

RELEASE OF DNA IN CIRCULATING BLOOD AND INDUCTION OF ANTI-DNA ANTIBODIES AFTER INJECTION OF BACTERIAL LIPOPOLYSACCHARIDES*

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Since 1957 (1-4) antibodies against nuclear antigens and particularly against DNA have been recognized in sera from patients with systemic lupus erythematosus. Such autoantibodies have also been found in sera from NZB × NZW F₁ hybrid mice (5, 6), which spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus (7, 8). The pathogenetic role of DNA and of antibodies directed against DNA (anti-DNA antibodies), particularly in kidney injury, has been suggested both in human (9) and in animal pathology (6). This hypothesis has been further supported by the finding that NZB × NZW F₁ hybrid mice that had been previously rendered tolerant to denatured DNA failed to develop glomerulonephritis (10).

The spontaneous development of anti-DNA autoantibodies is still poorly understood. The triggering of the immune response to DNA could result from antigenic stimulation by antigens containing DNA in a particularly immunogenic state. It is likely that in mice this response is genetically controlled (11, 12). However, it is possible that this response is modulated by thymus-dependent lymphocytes or by modifications of the immune status of the host, possibly induced by exogenous agents such as viruses or bacteria. Therefore, the spontaneous development of anti-DNA antibodies may be due to the release of immunogenic DNA in a host naturally, or rendered, responsive to this type of antigen. Many clinical or experimental conditions involving bacterial (13) or viral infections (11, 14, 15) have been reported to favor the formation of antinuclear antibodies. Such antibodies have also been found in sera from animals immunized with killed gram-negative bacteria (16, 17).

The purpose of the present study was to better define the mechanisms by which bacterial infections or bacterial products may favor the formation of anti-DNA antibodies. Particular attention was given to the study of the role of bacterial lipopolysaccharide (LPS)¹ on the immune response to DNA. Indeed, LPS

* This work has been supported by the Annette Kade Foundation, the Swiss National Research Foundation (grant no. 3.617.71 SR), and by a contract from INSERM, France.

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¹ *Abbreviations used in this paper:* DS DNA, double-stranded DNA; IFA, incomplete Freund's adjuvant; LPS, lipopolysaccharide; mBSA-DNA complexes, complexes of heat-denatured DNA and methylated bovine serum albumin; SS DNA, single-stranded DNA.

preparations are well known to have several effects on the immune system. In addition to its potent adjuvant effect on antibody formation (18), LPS was shown to have a mitogenic activity on B lymphocytes (19, 20). Moreover, it was demonstrated that in some instances LPS could favor the termination of a state of unresponsiveness due to tolerance maintained at T-cell level when LPS and antigen were injected simultaneously into mice with responsive B cells (21). Since it has been postulated that a similar state of unresponsiveness would exist for some autoantigens (22, 23), one may wonder whether bacterial LPS would favor the development of autoimmunity.

In the present study, it was demonstrated that the injection of bacterial LPS into mice, without any injection of exogenous DNA, could lead to the formation of anti-DNA antibodies. Furthermore, a significant release of DNA was demonstrated in circulating blood a few hours after the injection of LPS. Both of these effects were also observed after injection of the lipid A fraction of the LPS molecule.

Material and Methods

Mice. 6-10-wk-old mice were used throughout the study. C3H, C57BL/6, DBA/2, and Balb/c female mice were purchased from Charles River Breeding Laboratories, Inc., Elbeuf, France. NZB and NZW mice were originally provided by Dr F. J. Dixon from the Scripps Clinic and Research Foundation, La Jolla, Calif., and inbred in our own animal house. All NZB \times NZW F₁ hybrids were obtained by NZB male \times NZW female matings. Athymic Balb/c nude (nu/nu) female mice, athymic C57BL/6 nude (nu/nu) male mice, and littermate mice which were heterozygote for the mutant gene (nu/+) were purchased from the Laboratory Animals Breeding and Research Center (Gl. Bomholtgard Ltd., Ry, Denmark). OF₁ outbred female mice were purchased from IFFA CREDO (Centre de recherche et d'élevage 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.

Lipopolysaccharides. Polysaccharide B of: *Salmonella typhimurium* (lot 563628), *Salmonella enteritidis* (lot 586088), *Escherichia coli* 0127:B8 (lot 582337), and *E. coli* 0111:B4 (lot 587687) were obtained from Difco Laboratories, Detroit, Mich. LPS from the mutant strain *E. coli* 0111:B4, J5 (J5 LPS) and *Salmonella minnesota* Re 595 (Re 595 LPS) and lipid A extracted from *E. coli* 0111:B4 prepared as described by Chiller et al. (24) were kindly provided by David Morrison (Scripps Clinic and Research Foundation). All these LPS preparations were diluted to the desired concentration in sterile saline and were injected intraperitoneally in a final volume of 0.2 ml.

Immunization. Calf thymus DNA type V was purchased from Sigma Chemical Co., St. Louis, Mo. Denatured DNA was prepared by heating native DNA (0.5 mg/ml in phosphate-buffered saline, pH 7.0) at 100°C for 10 min and by transferring directly to an ice bath. Methylated bovine serum albumin was purchased from Calbiochem, San Diego, Calif. Incomplete Freund's adjuvant (IFA) was obtained from Difco Laboratories. Complexes of denatured DNA and methylated bovine serum albumin (mBSA-DNA complexes) were made by adding drop by drop methylated bovine serum albumin (10 mg/ml in distilled water) to an equal weight of DNA with constant stirring. After 10 min an emulsion was made with an equal volume of IFA. These complexes were injected intraperitoneally (0.3 ml containing 75 μg of DNA per mouse).

Separation of Double-Stranded from Single-Stranded DNA. The separation of double-stranded DNA (DS DNA) from single-stranded DNA (SS DNA) was performed using methylated albumin kieselguhr chromatography according to Sueoka and Cheng (25). 1.5×10 -cm columns were run by stepwise elution with solutions of increasing NaCl molarity (0.1, 0.25, 0.50, 1.0) buffered with 0.05 M PO_4 , pH 6.7. The last buffer was 1 M NaCl, 1.5 M NH_4OH . As reported by Tan and Natali (26), DS DNA was eluted with 0.5 M NaCl buffer, while SS DNA was eluted with 1 M NaCl, 1.5 M NH_4OH buffer.

