IN VITRO DEMONSTRATION OF A PARTICULAR AFFINITY OF GLOMERULAR BASEMENT MEMBRANE AND COLLAGEN FOR DNA*

A Possible Basis for a Local Formation of DNA-Anti-DNA Complexes in Systemic Lupus Erythematosus

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There is good evidence that the major pathological manifestations of systemic lupus erythematosus (SLE)[1] are related to the occurrence of immune complexes in blood or in tissues. Circulating immune complexes have been detected by various methods in serum from patients with SLE (1–4) and immune complexes localized in tissues have been demonstrated by immunofluorescence and electron microscopic studies (5–9).

The development of the acute clinical manifestations of SLE is generally associated with the presence in serum of a high titer of anti-DNA antibodies (10–12), and immune complexes formed of DNA, and anti-DNA antibodies have been shown to be the major component of the immune complexes localized in renal and skin lesions (11, 13, 14). However, such DNA-anti-DNA complexes seem to represent only a very small portion of the complexes detected in circulating blood (3). Therefore, one may wonder whether the tissue localization of DNA-anti-DNA complexes results directly from the localization of circulating complexes, as in serum sickness, or whether other mechanisms are involved. The latter hypothesis is also suggested by the fact that in many of the patients with SLE, the initial manifestations may be represented by only one type of lesion, such as a limited skin disease, with delayed appearance of other systemic manifestations.

The present investigation has been undertaken to define some of the factors which may favor the deposition or the local formation of DNA-anti-DNA complexes in tissue structures where such complexes are commonly localized during the course of SLE. In particular, the frequent concentration of DNA-anti-DNA

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DSDNA, double-stranded DNA; GBM, glomerular basement membrane; HGG, human immunoglobulin G; LPS, lipopolysaccharide; MAK, methylated albumin kieselguhr; mBSA, methylated bovine serum albumin; NMS, normal mouse serum; PBS, phosphate-buffered saline; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; SLE, systemic lupus erythematosus; SSDNA, single-stranded DNA.

complexes at the surface or inside basement membranes in kidney and in skin lead us to consider the possibility that basement membranes have a particular affinity for such DNA-anti-DNA complexes.

It was found that in vitro DNA alone can be efficiently bound to isolated glomerular basement membranes (GBM) and, in more general terms, to structures containing collagen. This GBM- or collagen-bound DNA can subsequently react with anti-DNA antibodies and form immune complexes which remain localized on these structures. Inversely, preformed DNA-anti-DNA complexes are not efficiently bound by GBM or collagen. These in vitro observations raise again the possibility that the systemic character of SLE might be partly related to the widespread distribution of collagen or collagen-like structures as previously proposed by Klemperer (15).

Materials and Methods

DNA. Highly polymerized calf thymus DNA (type V, Sigma Chemical Co., St. Louis, Mo.) was used without further purification. Mouse kidney DNA, bacterial DNA from *Micrococcus lysodeikticus,* viral DNA from SV 40, and plant DNA from tobacco purified by Marmur's technique (16) were kindly provided by Dr. Türler (Department of Molecular Biology, University of Geneva). Denatured DNA was prepared by heating native DNA (0.5 mg/ml in phosphate-buffered saline [PBS], pH 7.0) at 100°C for 10 min, followed by immediate cooling in an ice bath. DNA was fractionated on methylated albumin kieselguhr (MAK) columns according to Sueoka and Cheng (17) to separate double-stranded DNA (DSDNA) from single-stranded DNA (SSDNA). DSDNA was eluted with NaCl gradient of 0.5–0.9 M buffered with 0.05 M PO_4, pH 6.7, while SSDNA was eluted with 1.0 M NaCl–1.5 M NH_4OH. DNA fractions eluted from the column were immediately dialyzed against borate buffer (ionic strength 0.1, pH 8.4).

Proteins, Lipopolysaccharides and Antisera. Bovine serum albumin (BSA) and methylated BSA (mBSA) were obtained from Calbiochem, San Diego, Calif. Bacterial lipopolysaccharides (LPS) from *Salmonella typhimurium* (lot 563628), *Escherichia coli* 0127:B8 (lot 582337), and *E. coli* 0111:B4 (lot 587687) were obtained from Difco Laboratories, Detroit, Mich. They were further purified by the phenol-water extraction method, followed by fractionation with ethanol and ultracentrifugation (18). Human IgG (HGG) was prepared by chromatography through a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer at pH 8.0. Human C1q component of complement was prepared from fresh human serum according to Yonemasu and Stroud (19).

Normal mouse serum (NMS) was collected from 6 to 10-wk-old C57BL/6 mice, purchased from Charles River Breeding Laboratories, Inc., Elbeuf, France. Anti-DNA antisera were obtained from mice immunized with DNA-mBSA complexes as described previously (20), or NZB × NZW F_1 hybrid mice. NZB and NZW mice were originally provided by Dr. F. Dixon from the Scripps Clinic and Research Foundation, La Jolla, Calif., and inbred in our own animal house. Sera were stored at −20°C until used except when fresh serum was needed.

GBM and Collagen. GBM was isolated from normal human kidneys obtained from autopsies. The isolation of glomeruli was based on the method of Krakower and Greenspon (21) using stainless steel sieves and repeated sedimentation in 0.15 M NaCl until the materials on the sieves contained practically pure glomeruli as checked by light microscopy. GBM was obtained from these isolated glomeruli by ultrasonic disruption (MSE sonicator, Measuring & Scientific Equipment Ltd., Sussex, England), conducted until complete fragmentation of the glomeruli. The sonicated materials were washed five times with 0.15 M NaCl at 3,000 rpm. The sediments were used as GBM.

