Clin. exp. Immunol. (1984) **55**, 502–508.

Cross-reactivity of anti-DNA antibodies with proteoglycans

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(Accepted for publication 30 September 1983)

SUMMARY

Ten sera of patients with systemic lupus erythematosus (SLE) were tested in an enzyme linked immunosorbent assay for their ability to react with glycosaminoglycans, constituents of proteoglycans,in relation to their anti-DNA reactivity. The SLE sera reacted with hyaluronic acid and chondroitin sulphate and this reactivity correlated with the anti-DNA activity of these sera. By contrast, sera obtained from patients with other autoimmune diseases or normal sera lacked any of these reactivities. Anti-DNA antibodies purified by affinity chromatography with either oligo dT cellulose or Cibracon blue F3Ga Sepharose reacted with DNA as well as with hyaluronic acid. The cross-reactivity of anti-DNA antibodies could be confirmed by the reaction of a mouse monoclonal anti-DNA antibody with DNA, hyaluronic acid, and chondroitin sulphate. This pattern of cross-reactivities of anti-DNA antibodies suggests that several compounds can function as antigenic targets for these antibodies provided that their structures contain repeating negatively charged groups.

Keywords systemic lupus erythematosus anti-DNA antibodies proteoglycans

INTRODUCTION

In sera of patients with systemic lupus erythematosus (SLE) antibodies are present, reacting with a variety of autoantigens (Hahn, 1980). However, the reaction of antibodies with dsDNA is the major serological marker of this disease (Deicher, Holman & Kunkel, 1959; Stollar *et al.*, 1962; Arana & Seligmann, 1967; Koffler *et al.*, 1971). Recently it was demonstrated that monoclonal antibodies (MoAbs) directed against DNA, can cross-react with phospholipids, especially cardiolipin. This was shown with MoAbs derived from the MRL/1 mouse (Lafer *et al.*, 1981) and with MoAbs produced by human–human hybridomas (Shoenfeld *et al.*, 1983). Furthermore it was shown that sera of SLE patients contain antibodies directed against DNA, cross-reacting to phospholipids in micellar form (Koike, Tomioka & Kumagai, 1982). This cross-reactivity was thought to be due to the fact that the chemical structure of cardiolipin shows similarities to the sugar–phosphate backbone of nucleic acid by phosphodiester linked phosphate groups. Another common feature of DNA and phospholipids in micellar form is the presence of repeating negative units in these molecules. If structures with repeating negatively charged groups would form the antigenic determinant, that reacts with anti-DNA antibodies, other molecules fulfilling this criterium, should also react with anti-DNA. An important group of such molecules with repeating negatively charged units are the proteoglycans. Therefore we studied the cross-reactivity of anti-DNA antibodies with the glycosaminoglycans hyaluronic acid and chondroitin sulphate, that are constituents of the proteoglycans.

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MATERIALS AND METHODS

Sera. Blood samples were obtained from patients who fulfilled the criteria of the American Rheumatism Association for the classification of SLE (Tan *et al.*, 1982). After clotting in glass tubes at room temperature for 3 h and subsequent centrifugation for 5 min at 2,000*g*, the serum samples were stored at −20°C. All sera were heat inactivated for 30 min at 56°C before use.

DNA and glycosaminoglycans. Calf thymus DNA, purchased from Calbiochem, California, USA, was dissolved in 0·1 M phosphate buffer pH 7·4. Hyaluronic acid, obtained from Merck, Darmstadt, Federal Republic of Germany and chondroitin sulphate, obtained from Sigma, St Louis, Missouri, USA, were dissolved in 0·1 M phosphate buffer, pH 7·4.