Radioimmunological Detection of Anti-DNA Antibodies. The titration of anti-DNA antibody

was done according to Wold et al. (27), by precipitation of globulin-bound radiolabeled DNA with 50% saturated ammonium sulfate. DNA was labeled either externally with iodine-125 or internally with tritium. SS DNA was iodinated according to Commerford (28). [¹²⁵I]DNA had a specific activity of about 4×10^5 cpm/ μ g at 30% counting efficiency. [³H]DNA was prepared from human fibroblast cells L₁₃₂ grown in culture in presence of [³H]thymidine. DNA was isolated by Marmur's technique (29). The preparation of [³H]DNA had a specific activity of 3×10^5 cpm/ μ g at 33% counting efficiency. Purified DS [³H]DNA was obtained by fractionation on methylated albumin kieselguhr column. SS [³H]DNA was obtained by heat denaturation. Titrations were done as follows: 0.1 ml of serum diluted 1/10 in borate buffer (pH 8.4, ionic strength 0.1) was heated at 56°C for 30 min, then mixed with 0.2 μ g of [³H]DNA (DS [³H]DNA or SS [³H]DNA) or 0.01 μ g of [¹²⁵I]DNA in 0.1 ml of borate buffer. These mixtures were incubated overnight at 4°C; then 1.8 ml of 55.55% saturated ammonium sulfate was added. After an incubation at 4°C for 30 min, the precipitates were centrifuged at 1,000 *g* for 30 min, then washed once with 50% saturated ammonium sulfate. [¹²⁵I]DNA precipitated was directly counted in a gamma counter. [³H]DNA precipitated was dissolved in 0.5 ml of heated (70°C) distilled water and transferred into 15 ml of Dioxan scintillation fluid for counting in a LS 250 Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The results were expressed as a percentage of radiolabeled DNA precipitated and compared to the non-specific precipitation obtained with normal sera from the various strains of mice which were studied. Titration of anti-DNA antibodies was also done on sera fractionated by gel filtration on Sephadex G 200. 0.5 ml of pools of sera previously dialyzed for 24 h against borate buffer was applied to a Sephadex G 200 column and eluted with borate buffer. For titrations, 90 μ l of fraction sample was added to 10 μ l of normal mouse serum.

Detection of Other Antibodies. Antibodies to *S. typhimurium* LPS were titrated by passive hemagglutination as previously described (30). Antibodies to autologous erythrocytes were detected by direct Coombs' test (31).

Estimation of DNA Content in Serum Samples. DNA content in serum samples was determined by inhibition of radioimmunoassay. 0.1 ml of serum presumed to contain DNA, diluted 1/10 in borate buffer and heated at 56°C for 30 min, was added to 0.1 ml of [¹²⁵I]DNA (0.01 μ g) or to 0.1 ml of [³H]DNA (0.2 μ g) diluted in the same buffer. To these mixtures was added 0.1 ml of serum from a lupus patient which contained anti-DNA antibodies directed against both SS DNA and DS DNA diluted 1/50 in borate buffer and heated at 56°C for 30 min. After an overnight incubation at 4°C, 2.7 ml of 55.55% saturated ammonium sulfate were added and the percentage of radiolabeled DNA precipitated was measured. In the absence of cold DNA, about 40–60% specific precipitation was obtained. A standard inhibition curve was established with normal mouse serum containing increasing amounts of cold calf thymus DNA. Cold DS DNA and cold SS DNA were found to give a similar inhibition of the precipitation of radiolabeled DNA. In this system, DNA added to normal mouse serum at a concentration of 1 μ g/ml could be detected with a good reproducibility. The amount of DNA present in tested serum samples was estimated by reference to the standard curve. In some experiments, fractions eluted from methylated albumin kieselguhr columns were tested for DNA content. In these cases, after an overnight dialysis of the fraction against borate buffer, 90 μ l of these fractions were added to 10 μ l of normal mouse serum previously tested for the absence of detectable amounts of DNA. The DNA content was then measured in 0.1 ml as described above. When inhibition was observed, its specificity was controlled in each case by pretreatment of the tested samples with DNase. 0.1 ml of DNase (Deoxyribonuclease 1, 2600/mg, Worthington Biochemical Corp., Freehold, N. J.) at a concentration of 0.4 mg/ml in Veronal buffer, pH 7.2, (Tampon Veronal-magnesium-calcium, Institut Merieux, Marcy l'Etoile, Rhone, France) was added to 0.025 ml of the sample. Then, after an incubation of 3 h at 37°C, 0.025 ml of 1 M EDTA and 0.1 ml of borate buffer were added. After heating the mixture at 56°C for 30 min, 0.1 ml was used for DNA estimation. In the control tubes, EDTA was added before DNase in order to prevent its enzymatic action.

Results

Induction of Anti-DNA Antibodies by LPS. In the first group of experiments, the effects of *S. typhimurium* LPS were studied on the anti-DNA response induced by mBSA-DNA complexes in 6-wk old NZB \times NZW F₁ hybrid mice

known to be good responders to DNA (11). Four groups of seven NZB \times NZW females were studied: The first group was immunized by intraperitoneal injections of mBSA-DNA complexes in IFA (75 μ g of DNA) on days 0, 7, and 14. The second group was immunized as the first group with mBSA-DNA complexes but was injected intraperitoneally 4 h later with *S. typhimurium* LPS (50 μ g on day 0, 25 μ g on days 7 and 14). As a control, a third group was injected with the same amount of LPS but without any mBSA-DNA, and a fourth group with saline alone. The titers of serum anti-DNA antibodies, expressed in percent of [3 H]DNA precipitated, were measured on days 13, 21, 28, 35, and 55, and are represented in Fig. 1. It was found that the antibody response directed against SS DNA elicited by immunization with mBSA-DNA complexes in IFA was not increased by additional injections of LPS. However, a significant anti-DNA antibody response was induced by injections of LPS alone. Mice receiving saline

