glomerular fluorescence. In some sections, faint residual fluorescence was noted within nuclei after application of the anti-DNA conjugate to sections treated with 2 M

NaCl. Attempts at demonstrating DNA by pretreating sections with pH 3.2 citrate buffer and pH 2.4 glycine buffer for periods of 30 min to 3 hr were not successful.

Smaller deposits of DNA (Fig. 4) were also observed in a renal biopsy specimen (D.B.). These deposits were found in untreated sections but were more clearly demonstrable in sections incubated with 2 M NaCl for 5 min. This reaction was also inhibited by treating the anti-DNA conjugate with DNA. More prolonged treatment with NaCl resulted in complete extraction of DNA from the sections. One other autopsy specimen (case 9) and four additional biopsies studied did not exhibit DNA deposits along the basement membrane. These kidneys showed lesser quantities of γ-globulin distributed in a more membranous fashion in glomeruli. Efforts to demonstrate other nuclear antigens with a fluoresceinated globulin fraction containing antibodies to the Sm antigen and a fluorescein conjugated antinucleoprotein antibody were unsuccessful.

(*2*) *Studies on Isolated Glomeruli*

Smears of glomeruli isolated from the renal cortex (cases 1, 2, 9) and washed in physiological saline were stained with 1:10 dilutions of fluoresceinated antiserums to γG- and γM-globulins. The glomeruli retained these immunoglobulins following the concentration procedure (Fig. 5). A small aliquot of isolated glomeruli was embedded in agar and sectioned in order to demonstrate that the distribution of γG-globulin was similar to that observed in cryostat sections of kidney. Localization of γG-globulin in glomerular nuclei was not observed, but small focal cytoplasmic deposits were found in tubular cells.

After incubation of isolated glomeruli with acid buffer and DNase for the elution of antibody, smears and agar-embedded glomeruli were examined as described above. Fluorescence indicative of γG-globulin was considerably less intense in glomeruli subjected to acid buffer or DNase treatment. Nuclear deposits of γG-globulin were not observed, but fluorescent aggregates of protein persisted in occasional tubular cells.

(*3*) *Characterization of Glomerular Eluates*

Two main procedures were utilized for the elution of antibody from isolated glomeruli; treatment with acid buffer and treatment with DNase. The antibody activity of the eluates was assayed by immunofluorescence and agar gel precipitin techniques. In order to show the specificity of acid buffer and DNase for the elution of antibody from SLE glomeruli, pH 7.2 phosphate-buffered saline eluates were similarly examined. Control glomerular eluates from normal kidneys, with normal γ-globulin added in vitro and without further treatment, were also assayed for the presence of antibodies.

(*a*) *Immunofluorescence Studies.*—Anti-nuclear antibody was demonstrated in all eluates from SLE glomeruli by the indirect fluorescent antibody tech-

nique. Acid buffer eluates contained antibody reacting with hepatic nuclei to give an intense, diffuse, staining reaction (Fig. 6 *a*), in contrast to antibody obtained from DNase-treated glomeruli which stained hepatic nuclei in a finer, punctate pattern (Fig. 6 *b*).

The specificity of the reaction for various antigens was demonstrated by inhibition of the immunofluorescence reaction when eluates were incubated with

TABLE I

Localization of Immunoglobulins and Complement in Renal Glomeruli of Patients with SLE by the Fluorescent Antibody Technique

Case No.	γG-globulin	γM-globulin	γG-globulin (pH 3.2)*	γM-globulin (pH 3.2)*	β_{1C}-globulin
1	2+‡	1+	±	0	2+
2	2+	±	±	0	1+
4	3+	2+	±	0	1+
5	2+	0	1+	0	±
6	3+	±	±	0	3+
7	3+	1+	2+	1+	2+
9	2+	1+	1+	±	2+

* γ-globulin remaining after treatment of cryostat section with pH 3.2, 0.02 M citrate buffer for 2 hr.

‡ Relative number of glomeruli in a cryostat section stained by a fluorescein-conjugated antiserum. ±, rare; 1+, few; 2+, moderate number; and 3+, almost all glomeruli.

TABLE II

Inhibition of Immunofluorescence Reactions of Glomerular Eluates by Nuclear Antigens

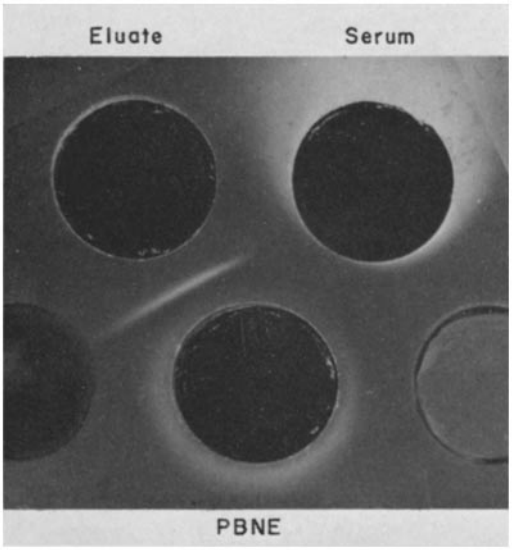
Eluate	No. of cases	Phosphate buffer extract	Nucleoprotein	DNA	Histone
Acid buffer	11	11	0	0	0
DNase	5	0	4	3	1
DNase + acid buffer	4	1	2	1	0

phosphate buffer nuclear extract, DNA, histone, and nucleoprotein (Table II). Antibody activity of acid buffer eluates was inhibited primarily by phosphate buffer extract, whereas antibodies in the DNase eluates were absorbed in various proportions by DNA (Fig. 6 *c*), nucleoprotein, or histone. Acid buffer treatment of glomeruli previously incubated with DNase eluted additional antibody reacting mainly with DNA and nucleoprotein, although in one instance a reaction with phosphate buffer nuclear extract was noted.