Type I collagen purified from calf skin, and type III collagen from fetal calf skin were kindly provided by Dr. C. M. Lapiére (Clinique dermatologique, Université de Liège, Belgium). These collagen preparations were solubilized with 0.1 M acetic acid at 4°C for 24 h. The soluble collagen obtained was dialyzed against potassium phosphate buffer, pH 7.6, at 4°C for 24 h, then dialyzed against 0.15 M NaCl at 4°C for 24 h. Insoluble collagen was prepared by incubating the soluble collagen at 37°C for about 20 min until the collagen fibers were formed. Type I collagen from

bovine achilles tendon was purchased from Sigma Chemical Co. The protein concentration was determined by the method of Lowry et al. (22). Insoluble GBM and collagen were solubilized with 0.1 N NaOH for the determination of protein concentration.

Radiolabeling Procedures. DNA was labeled either externally with iodine (^{125}I), or internally with tritium (^{3}H). Iodination of DNA was carried out according to the method of Commerford (23). [^{3}H]DNA was prepared from human fibroblasts L_{132} grown in culture in presence of [^{3}H]thymidine. DNA was isolated by Marmur's technique (16). These DNA preparations were shown to contain less than 1% of Pronase-sensitive material. These radiolabeled DNA were fractionated on MAK columns to obtain pure double-stranded and single-stranded forms. To remove selectively single-stranded regions within DSDNA, [^{125}I]DSDNA was treated with single-stranded specific S1 nuclease (Seikagaku Kogyo, Tokyo, Japan) in some experiments. The reaction mixtures consisted of 1 μg of [^{125}I]DSDNA and 2,000 U of S1 nuclease in 1.5 ml of 0.1 M KCl, 0.1 mM $ZnSO_4$, 0.025 M sodium acetate, pH 4.5. Incubation was at 37°C for 2 h.

HGG and BSA were labeled with iodine (^{125}I or ^{131}I) according to the procedure of McConahey and Dixon (24). The labeling of LPS with ^{51}Cr was carried out according to the method of Braude et al. (25).

Binding Assay. The binding of radiolabeled preparations to insoluble GBM and insoluble collagen was assayed using the following procedures: various amounts of insoluble GBM or collagen suspended in 0.1 ml of 0.15 M NaCl were incubated with 0.1 ml of radioactive preparations in the presence of 0.1 ml of 10% heat-inactivated NMS at 4°C overnight with constant shaking. As a control, radiolabeled preparations were incubated with NMS alone. All the reaction mixtures were centrifuged at 1,000 *g* for 20 min, then washed twice with PBS. Iodine-labeled preparations precipitated were directly counted in an automatic Beckman gamma counter (Beckman Instruments, Inc., Palo Alto, Calif.). The specific amount of radioactive preparations bound to GBM or collagen was obtained by subtracting the counts per minute in the control tubes from the counts per minute in the experimental tubes. The results were expressed as a percentage of radiolabeled preparations specifically bound to GBM or collagen. For [^{3}H]DNA, the supernatant from the reaction mixtures was transferred into 15 ml of Dioxan scintillation fluid for counting in an LS 250 Beckman liquid scintillation counter.

Inhibition of the binding of ^{125}I-labeled calf thymus DNA to the insoluble GBM or collagen by unlabeled DNA was studied by incubating various concentrations of unlabeled DNA with a constant amount of [^{125}I]DNA and GBM or collagen. Inhibition studies with anti-DNA antisera, fresh NMS, or C1q were conducted according to the same procedure.

The effect of pH on the binding of DNA to GBM was investigated as follows: 50 ng of [^{125}I]SSDNA in 0.01 ml of distilled water was incubated with 300 μg of GBM in 0.05 ml of distilled water containing 2% BSA in the presence of 0.2 ml of Britton-Robinson buffer at pH 3, 5, 7, 9, and 11. As control, [^{125}I]SSDNA was incubated alone with distilled water containing 2% BSA at the different pH values. The specific precipitation of [^{125}I]SSDNA was calculated for the different pH values described above. The effect of the salt concentration was studied as follows: 0.2 ml of various concentrations (0.01–2.0 M) of NaCl was added to [^{125}I]SSDNA and GBM mixtures as described in the above procedure instead of using Britton-Robinson buffer.

The binding of [^{125}I]DNA and [^{125}I]BSA to the soluble collagen was assayed by precipitation of [^{125}I]DNA protein or [^{125}I]BSA protein complexes with 10% polyethylene glycol (PEG), in which free radiolabeled DNA and BSA were soluble (26), while the soluble collagen was precipitated. 10 ng of radiolabeled preparations were incubated with 100 μg of soluble collagen (type I) in the presence of 10% heat-inactivated NMS at 37°C for 2 h. The mixtures were treated with PEG (final concentration: 10%) and incubated at 4°C overnight. The precipitates were centrifuged at 1,000 *g* for 30 min, then washed with 10% PEG. The results were expressed as a percentage of radiolabeled preparations specifically precipitated. The specific precipitation of radiolabeled preparations was calculated after correction for the nonspecific precipitation obtained in the presence of 10% heat-inactivated NMS. The formula described by Farr (27) was used for this correction.