Enzyme linked immunosorbent assay (ELISA). Antibodies to DNA or glycosaminoglycans were measured with a ELISA (Eaton, Schneider & Schur, 1983). Polystyrene microtitre plates (Costar, Cambridge, Massachussetts, USA) were precoated with 150 μl of 0·5 mg/ml protamine chloride (Kabi AB, Stockholm, Sweden) per well during a 2 h incubation and washed subsequently with phosphate-buffered saline (PBS). DNA (100 μl, 50 μg/ml, per well) or glycosaminoglycans (100 μl, 0·5 mg/ml, per well) were coated overnight at room temperature. Optimal concentrations of the coated antigens had been determined by incubation of serial dilutions of these antigens. This resulted in increased peroxidase staining until a plateau value was reached. An excess of the optimal antigen concentration was subsequently used for coating to ensure saturated binding to the wells. The plates were washed five times with PBS containing 0·05% vol./vol. Tween 20. To avoid non-specific binding the plates were subsequently coated for 2 h with 2% wt/vol. bovine serum albumin (BSA) dissolved in PBS and then washed five times with PBS/0·05% vol./vol. Tween 20. The sera were diluted in PBS, containing 2% wt/vol. BSA, in two-fold dilution (final volume 100 μl). After 1 h of incubation the plates were washed five times with PBS containing 0·1% wt/vol. BSA and 0·05% vol./vol. Tween 20 (wash buffer). Then, 100 μl of peroxidase conjugated rabbit anti-human light and heavy chain (Miles Laboratories Inc., California) diluted 1:500 in PBS containing 1% wt/vol. BSA were added followed by an incubation for 1 h. The plates were washed five times with wash buffer, and 100 μl freshly prepared substrate solution, 0·8 mg/ml 5′ aminosalicylic acid dissolved in 50 mM phosphate buffer, pH 6·0, containing 0·8 μl/ml 30% vol./vol. H_2O_2, were added to each well. After 30 min the signal was measured at 450 nm in a Titertek® multiskan. To determine blank values, wells were treated in the same way as mentioned above, but instead of serum 2% wt/vol. BSA dissolved in PBS was used. When mouse MoAbs were tested, peroxidase conjugated rabbit anti-mouse Ig (Miles Laboratories Inc.,) was used in a dilution of 1:500 in PBS containing 1% wt/vol. BSA. In each assay a positive SLE serum and a negative normal human serum were used as standards. The reproducibility of the assay as measured with the positive control serum was excellent.

Assays for anti-DNA antibodies. Crithidia luciliae and Farr assay were performed as described by Aarden and co-workers (Aarden, De Groot & Feltkamp, 1975; Aarden, Lakmaker & Feltkamp, 1976).

Purification of anti-DNA antibodies by affinity chromatography. Anti-DNA antibodies were isolated from an SLE serum by affinity column chromatography using oligo dT cellulose (Collaborative Research Inc., Waltham, Massachusetts, USA) or by Cibracon blue F3GA Sepharose (Pharmacia, Uppsala, Sweden). To the oligo dT column or blue sepharose column 0·5 ml serum was applied. The column was washed with PBS when oligo dT was used or with 50 mM Tris-HCl, pH 8·0, containing 50 mM NaCl when blue Sepharose was used. Bound anti-DNA antibodies were eluted with 20 ml PBS containing 1 M NaCl. The eluted fraction and the non-bound protein fraction were dialysed against PBS overnight and subsequently concentrated to the original volume. When the eluate was tested in ELISA, normal human serum (NHS) was added (final concentration 50% vol./vol.) to ensure a suitable protein concentration.

Radiolabelling of human Ig. Human IgG (Miles Laboratories) was radiolabelled with $Na^{125}I$ (Amersham, UK) by the chloramine-T method (Hunter & Greenwood, 1962). Specific activity was 2·5 μCi/μg ^{125}I-IgG.

RESULTS

Detection of antibodies against glycoaminoglycans

Ten SLE sera containing antibodies directed against DNA as determined with the *Crithidia luciliae* test and the Farr assay were tested in the ELISA with DNA, hyaluronic acid, or chondroitin sulphate as antigen. An example of the reaction pattern of an SLE serum with these antigens in the ELISA is shown in Fig. 1.

In all the SLE sera tested, there was a close correlation between the binding activity to DNA and to the glycosaminoglycans (Fig. 2), whereas normal human sera ($n=6$) or a pool of normal human sera ($n=40$) were negative with all antigens. Furthermore in sera from patients with other

