FEATURES OF SYSTEMIC LUPUS ERYTHEMATOSUS IN MICE INJECTED WITH BACTERIAL LIPOPOLYSACCHARIDES

Identification of Circulating DNA and Renal Localization of DNA-Anti-DNA Complexes*

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The development of renal disease in patients with systemic lupus erythematosus (SLE)[1] and in NZB × NZW F_1 hybrid mice is associated with a high titer of anti-DNA antibodies in serum (1–3). However, the presence of anti-DNA antibodies is not necessarily associated with tissue injury (4, 5). There is evidence that anti-DNA antibodies are involved in the pathogenesis of SLE through the formation of immune complexes with DNA released in circulating blood or in extravascular spaces (2, 3, 6, 7).

The origin of DNA participating in the formation of immune complexes in SLE and the mechanisms by which such DNA-anti-DNA complexes are localized in tissues are still unclear. Although circulating immune complexes have been detected by various methods in patients with SLE (8–12), only small amounts of circulating DNA-anti-DNA complexes have been found (10, 13). Particular mechanisms may be involved in their selective concentration in tissues as suggested by the demonstration of a high affinity of DNA for collagen or glomerular basement membrane (GBM) (14).

In view of the biological changes occurring in mice after the injection of bacterial lipopolysaccharides (LPS), this experimental model may be suitable for the investigation of these particular features of SLE. It is well known that in vivo LPS can cause various tissue lesions which are probably not related to immunopathological mechanisms (15–17). However, it has been observed that DNA appears in circulating blood soon after the injection of LPS and there is a subsequent formation of anti-DNA antibodies (18). In the present study, the

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[1] *Abbreviations used in this paper:* DSDNA, double-stranded DNA; GBM, glomerular basement membrane; LPS, lipopolysaccharides; 2-ME, 2-mercaptoethanol; NMS, normal mouse serum; PBS, phosphate-buffered saline; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; SLE, systemic lupus erythematosus; SSDNA; single-stranded DNA; VBS, veronal-buffered saline containing Mg^{++}.

possible interactions between released DNA and corresponding antibodies have been studied. Firstly, DNA occurring in circulating blood was purified and characterized; secondly, the possible formation and localization of DNA-anti-DNA complexes were investigated; and thirdly, the kinetics of serological and tissue changes have been followed in order to better define the possible mechanisms involved in the tissue deposition of DNA-anti-DNA complexes.

Materials and Methods

Mice. 6- to 10-wk-old mice were used throughout the study. C57BL/6, BALB/c, and DBA/2 female mice were purchased from Charles River Breeding Laboratories, Inc., Elbeuf, France. OF1 outbred female mice were purchased from IFFA CREDO (Centre de Recherche et d'Elevage des Oncins, St. Germain-sur-l'Arbresle, France). Blood samples were collected by orbital sinus puncture and the sera were stored at −20°C until used.

LPS. Polysaccharides B of *Salmonella typhimurium* (lot 563628), *S. enteriditis* (lot 586088), *Escherichia coli* 0127:B8 (lot 582337), and *E. coli* 0111:B4 (lot 587687) were obtained from Difco Laboratories, Detroit, Mich. Lipid A extracted from *E. coli* 0111:B4 were kindly provided by Dr. D. Morrison (Scripps Clinic and Research Foundation, La Jolla, Calif.). Alkali-treated LPS was prepared as follows: 1 ml of *S. typhimurium* LPS solution in distilled water was incubated with 0.5 ml of 0.25 N NaOH for 1 h at 37°C (19). After incubation, 0.5 ml of 0.25 N HCl was added to neutralize the sample and then dialyzed against saline. All the LPS preparations were diluted to the desired concentration in saline and were injected intraperitoneally in a final vol of 0.2 ml.

DNA. Highly polymerized calf thymus DNA (type V) was purchased from Sigma Chemical Co., St. Louis, Mo. Bacterial DNA from *Micrococcus lysodeikticus* was purified by Marmur's technique (20). Denatured DNA was prepared by heating native DNA [0.5 mg/ml in phosphate-buffered saline (PBS), 0.01 M, pH 7.0] at 100°C for 10 min and by transferring directly to an ice bath. Immunization of mice with DNA for preparation of anti-single-stranded DNA (SSDNA) antisera was carried out as described previously (18).

Radiolabeling Procedures. Calf thymus DNA was labeled with iodine (^{125}I) according to the method of Commerford (21). This DNA preparation was shown to contain less than 1% of pronase-sensitive material. The radiolabeled DNA was fractionated on methylated albumin kieselguhr columns in order to obtain pure SSDNA (22). Pure ^{125}I-labeled double-stranded DNA (DSDNA) was prepared by treating ^{125}I-labeled native DNA with single-strand specific S1 nuclease (Seikagaku Kogyo, Tokyo, Japan) (23). For labeling of LPS, LPS was further purified by the phenol-water extraction method, followed by fractionation with ethanol and ultracentrifugation (24). The labeling of LPS with ^{51}Cr was carried out according to the method of Braude et al. (25). This [^{51}Cr]LPS preparation was shown to contain less than 1% of pronase-sensitive material. Bovine serum albumin (Calbiochem, San Diego, Calif.) and human IgG were labeled with ^{125}I according to the procedure of McConahey and Dixon (26). C1q was purified from fresh human serum by the method of Yonemasu and Stroud (27) and was labeled with ^{125}I according to Heusser et al. (28).