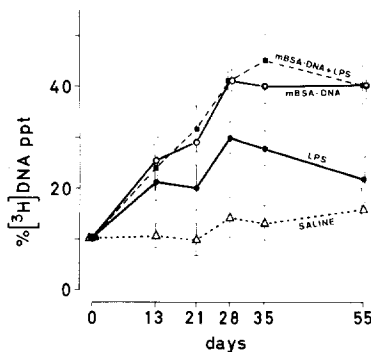


FIG. 1. Anti-DNA antibody levels of young NZB \times NZW F_1 hybrid female mice immunized with mBSA-DNA complexes (○) or mBSA-DNA + LPS (■), mice injected with LPS alone (●), or with saline alone (Δ). The injections were done on days 0, 7, and 14. Anti-DNA antibody levels are expressed as the mean percentage of SS [3 H]DNA precipitated obtained in each group. Vertical bars represent the limits of 1 SD.

did not show any significant modification of DNA-binding capacity during that period. The kinetics of the anti-DNA antibody response observed in the groups immunized with mBSA-DNA complexes were different from those observed in the group injected with LPS alone. In the former groups, levels of anti-DNA antibodies increased until day 28 and then remained high, while in the group receiving LPS alone, the anti-DNA levels increased similarly until day 28 but slowly decreased afterwards. In a similar experiment, a group of mice received injections of IFA alone. There was no increase of DNA-binding capacity through day 21, but a slight and transient increase was observed on days 28 and 35.

In the second group of experiments, this induction of anti-DNA antibodies by injections of LPS alone was studied in six strains of mice. 6-wk old female NZB \times NZW F_1 hybrids, C57BL/6, C3H, Balb/c, NZW, and DBA/2 mice were injected intraperitoneally with *S. typhimurium* LPS (50 μ g on day 0, 25 μ g on days 7 and 14). Individual serum samples were collected on days 0, 14, 28, 50, and 70 for measuring DNA-binding capacity. The mean values obtained in each group are shown in Fig. 2. One can see that five inbred strains of mice, as well as NZB \times

NZW hybrids, responded to LPS by formation of anti-DNA antibodies. At the doses used in this experiment, some strain-dependent differences existed in the anti-DNA response. NZB \times NZW and C57BL/6 mice showed an early and relatively high anti-DNA response, while Balb/c, NZW, and DBA/2 mice were relatively low responders. C3H mice showed an anti-DNA response which was halfway between the two previous groups. The kinetics of the anti-DNA antibody production in all the groups injected with LPS were similar. DNA-binding capacity of sera reached a peak on day 28 and then decreased. The hemagglutinating titers of antibodies to *S. typhimurium* LPS were measured on days 13 and 35 in these groups of mice injected with LPS. It was found that the anti-LPS response was quite similar in all strains except DBA/2, which exhibited a lower

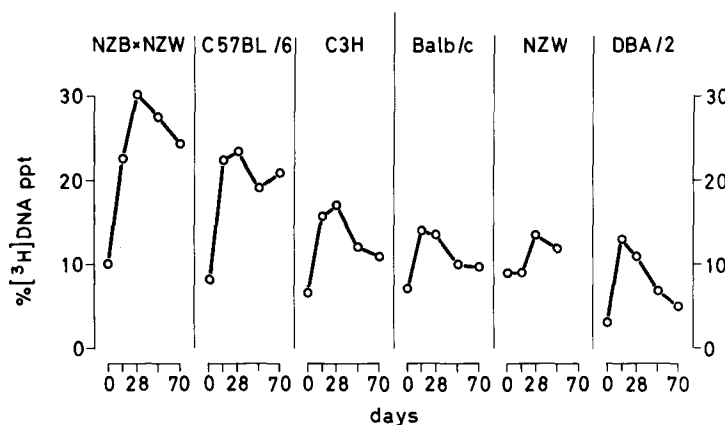


FIG. 2. Induction of anti-DNA antibodies in various strains of mice by injections of LPS. 50 μ g of *S. typhimurium* LPS was injected intraperitoneally on day 0, 25 μ g on days 7 and 14. Anti-DNA antibody levels are expressed as the mean percentage of SS [3 H]DNA precipitated obtained in each strain.

hemagglutinating titer. The Coombs' test, which was performed in NZB \times NZW and C57BL/6 mice on days 13 and 28, remained negative after the injections of LPS. In order to compare the anti-DNA response induced by injections of LPS with that induced by immunization with exogenous DNA, similar groups of the same strains of mice were immunized with mBSA-DNA complexes (75 μ g of DNA on days 0, 7, and 14). The levels of anti-DNA antibodies measured at day 28 after stimulation either with LPS or with mBSA-DNA are shown in Table I. It is evident that NZB \times NZW and C57BL/6 mice were good responders while DBA/2 were poor responders to both kinds of stimuli. However, C3H mice showed a better anti-DNA response after injections of LPS than after immunization with mBSA-DNA. Inversely, NZW mice showed a better anti-DNA response after immunization with mBSA-DNA than after injection of LPS. When serum samples obtained later (days 50 and 70) were analyzed, it appeared that anti-DNA levels were still increasing in all strains except DBA/2 after injection of mBSA-DNA, while they were generally decreasing slowly in mice receiving LPS.

In the third group of experiments, the effects of LPS were studied in athymic

nude mice. Homozygous (nu/nu) C57BL/6 nude mice, Balb/c (nu/nu) mice, and littermate heterozygous (nu/+) C57BL/6 and Balb/c mice were injected with *S. typhimurium* LPS (50 μ g on day 0, 25 μ g on days 7 and 14). As a control, similar groups of mice were injected with sterile saline. The mean values of SS [³H]DNA binding capacity obtained on serum samples collected on days 13 and 28 are shown in Table II. LPS induced a significant anti-DNA response in C57BL/6 nude mice and this response was similar to the anti-DNA response induced by LPS in C57BL/6 heterozygous mice. Such anti-DNA response was not observed in Balb/c nude mice, while a significant increase in DNA-binding capacity was observed in heterozygous (nu/+) Balb/c mice after the injection of LPS.