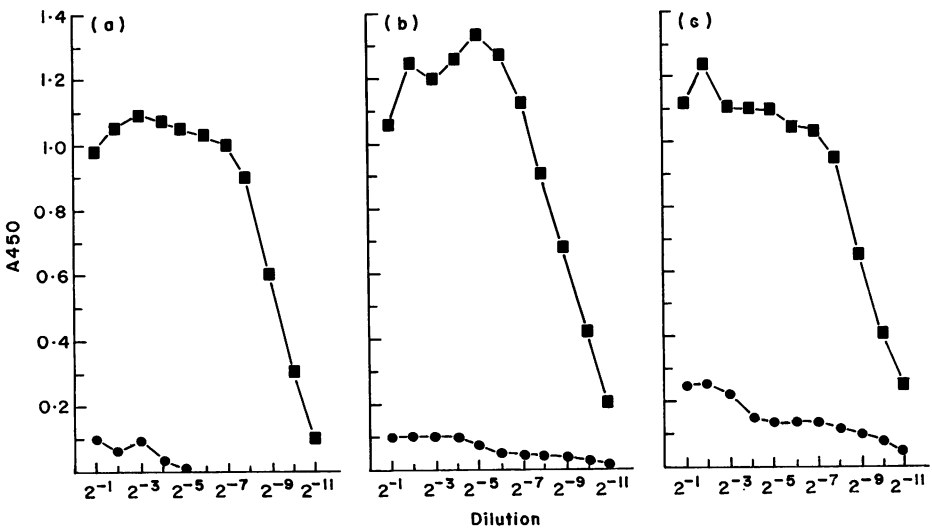

Fig. 1. Binding activity of an SLE serum with (a) DNA, (b) hyaluronic acid and (c) chondroitin sulphate as determined in an ELISA (■——■). Binding activity of normal human serum (●——●). The results are expressed as the absorption at 450 nm (A450).

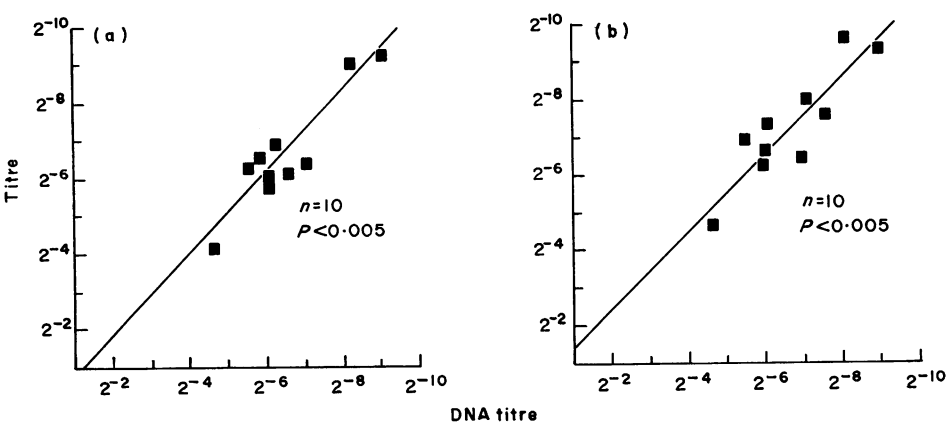

Fig. 2. Correlation of the DNA binding activity of SLE sera with the binding to (a) hyaluronic acid or (b) chondroitin sulphate. Titres of hyaluronic acid, chondroitin sulphate, and DNA, were defined as the dilution of each serum, that gave 50% of the maximal absorption at 450 nm with the appropriate antigen.

autoimmune diseases such as rheumatoid arthritis ($n = 12$), mixed connective tissues disease ($n = 12$) or thyreoiditis ($n = 12$) both anti-glycosaminoglycans and anti-DNA activities were absent in all cases. To exclude that the observed reactivity with hyaluronic acid was caused by a contamination of DNA, hyaluronic acid was treated with 10 μg/ml DNAase I (Boehringer, Mannheim, Federal Republic of Germany) during 60 min at 37°C in the presence of 5 mM $MgCl_2$ followed by extensive dialysis. After this treatment this antigen was used in the ELISA. The results with the DNAase treated and untreated preparations were similar, indicating that no contaminating DNA was present. When BSA was coated instead of DNA or the glycosaminoglycans, all sera were negative in the ELISA.

The reactivity of isolated anti-DNA antibodies with hyaluronic acid

Anti-DNA antibodies were isolated from an SLE serum, which reacted strongly in the *Crithidia luciliae* test, by oligo dT cellulose affinity chromatography. The unbound fraction and the eluate were, after dialysis and concentration to the original volume, tested in immunofluorescence on the *Crithidia luciliae*. Only in the eluted fraction anti-DNA antibodies were detectable. These fractions were also tested in the ELISA on DNA and hyaluronic acid and the results are given in Fig. 3. The eluted fraction reacts with both DNA and hyaluronic acid in a dose-dependent way, whereas the non-bound protein fraction gives only a minimal signal. When 2 μCi human ^{125}I-IgG was added to 0·5 ml NHS and handled in the same way as the SLE serum on the oligo dT column, 95% of the radioactivity was found in the non-bound protein fraction, which excludes that oligo dT cellulose binds human IgG in a non-specific way.

Anti-DNA antibodies were also isolated by blue Sepharose chromatography (Pollard & Webb, 1982). When the non-bound protein fraction and the eluted fraction were tested with immunofluorescence on the *Crithidia luciliae*, only in the eluted fraction anti-DNA antibodies were detected. Both fractions were tested in the ELISA with DNA or hyaluronic acid as antigen. The results given in Fig. 4 show that antibodies, reacting with both DNA and hyaluronic acid, are only present in the eluate. The non-specific binding of human IgG to blue Sepharose was also tested with 2 μCi ^{125}I-IgG, added to 0·5 ml NHS and handled in the same way as the SLE serum. 90% of the radioactivity was recovered in the non-bound protein fraction.