Collagenase Digestion of GBM. Collagenase from *Colstridium histolyticum* (type III, fraction A, 460 U/mg) was obtained from Sigma Chemical Co. A 1-mg/ml solution was prepared in 0.1 M Tris-acetate buffer, pH 7.4. For digestion of GBM with collagenase, 150 μg of GBM in 0.1 ml of Tris-acetate buffer was incubated with 100 μg of collagenase for 12 h at 37°C. The enzyme reaction was stopped by cooling in an ice bath. As undigested control, GBM was incubated with buffer alone.

Elution of Anti-DNA Antibodies. Elution of anti-DNA antibodies from GBM-DNA-anti-DNA mixtures was carried out as follows: 300 μg of GBM was incubated with 100 μg of DNA or PBS overnight at 4°C, then washed three times with PBS. 0.1 ml of heat-inactivated anti-DNA antisera or NMS was added and incubated overnight at 4°C, then washed three times with Veronal buffer pH 7.2. These mixtures were treated with 0.1 ml of 1 mg/ml DNase (deoxyribonuclease I, 2,600 U/mg, Worthington Biochemical Corp., Freehold, N. J.) in Veronal buffer at 37°C for 12 h to elute anti-DNA antibodies (11). 0.05 ml of 0.03 M EDTA was added to inhibit the DNase activity. The eluates obtained were examined for DNA-binding activity by a modification of the Farr test (28). 0.025 ml of eluates was incubated in 0.1 ml of 10 ng of [^{125}I]SSDNA in the presence of 0.1 ml of 10% heat-inactivated NMS. The radioactivity of [^{125}I]SSDNA precipitated with 50% saturated ammonium sulfate was measured in a gamma counter, and the results were expressed as a percentage of [^{125}I]SSDNA precipitated.

Intravenous Injection of Radiolabeled Preparations to Mice. 5.0 μg of ^{125}I-labeled SSDNA and ^{131}I-labeled BSA in 0.2 ml saline was injected to normal C57BL/6 female mice and to similar mice previously injected with 100 μg of *S. typhimurium* LPS. The amounts of injected radiolabeled preparations in kidneys and liver were calculated from radioactivity measurement. The results were expressed as the absolute amounts of injected SSDNA or BSA per gram of organ.

Results

Binding of DNA to GBM. Various sources of DNA, HGG, BSA, and bacterial LPS were tested for the binding to GBM. ^{125}I-labeled SSDNA from calf thymus, mouse kidneys, bacteria (*M. lysodeikticus*), virus (SV40) and plant (tobacco), ^{125}I-labeled DSDNA from calf thymus and ^{3}H-labeled SSDNA and DSDNA from human fibroblasts were used. 50 ng of each of these radiolabeled preparations was incubated with 300 μg of sonicated fragments of human GBM suspended in 10% heat-inactivated NMS. As a control, these radiolabeled preparations were incubated with NMS alone. All the preparations of DNA used were significantly bound by GBM, 40–80 times more than HGG and BSA, and 10–40 times more than LPS (Table I). After treatment of [^{125}I]DSDNA with S1 nuclease to selectively remove single-stranded regions within DSDNA, there was no significant difference in the binding to GBM as compared with nontreated [^{125}I]DSDNA (S1 treated: 36.6±2.6%, nontreated: 40.6±2.7%).

The possibility that DNA or basic nuclear proteins, such as histones, contaminating the GBM preparations could be involved in the binding of DNA to GBM was investigated. GBM pretreated with DNase at 37°C for 12 h or with 2.0 M NaCl at 4°C for 18 h (29) showed a higher DNA-binding activity (DNase-digested GBM: 32.4±4.4%; 2.0 M NaCl-treated GBM: 36.3±4.2%) than nontreated GBM (26.8±1.8%).

Increasing amounts (0.01–100 μg) of unlabeled calf thymus DNA were added to 100 μg of GBM with 5 ng of ^{125}I-labeled calf thymus DNA. There was a linear inhibition of the binding of [^{125}I]SSDNA in the presence of unlabeled SSDNA (Fig. 1*a*). 7 μg of unlabeled SSDNA was needed to obtain a 50% inhibition of the binding of 5 ng of [^{125}I]SSDNA. Only a very high concentration of DSDNA could inhibit the binding of [^{125}I]SSDNA. On the other hand, only 0.35 μg of SSDNA or DSDNA was sufficient to obtain a 50% inhibition of the binding of [^{125}I]DSDNA (Fig. 1*b*).

The maximum amounts of SSDNA and DSDNA, which can be bound to GBM, were determined by incubating a constant amount of GBM with increasing amounts of DNA. At saturation, approximately 4.0 μg of SSDNA and 0.5 μg of DSDNA were bound per 100 μg of GBM (Fig. 2).

