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Monoclonal antibody against bacterial lipopolysaccharide cross-reacts with DNA-histone

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SUMMARY

Monoclonal antibodies to bacterial lipopolysaccharide (LPS) were prepared by fusing spleen cells from BALB/c mice immunized with *Salmonella* Minnesota Re 595 LPS to the mouse myeloma cell line P3U1. One of them, designated RS01, revealed a strong positive antinuclear activity and reacted with DNA-histone. RS01 also bound specifically to *Salmonella* Minnesota Re 595 LPS and eliminated the biological activity of LPS. The *Salmonella* completely inhibited the ANA activity of RS01 and DNA-histone blocked the reactivity of RS01 with LPS. Thus, it is clear that an anti-LPS monoclonal antibody, RS01 cross-reacts with DNA-histone.

Keywords monoclonal antibody lipopolysaccharide antinuclear antibody DNA-histone cross-reactivity

INTRODUCTION

Systemic lupus erythematosus (SLE), an autoimmune disease, is characterized by the production of autoantibodies which can react with nucleic acids (Tan, 1982). It is as yet unclear why these antibodies are elicited. Investigations to clarify this point have been conducted previously. For example, vigorous immunization of native DNA (nDNA) failed to produce antibodies to these antigens in both normal and autoimmune animals (Madio *et al.*, 1984). However, cardiolipin-immunized mice and rabbits produced antibodies to cardiolipin that cross-react with DNA and a wide range of polynucleotides (Rauch *et al.*, 1984; Gaurnieri & Eisner, 1974), and also similar monoclonal antibodies were detected in autoimmune animals (Lafer *et al.*, 1981). These findings raise the possibility that antinuclear antibodies found in diseases such as SLE are originally directed against other molecules in which the epitope shares a strong structural analogy with nucleic acids. It is of great interest to know what types of molecule can cause these responses. During attempts to analyse anti-LPS antibodies by means of hybridoma technology, we found a monoclonal antibody that reacts not only with LPS but also with DNA-histone. This finding indicates that bacterial LPS can elicit antibodies which cross-react with host nuclear materials.

MATERIALS AND METHODS

Reagents and bacteria. Lipopolysaccharide (LPS) from *Salmonella* Minnesota Re 595 and

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Escherichia coli J5 were purchased from List Biological Laboratories, Inc., Cambell, CA, USA. This LPS contained less than 0·3% protein and less than 0·2% nucleic acid. DNA (Worthington Biochemical Corporation, Freehold, NJ) was dissolved in 1·5 mM sodium citrate containing 15 mM NaCl at 1 mg/ml and used as native DNA (nDNA). Denaturation of DNA (dDNA) was performed by heating nDNA solution for 10 min at 100°C, followed by rapid chilling. DNA-histone was isolated from calf thymus by the methods of Tan (1967), and analysed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The protein was stained only in the position corresponding to histones. The ratio of protein to DNA was 0·86.

Salmonella Minnesota Re 595 and *Escherichia coli* J5 were obtained from Dr S. Kanegasaki, University of Tokyo. *Streptococcus faecalis* was cultured from the blood of a patient at University Hospital, Tsukuba.

Monoclonal antibodies. Monoclonal antibodies against LPS were produced as described by Köhler & Milstein (1975). Two-month-old BALB/c mice were immunized with injections of 10 μg LPS from *Salmonella* Minnesota Re 595 incorporated into Freund's complete adjuvant. The mice were boostered with 10 μg LPS 2–3 weeks later; the spleens were removed 3 days later and used for fusion with the mouse myeloma cell line P3U1. The assay for detection of antibody to LPS secreted by the hybrids into culture medium was performed by enzyme-linked immunosorbent assay (ELISA). The cells yielding positive supernatant fractions were cloned by limiting dilution; the clones were expanded and then injected into Pristane-treated syngenic or nude mice to obtain ascites fluids. Monoclonal antibodies obtained were purified to homogeneity by ammonium sulphate precipitation, followed by molecular sieving by high performance liquid chromatography (h.p.l.c.). Antibody used in this study belonged to IgM, k isotype.