TABLE I
Comparative Studies of the Anti-DNA Response after LPS or mBSA-DNA

Mice strains*	% SS [³ H]DNA ppt \pm 1 SD \ddagger	
	LPS \S	mBSA-DNA \parallel
NZB \times NZW	29.8 \pm 3.0	41.0 \pm 2.1
C57BL/6	23.0 \pm 7.0	22.8 \pm 5.0
C3H	16.5 \pm 4.9	9.4 \pm 2.4
Balb/c	13.9 \pm 4.4	12.8 \pm 5.6
NZW	13.5 \pm 2.3	20.4 \pm 2.8
DBA/2	11.1 \pm 2.5	8.5 \pm 3.9

* 6-wk old female mice (5-8 in each group).

\ddagger DNA-binding test performed on day 28 with 0.2 μ g SS [³H]DNA and 0.1 ml of mouse serum diluted 1:10.

\S 50 μ g of *S. typhimurium* was injected on day 0, 25 μ g on days 7 and 14.

\parallel 75 μ g of mBSA-DNA complex was injected in IFA on days 0, 7, and 14.

TABLE II
Anti-DNA Antibody Response to Injections of LPS in Nude Mice

Genotype	Group	% SS [³ H]DNA ppt \pm 1 SD*			
		C57BL/6 \ddagger		Balb/c \S	
		D13	D28	D13	D28
nu/nu	LPS \parallel	13.0 \pm 2.9	18.0 \pm 2.0	9.8 \pm 1.7	8.4 \pm 3.4
nu/nu	Saline \P	9.9 \pm 4.0	11.9 \pm 1.0	9.2 \pm 2.3	8.0 \pm 2.0
nu/+	LPS \parallel	16.5 \pm 2.4	19.1 \pm 2.3	9.4 \pm 1.1	13.9 \pm 4.4
nu/+	Saline \P	13.0 \pm 2.9	11.9 \pm 3.3	6.5 \pm 0.7	5.3 \pm 1.0

* DNA-binding test performed with 0.2 μ g SS [³H] DNA and 0.1 ml of mouse serum diluted 1:10.

\ddagger 8-wk old C57BL/6 male mice (five in each group).

\S 8-wk old Balb/c female mice (seven in each group).

\parallel 50 μ g of *S. typhimurium* LPS was injected on day 0, 25 μ g on days 7 and 14 (in 0.2 ml sterile saline).

\P 0.2 ml sterile saline on days 0, 7, and 14.

Role of the Doses and of the Nature of the LPS Preparations. The induction of anti-DNA antibodies by injections of LPS was studied in relation to the doses of LPS injected. Four groups of C57BL/6 female mice were injected with different doses of *S. typhimurium* LPS ranging from a total dose of 20 μ g-800 μ g/mouse. One other group was immunized with mBSA-DNA complexes. It can be seen in Fig. 3 that the anti-DNA response induced by injections of LPS was related to the amount of LPS injected. At high doses of LPS, the titer of anti-DNA antibodies measured on day 28 was higher than that observed after immunization with mBSA-DNA complexes. The titers of anti-DNA antibodies induced by LPS of various origin was studied on 10-wk old NZB \times NZW females which were injected intraperitoneally with the same amount (50 μ g on day 0, 25 μ g on days 7 and 14) of LPS from *S. typhimurium*, *E. coli* 0127:B8 and *S. enteritidis*. All the

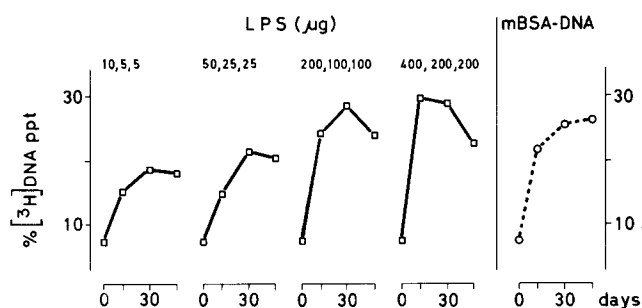


FIG. 3. Anti-DNA antibody response of 6-wk old C57BL/6 female mice after injections of various doses of *S. typhimurium* LPS (\square) or after immunization with mBSA-DNA complexes (75 μ g DNA) (\circ). Groups of seven mice were injected intraperitoneally on days 0, 7, and 14. Titers of anti-DNA antibodies are expressed as the mean percentage of SS [3 H]DNA precipitated obtained in each group.

LPS tested were able to induce an anti-DNA response, but the kinetics of this response were dependent on the origin of the LPS injected. Indeed, there was no significant response induced by *S. enteritidis* LPS until day 45 (Table III).

To better define the active part of the molecule of LPS, LPS preparations obtained from mutant bacteria and lipid A fraction extracted from *E. coli* were tested for their ability to induce anti-DNA antibodies in C57BL/6 mice. Groups of 8-wk old mice were injected with the same amount (50 μ g on day 0, 25 μ g on days 7 and 14) of LPS from *E. coli* 0111:B4, or from the mutant strains *E. coli* 0111:B4, J5 and *S. minnesota* Re 595, or of lipid A extracted from *E. coli* 0111:B4. As a control, a group of mice was injected with saline alone. The individual titers of anti-DNA antibodies measured on day 13 are shown in Fig. 4. All the LPS preparations injected induced a significant increase in the DNA-binding capacity. The highest response was obtained with mutants with a relatively high content of lipid A (Re 595 LPS) and with purified lipid A. A lower response was observed with a more complete molecule of LPS (J5 LPS), which, on a weight basis, contains a lower amount of lipid A. The lowest response was observed with the LPS preparation which, on a weight basis, contains the lowest amount of lipid A.