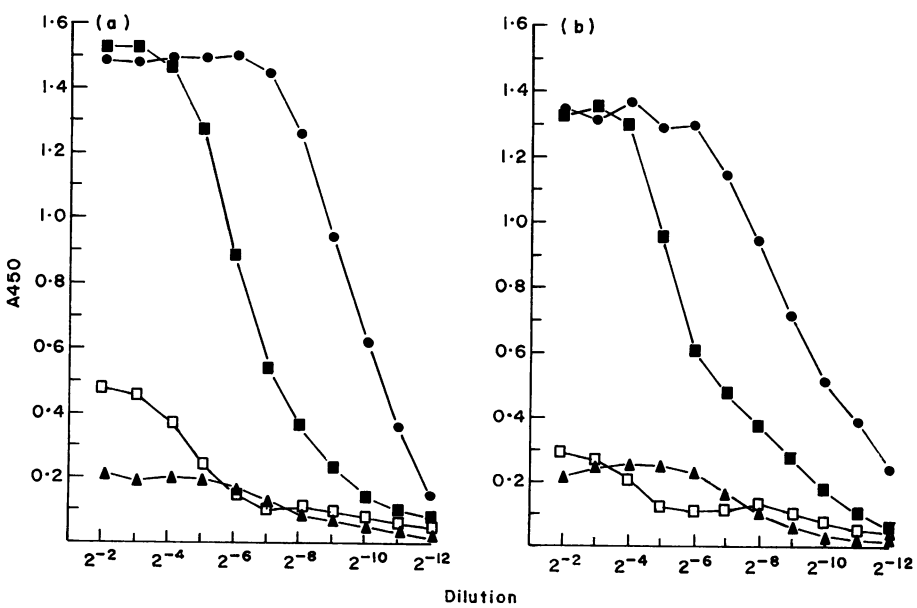

Fig. 3. Reaction pattern of anti-DNA antibodies, isolated by oligo dT cellulose chromatography, with (a) DNA and (b) hyaluronic acid. Original SLE serum (●——●), eluted fraction (■——■), the non-bound fraction (□——□) and normal human serum (▲——▲). The results are expressed as the absorption at 450 nm (A450).

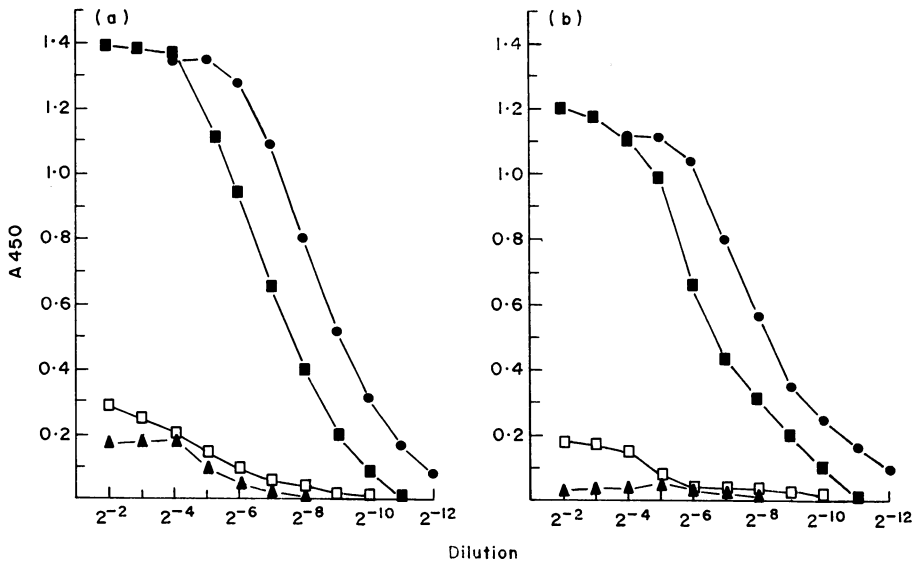

Fig. 4. Reaction pattern of anti-DNA antibodies, isolated by Cibracon blue F3GA Sepharose, with (a) DNA and (b) hyaluronic acid. Original SLE serum (●——●), eluted fraction (■——■), non-bound fraction (□——□) and normal human serum (▲——▲). The results are expressed as the absorption at 450 nm (A450).