A comparison of serum and eluate γG-globulin concentrations revealed that the antinuclear antibody titer per unit of γ-globulin was significantly higher in

most eluates than that observed in corresponding serum specimens (Table III). Only trace amounts of γM- and γA-globulins were demonstrable in several eluates. The ratios of the minimal quantity of serum γG-globulin to eluate γG-globulin which gave positive antinuclear fluorescence reactions ranged from 4 to 400. In one instance (case 10), the serum contained free DNA and no antinuclear antibodies, although the glomerular eluates contained antibody reacting with phosphate buffer extract of nuclei, nucleoprotein, and DNA.

Control eluates from normal kidneys, three acid buffer eluates (cases 2, 3, and 11), and one DNase eluate obtained from liver tissues (case 11) treated in a



TEXT-FIG. 1. Case 9. Agar gel precipitin reaction between phosphate buffer nuclear extract (PBNE) and eluate from glomeruli treated with acid buffer. The serum shows no reaction; γG-globulin concentration of the eluate is 0.20 mg/ml and of the serum 3.2 mg/ml.

manner identical to renal cortical tissues showed no antinuclear activity. One of two eluates (cases 7 and 8) obtained from SLE glomeruli treated with pH 7.2 phosphate-buffered saline showed weak antinuclear activity in the undiluted specimens. In contrast, acid buffer eluates from these kidneys had antinuclear antibody reactions at 1:50 dilutions.

Complement-fixing antibodies to nuclear and cytoplasmic antigens were measured by the indirect immunofluorescence technique. Antinuclear antibodies uniformly fixed complement. One eluate (case 1) contained complement-fixing antibody reacting with thyroid epithelium, distal tubular epithelium of kidney, and gastric parietal cell cytoplasm, and another (case 4) contained antibody reacting with renal and gastric epithelium.

Incubation of eluates with sections of normal kidney revealed a faint, diffuse uptake of γ-globulin by normal glomeruli, but not by tubular basement membrane. A similar reaction was observed with eluates from normal kidneys to

TABLE III

Comparison of the Minimum γG-Globulin Concentrations of Serums and Eluates Giving Antinuclear Fluorescence Reactions

Case No.	Eluate	Eluate	Serum	Ratio of serum γG-globulin/eluate γG-globulin
		μgγG-globulin/ml	*μgγG-globulin/ml*	
1	A*	5	—	—
2	A	5	256	51
3	A	20	260	13
4	A	10	720	72
5‡	A	60	2400	40
6	A	200	660	3
7	A	90	3600	40
	B§	9	3600	400
8	A	32	200	6
	B	54	200	4
9	A	4	1100	275
	B	12	1100	92
10	A	9	‖	—
	B	5	‖	—
11	A	70	1400	20
	B	50	1400	28

* Acid buffer eluate.
‡ Subacute glomerulonephritis.
§ DNase eluate.
‖ Negative nuclear fluorescence reaction.

TABLE IV

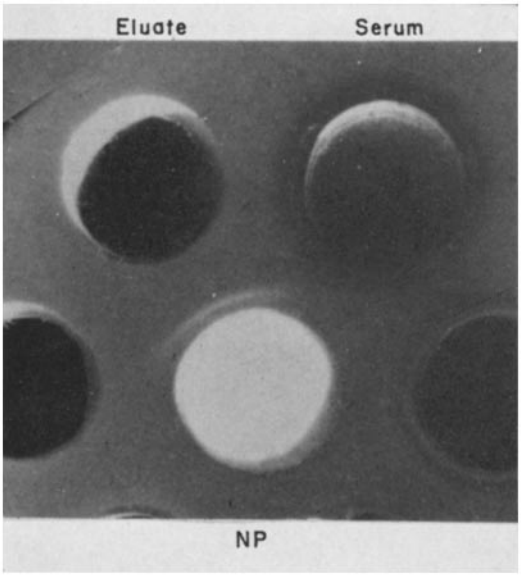
Precipitin Reactions Between Nuclear Antigens and Glomerular Eluates

Eluate	No. of cases	Phosphate buffer extract	Nucleoprotein
Acid buffer	10	7	0
DNase	5	0	3
DNase + acid buffer	4	0	1

which fraction II had been added prior to the stage of acid buffer elution. Complement-fixing antibody to basement membrane was not detectable by immunofluorescence.