Characteristics of Anti-DNA Antibodies Induced by LPS. The specificity of anti-DNA antibodies induced by LPS was studied by using SS [^3H]DNA and purified DS [^3H]DNA. As shown in Table IV, sera from mice injected with *S. typhimurium* LPS had a DNA-binding capacity directed mainly against SS DNA. However, a significant increase of DS DNA-binding capacity was also

TABLE III
Effect of LPS from Various Sources on Anti-DNA Antibody Production in NZB \times NZW Mice

Group*	% SS [^3H]DNA ppt \pm 1 SD \ddagger		
	D21	D28	D45
<i>S. typhimurium</i> LPS \S	26.5 \pm 3.9	29.6 \pm 4.9	32.9 \pm 4.2
<i>E. coli</i> O127:B8 LPS \S	22.2 \pm 6.2	26.9 \pm 8.4	36.1 \pm 4.9
<i>S. enteritidis</i> LPS \S	14.6 \pm 5.1	20.4 \pm 4.2	29.6 \pm 5.2
Saline \parallel	12.5 \pm 4.8	17.7 \pm 2.7	18.0 \pm 1.4

* 10-wk old NZB \times NZW F₁ hybrid female mice (5-7 mice in each group).

\ddagger DNA-binding test performed with 0.2 μg SS [^3H]DNA and 0.1 ml mouse serum diluted 1:10.

\S 50 μg of LPS was injected on day 0, 25 μg on days 7 and 14 (in 0.2 ml sterile saline).

\parallel 0.2 ml sterile saline on days 0, 7, and 14.

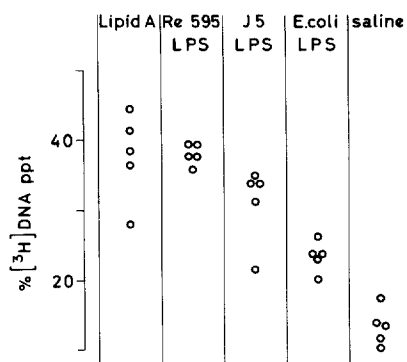


FIG. 4. Induction of anti-DNA antibodies in 6-wk old C57BL/6 female mice by injections of various LPS preparations. Five mice in each group were injected with 50 μg of the LPS preparation on day 0, 25 μg on days 7 and 14. Individual anti-DNA antibody levels obtained on day 13 are represented by the percentage of SS [^3H]DNA precipitated.

observed. In mice injected with lipid A, the DNA-binding capacity was high, not only for SS [^3H]DNA, but also for DS [^3H]DNA. The kinetics of antibodies directed against DS DNA were similar to the kinetics of antibodies directed against SS DNA. The specificity of these anti-DNA antibodies was further confirmed by inhibition studies with cold DS DNA and cold SS DNA from calf thymus. With DS [^3H]DNA as antigen (0.2 μg), the binding capacity of anti-DNA antibodies induced by lipid A was strongly inhibited by prior

incubation with 2.5 μg of either DS DNA or SS DNA. With SS [^3H]DNA as antigen (0.2 μg), the binding capacity of the same antibodies was not inhibited at all by prior incubation with 2.5 μg of DS DNA, but was strongly inhibited by the addition of 2.5 μg of SS DNA. These studies indicate that the sera from mice injected with lipid A did contain antibodies with two types of specificity: some reacting only with the antigenic determinants of SS DNA and some reacting with antigenic determinants common to SS DNA and to DS DNA.

The anti-DNA antibodies induced by LPS were further defined by gel filtration on Sephadex G 200. By using pools of sera collected on day 28 from C57BL/6 mice

TABLE IV
Specificity of Anti-DNA Antibodies Induced by LPS and Lipid A

Group*	% [^3H]DNA ppt ± 1 SD \ddagger	
	DS [^3H]DNA	SS(^3H]DNA
LPS \S	9.6 \pm 2.4	23.1 \pm 2.8
Lipid A \parallel	22.1 \pm 3.4	37.9 \pm 6.2
Saline ∇	4.4 \pm 1.4	7.1 \pm 0.9

* 6-wk old C57 BL/6 females (5-7 in each group).

\ddagger Measured on day 13 by using 0.2 μg of [^3H]DNA and 0.1 ml mouse serum diluted 1:10.

\S 50 μg of *S. typhimurium* was injected on day 0, 25 μg on day 7.

\parallel 50 μg of lipid A extracted from *E. coli* 0111:B4 was injected on day 0, 25 μg on day 7.

∇ 0.2 ml sterile saline on days 0 and 7.

injected with *S. typhimurium* LPS, a [^{125}I]DNA-binding activity was detected in both the first and second peaks eluted from the Sephadex G 200 column. From quantitative estimation, it was found that the amount of antibody contained in the first peak was higher than that of the second peak. When anti-DNA antibodies were induced in mice of the same strain by immunization with mBSA-DNA complexes and then fractionated in a similar way, a larger part of the DNA-binding activity was found in the second peak of elution. The characteristics of anti-DNA antibodies induced in poor responder mice may be different. Indeed, pools of sera collected on day 28 from DBA/2 mice injected with either LPS or with mBSA-DNA complexes were shown to contain anti-DNA antibodies which were eluted only in the first peak of the Sephadex G 200 column.

Release of DNA in Circulating Blood after Injections of LPS. Since it is known that bacterial LPS can lead in vivo to tissue destruction and cellular disruption, the possibility that endogenous nuclear material might be released and play a role in the production of anti-DNA antibodies was investigated. For this purpose, 10 C57BL/6 mice were injected intraperitoneally with 300 μg of *S. typhimurium* LPS and bled at various times after injection. Sera were collected

very carefully to prevent any cell destruction, and the amount of DNA present in serum was evaluated by inhibition of DNA-binding test. A very significant inhibition of the binding of SS [^3H]DNA and of [^{125}I]DNA by anti-DNA antibodies from the lupus patient serum used was observed in presence of serum samples collected between 4 and 20 h after the injection of LPS. By using a standard inhibition curve, the amount of circulating DNA was estimated (Fig. 5). With reference to calf thymus DNA, a peak of approximately 40 $\mu\text{g}/\text{ml}$ of DNA was found in circulating blood after 11 h. This experiment was repeated several times and the highest concentration of DNA in serum was usually detected 8-10 h after the injection of LPS. Prior treatment of the tested sera with DNase resulted in an almost complete disappearance from the sera of the material responsible for the inhibition of the DNA binding (Fig. 5). The presence of a significant amount of DNA in the preparation of LPS injected was excluded