Purification and Characterization of DNA in Circulating Blood. The amount of DNA released into circulating blood after injection of LPS was determined by inhibition of radioimmunoassay as described previously (18). To avoid the contamination of nuclear DNA from leukocytes, blood samples were carefully collected into EDTA or heparinized tubes. Circulating DNA was purified by affinity column chromatography and CsCl density gradient centrifugation. 2 ml of pooled plasma collected 12 h after a single injection of 100 μg *S. typhimurium* LPS was applied to a CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column coated with anti-DNA antibodies which reacted with both SSDNA and DSDNA. Bound DNA was eluted by 2.5 M KSCN (pH 6.0) and dialyzed against 0.15 M NaCl containing 3 mM EDTA. This crude DNA fraction was treated with pronase (1 mg/ml: 45,000 proteolytic units/g; Calbiochem) at 37°C overnight and mixed with CsCl (Merck, A., G., Inc., Darmstadt, W. Germany) in 0.02 M Tris-HCl buffer (pH 8.5). The density was adjusted to 1.700 g/cm^3 with either solid CsCl or Tris-HCl buffer. This solution was centrifuged at 36,000 rpm at 18°C for 60 h with a SW65-Ti rotor in a Spinco L2-65B preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). DNA in fractions was detected by inhibition of radioimmunoassay. DNA-containing fractions were concentrated

and some of this purified DNA was labeled with ^{125}I. This labeled DNA contained less than 5% of pronase-sensitive material and had a sp act of about 5×10^4 cpm/μg.

To determine the density of the released DNA, ^{125}I-labeled purified DNA in CsCl was centrifuged at 36,000 rpm at 18°C for 60 h. Four-drop fractions were collected. Some fractions were used to measure refractive index, the remaining fractions being used to determine radioactivity in an automatic Beckman gamma counter. The molecular size of DNA was determined by using sucrose density gradients. 0.5 ml of plasma diluted $^1/_{10}$ in borate buffer (ionic strength 0.1, pH 8.4) or purified DNA was layered on 4.5 ml of 10–40% (wt/vol) linear sucrose gradient in borate buffer containing 3 mM EDTA. The samples were centrifuged at 40,000 rpm at 4°C for 18 h with a SW65-Ti rotor. Serial fractions were collected and optical density patterns were recorded by a Uvicord (LKB-Produkter, Bromma, Sweden). The 19 S peak of plasma was used one reference marker. [^{125}I]IgG or [^{125}I]BSA was centrifuged in control tubes and used as a 7 S or 4.5 S reference marker. The apparent S values were calculated according to the method of Martin and Ames (29). The gradients were divided into nine fractions which were dialyzed against borate buffer before the examination of DNA content by the radioimmunoassay. To control the specificity, 0.1 ml of each fraction was treated with 0.05 ml of 1 mg/ml DNase (deoxyribonuclease I, 2,600 U/mg; Worthington Biochemical Corp., Freehold, N. J.) in Veronal-buffered saline containing Mg^{++} (VBS), pH 7.2, for 3 h at 37°C. Then, 0.025 ml of 0.03 M EDTA (pH 7.0) was added. As undigested control, the same fractions were incubated with DNase in the presence of EDTA.

Radioimmunological Detection of Anti-DNA and Anti-LPS Antibodies. The titration of anti-DNA antibodies was carried out using a modification of the Farr DNA-binding radioimmunoassay. 0.1 ml of sera diluted $^1/_{10}$ in borate buffer was heated at 56°C for 30 min, then mixed with 10 ng of ^{125}I-labeled calf thymus SSDNA in the presence of sodium dodecyl sulfate (SDS; final concentration, 0.025%) in order to eliminate the nonspecific binding of DNA by nonimmunoglobulin basic proteins. The details were described previously (30). Titration of anti-DNA antibodies was also carried out on sera fractionated by gel filtration on Sephadex G-200 column (18).

For the titration of anti-LPS antibodies, a radioimmunological method was developed. Briefly, 0.1 ml of sera diluted $^1/_{10}$ in borate buffer was heated at 56°C for 30 min, then mixed with 0.1 μg of ^{51}Cr-labeled LPS in 0.2 ml of borate buffer in the presence of 0.1 ml of 0.1% SDS (final concentration, 0.025%). The mixture was incubated for 2 h at 37°C, then 1.2 ml of 20% polyethylene glycol (PEG, average mol wt 6,000; Siegfried, Zofingen, Switzerland) in borate buffer was added to yield a final concentration of 15%. After an overnight incubation at 4°C, the tubes were centrifuged at 1,000 *g* for 30 min, then washed with 3 ml of 15% PEG. The results were expressed as a percentage of [^{51}Cr]LPS precipitated. In this method, free labeled LPS is soluble in 15% PEG, while LPS bound to antibodies is precipitated. Analysis of numerous immune sera indicated that the sensitivity and specificity of this new method was comparable to that of the passive hemagglutination test for detection of 19 S anti-LPS antibodies but better for the detection of corresponding 7 S antibodies.

Detection of Circulating Immune Complexes. The presence of circulating DNA-anti-DNA complexes was examined according to the method of Herbeck et al. (9). Using the antigen-nonspecific method, the presence of immune complex-like material in circulation was examined by a modified [^{125}I]C1q-binding test (11). The effect of DNase treatment or reduction by 2-mercaptoethanol (2-ME) on the serum [^{125}I]C1q-binding activity was carried out according to Zubler et al. (31).

Determination of C3 Level in Sera. Blood C3 level was determined by radial immunodiffusion in agar by the method of Mancini et al. (32) and was expressed as a percentage of the normal pool values. Sera were assayed immediately after collection.