(*b*) *Precipitating Antibodies* (*Table IV*).—7 of 10 acid buffer eluates contained precipitating antibodies reacting only with phosphate buffer extract of nuclei (Text-fig. 1). Although corresponding serums showed precipitin reactions in all

but one instance (case 4), the relative titers of precipitating antibodies in several eluates were more than tenfold that of the serum. Precipitin lines between DNase eluates and nucleoprotein, but not with phosphate buffer extract of nuclei, were also observed (Text-Fig. 2). Additional precipitating antibody to



TEXT-FIG. 2. Agar gel precipitin reaction between sonicated nucleoprotein (NP) and an eluate from glomeruli treated with DNase and acid buffer. The serum shows no reaction. γG-globulin concentration of the eluate is 0.09 mg/ml and of the serum 7.0 mg/ml.

TABLE V

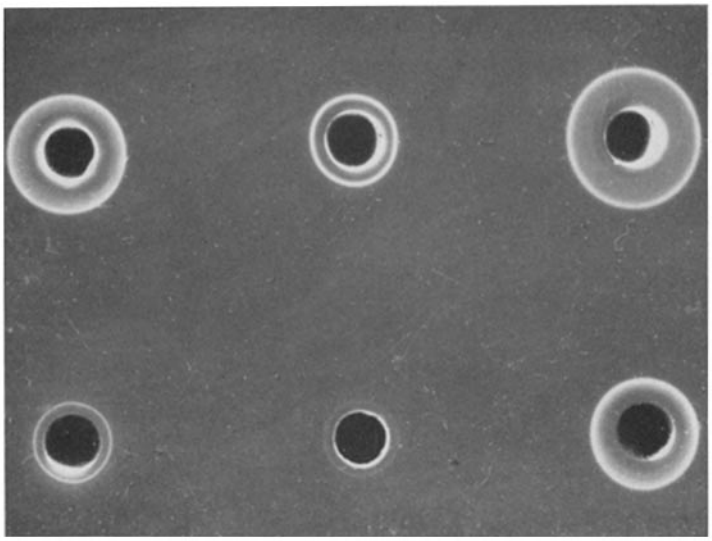
Comparison of Antibody Titers in Serums and Glomerular Eluates

Case No.	Antinuclear antibody	Anti-γ-globulin*	Anti-A antibody	Anti-B antibody
7—Serum	1:10	0	1:16	1:4
Eluate A	1:50	0	0	0
Eluate B	1:10	0	0	0
9—Serum	1:100	1:128	1:128	1:128
Eluate A	1:50	0	0	0
Eluate B	1:50	0	0	0
10—Serum	0	1:128	0	0
Eluate A	1:10	0	0	0
Eluate B	1:10	0	0	0

* Pepsin-digested human γG-globulin antigen.

nucleoprotein was obtained from glomeruli treated with DNase and then with acid buffer. Precipitin reactions with DNA and denatured DNA were not observed.

Nuclear antigens could not be studied in the eluates because DNA and other nuclear materials appeared from cell breakdown and were detectable in eluates from normal as well as from nephritic kidneys.



TEXT-FIG. 3. Radial immunodiffusion study of three eluates obtained by treatment of glomeruli with DNase and acid buffer. In the upper row, precipitin rings of eluates from cases 7, 10, and 9 representing γG-globulin concentrations of 88, 57, and 125 μg/ml. In the lower row, precipitin rings of decreased size from the same eluates after absorption with nucleoprotein strands, representing γG-globulin concentrations of 15, 10, and 75 μg/ml. The decrease in γ-globulin concentration indicates specific absorption of eluate antibody by nucleoprotein. In the two eluates with marked decrease in γ-globulin concentrations, a parallel inhibition of the antinuclear fluorescence reaction was noted.

Precipitating antibodies reacting with ribosomes were noted in two of three acid buffer eluates tested (case 1 and 3) and in two eluates from glomeruli treated with acid buffer and then incubated with RNase (case 7 and 9). Attempts to elute antiribosomal antibody by treatment of glomeruli with RNase alone were not successful.

(*c*) *Hemagglutinating Antibodies.*—In order to show that selective elution of glomerular-bound antibody resulted from acid buffer and enzyme treatments of glomeruli, several antibodies present in high titer in serums were assayed in eluates. Hemagglutinating antibody titers to γ-globulin and blood group substances A and B were compared to the antinuclear antibody titers determined by immunofluorescence for glomerular eluates and corresponding serums (Table

V). In contrast to the high relative titers of antinuclear antibodies, anti-γ-globulin, anti-A, and anti-B antibodies were not found in the glomerular eluates.

Eluates from each kidney were tested for rheumatoid factor activity because of the possibility that cryoglobulin containing rheumatoid factor (34, 35) was deposited in the glomerular lesions. Rheumatoid factor was not demonstrable in the acid buffer eluates from the 11 cases studied.

(*d*) *Quantitation of Antinuclear Antibody.*—γG-globulin eluted from two kidneys by DNase and acid buffer treatment of isolated glomeruli was predominantly antinuclear antibody. After absorption of the eluates with insoluble nucleoprotein strands, γ-globulin concentration of the eluates was markedly reduced (Text-fig. 3). A parallel inhibition of the antinuclear fluorescence reaction after absorption with nucleoprotein was noted. One eluate showed only a minimal reduction of γG-globulin concentration and no inhibition of the antinuclear fluorescence reaction. Normal γ-globulin controls showed no reduction of γ-globulin concentrations after absorption with nucleoprotein strands.