ELISA of anti-LPS antibody. The technique described by Ito *et al.*, (1980) was used as the basis for the assay. The wells of the plastic plates (Immulon II, Dynatech Laboratories, Inc., Alexandria, VA, USA) were coated with 50 μl of LPS (200 μg/ml) in 0·15 M NaCl in the presence of 50 μl of 0·02 M $MgCl_2$ by incubation at 37°C for 1 h. After discarding the LPS solution, 200 μl of 0·02 M $MgCl_2$ containing 0·5% bovine serum albumin (BSA) was added to each well and incubated for 1 h at 37°C to block non-specific binding to each well surface. This solution was hereafter used for sample dilution and all washing steps. After aspirating the solution, 100 μl of the sample was added to the individual wells and incubated for 1 h at 37°C, followed by three washes. Then, 100 μl of HRP-labelled affinity purified goat anti-mouse immunoglobulins (Cappel Laboratories Inc., Cochranville, PA, USA) was added to each well and the plate was incubated for 1 h. After seven washes, 100 μl of *o*-phenylene-diamine (0·4 mg/ml in citrate-phosphate buffer, pH 5·0 containing 0·001 M $MgCl_2$) was dispensed into the wells and incubated for 30 min at 37°C. The reaction was stopped by addition of 2 M H_2SO_4 and the plate was read at 490 nm in a microplate photometer.

Competition inhibition ELISA was performed in Immulon II microtitre plates sensitized with LPS purified from *Salmonella* Minnesota Re 595. The monoclonal antibody was diluted to a concentration that gave 30–70% of the maximal absorbance in a direct ELISA. The mixtures containing inhibitor and monoclonal antibody were added to the washed, sensitized microtitre plates and allowed to react for 1 h. Plates were then processed as described for the ELISA.

Double diffusion method. For the demonstration of precipitin reactions between monoclonal antibody and DNA-histone, a double diffusion method was used according to the method previously described by Tan (1967), using 0·6% agarose gel.

Immunofluorescent techniques. Antinuclear antibody (ANA) activity was detected by indirect immunofluorescence method, with methanol-fixed frozen sections of rat liver as sources of nuclear antigen. To assess inhibition of ANA reaction, various inhibitors indicated in results were mixed with an equal volume of hybridoma culture fluid (10 μg/ml) and incubated for 1 h at 37°C. The samples were then tested for ANA reactivity. The reactivity of RS01 with histone was investigated by the technique of Fritzler & Tan (1978). Briefly, acetone-fixed rat liver section were treated with 0·1 M HCl to elute histone and the acid-eluted section was reconstituted by incubation with calf thymus histone (Worthington Biochemical Corporation, Freedhold, NJ, USA) at a concentration of 25 μg/ml. Untreated, acid-eluted, and reconstituted sections were used as substrate for ANA assay.

Absorption of LPS activity by RS01. Purified RS01 (3 mg of IgM) was coupled to 0·5 g CNBr-

activated Sepharose 4B (Pharmacia Fine Chemicals, Pharmacia Japan Inc., Japan) as suggested by the manufacturer using endotoxin free buffers. As a control, another mouse IgM monoclonal antibody (anti-human C4) was coupled to Sepharose 4B in the same way. Serially diluted gels were added to 2 ng of LPS extracted from *Salmonella* Minnesota Re 595 in a total volume of 0·2 ml and the mixtures were incubated for 30 min at room temperature. After centrifugation, the supernatant fractions were assayed for endotoxin activity by the modified Limulus amebocytes assay (Iwanaga *et al.*, 1978) using colorimetric endotoxin determination reagents (Pyrodick; Seikaguku Kogyo Co., Ltd., Japan). A 1·0 ml sample was added to 0·1 ml of the endotoxin determination reagent and incubated at 37°C for 20 min. The reaction was stopped by adding 1 ml of 0·6 M acetic acid and the o.d. of the mixtures were read at 405 nm. The percentage of absorption of LPS activity by gels was calculated.