Studies of Kidney Tissues. Samples of renal tissue from mice injected with LPS were studied for routine histology with hematoxylin-eosin, trichrome Masson, and periodic acid-Schiff stains, and by immunofluorescence. For immunofluorescence, kidney specimens were snap-frozen in liquid nitrogen and sections of 4-μm thickness were used. Goat anti-mouse 7 S IgG antisera and monospecific anti-mouse IgM antisera were purchased from Meloy Laboratories Inc., Springfield, Va. Rabbit anti-rat C3 antisera, which cross-react with mouse C3, were obtained from Nordic Immunology Laboratories, Tilburg, The Netherlands. The above antisera were conjugated to fluorescein isothiocyanate (Baltimore Biological Laboratories, Cockeysville, Md.) following the method of Clark and Shepard (33). To elute immunoglobulins from kidney sections, unfixed cryostat sections were treated with 0.12 M glycine-HCl buffer (pH 3.0) for 1 h at 37°C, or with 2.5

M KSCN (pH 6.0) for 30 min at room temperature. After elution, these sections were washed for 30 min in PBS (pH 7.2) and then incubated with the appropriate fluorescent reagents. For electron microscope studies, kidneys were fixed in phosphate-buffered 2% glutaraldehyde, post-fixed in osmic acid, dehydrated, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate.

Elution of Kidney-Fixed Immunoglobulins. Kidney-fixed immunoglobulins were eluted by the method of Lambert and Dixon (3) with a modification. For elution, saline-insoluble sediments of kidney homogenate were treated with equal volume of DNase (1 mg/ml in VBS) for 1 h at 37°C and overnight at 4°C, followed by elution with 2.5 M KSCN (pH 6.0) at room temperature for 1 h. Eluates obtained were dialyzed against 0.15 M NaCl. The immunoglobulins in the eluates were isolated by precipitation in ammonium sulfate at 50% saturation at pH 7.0. The eluates were studied by immunoelectrophoresis analysis. The amount of IgG and IgM in the eluates was estimated by a radial immunodiffusion in agar and was expressed in micrograms of immunoglobulins per gram of kidneys. For standard immunoglobulins, mouse IgG and IgM myeloma proteins were purchased from Litton Bionetics, Kensington, Md. The amount of anti-DNA antibodies in the eluates was determined by quantitative absorption at 4°C with CNBr-activated Sepharose 4B coated with both SSDNA and DSDNA. The immunoglobulin concentration was determined by quantitative radial immunodiffusion before and after absorption. As control, equivalent amounts of purified mouse myeloma IgG and IgM were subjected to absorption experiment. Antibody activities against SSDNA and LPS in the eluates were determined by radioimmunoassays. For titration, different amounts of heat-inactivated eluates were incubated with radiolabeled antigens in the presence of 0.1 ml of 10% heat-inactivated normal mouse serum (NMS) containing 3 mM EDTA. As control, equivalent amounts of purified mouse myeloma immunoglobulins were incubated with radiolabeled antigens in the presence of NMS.

Results

Purification and Characterization of Circulating DNA in Mice Injected with LPS. A relatively large amount of DNA, 0.3–2 μg/ml of plasma (mean ± 1 SD; 0.83 ± 0.68 μg/ml), has been detected in circulating blood 12 h after a single injection of 100 μg *S. typhimurium* LPS into 10 C57BL/6 mice, while no DNA could be detected in plasma of control C57BL/6 mice. Such plasma samples have been pooled and used for the purification of DNA. The purification was achieved by affinity column chromatography and CsCl density gradient ultracentrifugation, and the DNA obtained was then further characterized.

In order to determine the size of this DNA, the purified fraction was subjected to sedimentation velocity analysis in a sucrose density gradient (10–40% wt/vol). After ultracentrifugation, the presence of DNA was determined in each fraction by radioimmunoassay. The specificity was controlled by treatment with DNase. DNA was demonstrated in gradient fractions containing 4–6 S material. When ^{125}I-labeled purified DNA was examined, the peak of radioactivity was also found in tubes containing 4–6 S material. The same methodology was applied to a plasma pool from mice injected with LPS, and DNA was again demonstrated in gradient fractions containing 4–6 S material.

The released DNA has been characterized by CsCl buoyant density gradient analysis for comparison with mammalian cellular DNA. For this purpose, the ^{125}I-labeled DNA purified from the plasma pool of mice injected with LPS was subjected to CsCl density gradient analysis. This released DNA banded in a broader peak than that obtained in separate control experiments with marker *M. lysodeikticus* (ρ = 1.731 g/cm^3) and calf thymus (ρ = 1.700 g/cm^3) DNA. The peak of radioactivity was found at a density of approximately 1.700 g/cm^3, indicating its general similarity to double-stranded mammalian cellular DNA.

TABLE I
Immunochemical Property of DNA Released after Injection of LPS

Added DNA	[^{125}I]SSDNA ppt*	[^{125}I]DSDNA ppt*
	%	%
NMS‡	49§	2
NMS + purified DNA‖	45	NT¶
NMS + heat-denatured purified DNA	34	NT

* 0.1 ml of mouse anti-SSDNA antiserum (1:300) diluted in borate buffer was added to 0.1 ml of tested DNA sample in 10% NMS, then to 10 ng of [^{125}I]SSDNA or [^{125}I]DSDNA in a direct binding test.

‡ Pool of serum from 8-wk-old normal C57BL/6 mice.

§ Mean of duplicate.

‖ DNA purified from a plasma pool of C57BL/6 mice injected with *S. typhimurium* LPS.

¶ NT, not tested.

To exclude the possibility that DNA contaminating the LPS preparations could appear in the circulating blood after injection of LPS, the amount of DNA present in the preparations of LPS was determined by inhibition of radioimmunoassay. No DNA could be detected in the preparations of *S. typhimurium* LPS which had been used.