DISCUSSION

Antibodies reacting with multiple nuclear antigens were obtained from the isolated glomeruli of kidneys from patients with SLE nephritis. Acid buffer treatment eluted antibodies reacting with phosphate buffer–soluble nuclear antigens and ribosomes, whereas deoxyribonuclease treatment eluted antibodies with specificity mainly for DNA and nucleoprotein. The ability of the latter treatment to preferentially elute several types of antibody is probably related to the enzymatic breakdown of the antigens to which they were combined. It is known that deoxyribonuclease will release DNA antibody from immune precipitates and therefore glomerular deposits of antigen-antibody complexes of the DNA–anti-DNA type would be dissociated by incubation of glomeruli with this enzyme. In several eluates obtained by deoxyribonuclease treatment, the eluate γ-globulin was composed predominantly of antibody reacting with DNA and nucleoprotein, further demonstrating the selective action of deoxyribonuclease. These studies offer strong evidence for the concept that the eluted anti-DNA antibody was bound to DNA antigen in the renal glomerulus.

Quantitative immunochemical studies suggest that antibodies derived from both acid buffer and deoxyribonuclease-treated glomeruli are markedly concentrated in the kidney. The activity of anti-nuclear antibodies in the eluates per milligram of γ-globulin was significantly increased over that found in corresponding serum specimens. Two eluates contained antinuclear antibodies which were not demonstrable in serums. Similar results were obtained when antibody activity was assayed by either immunofluorescence or agar gel precipitin techniques.

Evidence was obtained that certain antibodies to cytoplasmic antigens are also concentrated in glomeruli. Ribosomal antibodies have been found in the serums of

patients with SLE, with highest incidence in those patients with glomerulonephritis (27). Precipitating antibodies to ribosomes and cytoplasmic antibodies demonstrable by immunofluorescence were found in several glomerular eluates. Although present in lower incidence than antinuclear antibodies, such anticytoplasmic antibodies may have significance in selected cases.

The observation that multiple antibodies to nuclear and cytoplasmic antigens are concentrated in glomeruli raised the possibility that all γ-globulin is concentrated in previously damaged glomeruli. Therefore, the glomerular eluates were assayed for antibodies known to be present in high titers in the serums of these patients in order to determine if these antibodies as well as antinuclear antibodies were concentrated in the eluates. Anti-γ-globulin antibodies, which are present in the γG-globulin fraction of serum and blood group antibodies, were observed in selected serums, but were not found in the glomerular eluates. The previous demonstration by the fluorescent antibody technique that γG- and γM-globulins are localized in glomeruli (13) without significant deposits of γA-globulin is an additional indication that γ-globulins are not randomly concentrated in diseased glomeruli.

A limited number of in vivo mechanisms offer an explanation for the selective concentration of antinuclear antibodies in the glomerular eluates. Certainly, antigen-antibody complexes deposited along the glomerular basement membrane appear to be the most likely source. However, the presence of γ-globulin complexes of some other type, possibly from cryoprecipitates, cannot be excluded (34). Rheumatoid factor activity, which has been associated with cryoglobulins in the serums of some of the patients (35), was not found in the eluates. Although the test system utilized in this study for assay of rheumatoid factors has broad specificity (4), it is possible that rheumatoid factors with unique determinants were not identified.

The strongest evidence obtained from the present studies for the renal deposition of antigen-antibody complexes was the demonstration of DNA antigen on the glomerular basement membrane of a kidney from which anti-DNA antibodies were eluted. Glomerular DNA antigen was also found in a renal biopsy specimen taken from a patient with circulating anti-DNA antibodies. Evidence for localization of DNA has also been obtained in two other laboratories.[1] The in vivo formation of DNA–anti-DNA complexes is also supported by the finding of circulating DNA antigen and anti-DNA antibodies in a few patients with active SLE (19).

Previous histochemical studies have provided only equivocal evidence for the presence of DNA antigen in protein deposits of diseased glomeruli. Weakly Feulgen-positive material has been noted in the fibrinoid deposits and thrombi of kidneys with SLE nephritis (36, 37). In the present studies, initial attempts to demonstrate DNA antigen in glomeruli by the fluorescent antibody technique in untreated or acid buffer–treated sections were unsuccessful. However, DNA was localized by means of isolated antibody to DNA labeled with fluorescein, particularly after treatment of tissues with 2 M sodium chloride. The concentrated salt solution was utilized in an attempt to elute antibodies which might block reactive sites on the antigen preventing its detection. It also removed the major portion of intranuclear DNA which interfered with the demonstration of small quantities of extranuclear DNA by the fluores-

[1] Personal communications from Dr. E. M. Tan and from Dr. E. V. Barnett.

cent antibody technique. Recently, 2.5 M potassium thiocyanate has been used to elute antibody from immune complexes in the glomeruli of rats immunized with renal tubular antigen (38). The technique and time of elution for the removal of blocking antibodies must be adapted to each antigen-antibody complex system studied. For the demonstration of DNA antigen, other procedures such as acid buffer elution were not effective. Prolonged treatment of cryostat sections with concentrated salt solution resulted in the removal of DNA antigen as well as antibody from the glomeruli.