RESULTS

Reactivity of RS01 with nuclear antigens. The spleen cells from a mouse immunized with bacterial LPS were fused with the mouse myeloma cell line P3U1. Twenty wells (14·3%) of 140 growing cultures were positive for anti-LPS antibody production. These cells were cloned by limiting dilution and the clones were expanded. One of them, designated RS01, revealed a strong positive antinuclear antibody (ANA) reaction. It showed a homogenous pattern of nuclear staining, which suggests that the RS01 reacts with DNA-histone. This serological reaction was completely inhibited by DNA-histone (100 μg/ml), whereas both nDNA and dDNA (1 mg/ml) failed to inhibit the reaction. The nature of the nuclear antigen reactive with RS01 was further characterized by

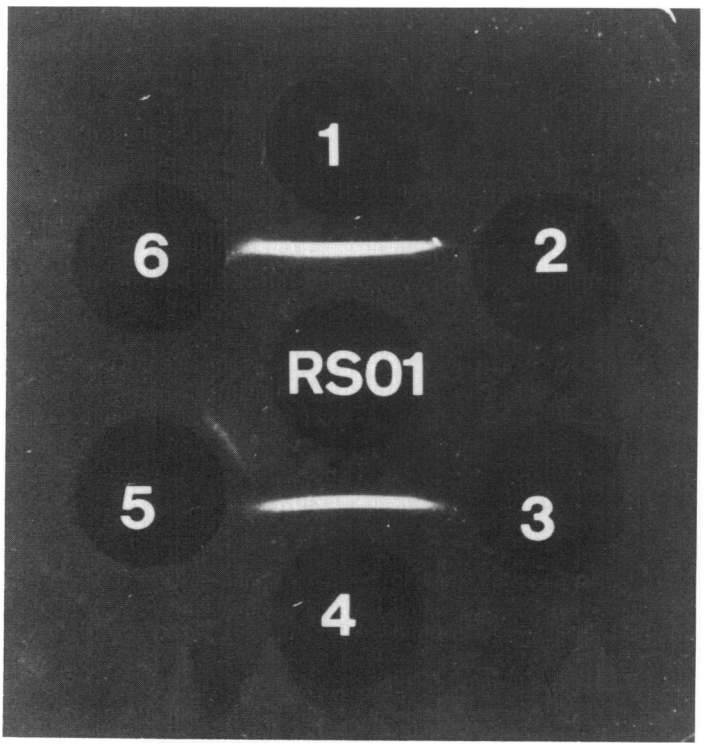

Fig. 1. Double diffusion in agarose. RS01 formed a clear precipitin line with DNA-histone (1 mg/ml; well 1, 4), a faint line with dDNA (0·5 mg/ml; well 5), and no line with nDNA (well 3). Either trypsin (well 2) or deoxyribonuclease (DNase; well 6) destroyed the antigenicity of DNA-histone, showing the specificity of this reaction.