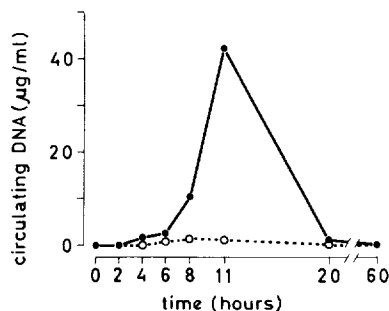


FIG. 5. DNA released in circulating blood after the injection of 300 μg of *S. typhimurium* LPS in 10 C57BL/6 mice. Pools of sera were collected at various times after this injection and assayed for DNA content by inhibition of [^3H]DNA binding. Closed circles represent the amount of circulating DNA. Open circles represent the amount of circulating DNA still detectable after pretreatment of the serum samples with DNase.

since LPS did not inhibit the binding of SS [^3H]DNA by the anti-DNA antibodies from the lupus patient serum. In similar experiments, it was found that *E. coli* 0111:B4 LPS and lipid A could induce a release of DNA in circulating blood like *S. typhimurium* LPS. Moreover, a second injection of LPS, 7 days after the first injection, again induced a similar release of DNA. In another group of experiments, other strains of mice were studied. Circulating DNA was detected in serum after the injection of LPS in all the strains tested: NZB \times NZW F₁ hybrids, Balb/c, homozygous nude Balb/c, and OF₁ outbred mice.

Characteristics of the DNA Released after Injection of LPS. The physico-chemical nature of DNA circulating in mouse blood 7 h after the injection of 300 μg of *S. typhimurium* LPS was studied by chromatography on methylated albumin kieselguhr column. Preliminary studies using [^3H]DNA in its native form and after heat denaturation indicated that in our experimental conditions native DNA was mostly eluted in 0.5 M NaCl, while heat-denatured DNA was eluted in 1 M NaCl, 1.5 M NH_4OH phosphate buffer (Fig. 6). DNA-containing serum, obtained after injections of LPS, was processed in similar conditions and the fractions eluted at various salt concentrations were checked after dialysis in

borate buffer for their content of DNA by inhibition of [^3H]DNA or [^{125}I]DNA-binding test. The amount of DNA was estimated with reference to a standard inhibition curve performed with calf thymus DNA. As shown in Fig. 6, there is a small amount of DNA present in normal mouse serum which is eluted at the molarity corresponding to the elution of SS DNA. Serum from mice injected with LPS contained a much greater amount of DNA, which is eluted in two peaks. The smaller peak is eluted at the molarity corresponding to the elution of DS DNA

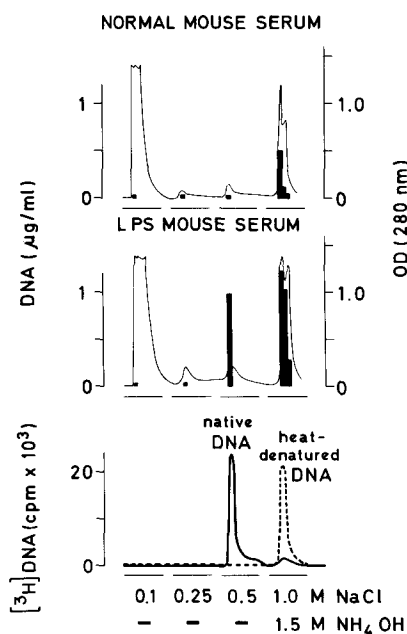


FIG. 6. Physicochemical characteristics of circulating DNA on methylated albumin kieselguhr column. The upper part of the graph represents the pattern of elution of DNA detected either in normal mouse serum or in serum from mice injected with LPS. The vertical bars correspond to the concentration of DNA in each fraction studied. The optical density of these fractions is indicated by the continuous lines. The lower part of the graph represents the pattern of elution obtained with native [^3H]DNA and with heat-denatured [^3H]DNA.

and the larger one at the molarity corresponding to the elution of SS DNA. Prior treatment of the fraction samples by DNase prevented their inhibitory effect on [^3H]DNA-binding by anti-DNA antibodies.

The DNA released in circulating blood after injection of LPS was further characterized by immunochemical analysis. The inhibitory activity of a serum pool from mice injected with LPS was compared to that of cold SS DNA and cold DS DNA by using as antibody a lupus patient serum and as antigen either SS [^3H]DNA or DS [^3H]DNA. This anti-DNA antibody was selected for its specificity which allowed for a differentiation between SS DNA and DS DNA (with DS [^3H]DNA as antigen) and for an estimation of the total content of DNA in serum (with SS [^3H]DNA as antigen). Indeed, this antibody had a higher avidity for DS DNA than for SS DNA. The ratio of the absolute amounts of DNA

bound in presence of 0.225 μg or 2.7 μg of DNA was 0.15 for SS [^3H]DNA and 0.42 for DS [^3H]DNA. With this particular anti-DNA antibody, in the conditions of the experiment, the addition of cold SS DNA inhibited equally the binding of SS [^3H]DNA and of DS [^3H]DNA (Table V). On the other hand, the addition of cold DS DNA inhibited more strongly the binding of DS [^3H]DNA than that of SS [^3H]DNA. When the inhibitory activity of serum from mice injected with LPS was evaluated, it was found that the pattern of inhibition was largely related to that obtained with cold SS DNA. Indeed, for this particular pool of mouse serum, the amount of DNA estimated by inhibition of the binding of SS [^3H]DNA would be approximately equivalent to 25 $\mu\text{g}/\text{ml}$ of either cold DS DNA or cold SS DNA. In contrast, the values obtained by inhibition of DS [^3H]DNA binding would be

TABLE V
Immunochemical Study of DNA Released after Injection of LPS

Added serum	% [^3H]DNA ppt*	
	SS [^3H]DNA	DS [^3H]DNA
NMS‡	39	41
LPS-MS§	29	26
NMS + SS DNA (25 $\mu\text{g}/\text{ml}$)	31	28
NMS + DS DNA (25 $\mu\text{g}/\text{ml}$)	29	17

* 100 μl of anti-DNA systemic lupus erythematosus serum 1:50 added to 100 μl of tested serum 1:10 then to 0.2 μg [^3H]DNA in a direct-binding test.