TABLE I
Binding of DNA to GBM

Radiolabeled preparations*	Origin	Binding‡
		%
[^{3}H]SSDNA	Human fibroblasts	55.9 ± 1.3§
[^{3}H]DSDNA		39.2 ± 6.0
[^{125}I]SSDNA	Calf thymus	40.4 ± 1.0
[^{125}I]DSDNA		50.7 ± 2.1
[^{125}I]SSDNA	Mouse kidneys	31.2 ± 3.4
[^{125}I]SSDNA	Bacteria (*M. lysodeikticus*)	36.3 ± 4.9
[^{125}I]SSDNA	Virus (SV40)	26.7 ± 5.5
[^{125}I]SSDNA	Plant (tobacco)	44.8 ± 0.9
[^{125}I]BSA		0.7 ± 0.4
[^{125}I]HGG		0.6 ± 0.3
[^{51}Cr]LPS	*S. typhimurium*	1.4 ± 0.7
	E. coli 0127:B8	1.9 ± 0.8
	E. coli 0111:B4	3.0 ± 0.6

* 50 ng of radiolabeled preparations was incubated with 300 μg of insoluble human GBM in the presence of 0.1 ml of 10% heat-inactivated NMS.
‡ Specific precipitation of radiolabeled preparations with GBM.
§ Mean of triplicates ±1 SD.

The dissociation rate of [^{125}I]DNA-GBM complexes was measured in the presence of an excess of unlabeled DNA. 50 ng of [^{125}I]DNA was incubated with 300 μg of GBM overnight at 4°C, then 50 μg of unlabeled DNA was added. Tubes were taken at timed intervals thereafter. The percentage of [^{125}I]DNA bound to GBM was determined and compared to that of a control tube to which unlabeled DNA had not been added. In the presence of unlabeled SSDNA, only 30% of [^{125}I]SSDNA was released from the [^{125}I]SSDNA-GBM complexes after 12 h. In the presence of unlabeled DSDNA, there was no apparent dissociation of [^{125}I]SSDNA from GBM (Fig. 3). On the other hand, [^{125}I]DSDNA-GBM complexes were rapidly dissociated after the addition of unlabeled SSDNA as well as DSDNA.

To find the optimal conditions under which DNA would be bound by GBM, the binding activity was examined at various temperatures, pH values, and salt concentrations. The highest binding of [^{125}I]SSDNA to GBM was observed at 4°C (36.5±6.5%). Less, but still significant, binding was seen at 20°C and 37°C, respectively (25.2±1.9 and 10.3±1.9%). A maximal binding of [^{125}I]SSDNA to GBM was observed between pH 7.0 and 9.0 and between 0.05 and 0.3 M NaCl. In the presence of an anionic detergent, 0.03% sodium dodecyl sulfate (SDS), a strong inhibition of the binding of [^{125}I]SSDNA to GBM was also observed (with SDS: 2.0±0.5%; without SDS: 18.3±2.7%).

The relationship between the reaction of DNA with GBM and that of DNA with C1q (30) was investigated. Various amounts of purified human C1q were added to heat-inactivated serum and incubated with [^{125}I]SSDNA and GBM. The binding of [^{125}I]SSDNA to GBM was strongly inhibited by the addition of

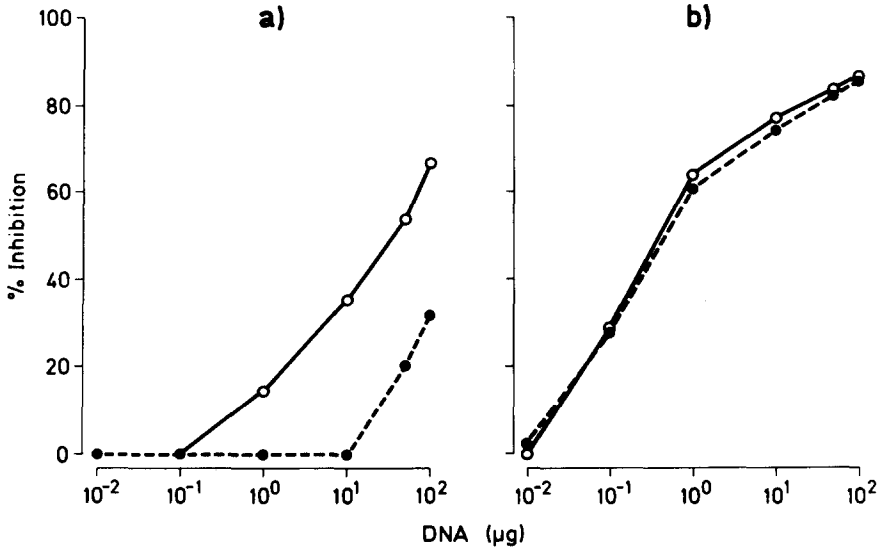

FIG. 1. Inhibition of the binding of [^{125}I]DNA to GBM by unlabeled DNA. Increasing amounts of SSDNA (○) and DSDNA (●) were added to a solution of 100 μg of GBM which can fix specifically about 30% of [^{125}I]DNA (5 ng). (*a*) Percent inhibition of the binding of [^{125}I]SSDNA to GBM with unlabeled DNA. (*b*) Percent inhibition of the binding of [^{125}I]DSDNA.

C1q and the inhibitory activity was dependent on the amount of C1q added to the reaction mixtures. This reaction may be responsible for the interference of fresh serum with the binding of DNA to GBM. Indeed, when a mixture of [^{125}I]SSDNA and GBM was incubated in the presence of fresh NMS, the binding of [^{125}I]SSDNA was about two times lower than in the presence of heat-inactivated serum.

To determine the participation of the collagen-like component of GBM (31) in the binding of DNA, GBM was treated with collagenase. In the conditions used, about 70% of GBM proteins, but less than 10% of [^{125}I]BSA, were digested. As compared with the undigested control, the binding of both types of DNA to GBM was almost completely abolished by the collagenase digestion of GBM (Table II). Most of [^{125}I]DNA could also be released from preformed [^{125}I]DNA-GBM complexes as a result of a similar collagenase digestion.

