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① 主論文

A Study on Cross-Reactivity of Anti-DNA Antibody with Glycosaminoglycans

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To study the pathogenesis of lupus nephritis, the cross reactivity between anti-DNA antibody and glycosaminoglycans (GAGs) was investigated. Monoclonal anti-DNA antibodies were obtained from hybridomas by the fusion of MRL/lpr/lpr splenocytes with murine myeloma cells. Some of these monoclonal anti-DNA antibodies showed cross reactivity with GAGs, such as hyaluronic acid, chondroitin sulfate and heparan sulfate. To elucidate the mechanism of cross reactivity, inhibition assays with propanol and polyethylenimine (PEI), a cationic agent, were carried out. Increase of the concentration of PEI (0.6-2.0 % vol/vol) resulted in a dose dependent decrease in the binding ability of anti-DNA antibody to GAGs. Propanol, an organic reagent which disrupts the van der Waals bonds between epitopes and paratopes, showed little inhibitory effect on the binding activity of monoclonal anti-DNA antibody to GAGs. These results indicate that the binding of anti-DNA antibody to GAGs is due to a charge interaction rather than van der Waals forces. Anti-DNA antibody which can react with GAGs in the glomerular basement membrane seems to play an important role in the pathogenesis of lupus nephritis.

Key words : anti-DNA antibody, cross-reactivity, glycosaminoglycan, lupus nephritis

High titer of anti-DNA antibody, especially anti-double stranded DNA antibody activity in the sera is specific for the patients of systemic lupus erythematosus (SLE) and correlates with clinical findings and severity of renal involvement (1). However, the pathogenic significance of anti-DNA antibody in lupus nephritis remains uncertain.

There are two proposed mechanisms by which anti-DNA antibody could cause lupus nephritis. One is the circulating immune complex (CIC) mechanism: circulating DNA/anti-DNA antibody immune complex is present in the sera of patients with lupus nephritis. However, the direct demonstration of DNA both in CIC and glomeruli has been difficult, and reported only in a few studies (2, 3). Another mechanism is the *in situ* IC formation mechanism: Izui demonstrated that DNA had a high affinity for collagen molecule in GBM *in vitro* (4). They proposed that circulating anti-DNA antibodies would bind

to DNA bound to GBM and form the immune complex *in situ*. However, there is a lack of direct and concrete evidence which supports either of these two hypotheses.

Recently, Faaber *et al.* showed the cross reactivity of anti-DNA antibody with GAGs (5, 6), and proposed a new hypothesis with respect to the pathogenesis of lupus nephritis. They suggest that anti-DNA antibody binds directly to the GAGs to form the immune complex *in situ* and causes lupus nephritis.

We cultivated the monoclonal anti-DNA antibodies and studied the cross reactivity with GAGs to investigate the precise mechanism of this cross reactivity.

Materials and Methods

Mice. MRL-lpr/lpr mice were purchased from Kiwa laboratory, Japan and BALB/C mice were obtained from the animal colony of our institute.

Cell fusion and cloning. Somatic cell hybridization was carried

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out as described by Köhler and Milstein (7). Spleen cells from MRL-lpr/lpr mice were fused with the non-immunoglobulin secreting aminopterin sensitive murine myeloma cell line, NS-1. Anti-DNA antibody-producing hybridomas were cloned by repeating the procedure of limiting dilutions.

DNA and glycosaminoglycans. Calf thymus DNA, purchased from Worthington Biochemical Corporation, New Jersey, USA, was dissolved in 0.01M Tris-HCl buffer, pH8.0 containing 0.001M EDTA. Chondroitin sulfate and hyaluronic acid were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Heparan sulfate was obtained from Seikagaku Kogyo, Tokyo, Japan. These GAGs were dissolved in phosphate buffered saline (PBS), pH7.4.