‡ Pool of sera from 6-wk old normal C57BL/6 female mice.

§ Pool of sera from 6-wk old C57BL/6 female mice collected 8 h after an intraperitoneal injection of 200 μg of *S. typhimurium* LPS.

|| 10 μl of calf thymus DNA (25 $\mu\text{g}/\text{ml}$) in 100 μl of normal mouse serum diluted 1:10.

approximately equivalent to either 25 $\mu\text{g}/\text{ml}$ of cold SS DNA or to 5 $\mu\text{g}/\text{ml}$ of cold DS DNA. Therefore, it is likely that most of this circulating DNA reacts immunochemically in a similar way to SS DNA.

Discussion

The present data are concerned with two different effects of bacterial LPS observed in mice: first, a release of DNA in circulating blood, and secondly, an induction of anti-DNA antibodies. The relationship between these two phenomena and their pathogenic significance will be discussed.

In the early phase after injection of LPS into mice, a release of DNA in circulating blood was demonstrated in several experiments. The appearance of DNA in the circulating blood a few hours after the injection of LPS is not a phenomenon restricted to some particular mice, since it was found in several inbred and outbred strains of mice. It should be pointed out that the DNA

estimations were done with reference to DNA from a different species and therefore the estimations should be considered as approximate and likely to be influenced by the molecular size and by the nature of released DNA, rather than absolute quantitation. The physicochemical and immunochemical data suggested that most of the DNA circulating after the injection of LPS had several characteristics of SS DNA. Indeed, the elution profile of this released DNA on methylated albumin kieselguhr columns is similar to that of a mixture containing a small amount of DS DNA and a large amount of SS DNA. It is also possible that released DS DNA would acquire a high affinity for methylated albumin kieselguhr, through the uncoiling of one part of the DNA molecule or through the binding to cellular components or to serum proteins. The immunochemical analysis also indicates that the specificity of the released DNA corresponds largely to that of SS DNA. The antibody used in these inhibition studies reacted with antigenic determinants present in both SS DNA and DS DNA but had a higher avidity for DS DNA. Our data did not exclude the presence of DS DNA in sera from mice injected with LPS, but suggested strongly that DNA with the characteristics of SS DNA was responsible for most of the inhibition of the DNA binding by the anti-DNA antibodies used in these experiments.

The mechanism responsible for the release of DNA into circulating blood after the injection of LPS has not been elucidated, but several hypotheses may be considered. First, it is well known that bacterial endotoxins have a cytotoxic effect on nucleated cells *in vitro* (32, 33). A similar effect may lead to a release of endogenous nuclear material from cells disrupted "in vivo." Secondly, severe generalized reactions may be induced in the host by the injection of LPS. When injected in sufficient amounts, endotoxins may cause a hemorrhagic shock, and smaller doses would cause fever, leukopenia, hemorrhagic necrosis, and a wide variety of circulatory disturbances (32, 34). These manifestations lead indirectly to disseminated cell disruptions which may also account for a release of endogenous nuclear material. Thirdly, it was shown that during endotoxin hemorrhagic shock, there is an absorption of bacterial material from the bowel (35), which might lead to the appearance of exogenous nuclear material in serum. Apart from the questionable origin of DNA released in serum, the persistence of a relatively high concentration of DNA in the circulating blood during a few hours can only be explained either by a continuous and intense release of DNA during this period or by a particular form of this DNA, since it is known that the clearance of circulating DNA in mice is very fast, with a half-life of a few minutes (36).

It was surprising to see that the injection of LPS alone induced an immune response to DNA. In the first experiment, young NZB \times NZW F₁ hybrid mice, which would later naturally develop such anti-DNA antibodies, were used, and therefore the effect of LPS might be interpreted as an enhancement of a natural phenomenon. However, it was clear that the injections of LPS were inducing similar anti-DNA antibodies in other strains of mice such as C57BL/6 or C3H, which do not spontaneously develop a significant level of anti-DNA antibodies. This induction of anti-DNA antibodies by LPS was quite efficient since C57BL/6 mice developed even higher titers of anti-DNA antibodies after injections of a high dose of LPS than after immunization with mBSA-DNA complexes. The increase in [³H]DNA binding induced by LPS is due to the presence in serum of

antibodies with two types of specificity. Most of them react with antigenic determinants present only on SS DNA, but it is of particular importance to note that antibodies reacting with DS DNA appeared in the serum of mice (C57BL/6) injected with LPS or with lipid A. This observation is of interest since it has been frequently stated that anti-DS DNA antibodies appear only in systemic lupus erythematosus or in the natural disease of NZB \times NZW F₁ hybrid mice (37, 38). It should be noted that the DS [³H]DNA used in these studies has been previously purified on a methylated albumin kieselguhr column and was shown to contain less than 1% of [³H]DNA reacting with anti-SS DNA antibodies. Moreover, the specificity of these anti-DS DNA antibodies has been confirmed by inhibition experiments. The elution profile of anti-DNA antibodies induced by LPS on Sephadex G 200 suggests that both 19S and 7S antibodies are present in the sera obtained after 4 wk. In some strains of mice such as DBA/2 mice, most of the anti-DNA antibody activity found after injections of LPS is present in the 19S peak. Similar patterns have been previously described in DBA/2 mice immunized with mBSA-DNA complexes (39). The active part of the LPS molecule in the induction of anti-DNA antibodies seems to be common to various gram-negative bacterial endotoxins, since similar effects were observed with LPS extracted from *S. typhimurium*, *S. enteritidis*, or *E. coli*. It appears to be the phospholipid fraction containing lipid A since there is a positive correlation between the titers of anti-DNA antibodies induced in mice by injections of equivalent amounts of lipid A or of LPS from various mutant bacteria and the absolute concentration of lipid A per mole in these LPS preparations. Lipid A was also shown to be responsible for the ability of LPS to induce lymphocyte mitogenesis and to act as an adjuvant on antibody formation (24).