The mechanism by which the spectrum of different antibodies are produced in patients with systemic lupus erythematosus remains an unsolved problem. A virus etiology may be suggested in the human disease similar to that proposed for the disease of NZB mice (39). Lymphoid cell hyperplasia and a hyperimmune response to endogenous and exogenous antigens may be directly related to a viral infection. Genetic factors may also be operative in patients with this disease. An intrinsic immunological hyperreactivity to normal cellular breakdown products may result in the formation of multiple antibodies to autologous constituents. The presence of a primary or induced defect in the stability of cells is less likely, although not excluded. Physical or chemical stimuli may initiate a process which releases cellular antigen from such cells, causing intensive stimulation of the lymphoid system.

The emphasis on the deposition of DNA–anti-DNA immune complexes in glomeruli does not imply that other potential antigen-antibody complex systems are of lesser importance. It is possible that the elution procedure extracts antigens from both normal and nephritic tissues which may partially or completely inhibit important antibodies in the eluates. This was found to be the case with DNA which was extracted from both normal and diseased glomeruli. However, here the use of DNase permitted detection of the antibody.

The failure to demonstrate nucleoprotein and Sm antigen in glomeruli may reflect technical difficulties of the elution procedure. A high incidence of antibodies to the Sm antigen has been noted in the serums (26) and the antigen has been found in the urine of some patients (26). The Sm antigen may be less firmly bound to tissues than the DNA antigen and therefore more easily eluted when sections are washed in acid buffer. Present studies are directed toward identifying these and other antigens in an attempt to determine their relative significance.

Despite the evidence for antigen-antibody complexes in the glomeruli of patients with SLE, the possibility still remains that other factors might play a more basic role in the glomerular injury. Antibodies to basement membrane or some other key area that might exert direct injury require consideration. A faint uptake of eluted γ-globulin by normal glomeruli as previously described (15) was observed with eluates from both SLE kidneys and normal kidneys. The failure to demonstrate reactions with tubular basement membrane and the absence of complement fixation reactions speak against specific antibody. It appeared likely that the elution procedure resulted in the formation of some altered γ-globulin which has been shown to have affinity for glomerular tissues (40).

SUMMARY AND CONCLUSIONS

Antibodies were eluted from the isolated glomeruli prepared from the kidneys of 10 patients with the nephritis of systemic lupus erythematosus. Antibodies re-

acting primarily with buffer extracts of nuclei were eluted by acid treatment, and antibodies reacting mainly with DNA and nucleoprotein were eluted with deoxyribonuclease. Quantitative immunochemical studies revealed a high concentration of antinuclear antibody per milligram of γ-globulin in glomerular eluates compared with that in the corresponding serums. The γ-globulin of two eluates was found to consist predominantly of antinucleoprotein antibody. The selective elution of antinuclear antibodies was also indicated by the absence of other serum antibodies in the eluates.

DNA antigen was demonstrated in the glomeruli of two kidneys with nephritis by means of isolated anti-DNA antibody labeled with fluorescein. In one of these cases, anti-DNA antibodies were also found concentrated in the glomeruli and, in the second, circulating anti-DNA antibodies were demonstrated in the patient's serum.

The immunochemical evidence for the high specific activity of antinuclear antibodies and the association of DNA antigen with DNA antibody in glomeruli add further support for the antigen-antibody complex hypothesis for renal injury in systemic lupus erythematosus.