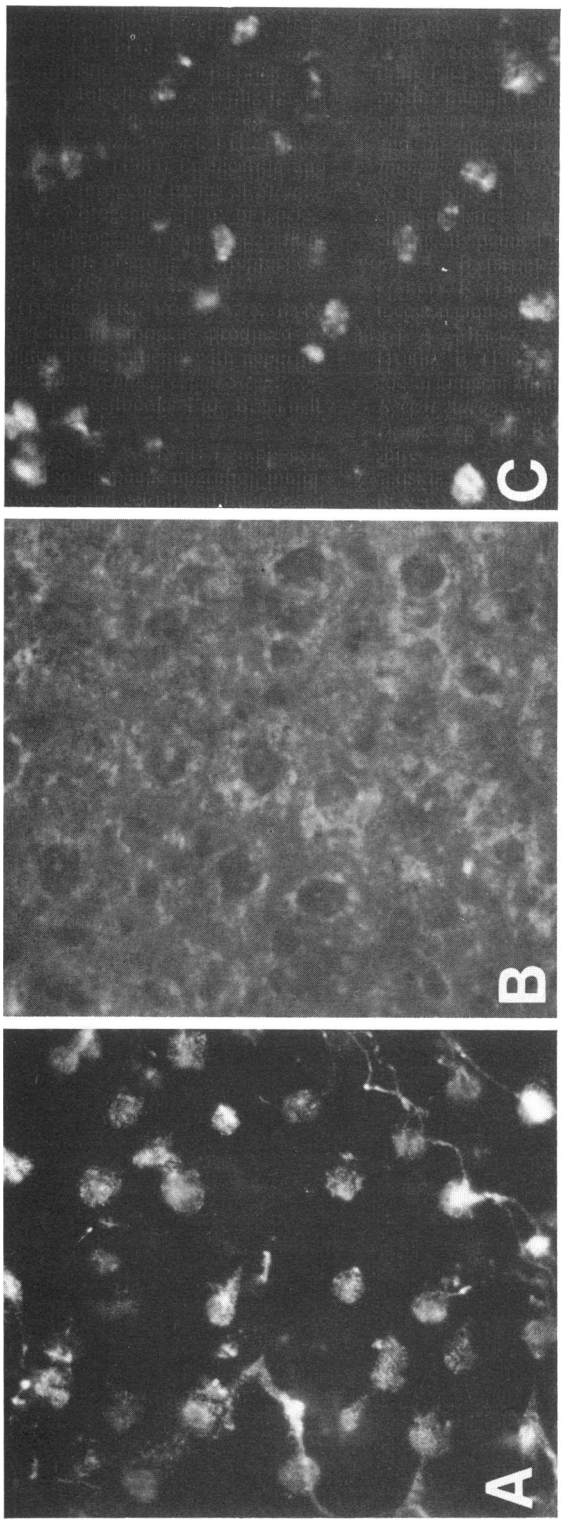

Fig. 2. ANA reactivity of RS01 when rat liver section was treated with acid and reconstituted with histone. (A) Control: an untreated section showing homogenous pattern of staining; (B) a section extracted with 0·1 M HCl, then used as a substrate for ANA determination. There was no nuclear staining; (C) a section extracted, and reconstituted with histone. There was restoration of nuclear fluorescence.

Table 1. Specificity of RS01 for various lipopolysaccharides

LPS* from	Inhibition of RS01 binding (%)
Salmonella	
Minnesota Re 595	88·9
typhimurium	3·7
enteritidis	0
typhi	5·0
Shigella flexneri	0
Escherichia coli	
J5	52·4
0128:B12	14·3
026:B6	0

* Concentration of 100 μg/ml.

double diffusion method and immunofluorescent technique. RS01 formed a clear precipitin line with DNA-histone and a faint line with dDNA. The line of RS01 and DNA-histone was abolished by either trypsin or deoxyribonuclease treatment of DNA-histone (Fig. 1). The reactivity of RS01 with histone was shown in Fig. 2. The ANA activity of RS01 was eliminated by acid-treatment and reconstituted by isolated histone. From these results, it is clear that RS01 reacts with DNA-histone.

Interestingly, RS01 induced the formation of lupus erythematosus (LE) bodies by the method described by Lachman (1961), which agrees with other anti-DNA-histone antibodies in human SLE.

Reactivity of RS01 with bacterial LPS. Purified RS01 (3 μg/ml) was incubated with several kinds of bacterial LPS at 37°C for 1 h and the inhibition of binding of RS01 to LPS purified from *Salmonella* Minnesota Re 595 was examined by the competitive inhibition ELISA method (Table 1). The binding was almost completely blocked by LPS from *Salmonella* Minnesota Re 595. This