Enzyme-linked immunosorbent assay. Anti-DNA and anti-GAG antibody assays were performed by enzyme-linked immunosorbent assay (ELISA). Each well of polystyrene microtiter plate (Sumitomo, Tokyo, Japan) was precoated with 100 μ l of 0.75 mg/ml of protamine sulfate and incubated overnight at 4 $^{\circ}$ C. After washing with PBS, 100 μ l/well of DNA (200 μ g/ml) or GAG (500 μ g/ml) were added to each well and incubated overnight at 4 $^{\circ}$ C. The plates were washed three times with PBS to remove excess antigen. Unbound sites on the plates were subsequently blocked for 1h at room temperature with 1% (wt/vol) bovine serum albumin (BSA) in PBS and then washed three times with PBS. One hundred μ l of a monoclonal antibody to be tested was added to each well and incubated for 1h at room temperature. After washing three times with PBS, 100 μ l of peroxidase-conjugated goat anti-mouse IgG (Cappel, Pennsylvania, USA), diluted 1:3,000 in PBS containing 1% BSA were added and incubated again for 1h at room temperature. Then 100 μ l of substrate solution, 1mg/ml o-phenylenediamine (Wako Pure Chemical Industries, Tokyo, Japan) dissolved in 0.1M citrate buffer, pH5.0, containing 1 μ l/ml of 35% (vol/vol) H₂O₂ was added to each well. After 10min, the reaction was stopped by adding 50 μ l of 3N HCl. The optical absorbance was measured at 492 nm by an EIA READER, BIO-RAD MODEL 2550.

Crithidia assay. Crithidia assay was carried out to verify anti-double stranded DNA activity. Crithidia lucillae was plated on glass slide. Monoclonal antibodies were placed on the slides and FITC-conjugated goat anti-mouse IgG antibodies were applied. Fluorescence of kinetoplasts was observed by immunofluorescent microscopy.

ELISA with different salt concentration. To investigate the influence of ionic strength on the interaction of monoclonal antibodies and DNA or GAGs, ELISAs were performed with various concentrations of NaCl. Monoclonal anti-DNA antibodies were applied to the antigen-coated wells in the presence of different NaCl concentrations (0.03–1M).

Inhibition studies. To elucidate the mechanism of interactions between anti-DNA antibodies and DNA or GAGs, ELISA were performed in the presence of a cationic agent, PEI or propanol. Monoclonal antibodies were applied to antigen-coated wells in the presence of increasing concentration of PEI (0.03–1% vol/vol) or propanol (0.03–4.00% vol/vol). The following procedures were

performed under standard conditions. As a control study, the same inhibition studies were performed using thyroid microsomal antigen and monoclonal anti-microsomal antibody, which were produced in our laboratory (8).

Results

Production of monoclonal anti-DNA antibodies. The cell-fusion procedure was carried out twice and yielded 98 cell lines producing anti-DNA antibody.

Cross-reactivity of monoclonal anti-DNA antibodies with GAGs. Seven clones of anti-DNA antibody producing hybridomas were able to cross-react with GAGs (Table 1). Anti-DNA antibody activity of these clones was also confirmed with Crithidia assay. There was a correlation between anti-DNA activity and anti-GAG activity in these monoclonal antibodies (Figs. 1, 2)

Influence of the salt concentration on the binding of monoclonal anti-DNA antibodies to DNA and GAG. Decrease of binding activity to DNA and GAG in the presence of increasing concentration of NaCl in a dose-dependent manner was demonstrated (Fig. 3). These data indicate that bindings of these monoclonal antibodies to DNA and GAGs were not mediated with non-specific charge interaction because binding activities were not significantly reduced with 0.3M NaCl in which concentration, non-specific charge interaction was inhibited (9). It was also demonstrated that NaCl at the concentrations used in this study did not dissociate the DNA or GAG from the plate (Data was not shown).

Effect of PEI on the binding to DNA and GAG. The presence of increasing amount of PEI caused dose-

Table 1 Binding activity of monoclonal anti-DNA antibodies to glycosaminoglycans

Hybridoma clones	Antigen			
	Heparan sulfate	Chondroitin sulfate	Hyaluronic acid	DNA
1G3	0.599	0.413	0.609	0.409
1A9	1.273	1.820	1.523	1.667
3G2	0.380	0.348	0.351	0.647
3B4	1.033	1.144	1.096	1.015
3B9	1.778	1.092	1.351	1.746
9C4	0.428	0.905	0.894	1.989
7C1	0.759	1.664	0.326	1.345

All values represent optical absorbance at 492 nm.

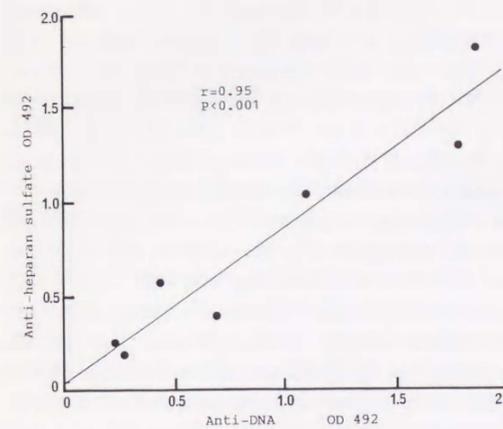


Fig. 1 Correlation between anti-DNA activity and anti-heparan sulfate activity of monoclonal anti-DNA antibodies.