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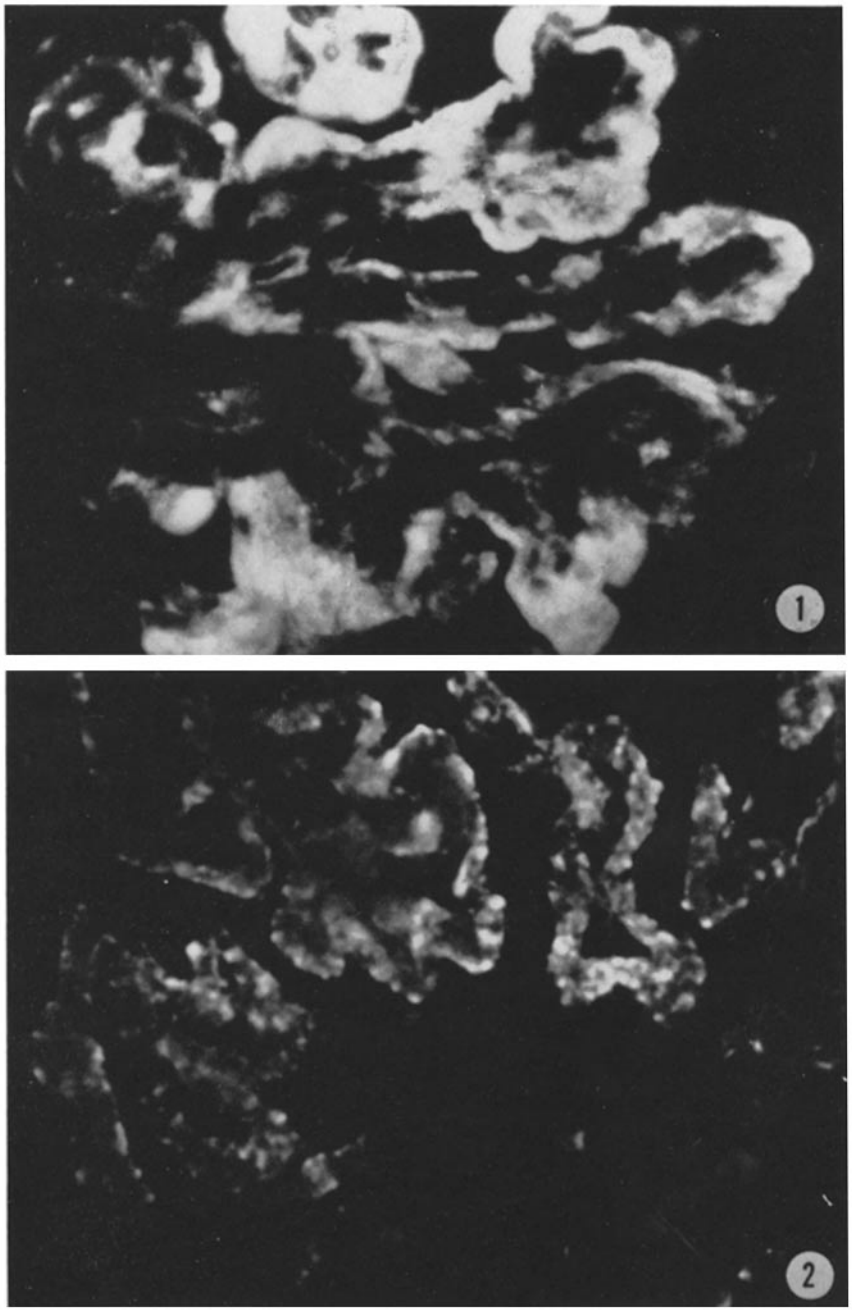
EXPLANATION OF PLATES

PLATE 47

FIG. 1. Case 7. Lumpy deposits of γG-globulin outlining tufts of a glomerulus from a kidney with severe SLE nephritis. The section was incubated with a fluorescein-labeled anti-γG-globulin serum. × 400.

FIG. 2. Biopsy (D.M.). Granular deposits of the C'3 component of complement along the basement membrane of a kidney showing early changes of SLE. The section was incubated with a fluorescein-labeled anti-β_{1C}-globulin serum. × 540.



(Koffler et al.: Nephritis of systemic lupus erythematosus)