Binding of DNA to Collagen. The possibility that DNA could react with collagen molecules was investigated, using type I and type III collagen purified from bovine skin. 100 μg of soluble and insoluble collagen was incubated with 10 ng of [^{125}]SSDNA, [^{125}I]DSDNA, or [^{125}I]BSA in the presence of heat-inactivated NMS. Significant amounts of [^{125}I]SSDNA and [^{125}I]DSDNA were bound to soluble collagen as well as insoluble collagen, while [^{125}I]BSA was not significantly precipitated (Table III). It was also found that DNA from other sources can bind collagen.

The DNA-binding capacity of insoluble GBM and collagen (type I) were compared (Fig. 4). At saturation, approximately 8 μg of SSDNA was bound to 100 μg of collagen, while 4 μg of SSDNA was bound to 100 μg of GBM.

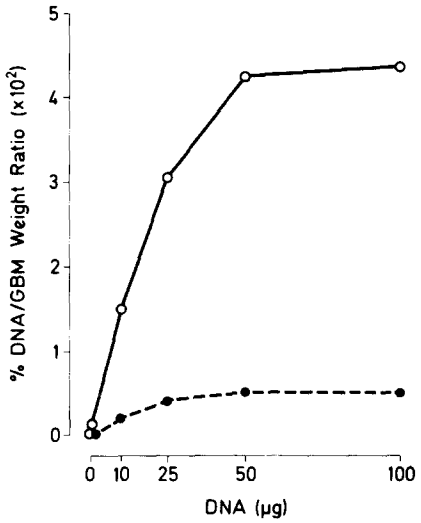

FIG. 2. Binding curves of SSDNA (○) and DSDNA (●) to GBM. Increasing amounts of SSDNA and DSDNA were incubated with 100 μg of GBM. The DNA-binding capacity of GBM is expressed on the vertical axis as weight ratio of DNA bound to GBM.

Binding of Preformed DNA-Anti-DNA Complexes to GBM. Experiments were performed to determine whether or not anti-DNA antibodies could influence the binding of DNA to GBM. First, the effect of anti-DNA antibodies on the binding of [^{125}I]DNA to GBM was examined. For this purpose, two different antisera were used: anti-SSDNA antibodies were obtained from mice immunized with DNA-mBSA complexes, and antibodies which were directed against both SSDNA and DSDNA were obtained from NZB × NZW F_1 hybrid mice. 0.1 ml of 10% heat-inactivated antisera was incubated with 50 ng of [^{125}I]DNA at 4°C overnight, then 300 μg of GBM was added. As control, [^{125}I]DNA was incubated with NMS. Antisera specific for SSDNA could specifically inhibit the binding of [^{125}I]SSDNA but not that of [^{125}I]DSDNA (Table IV). On the other hand, antisera which reacted with both DNAs could inhibit the binding of both types of [^{125}I]DNA to GBM. These results indicated that anti-DNA antibodies could interfere with the binding of DNA to GBM, presumably through the formation of immune complexes.

Secondly, to see the effect of anti-DNA antibodies on the preformed [^{125}I]DNA-GBM complexes, the following experiments were carried out. (*a*) [^{125}I]SSDNA was incubated with anti-DNA antibodies overnight at 4°C, then GBM was added, and the mixture was further incubated overnight at 4°C. (*b*) [^{125}I]SSDNA, antibodies, and GBM were incubated simultaneously. (*c*) [^{125}I]SSDNA was first incubated with GBM, then antibodies were added. (*d*) As a control, [^{125}I]SSDNA and GBM were incubated with NMS. Table V shows the effect of anti-DNA antibodies on the binding of [^{125}I]SSDNA to GBM. A strong inhibition of the binding could be observed in experiment *a*, in which most of the [^{125}I]SSDNA was complexed with antibodies before the reaction with GBM occurred. In experiment *b*, significant inhibition of the binding of [^{125}I]SSDNA was also seen. However, in experiment *c*, in which [^{125}I]SSDNA was already

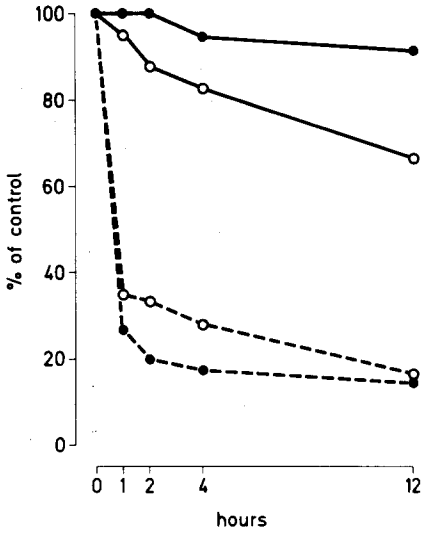

FIG. 3. Dissociation curves of [^{125}I]DNA-GBM complexes. [^{125}I]DNA (50 ng) was mixed with 300 μg of GBM overnight at 4°C, then 50 μg of unlabeled DNA was added, and after 1, 2, 4, or 12 h, the percentage of [^{125}I]DNA bound to GBM was determined. As control, the same procedures were used, but borate buffer was added to the mixtures instead of unlabeled DNA. Solid line represents the percentage of control values of [^{125}I]SSDNA bound to GBM after the addition of unlabeled SSDNA (○) and DSDNA (●). Interrupted line represents that of [^{125}I]DSDNA bound to GBM after the addition of unlabeled SSDNA (○- - -○) and DSDNA (●- - -●).