The purified DNA was further characterized by immunochemical analysis. The purified DNA slightly inhibited the reactivity of mouse anti-SSDNA antibodies with [^{125}I]SSDNA but this inhibition was considerably increased after heat denaturation of that DNA (Table I). The purified DNA also inhibited the reactivity of human anti-(DS+SS)DNA antibodies with [^{125}I]DSDNA. Therefore, DNA purified from plasma of mice injected with LPS may react immunochemically as a mixture of SSDNA and DSDNA or DSDNA with some single-stranded regions.

Anti-DNA, Anti-LPS Antibodies, and Circulating Immune Complexes after Injection of LPS. Anti-DNA and anti-LPS responses were followed in C57BL/6 mice after a single intraperitoneal injection with 100 μg of *S. typhimurium* LPS. The titers of serum anti-DNA antibodies and anti-LPS antibodies, expressed in percentage of [^{125}I]SSDNA and [^{51}Cr]LPS precipitated, were measured on days 1, 2, 3, 8, 15, and 30. A shown in Fig. 1, on the 3rd day after a single injection of LPS, a significant anti-DNA response was induced. The peak response was observed on day 8 and serum DNA-binding activity decreased slowly afterwards. On the other hand, anti-LPS antibodies developed more slowly than anti-DNA antibodies. A high level of anti-LPS antibodies remained until day 30. Mice receiving saline did not show any significant binding activity to DNA or to LPS during that period. Anti-DNA antibodies induced by a single injection of LPS were shown by Sephadex G-200 gel filtration analysis to belong to both the IgM and IgG classes.

The reactivity of anti-DNA antibodies induced by LPS to the DNA released after the injection of LPS was investigated. Sera from mice injected with LPS showed a significant binding activity to [^{125}I]DNA purified from circulating blood of LPS-injected mice (% binding ± 1 SD: sera on day 8, 15.0 ± 0.9%; control sera, 8.5 ± 1.3%).

In view of the association of (*a*) the release of DNA and (*b*) the appearance of

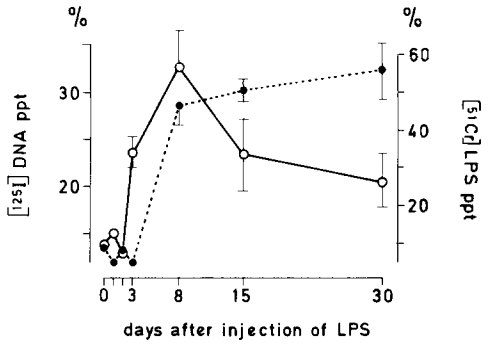

FIG. 1. Induction of anti-DNA antibodies in C57BL/6 mice after a single injection of bacterial LPS. 100 μg of *S. typhimurium* LPS was injected intraperitoneally on day 0. Anti-DNA level is expressed as the mean percentage (7–10 mice in each examination) of [^{125}I]SSDNA precipitated (○—○). Anti-LPS level is expressed as the mean percentage of [^{51}Cr]LPS precipitated (●---●). Vertical bars represent the limits of 1 SD.

TABLE II
Effect of DNase Treatment on Serum DNA-Binding Activity

Sera	Treatment	[^{125}I]SSDNA ppt*		
		Day 2	Day 3	Day 8
		%	%	%
LPS injected‡	Nontreated	11 ± 3§	24 ± 2	36 ± 6
	DNase-treated	11 ± 4	25 ± 2	35 ± 6
Control	Nontreated	NT‖	10 ± 2	11 ± 4
	DNase-treated	NT	9 ± 2	10 ± 3

* 0.025 ml of serum was treated with 50 μg of DNase in 0.05 ml VBS at 37°C for 3 h. Then, 0.05 ml of 0.015 M EDTA (pH 7.0) and 0.125 ml of borate buffer were added. After heating the mixture at 56°C for 30 min, 0.1 ml was tested for the DNA-binding activity. In the control tubes, EDTA was added before DNase in order to prevent its enzyme action.

‡ 100 μg *S. typhimurium* LPS were injected intraperitoneally into C57BL/6 mice on day 0.

§ Mean of seven mice ± 1 SD.

‖ NT, not tested.

anti-DNA antibodies, the presence of DNA-anti-DNA complexes in circulating blood was investigated. The level of anti-DNA antibodies was assessed in sera collected 12 h to 15 days after injection of LPS, with or without treatment of DNase. There was no increase in the serum DNA-binding activity after the digestion of sera with DNase (Table II).

The presence of circulating immune complexes after injection of LPS was also studied by measurement of the serum [^{125}I]C1q-binding activity. During day 1 and day 3 after injection of LPS, there was no increase in the [^{125}I]C1q-binding activity (Fig. 2). From day 5 to day 30, a slight, but significant, increase in the [^{125}I]C1q-binding activity was observed. In order to characterize the C1q-binding material in sera of mice injected with LPS, sera which exhibited high C1q-binding activity were pooled and subjected to various treatments. One serum pool was subjected to reduction with 0.2 M 2-ME and alkylation with iodoacetamide. This treatment abolished most of the C1q-binding activity (% binding: 2-

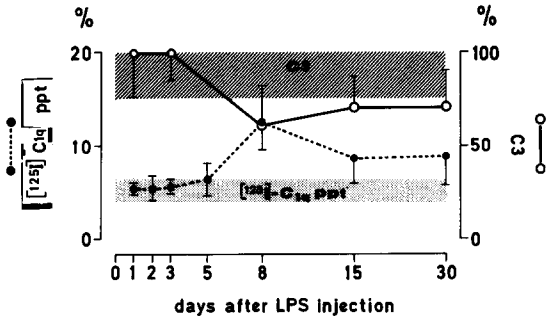

FIG. 2. Circulating immune complexes and C3 levels in mice injected with LPS. 100 μg of *S. typhimurium* LPS was injected intraperitoneally on day 0 into C57BL/6 mice. The level of circulating immune complexes (●---●) was measured by the [^{125}I]C1q-binding test. Results are expressed as the mean percentage of [^{125}I]C1q precipitated (7–10 mice in each examination). Blood C3 level (○—○) was measured by quantitative radial immunodiffusion. Results are expressed as the mean percentage of normal pooled values. Vertical bars represent the limits of 1 SD. The upper shadowed area in the figure indicates the mean −1 SD range for C3 level in the control mice. The lower shadowed area indicates the mean ± 1 SD range for [^{125}I]C1q-binding activity in the control mice.