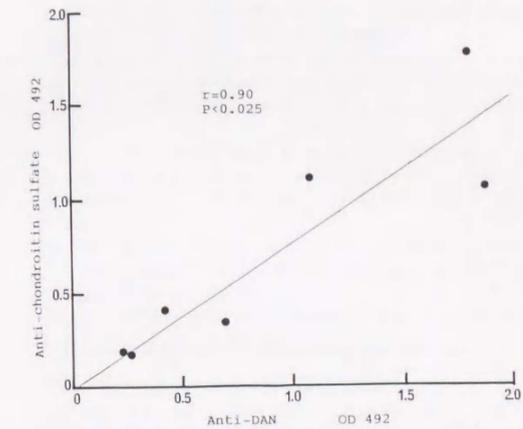


Fig. 2 Correlation between anti-DNA activity and anti-chondroitin sulfate activity of monoclonal anti-DNA antibodies.

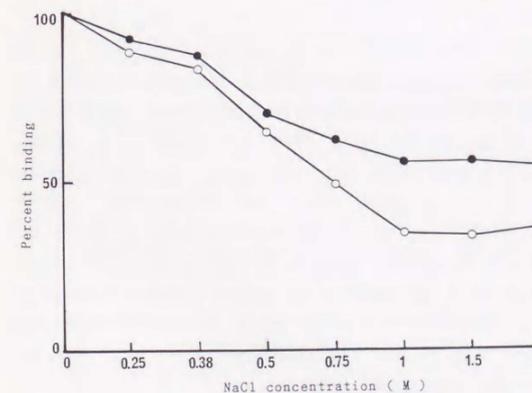


Fig. 3 Influence of ionic strength on the binding activities of monoclonal anti-DNA antibody (1A9 clone) to DNA and glycosaminoglycan. Binding of monoclonal antibody to DNA (○—○) and heparan sulfate (●—●).

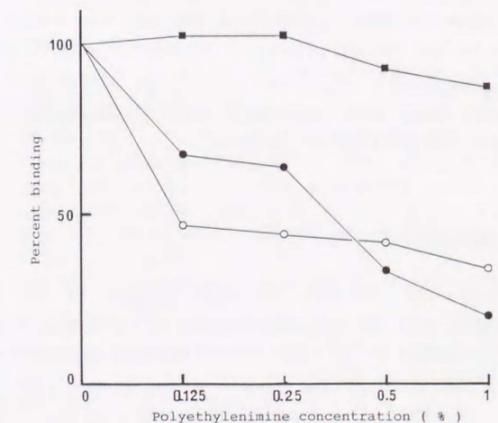


Fig. 4 Influence of polyethylenimine on the binding of monoclonal antibodies to DNA, glycosaminoglycan and thyroid microsomal antigen. Binding of monoclonal anti-DNA antibody (1A9 clone) to DNA (○—○) and heparan sulfate (●—●). Binding of monoclonal anti-microsomal antibody to thyroid microsomal antigen (■—■).

dependent reduction in anti-DNA and anti-GAG activity (Fig. 4). Five% (vol/vol) of PEI reduced anti-DNA activity and anti-GAG activity by 50% and 65%, respectively. But PEI did not show an inhibitory effect on

the other antigen-antibody interaction; microsomal antigen and monoclonal anti-microsomal antibody.

Effect of propanol on the binding to DNA and GAG. Propanol is an organic agent which inhibit van der Waals

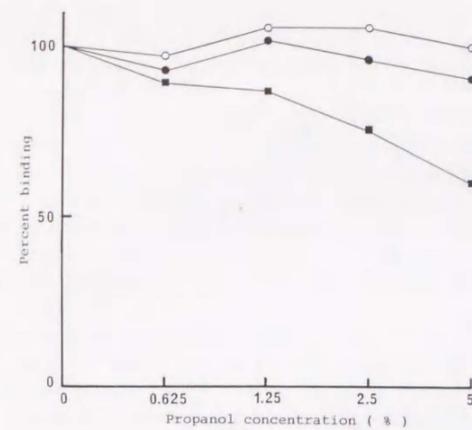


Fig. 5 Influence of propanol on the binding of monoclonal antibodies to DNA, glycosaminoglycan and thyroid microsomal antigen. Binding of monoclonal anti-DNA antibody (1A9 clone) to DNA (○—○) and heparan sulfate (●—●). Binding of monoclonal anti-microsomal antibody to thyroid microsomal antigen (■—■).

force between antigen and antibody (10, 11). As shown in Fig. 5, increase of propanol concentration had little effect on the binding activity of monoclonal antibodies to DNA and GAG. On the other hand, the binding activity of monoclonal anti-microsomal antibody to microsomal antigen was inhibited by propanol.