TABLE II
Effect of Collagenase on Binding of DNA to GBM

Treatment	[^{125}I]SSDNA ppt.*	[^{125}I]DSDNA ppt.*
	%	%
GBM‡	22.4 ± 4.6‖	21.8 ± 6.2
Collagenase-treated GBM§	2.3 ± 0.3	2.2 ± 0.3

* 50 ng of ^{125}I-labeled calf thymus DNA.
‡ 150 μg of insoluble human GBM in 0.1 M Tris-acetate buffer.
§ Incubated with 100 μg of collagenase in 0.1 M Tris-acetate buffer for 12 h at 37°C.
‖ Mean of triplicates ±1 SD.

attached to GBM before the addition of antibodies, the antibodies did not have any dissociating effect on the [^{125}I]SSDNA-GBM complexes.

Binding of Anti-DNA Antibodies to GBM-Bound DNA. The previous findings suggested that DNA-anti-DNA complexes could not react efficiently with GBM, and raised the possibility that anti-DNA antibodies could react with DNA bound to GBM, resulting in a local formation of DNA-anti-DNA immune complexes on GBM.

To investigate this possibility, GBM was first incubated with 100 μg of SSDNA or DSDNA overnight at 4°C. After incubation and washing, anti-SSDNA antibodies or NMS were added and incubated overnight at 4°C. After

TABLE III
Binding of DNA to Soluble and Insoluble Collagen

Collagen	[^{125}I]SSDNA ppt.	[^{125}I]DSDNA ppt.	[^{125}I]BSA ppt.
	%	%	%
Calf skin (type I)			
Soluble*	68.2 ± 0.1§	29.0 ± 0.9	3.2 ± 0.7
Insoluble‡	68.4 ± 1.6	23.4 ± 6.9	1.8 ± 0.6
Fetal calf skin (type III)			
Insoluble	82.3 ± 3.0	66.7 ± 2.8	0.1 ± 1.6

* 10 ng of radiolabeled preparations was incubated with 100 μg of soluble collagen in the presence of 10% heat-inactivated NMS at 37°C for 2 h.

‡ 100 μg of insoluble collagen suspended in 0.1 ml of 0.15 M NaCl were incubated with 10 ng of radiolabeled preparations in the presence of 10% heat-inactivated NMS at 4°C overnight.

§ Mean of triplicates ±1 SD.

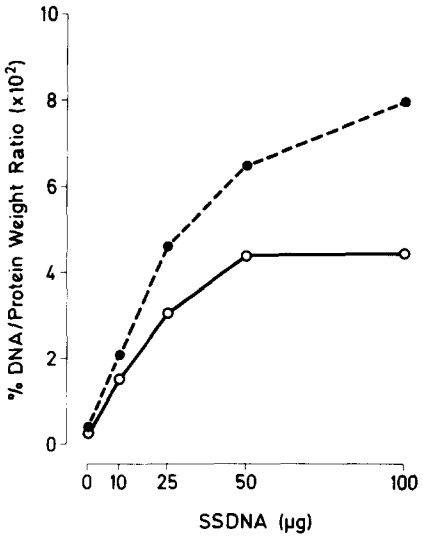

FIG. 4. Binding curves of SSDNA to GBM (○) and collagen (●). Increasing amounts of SSDNA were incubated with 100 μg of GBM or collagen. The DNA-binding capacity of these proteins is expressed on the vertical axis as weight ratio of DNA bound to GBM or collagen.

washing, the mixtures were treated with DNase at 37°C for 12 h to dissociate DNA-anti-DNA complexes. The eluates obtained were tested for anti-SSDNA activity by a modification of the Farr test. Eluates from GBM-SSDNA-anti-SSDNA mixtures showed a significant SSDNA-binding activity (Table VI). Eluates from mixtures of GBM with anti-SSDNA serum without added SSDNA did not exhibit a significant DNA-binding activity. A similar experiment was performed using collagen instead of GBM and similar results were obtained.

In Vivo Localization of Injected [^{125}I]SSDNA in Kidneys. The previous experiments suggested that under certain circumstances, some DNA may bind to GBM. When [^{125}I]SSDNA was injected intravenously to normal C57BL/6

TABLE IV
Binding of Preformed DNA-anti-DNA Complexes to GBM

Serum	Serum DNA-binding activity*		Binding of DNA to GBM‡	
	[^{125}I]SSDNA	[^{125}I]DSDNA	[^{125}I]SSDNA	[^{125}I]DSDNA
	%		%	
NMS	12.4	1.2	42.0 ± 3.5‖	55.4 ± 6.0
Anti-SSDNA§	65.6	2.4	4.5 ± 1.0	53.2 ± 4.4
NZB/W sera	46.5	46.2	22.1 ± 2.9	29.0 ± 1.4

* Serum [^{125}I]DNA binding activity was determined by the modified Farr test. 0.1 ml of 10% heat-inactivated sera was incubated with 50 ng of [^{125}I]DNA precipitated with 50% saturated ammonium sulfate.