TABLE III
Effect of DNase Treatment on Serum C1q-Binding Activity

Sera	Treatment	[^{125}I]C1q ppt,* day 8
LPS-injected‡	Nontreated	21.9§
	DNase-treated·	23.7
Control	Nontreated	5.1

* 0.05 ml of pooled serum was treated with 50 μg of DNase in 0.05 ml VBS at 37°C for 3 h followed by the incubation with 0.2 ml of 0.2 M EDTA (pH 7.0) at 37°C for 30 min. Then, 0.05 ml of [^{125}I]C1q and 1.75 ml of 3% PEG in borate buffer were added and incubated at 4°C for 1 h. In the control tubes, EDTA was added before DNase in order to prevent its enzyme action.

‡ 100 μg *S. typhimurium* LPS were injected intraperitoneally into C57BL/6 mice on day 0.

§ Mean of duplicate.

ME treated, 8.9%; nontreated, 26.7%). The same treatment led to a decrease in the C1q-binding activity of aggregated human IgG (0.5 mg/ml) from 52.9 to 9.0%. It was found that DNA was not part of the C1q-binding material since the C1q-binding activity of the same serum pool did not change after treatment with DNase (Table III). One should note that blood C3 levels dropped significantly after the appearance of the C1q-binding material in circulating blood (Fig. 2).

Immune Complexes in Renal Glomeruli after Injection of LPS. The glomeruli of C57BL/6 mice injected intraperitoneally with 100 μg *S. typhimurium* LPS showed minimal histologic alterations. 2 wk after injection of LPS, abnormal segmentation of the glomerular tufts, focal increase in mesangial substance and, in some glomeruli, mesangial cell proliferations were observed. Electron microscopically, all glomeruli showed segmental or focal intercapillary cell proliferations. Some glomeruli showed extensive focal thickening of GBM and the presence of subepithelial nodules. These nodules had the same apparent

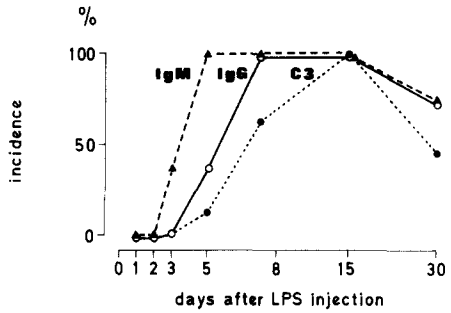

FIG. 3. Time-course of cumulative incidence of glomerular IgM (▲), IgG (○), and C3 (●) deposits after a single injection of LPS. 100 μg of *S. typhimurium* LPS was injected intraperitoneally on day 0 in 8- to 10-wk-old C57BL/6 mice. The total number of mice with the glomerular deposits is expressed as percent of all mice (7–10 mice in each examination).

density as the basement membrane and were often associated with fusion of the epithelial cell foot processes.

Immunohistochemical studies were carried out on mouse kidneys obtained 1–30 days after injection with 100 μg *S. typhimurium* LPS (Fig. 3). 3 days after injection with LPS, a small amount of IgM was found in the mesangial areas and along the glomerular capillary walls in three out of eight mice. No glomerular localization of IgG and C3 was observed at this time. From day 5 to day 15, all mice examined showed fine granular IgM deposits along the glomerular capillary walls as well as in mesangial areas (Fig. 4*a*). Less intense, granular deposits of IgG and C3 were found in similar locations to the IgM deposits (Fig. 4*b*). Only 2 out of 20 control mice showed deposits of immunoglobulins (IgG and IgM) and C3 in glomeruli. In similar experiments, it was found that a single injection of *S. typhimurium* LPS induced glomerular deposits as well as anti-DNA antibodies in other strains tested: BALB/c, DBA/2, and OF1 outbred mice.

Involvement of Anti-DNA Antibodies in Glomerular Deposits. The induction of glomerular deposits of immunoglobulins by a single injection of LPS was studied in relation to the doses of LPS injected. Five groups of C57BL/6 mice were injected with different doses of *S. typhimurium* LPS ranging from 0.1 to 100 μg/mouse. Mice injected with more than 10 μg of LPS developed significant amounts of anti-DNA antibodies as well as granular immunoglobulin deposits in the glomeruli (Table IV). Less than 1 μg of LPS did not induce anti-DNA antibodies nor renal deposits, although anti-LPS antibodies were detected. LPS of other origins, either 100 μg of LPS from *S. enteriditis*, *E. coli* 0127:B8, or *E. coli* 0111:B4, were injected intraperitoneally into C57BL/6 mice. All the LPS tested were able to induce similar glomerular deposits as well as an anti-DNA response. A single injection of 50 μg of lipid A, the active part of the LPS molecule, had similar effects. LPS from *S. typhimurium* was mildly treated with alkali to reduce the immunogenicity of LPS (20). 100 μg of alkali-treated LPS was injected into seven C57BL/6 mice. Mice injected with alkali-treated LPS developed low, but still significant, titers of anti-DNA antibodies (Table IV), but they did not show a significant increase in serum LPS-binding activity. Six out of seven mice showed granular immunoglobulin deposits in glomeruli of kidneys taken on day 15.