Discussion

Anti-DNA antibody has been believed to play an important role in the pathogenesis of systemic lupus erythematosus (SLE). But the mechanism that involves anti-DNA antibody that causes tissue injuries has not been elucidated. Development of monoclonal anti-DNA antibodies by somatic cell hybridization techniques has facilitated the analysis of the character of anti-DNA antibody and its target antigen.

Studies using monoclonal anti-DNA antibody revealed that anti-DNA antibody could react to a broad spectrum of antigens including those with molecular structures that were apparently different from DNA. Cross-reactivity with the synthetic polynucleotides of different composition has been reported. The cross-reactive antigenic determinant seemed to be in the sugar-phosphate backbone (12). Anti-DNA antibody were able to react with cardiolipin

and phospholipids. Phosphodiester linked phosphate groups were identified as possible cross-reactive moieties (13–15). Furthermore, cross-reactivity of anti-DNA antibody extended to a variety of antigens, such as IgG, platelet, Raji cell and endogenous bacteria (16–18). Faaber *et al.* also reported the cross-reactivity with GAGs (5, 6). The wide variety of cross-reactions is difficult to explain with the existing data. This situation seems rich in possibilities for further investigations.

The repulsive and attractive forces that constitute the non-covalent interaction between antigens and antibodies (10) are characterized by a) Dispersion (van der Waals) force and b) Electrostatic (Coulombic) interaction. The organic reagents, propanol and acetic acid, decrease the surface tension of the liquid medium and disrupt the van der Waals bond to dissociate the antigen-antibody complex. Microsomal antigen and anti-microsomal antibody complex is efficiently dissociated by propanol. However, the bindings of monoclonal anti-DNA antibodies to DNA and GAG were not inhibited by propanol at the concentration in which microsomal antigen and anti-microsomal antibody was dissociated. From these data, it may be concluded that van der Waals force does not contribute the interaction between anti-DNA antibody and DNA or GAG.

Both DNA and GAG are negatively charged. Ebling and Hahn reported that anti-DNA antibodies eluted from NZB/W F1 mouse kidneys had higher isoelectric points than those in the sera. They speculated that cationic anti-DNA antibodies were pathogenic in lupus nephritis (19). In the present study, the cationic agent, PEI, inhibited the binding of anti-DNA antibody to DNA and GAG. It is possible that the binding of anti-DNA antibody to GAG is mediated by simple charge-charge interaction. Because the 0.3M ionic strength, which can prevent non-specific charge-charge interaction did not affect the interaction between anti-DNA antibody and GAG, this interaction does not seem to be mediated with a simple force between opposite charges. From these results, it appears that this charge-charge interaction exists between epitope and paratope.

Van der Waals force decreases at a rate inverse to the 7th power of the intermolecular distance. The distance between two molecules should be very close for van der Waals force to work efficiently. On the other hand, electrostatic force is inversely proportional to the second power of the intermolecular distance and could work at distances where van der Waals force could not work.

There was a significant correlation between anti-DNA activity and anti-GAG activity in these monoclonal antibodies. However, DNA and GAG do not have similar molecular structures other than repeating negative charged residues. It seems to be reasonable that van der Waals force does not work effectively between such molecules.

In this study, the cross-reactivity of monoclonal anti-DNA antibody with GAG was demonstrated under various conditions. Heparan sulfate proteoglycan, which is one of the GAGs in glomeruli, is an important component of anionic site in the GBM and necessary to maintain both charge and size selective barriers (20, 21). The cross-reaction between anti-DNA antibody with GAG may play two possible roles in the pathogenesis of lupus nephritis. Direct binding of anti-DNA antibody to GAG may lead to the formation of the immune complex *in situ* and cause the activation of complements and tissue damage. Another possibility is that anti-DNA antibody may neutralize the negative charge of GBM to disturb the charge selective barrier and result in proteinuria.

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