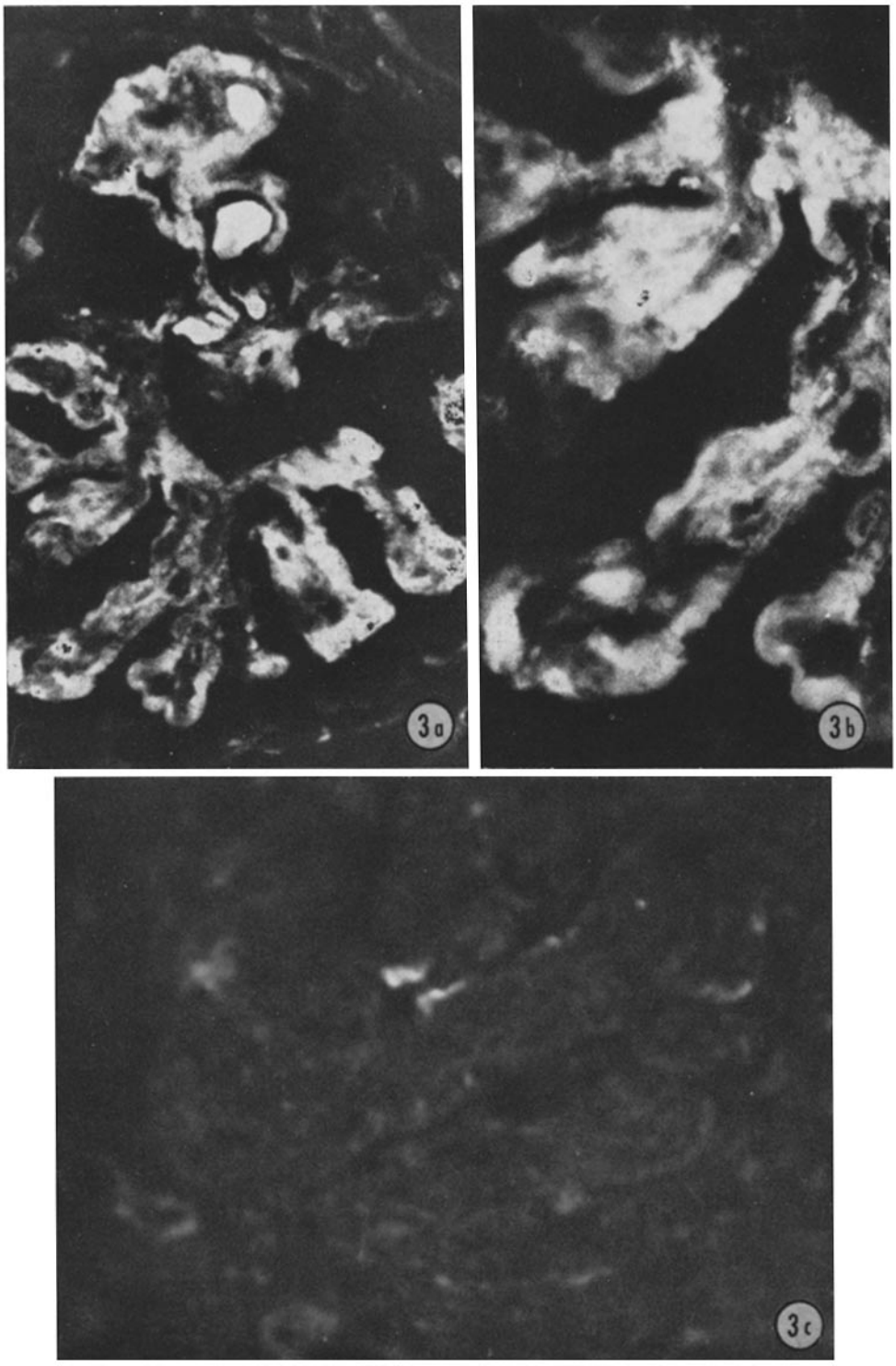
PLATE 48

FIG. 3. Case 7. Localization of DNA antigen in a renal glomerulus of a kidney with severe SLE nephritis.

FIG. *a*. Deposits of DNA along the glomerular basement membrane demonstrated after treatment of a cryostat section with 2 M NaCl for 120 min followed by incubation with fluorescein-labeled anti-DNA antibody. × 250.

FIG. *b*. Granular and lumpy deposits of DNA visible at higher magnification. × 540.

FIG. *c*. Serial section of the same kidney incubated with fluorescein-labeled anti-DNA antibody after inhibition with DNA. × 250.

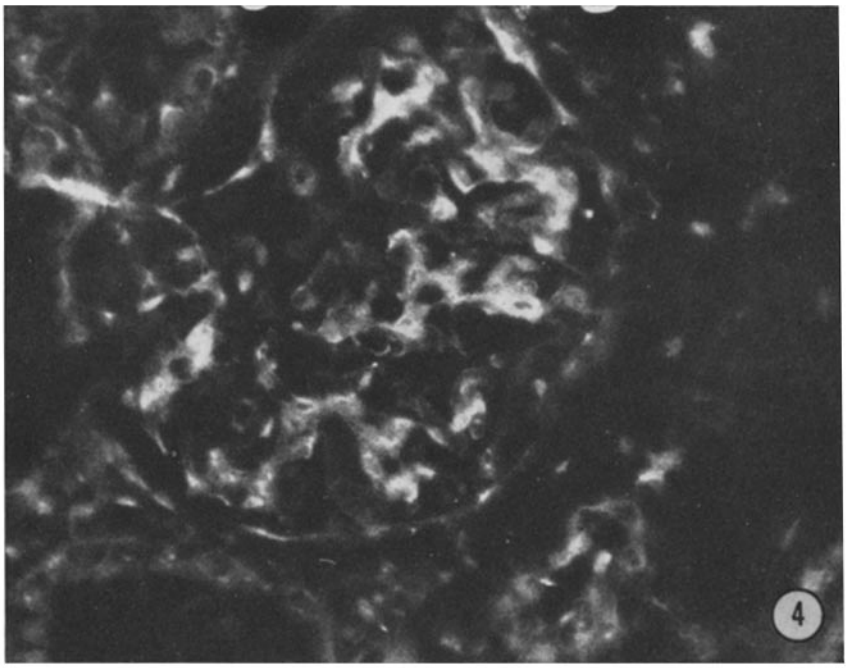


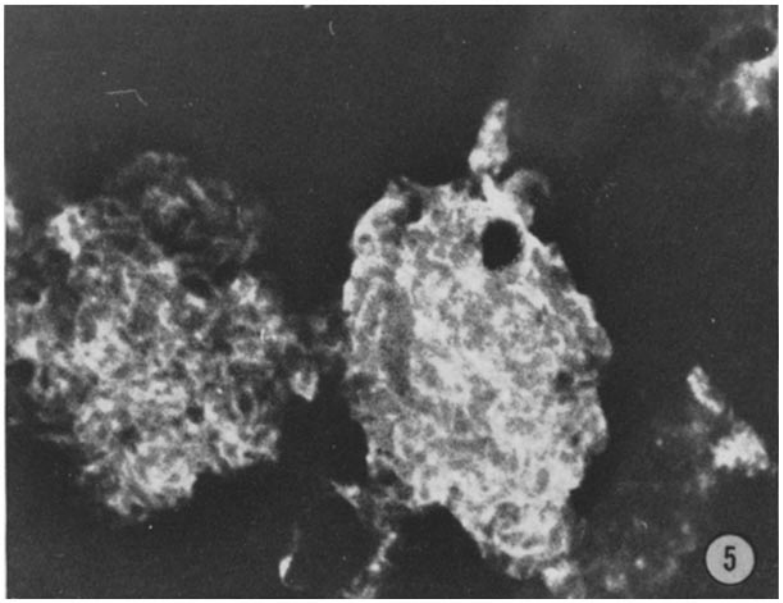
(Koffler et al.: Nephritis of systemic lupus erythematosus)

PLATE 49

FIG. 4. Biopsy (D.B.). Localization of DNA antigen in a renal glomerulus of a kidney with early SLE nephritis. Focal deposits of DNA in a glomerulus of a section treated with fluorescein-labeled anti-DNA antibody after incubation with 2 M NaCl for 5 min. Note staining of residual DNA in nuclei as well. × 250.