Several mechanisms can be proposed in order to explain the induction of anti-DNA antibodies by LPS. The effect of LPS on the immune system should be considered as well as the release of DNA which has been demonstrated in circulating blood. In addition, a genetic background probably modulates the intensity of the anti-DNA response induced by LPS. The studies performed in several inbred strains of mice show that some strains developed a good anti-DNA response after injections of LPS, while others are poor responders.

The pattern of the anti-DNA response induced by LPS corresponds largely to the pattern of the anti-DNA response induced by immunization with mBSA-DNA in the same strains of mice. Since the immune response to DNA has been shown to be under genetic control (11), it is likely that there is a similar control for the anti-DNA response induced by LPS. However, it should be pointed out that in C3H mice, which respond poorly to mBSA-DNA, a better response could be obtained after injections of LPS, indicating that other control mechanisms may be involved in this response. On the other hand, there is no correlation of the intensity of the anti-DNA response induced by LPS with the level of antibody directed against the antigenic part of the PLS molecule, nor with the *H-2* histocompatibility locus, since NZB, Balb/c, and DBA/2 mice have the same *H-2^d* locus.

The effects of LPS on the immune system may proceed through several channels. First, high doses of LPS may depress the host resistance to viral or bacterial infections (40), and therefore favor the development of a natural infection eventually responsible for the triggering of the production of anti-DNA antibodies. However, this effect on LPS on host resistance is very much dose dependent, and small doses of LPS in fact increase the

resistance to infections (41, 42). The induction of anti-DNA antibodies by LPS does not depend on a similar dose effect relationship since the titer of anti-DNA antibodies is directly related to the amount of LPS injected. Secondly, it is well known that LPS is a potent mitogen for B lymphocytes (19, 20) and it has been shown, *in vitro*, that LPS could induce a polyclonal stimulation of such lymphocytes and lead to the appearance of plaque-forming cells reacting with various antigens (43). A similar polyclonal effect might be expected *in vivo*, but it has not been reported as yet. Moreover, in our experiments, the injection of LPS in NZB \times NZW F₁ hybrid mice is followed by a production of anti-DNA antibodies, but not of autoantibodies reacting with autologous red cells. Thirdly, the nonspecific adjuvant effect of LPS does not seem to play a major role in the anti-DNA response since LPS did not enhance the immune response to mBSA-DNA. Furthermore, the injection of IFA alone in NZB \times NZW mice only induced a late, very slight, and transient response to DNA. Fourthly, LPS was shown to favor the immune response to thymus-dependent antigens in animals deprived of T lymphocytes (44). This effect may be of particular importance in the immune response to autologous antigens. An immune response to human IgG globulins can be elicited by simultaneous injection of this antigen and of LPS in mice previously rendered unresponsive to this antigen, but characterized by a state of T-cell tolerance or of B-cell responsiveness (21). Moreover, as shown by Louis et al. (45), LPS interferes with the induction of tolerance by injection of soluble IgG and converts it to an immunization process. Since it has been shown that there is a release of DNA into circulating blood which occurs a few hours after the injection of LPS, one may wonder whether the simultaneous action of LPS and of soluble DNA on B lymphocytes would not lead to a state of immunity comparable to that obtained in the IgG system. The role of a stimulation of B lymphocytes in the induction of anti-DNA antibodies by LPS is further suggested by the fact that the injection of LPS into athymic homozygous nude C57BL/6 mice results in a production of anti-DNA antibodies similar to that observed in heterozygous (nu/+) C57BL/6 mice. However, since it is known that a few T lymphocytes may persist in nude mice (46, 47), one cannot exclude, as suggested by recent observation (48, 49), that the effect of LPS on anti-DNA antibody production also involves thymus-derived lymphocytes. Fifthly, one should also consider the possibility that the DNA which is released in circulating blood after the injection of LPS would be in a particularly good immunogenic form.

The present observation may have a double pathogenic significance. The fact that LPS can induce anti-DNA antibodies in mice suggests that these phenomena may play a role in the triggering or in the enhancement of the production of anti-DNA antibodies occurring occasionally during infections with gram-negative bacteria. Analogous mechanisms might be involved in the induction of anti-DNA antibodies during some viral infections and, perhaps, in the development or exacerbation of systemic lupus erythematosus. A chronic release of bacterial endotoxins *in vivo* might also lead to a chronic release of DNA in the presence of anti-DNA antibodies. Therefore, DNA-anti-DNA complexes might be formed and prepare the way for the development of a lupus type immune complex disease.

Summary

The present data demonstrate the induction of antisingle-stranded (SS) DNA and antidouble-stranded DNA antibodies in various strains of mice, including athymic C57BL/6 nude mice, after the injection of bacterial lipopolysaccharide

(LPS). This anti-DNA response is dose dependent and varies quantitatively according to the strain of the injected mice. It is not correlated to the *H-2* histocompatibility locus nor to the immune response to LPS. The lipid A fraction appears to be the active part of the LPS molecule for this particular effect. In addition, it was found that DNA is released in circulating blood a few hours after the injection of LPS. Most of the DNA released has physicochemical and immunochemical characteristics of SS DNA. Therefore, the anti-DNA response induced by injections of LPS may be the result of a release of DNA in a particularly immunogenic form at a time when the immune system, in particular the B lymphocytes, is rendered capable by LPS of developing an immune response to such a soluble antigen. These effects of LPS may account for the triggering or the exacerbation of anti-DNA antibodies during infections with gram-negative bacteria, and a similar mechanism may be involved in the pathogenesis of systemic lupus erythematosus.

The authors wish to thank Miss Edith Mödder and Mr. Guy Brighthouse for their excellent technical assistance.

Received for publication 8 July 1974.

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