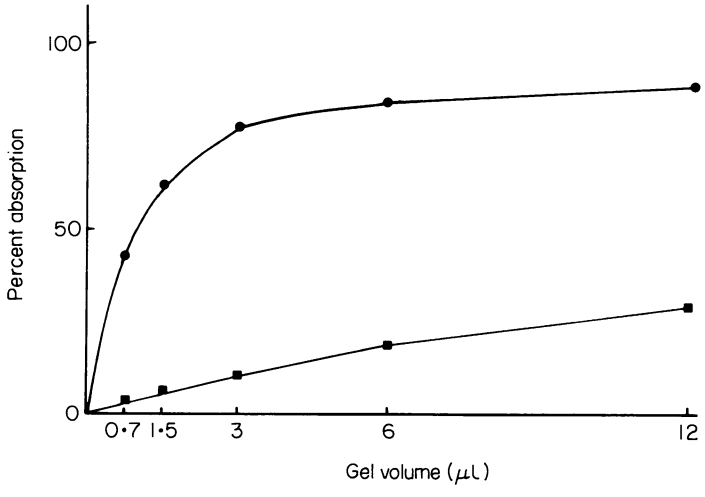

Fig. 3. Absorption of LPS activity with RS01. Various amounts of RS01-coupled Sepharose 4B (●) and mouse IgM-coupled Sepharose 4B (■), as control (expressed as a volume of beads added), were incubated with LPS. After centrifugation, the supernatant fractions were assessed for endotoxin activity as described in Materials and Methods. The percentage of absorption of LPS activity was calculated.

reaction was partially inhibited by LPS from *Escherichia coli* J5, and other bacterial LPS had little effect on the reaction. These results indicate that RS01 is specific for the LPS which was used as the immunogen.

To rule out the possibility that RS01 reacted with some contaminants of the LPS obtained commercially, we examined whether RS01 inhibits the biological activity of bacterial LPS using the modified Limulus amebocyte lysate assay. RS01 did not directly inhibit this colorimetric reaction. As shown in Fig. 3, however, RS01-coupled Sepharose 4B eliminated the LPS activity in a dose-dependent fashion, while the control had little effect.

The specificity of RS01 for DNA-histone and LPS. Next, we investigated the specificity of interaction of RS01 with both antigens. Preliminary studies showed that radiolabelled RS01 bound specifically to *Salmonella* Minnesota Re 595. Various numbers of *Salmonella* Minnesota Re 595 and *Streptococcus faecalis* as a control were incubated with a constant amount of RS01 (10 μg/ml) at 37°C for 1 h. After centrifugation, the ANA activity of supernatant fractions was assessed (Table 2). The reactivity of RS01 was completely inhibited by 10^9/ml of *Salmonella*. *Streptococcus faecalis*, a Gram-positive cocci, in contrast, had no effect at the concentration of 10^{10}/ml.

We also examined whether DNA-histone blocked the reactivity of RS01 with LPS using ELISA. Controls consisted of dDNA and nDNA. As shown in Fig. 4, DNA-histone strongly inhibited the

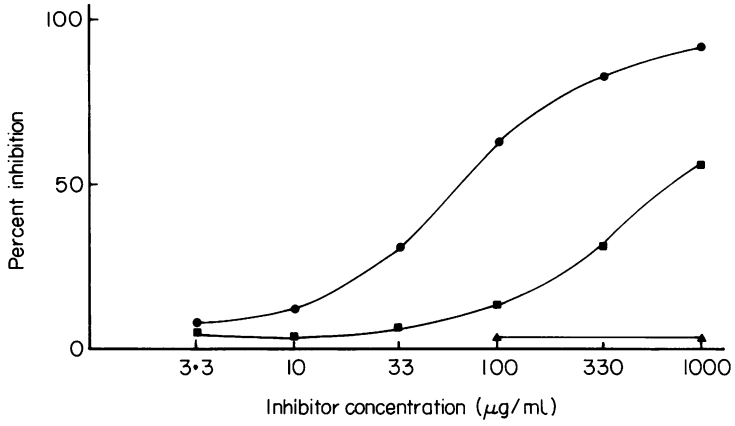

Fig. 4. Inhibition of RS01 binding to LPS by nuclear antigens. The specificity of RS01 antibody was determined by a competitive inhibition ELISA. Values represent the mean percentage of inhibition of RS01 binding activity for duplicate samples in the presence of inhibitors. DNA-histone (●); nDNA (▲); dDNA (■).

binding of RS01 to LPS; 50% inhibition was achieved at about 65 μg/ml, while dDNA weakly inhibited this reaction and 50% inhibition required about 700 μg/ml. Together with the results of Fig. 1, it is possible that dDNA and DNA-histone share some epitope.