‡ 50 ng of [^{125}I]DNA was first incubated with 0.1 ml of 10% heat-inactivated antisera overnight at 4°C, then 300 μg of GBM was added and incubated overnight at 4°C.

§ Anti-SSDNA antisera were obtained from mice immunized with DNA-mBSA complexes in Freund's incomplete adjuvant.

‖ Mean of triplicates ±1 SD.

mice, a small amount of DNA was still detected in kidneys after 2 and 8 days, but this binding did not differ significantly from that of similarly injected [^{131}I]BSA. However, when this experiment was performed in mice which were injected with 100 μg of *S. typhimurium* LPS (2 days before the injection of DNA or BSA), a very significant binding of DNA was observed in kidneys studied at 48 h. This [^{125}I]SSDNA largely persisted in kidneys for several days as demonstrated in kidneys studied after 8 days (Table VII). These results strikingly differ from those obtained when the persistence of injected [^{125}I]SSDNA was investigated on plasma samples. Indeed, no [^{125}I]SSDNA could be detected in plasma 2 h after the injection.

Discussion

The present data demonstrate that, in vitro, collagen and collagen-like structures in GBM can bind DNA 40–80 times more than HGG or BSA, and 10–40 times more than LPS. This phenomenon appeared to be unrelated to the origin of the DNA tested since mammalian, bacterial, viral, and plant DNA reacted similarly. The fact that the digestion with collagenase almost completely abolished the binding of DNA, and that most of the bound DNA was released from a preformed [^{125}I]DNA–GBM complex after treatment with collagenase, indicated that collagen was the DNA-binding protein in the preparations of collagen or of GBM which were studied. One should note that the pretreatment of GBM with 2.0 M NaCl or with DNase, to remove contaminating histones (29) or DNA, resulted in an increase and not in a decrease of the DNA-binding capacity.

The previous observation of binding of DNA to C1q may reflect the particular affinity of collagen-containing substances for DNA, since it is known that C1q contains collagen-like polypeptide chains (32) and is also affected by collagenase (33). The mechanism of this binding is probably related to an interaction between highly negatively charged DNA with the basic sites on collagen, GBM, or C1q. Such interaction actually occurs between DNA and other basic proteins

TABLE V
Effect of Anti-DNA Antibodies on Binding of DNA to GBM

First reaction	Second reaction with:	[^{125}I]SSDNA ppt.	Inhibition
		%	%
[^{125}I]SSDNA + Anti-DNA*	GBM‡	16.8§	54.2
[^{125}I]SSDNA + Anti-DNA + GBM	(−)	28.0	23.7
[^{125}I]SSDNA + GBM	Anti-DNA	36.4	0.8
[^{125}I]SSDNA + GBM + NMS	(−)	36.7	−

* 50 ng of [^{125}I]SSDNA was incubated with 0.1 ml of 10% heat-inactivated anti-SSDNA antisera. Anti-SSDNA antisera were prepared by immunizing DNA-mBSA complexes in Freund's incomplete adjuvant in mice. These antisera showed about 60% of [^{125}I]SSDNA-binding activity determined by the Farr test.

‡ 300 μg of GBM was suspended in 0.1 ml of 10% heat-inactivated NMS.

§ Mean of duplicates.

TABLE VI
Elution of Anti-DNA Antibodies from GBM-DNA-Anti-DNA Complexes

First reaction	Second reaction with:	[^{125}I]SSDNA binding activity of eluates*
GBM + SSDNA‡	Anti-SSDNA§	49.4 ± 1.8‖
GBM + DSDNA	Anti-SSDNA	17.7 ± 0.5
GBM + PBS	Anti-SSDNA	13.6 ± 0.2
GBM + SSDNA	NMS	13.8 ± 0.3
GBM + DSDNA	NMS	15.6 ± 0.5
GBM + PBS	NMS	17.9 ± 0.8
PBS + SSDNA	Anti-SSDNA	14.4 ± 0.4

* 0.025 ml of eluates was incubated with 10 ng of [^{125}I]SSDNA in the presence of 10% heat-inactivated NMS. [^{125}I]SSDNA-binding activity was determined by the Farr test.

‡ 300 μg of GBM was incubated with 100 μg of SSDNA or DSDNA overnight at 4°C.

§ 0.1 ml of heat-inactivated anti-SSDNA antisera obtained from mice immunized with DNA-mBSA complexes in Freund's incomplete adjuvant.

‖ Mean of triplicates ±1 SD.

such as histones, egg white lysozyme, mBSA (34, 35, 17). This type of interaction would also explain the inhibiting effect of low pH, low ionic strength, and of anionic detergents such as SDS on the DNA-binding capacity of GBM. The competitive effect of C1q on this binding may proceed through a similar interaction. The interference with the binding of DNA by anti-DNA antibodies may result from a change in the charge configuration of DNA after the binding of antibodies, although one cannot exclude the possibility that antibodies masked the binding site present in DNA or changed the spatial configuration of DNA.