The presence of granular deposits of immunoglobulins and C3 in renal glo-

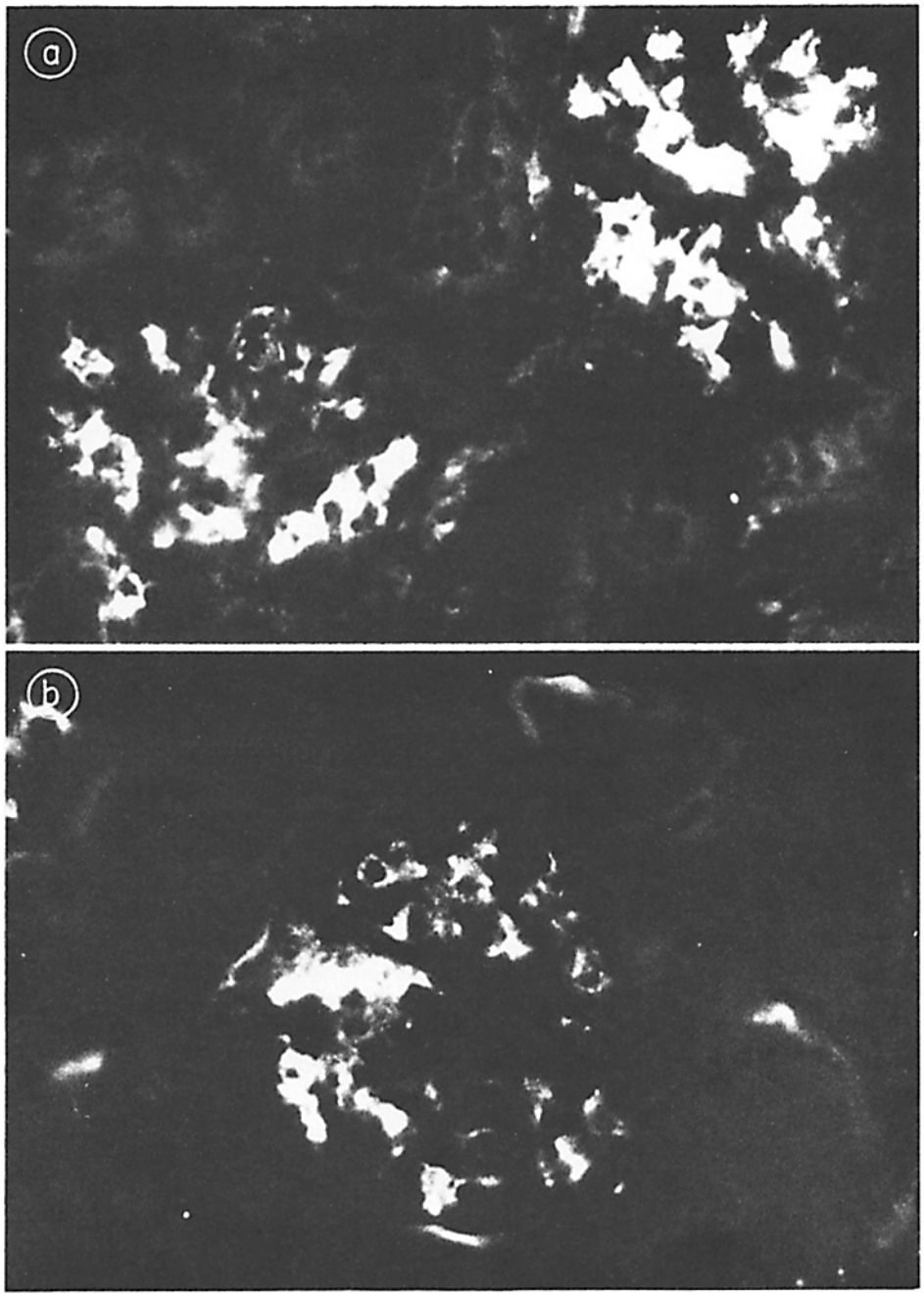

FIG. 4. Glomerular IgM (*a*) and C3 (*b*) deposits in the mesangial areas and along the glomerular capillary walls 15 days after a single intraperitoneal injection of 100 μg *S. typhimurium* LPS. Original magnification, 200.

meruli of mice suggested immune complex deposition. Kidney sections were treated with an acid buffer (pH 3.0) or 2.5 M KSCN (pH 6.0) followed by immunofluorescence analysis. These treatments caused a marked reduction in fluorescence of glomerular deposits. In contrast, immunoglobulins were not

TABLE IV

Anti-DNA, Anti-LPS Antibodies, and Glomerular Deposits after Injection of Various Doses of LPS or Alkali-Treated LPS

Group	Dose	[^{125}I]SSDNA ppt*	[^{51}Cr]LPS ppt‡	Glomerular deposits§
	μg	%	%	
LPS‖	0.1	14 ± 3	15 ± 5	0/7¶
	1	14 ± 3	20 ± 3	1/7
	10	21 ± 4	31 ± 6	7/7
	50	22 ± 5	44 ± 10	7/7
	100	24 ± 2	28 ± 8	7/7
Alkali-LPS**	100	20 ± 3	6 ± 1	6/7
Control		13 ± 3	7 ± 1	1/14

* Serum [^{125}I]SSDNA-binding activity (mean ± 1 SD) examined on day 3.