In conclusion, a monoclonal antibody, RS01, which directs to LPS, cross-reacts with DNA-histone.

DISCUSSION

This is the first demonstration that an anti-LPS monoclonal antibody cross-reacts with DNA-histone. Hybridoma antibodies were selected on the basis of binding to LPS from *Salmonella* Minnesota Re 595 which we used as the immunogen. One of these monoclonal antibodies, RS01 reacted with DNA-histone (Fig. 1 and 2). This monoclonal antibody also bound to *Salmonella* Minnesota Re 595 LPS (Table 1) and eliminated the biological activity of LPS (Fig. 3). In addition, the reactivities of RS01 with nuclear antigens and LPS were inhibited by the *Salmonella* and DNA-histone, respectively (Table 2 and Fig. 4). On the basis of these results, it is clear that RS01 has the strict specificity for both LPS and DNA-histone.

Table 2. Effect of bacteria on the antinuclear antibody reactivity of RS01 antibody

	Fluorescence reaction* at concentrations of (bacteria/ml)							
Bacteria	0	10^7	$3{\cdot}3 \times 10^7$	10^8	$3{\cdot}3 \times 10^8$	10^9	$3{\cdot}3 \times 10^9$	10^{10}
Salmonella Minnesota Re 595	3+	3+	3+	2+	2+	1+	0	0
Streptococcus faecalis	3+	ND	ND	ND	ND	3+	ND	3+

* 3+, 2+, 1+ are a qualitative estimate of the intensity of fluorescence staining from the most positive reaction (3+) to least intense positive antinuclear antibody (1+). 0 is no reaction.

ND, not done.

Our results suggest that bacterial LPS and DNA-histone share some antigenic determinant. Lafer *et al.* (1981) have shown that the diester-linked phosphate groups, e.g. as found in the phospholipid cardiolipin, form the epitope for murine lupus anti-DNA monoclonal antibodies which was reacted with a wide range of polynucleotides. Similarly, LPS derived from *Salmonella* Minnesota Re 595 consists of 2-keto-3-deoxyoctonate (KDO) and lipid A which is a phospholipid containing diphosphate ester (Rietschel *et al.*, 1982). At present, it is not clear what is the antigenic determinant on LPS and DNA-histone, but based on the experiments with phospholipids described above, it is possible that a chemically similar structure between LPS and DNA-histone may constitute the epitope.

Previous studies have shown that bacterial LPS induces anti-DNA antibodies (Fournié, Lambert & Miescher, 1974). This phenomenon has been considered to be a result of the polyclonal B cell activation by LPS (Izui *et al.*, 1977), although the precise mechanism is unclear. In addition, we show here that LPS serves as the immunogen, resulting in the production of antibody which exhibits the cross-reactivity. The observation (Table 1) that the specificity of RS01 directs strictly to the immunized LPS supports our conclusion. Of course, LPS has some non-specific effect on the antibody response as already reported (Coutinho & Möller, 1975).

Another important finding is that antinuclear antibody derives from normal mice that were immunized with an exogenous substance, bacterial LPS. This is compatible with cardiolipin-immunized normal mice producing anti-DNA antibodies (Rauch *et al.*, 1984). Considering that vigorous immunization of native DNA failed to produce antinuclear antibodies, it seems probable that some antinuclear antibodies are not directed originally against nuclear antigens, but cross-react with structurally similar regions shared by these molecules and the primary antigens. In this respect, bacterial LPS may serve as one of these antigens. Therefore, it is important to know whether antinuclear antibodies in SLE cross-react with LPS. This is currently under investigation.

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