The fact that 100 μg of GBM can bind up to 4 μg of SSDNA but only 0.5 μg of DSDNA suggested a higher affinity of GBM for SSDNA than for DSDNA. This

TABLE VII
In Vivo Renal Localization of [^{125}I]SSDNA in Normal Mice and in LPS-Treated Mice

Group*	Organ	[^{125}I]SSDNA‡	[^{131}I]BSA‡
Normal	Kidneys	12.7 ± 4.6‖	16.4 ± 3.3
	Liver	23.7 ± 5.7	22.0 ± 5.8
	Kidneys/liver	0.5 ± 0.1	0.8 ± 0.1
LPS§	Kidneys	197.2 ± 45.8	12.5 ± 3.0
	Liver	23.2 ± 1.2	16.2 ± 3.0
	Kidneys/liver	8.5 ± 1.6	0.8 ± 0.1

* 8-wk-old C57BL/6 female mice (five mice in each group).
‡ 5.0 μg of radiolabeled preparations was injected intravenously.
§ 100 μg of *S. typhimurium* LPS was injected intraperitoneally at day 0. Radiolabeled preparations were given at day 2. Mice were sacrificed at day 10.
‖ Nanograms of injected radiolabeled preparations per gram of organ (mean ±1 SD).

difference was confirmed in dissociation experiments and is similar to that described for the interaction between DNA and mBSA (17). Moreover, inhibition experiments indicated that unlabeled SSDNA was a strong inhibitor of the binding of [^{125}I]DSDNA as well as [^{125}I]SSDNA, whereas unlabeled DSDNA had a strong inhibitory effect only on the binding of [^{125}I]DSDNA. The different binding properties of SSDNA and DSDNA may be due to the difference of charge density in their secondary structures. Although some [^{125}I]DSDNA was still bound by GBM after selective removal of most of the single-stranded regions with S1 nuclease, one cannot exclude that single-stranded regions present within DSDNA (36) may be responsible for the binding of [^{125}I]DSDNA to GBM. Alternatively, DNA may have two reactive sites for the binding to GBM. One site may be present on the side of the basis, exposed only in SSDNA, and another on the side of the backbone which is exposed in both SSDNA and DSDNA.

The biological significance of the high affinity of collagen and GBM for DNA should be considered in relation to the frequent tissue deposition of DNA-anti-DNA antibody complexes during the course of some human or animal diseases. Such complexes have been found in GBM (11, 13, 37), epidermal basement membrane (7, 14) and in interstitial tissues (38), and their pathogenic significance has been demonstrated (11, 37, 39, 40). It is generally proposed that the tissue localization of DNA-anti-DNA complexes would result from the formation of such complexes in circulating blood with a secondary localization in filtering membranes or in perivascular areas (41). However, although immune complexes are likely to exist in the plasma from patients with SLE, only occasional and minute amounts of DNA-anti-DNA complexes could be detected in the samples (3). Furthermore, in rabbits and in mice it was found that DNA-anti-DNA complexes have a very short half-life in circulating blood (42), which should not favor their deposition in filtering membranes. Since it was found in vitro: first, that DNA can be actively bound by collagen or GBM and, secondly, that DNA thus bound can act as an immunoabsorbant and react efficiently with free anti-DNA antibodies, one can imagine an alternative hypothesis to explain the localization of DNA-anti-DNA complexes in basement membranes or in

interstitial tissues. Accordingly, free DNA released in blood (10, 43) or in extravascular spaces (44) from disrupted autologous cells or from bacteria or DNA viruses could, in a first phase, be bound by collagen fibers or basement membranes. When this occurs in an individual developing anti-DNA antibodies, DNA-anti-DNA complexes would easily be formed locally at the site of DNA localization without any requirement for circulating DNA-anti-DNA complexes.

In vivo, some factors may influence the binding of DNA by collagen-like substances. Lower peripheral temperatures, increased vascular permeability, decrease of blood DNase activity and decrease of C1q level would favor this binding. Some of these conditions are observed in patients with SLE (45) or in New Zealand mice (46). Our experimental data suggest that this may also occur in mice treated with LPS. Indeed, a significant renal binding of injected DNA was observed in mice treated with LPS 2 days before this injection. Although the morphological distribution of this kidney-bound DNA has still to be defined, this phenomenon may result from the high avidity of GBM structures for DNA. Furthermore, it has been shown that in mice injected with LPS there is first a release of DNA in circulating blood and then an induction of anti-DNA antibodies (20). Recent observations indicate that these mice rapidly undergo a deposition of immune complexes in renal glomeruli in which DNA-anti-DNA antibody complexes have been identified.[2]

Summary

In vitro, collagen and collagen-like material in GBM, were demonstrated to have a particular high affinity for any DNA tested (mammalian, bacterial, viral, and plant). GBM fixed DNA 40–80 times more than HGG and BSA and 10–40 times more than bacterial LPS. GBM has a higher affinity for SSDNA than for DSDNA. This binding was inhibited at low pH, low ionic strength, and in the presence of anionic detergents, indicating that the highly negatively charged DNA may interact with the basic site on collagen or GBM by electrostatic forces. This interaction was competitively interfered with by DNA-binding proteins such as C1q.

Complexes formed of DNA and anti-DNA antibodies did not exhibit the same binding property as free DNA. However, DNA which was already bound to GBM or to collagen could very efficiently bind anti-DNA antibodies and form immune complexes which would remain on these structures.

The biological significance of the binding of DNA to GBM or to collagen should be particularly considered in relation to the pathogenesis of SLE. It is possible that DNA released from disrupted or degenerating cells would bind to surrounding collagen fibers or to basement membranes and then act as an immunoabsorbant for circulating anti-DNA antibodies. Some evidence for an in vivo binding of SSDNA to renal structures was obtained in mice treated with bacterial LPS 2 days before the injection of SSDNA.

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