‡ Serum [^{51}Cr]LPS-binding activity (mean ± 1 SD) examined on day 15.

§ Glomerular immunoglobulin deposits examined on day 15.

‖ Various amounts of *S. typhimurium* LPS were injected intraperitoneally into C57BL/6 mice on day 0.

¶ Number of positive/number tested.

** Alkali-treated LPS was prepared by incubating *S. typhimurium* LPS in distilled water with half volume of 0.25 N NaOH for 1 h at 37°C.

eluted from glomeruli of sections treated with PBS (pH 7.2) under similar conditions.

To characterize more directly the possible involvement of DNA-anti-DNA complexes in the immune complex deposits, the immunoglobulins were eluted from kidneys and the nature of immunoglobulins in glomerular deposits were examined. For elution, kidneys from 30 mice were collected 15 days after a single injection of 100 μg *S. typhimurium* LPS. The eluates were shown by immunoelectrophoretic analysis to contain mouse immunoglobulins but not other mouse serum proteins. Considerable amounts of IgM (5.6 μg/of kidneys) and IgG (28 μg/g of kidneys) were eluted from kidneys with DNase treatment. Further treatment with KSCN resulted in the elution of significant amounts of IgM (2.4 μg/g of kidneys) and IgG (12 μg/g of kidneys) from kidneys. Both eluates were pooled and tested for the antibody activity against DNA and LPS. Results were compared with those obtained from a serum pool of the same animals on day 15 after injection of LPS. The eluates exhibited a high binding activity to [^{125}I]SSDNA but not to [^{51}Cr]LPS, while the serum pool showed higher LPS-binding activity than DNA-binding activity (Table V). A quantitative study, by serial dilution of the eluates, showed that at the same concentration of immunoglobulins, the DNA-binding activity in the eluates could be as much as 50 times higher than that given by the serum. The presence of anti-DNA antibodies in the kidneys was also determined by fixing the eluted immunoglobulins to insoluble Sepharose 4B coated with DNA. Approximately 40% of the immunoglobulins eluted were capable of binding to the DNA-Sepharose (Table V). Absorption of the eluates with DNA before incubation with [^{125}I]SSDNA abolished most of the DNA-binding activity. The reactivity of the renal eluates to the DNA released into circulating blood after injection of

TABLE V
Specificity of Immunoglobulins Eluted from Kidneys

Immunoglobulins	Treatment	Ig concentration (μg/ml)*		[125I]-SSDNA ppt‡	[51Cr]LPS ppt‡
		IgM	IgG		
				%	%
Eluates§	Unabsorbed	83	400	48	8
	Absorbed‖	54	285	15	6
IgM Control¶	Unabsorbed	94	NT**	11	6
	Absorbed	93	NT	12	5
IgG Control¶	Unabsorbed	NT	385	10	5
	Absorbed	NT	350	12	5
Serum‡‡					
LPS injected	Unabsorbed	1,800	14,000	22	51
Control	Unabsorbed	900	9,200	11	3

* The concentration of immunoglobulins was estimated by a radial immunodiffusion in agar.
‡ 10-μl eluates or control immunoglobulins were incubated with 10 ng of [125I]SSDNA or 100 ng of [51Cr]LPS in the presence of 10 μl of heat-inactivated NMS containing 3 mM EDTA. For serum, 10 μl of heat-inactivated sera were incubated with either [125I]SSDNA or [51Cr]LPS.
§ Immunoglobulins eluted from mouse kidneys obtained 15 days after injection of 100 μg *S. typhimurium* LPS.
‖ Absorbed with Sepharose 4B coated with DNA.
¶ Purified mouse myeloma proteins.
** NT, not tested.
‡‡ Collected on day 15 from the same mice for elution experiments.

LPS was also investigated. The eluates exhibited a significant binding to [125I]DNA purified from circulating blood of LPS-injected mice (% binding ± 1 SD: eluates diluted 1/10 in 10% NMS, 19.4 ± 0.1%; NMS, 8.5 ± 1.3%). From the results obtained in the elution experiments, we concluded that at least part of the immune complex deposits in the glomeruli were DNA-anti-DNA complexes.

Discussion

DNA has been demonstrated in circulating blood in various clinical conditions (1, 6, 7), but the nature and the origin of this DNA has not been systemically defined. In mice injected with LPS, it has been suggested that DNA might be released from endogenous cells or from infectious agents (18). The present data demonstrate that the circulating DNA released after injection of LPS has a similar density to mammalian cellular DNA. In the preliminary hybridization experiments, it was found that this released DNA contained mainly mouse DNA sequences. One should also note that no detectable DNA was present in the preparations of LPS which has been used. It is likely that LPS caused the release of DNA from host cells into circulating blood. Although cellular origin of DNA is difficult to define, cells which are damaged, directly or indirectly, by LPS may be responsible for the release of DNA (34). One should note that this DNA was of relatively small size (4–6S). DNA may have been excreted as a small fragment of DNA from intact cells (35). Alternatively, DNA may have

been partially digested by serum or cellular DNase. Immunochemical analysis indicates that the specificity of the purified DNA corresponds to a mixture of SSDNA and DSDNA or DSDNA with some single-stranded regions. A broader peak of released DNA in CsCl density gradient may be due to the low molecular weight of this released DNA and may also represent a mixture of DSDNA and SSDNA. Since SSDNA is iodinated more efficiently than DSDNA (21), a mixture of DSDNA and SSDNA which had been iodinated together would be overrepresented in relation to labeled molecules for SSDNA.