FIG. 5. Case 9. Isolated glomeruli prior to elution showing bright staining with fluorescein-labeled anti-γG-globulin serum. × 250.





(Koffler et al.: Nephritis of systemic lupus erythematosus)

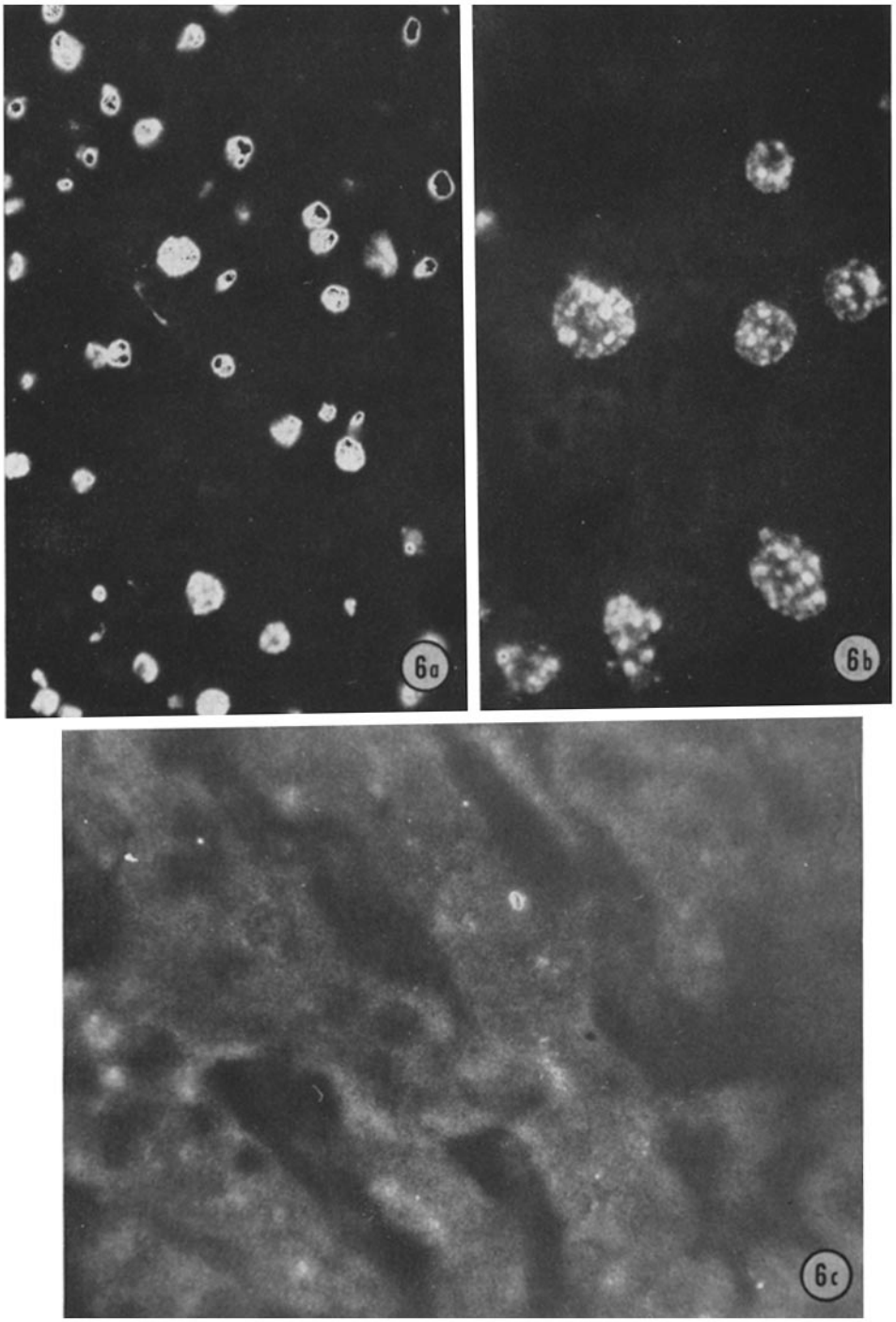
PLATE 50

FIG. 6. Section of human liver incubated with an eluate followed by a fluorescein-labeled anti-γ-globulin serum.

FIG. *a*. Case 3. Eluate from glomeruli treated with acid buffer. Note intense homogeneous nuclear staining pattern. × 250.

FIG. *b*. Case 10. Eluate from glomeruli treated with DNase and acid buffer. Note coarse, punctate nuclear staining pattern. × 400.

FIG. *c*. Case 10. Same eluate as in Fig. 6 *b* after absorption with DNA. Note absence of nuclear staining. × 400. Exposure time, 3 times that used for demonstrating nuclear fluorescence.



(Koffler et al.: Nephritis of systemic lupus erythematosus)