It was confirmed that anti-DNA antibodies are induced within a few days after a single injection of LPS. These antibodies react largely with SSDNA but can also react with the DNA which was purified from the plasma of mice injected with LPS. The possible mechanisms involved in the formation of these anti-DNA antibodies have been previously discussed (18). We consider that this results either from a polyclonal stimulation of B lymphocytes or from a concomitant stimulation of B lymphocytes by LPS and by DNA released from host cells.

The two phenomena observed, (*a*) release of DNA and (*b*) formation of anti-DNA antibodies, suggest the possible occurrence of DNA-anti-DNA complexes in serum or in tissues. In serum, DNA-anti-DNA complexes were not detected using a direct antigen-specific method nor an indirect C1q-binding test combined with DNase treatment of the sample. The results may be explained by the fact that the half life of the DNA released into circulating blood appears to be very short (18, 36), while anti-DNA antibodies are not detected before 3 days after the injection of LPS. There is no apparent coexistence of DNA and anti-DNA antibodies in plasma. However, unidentified immune complex-like material was demonstrated in serum, using the C1q-binding test, 5–8 days after the injection of LPS in mice.

In tissues, the deposition of immune complex-like material after injection of LPS seems to occur, particularly in renal glomeruli. Indeed, the presence of immunoglobulin deposits, in association with C3, in a granular pattern within glomerular capillary walls and mesangium is suggestive of a deposition of immune complexes. Such deposits may also correspond to the subepithelial nodules observed in electronmicroscope studies. There is good evidence that DNA-anti-DNA antibody complexes are involved in these glomerular deposits. (*a*) The granular immunoglobulin deposits which were found as early as 3 days after the injection of LPS were simultaneous with the appearance of anti-DNA antibodies in circulating blood. A direct correlation was observed between the level of circulating anti-DNA antibodies and the intensity of the glomerular deposits. (*b*) Immunoglobulins with anti-DNA activity were eluted from the kidneys of mice injected with LPS. Quantitatively, about 40% of the immunoglobulins eluted were anti-DNA antibodies. The DNA-binding activity in the eluates was more than 50 times higher than that of the serum, at the same concentration of immunoglobulins. It was noteworthy that anti-DNA antibodies eluted from kidneys reacted with the DNA purified from plasma of mice injected with LPS. The presence of other types of immune complexes in the glomerular deposits was also suggested by the fact that about 60% of immunoglobulins in the renal eluates could not be absorbed with DNA. Such unidentified complexes may involve endogenous antigens such as immunoglobulins, or microbial antigens such as C-type virus-associated proteins. Indeed, LPS is known to activate

endogenous C-type virus in mouse spleen cell cultures (37) and this type of immune complex is found to be associated with DNA-anti-DNA complex deposits in the renal lesions of NZB × NZW F_1 hybrid mice (38). The present data do not support the hypothesis that LPS-anti-LPS complexes are involved in the renal deposits since the renal deposits also occurred in conditions where no antibodies to LPS were detectable.

The fact that DNA-anti-DNA complexes were not detected in circulating blood, and that the glomerular deposits preceded the occurrence of circulating immune complex-like material, would be in agreement with the hypothesis that the deposition of DNA-anti-DNA complexes in the glomeruli would not be due to a direct deposition of circulating complexes. Recently, it was demonstrated that, in vitro, DNA alone can bind to GBM and subsequently react with circulating free anti-DNA antibodies thus forming immune complexes directly on GBM (14). In vivo, the treatment of mice with LPS was shown to favor the binding of injected DNA to renal structures. Therefore, one can imagine that some of the DNA released into the general circulation or within renal arterioles or capillaries would first bind to GBM. This binding would be favored by the increased vascular permeability or the endothelial damage induced by LPS (34, 39, 40). In a second step, the anti-DNA antibodies appearing in circulating blood 3 days later may react with such bound DNA and form immune complexes.

Similar mechanisms may be involved in the pathogenesis of SLE, as for the deposition of immune complexes in kidneys and also for the development of lesions in other tissues. The present observations also suggest that a release of bacterial products may be partly responsible for the exacerbation of SLE during gram-negative bacterial infections.

Summary

After injection of lipopolysaccharides (LPS) in mice, there is first a release of DNA into plasma and secondly an induction of anti-DNA antibodies. The circulating DNA was purified from plasma and physico-immunochemically characterized. This DNA has a similar density to mammalian cellular DNA, is 4–6S in size, and probably represents a mixture of single-stranded DNA (SSDNA) and double-stranded DNA (DSDNA) or DSDNA with some single-stranded regions. This purified DNA was shown to react with anti-DNA antibodies which appeared as early as 3 days after a single injection of LPS in mice. In serum, DNA-anti-DNA complexes were not detected, although unidentified circulating immune complex-like material was demonstrated 5–8 days after the injection of LPS. In tissues, particularly in renal glomeruli, fine granular immune complex-type immunoglobulin deposits appeared along the glomerular capillary walls and in the mesangium 3 days after the injection of LPS. There is a direct correlation between the level of anti-DNA antibodies and the intensity of glomerular deposits and about 40% of immunoglobulins eluted from kidneys are anti-DNA antibodies, indicating that some of the immune complexes localized in kidneys are DNA-anti-DNA complexes.

Based on these observations, the following hypothetical mechanism for the glomerular localization of DNA-anti-DNA complexes after the injection of LPS in mice is proposed. First, DNA, which has been released in circulating blood after injection of LPS, might bind to renal glomeruli, probably on glomerular

basement membranes (GBM) through a high affinity of GBM for DNA; secondly, circulating anti-DNA antibodies, which appear later, might react with the glomerular-bound DNA and form immune complexes independently of circulating immune complexes. However, the possibility of direct deposition of immune complexes is not ruled out.

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