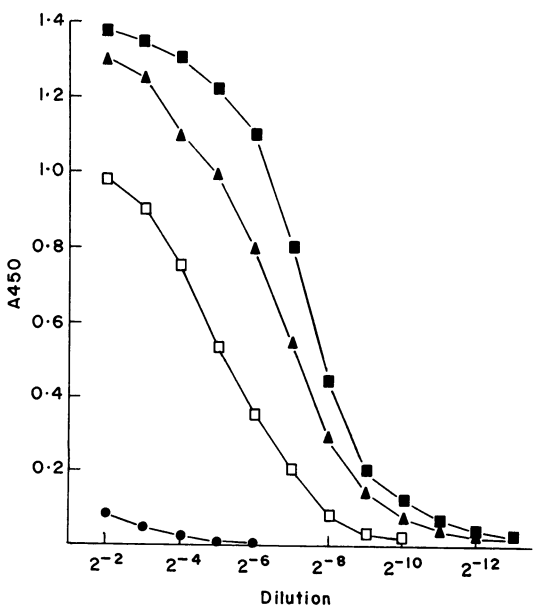

Fig. 5. Binding activity of a mouse anti-DNA MoAb with DNA (■——■), hyaluronic acid (▲——▲), chondroitin sulphate (□——□). A mouse MoAb of the same subclass but without anti-DNA activity was used as a control (●——●). The results are expressed as the absorption at 450 nm (A450).

Cross-reactivity of monoclonal anti-DNA antibody with hyaluronic acid

Finally, to prove that the observed cross-reactivity is a property of anti-DNA antibodies, we used a mouse anti-DNA MoAb (provided by T. Westgeest and L. Aarden). This MoAb was obtained from $(C57BL6 \times DBA_2)F_1$ mice, after induction of graft versus host disease (GVHD) by injecting DBA/2 lymphocytes. In this GVHD model the mice developed a series of autoantibodies including anti-DNA. After fusion with Sp 2/0 myeloma cells an anti-DNA MoAb that reacted positively in the *C. luciliae* test was obtained. This antibody was tested in ELISA with DNA, hyaluronic acid, chrondroitin sulphate. As shown in Fig. 5, this MoAb reacts with all three antigens tested.

DISCUSSION

Our results show that in SLE sera antibodies are present, which are directed against two constituents of proteoglycans, i.e. hyaluronic acid and chondroitin sulphate. The anti-glycosaminoglycan activity was only found in sera with anti-DNA antibody activity and not in sera of patients with other autoimmune diseases, such as rheumatoid arthritis, mixed connective tissue disease or thyroiditis. By isolating anti-DNA antibodies from SLE sera by affinity chromatography with oligo dT cellulose or blue Sepharose, it could be demonstrated that it indeed were anti-DNA antibodies in the SLE sera that cross-reacted with hyaluronic acid. Furthermore, that the observed cross-reactivity was a property of anti-DNA antibodies could be confirmed by using a MoAb directed against DNA, that also reacted with hyaluronic acid and chondroitin sulphate. The fact that anti-DNA antibodies can bind to the sulphonated polyaromatic dye Cibracon blue F3GA covalently coupled to sepharose even extends the number of cross-reactive antigens. This latter binding is broken under high salt conditions, and this indicated that the antigen–antibody interaction is of an ionic nature.

It was recently described, that monoclonal and polyclonal anti-DNA antibodies reacted with phospholipids especially with cardiolipin and phosphatidic acid (Lafer *et al.*, 1981; Koike *et al.*, 1982; Shoenfeld *et al.*, 1983). A common feature of DNA, hyaluronic acid, chondroitin sulphate, Cibracon blue F3GA, and the phospholipids is that they are molecules with repeating negatively charged groups. These data together with our findings of the reactivity with proteoglycans suggest that anti-DNA antibodies recognize a variety of structures which contain repeating negative units as functional epitopes. DNA is also a molecule with repeating negative phosphate groups. Furthermore, these data could give another explanation for the diversity of autoantibodies found in SLE sera. This apparent diversity might be generated by only a small group of autoantibodies which are reactive with a variety of antigens sharing a common feature as functional epitope. The affinity for such antigens may vary and our preliminary data on the determination of binding to the various antigens suggest that the affinity to glycosaminoglycans is lower than that to DNA. These differences certainly require further study.

From a clinical point of view the present data may help to explain some of the symptoms and signs of SLE. Proteoglycans form an essential part of connective tissue, being present in large amounts, especially in the joints and skin. The presence of antibodies with anti-proteoglycan activity may lead to inflammatory changes in these tissues, leading to arthralgia, arthritis, and skin rashes, which are the most common abnormalities in SLE (Tan *et al.*, 1982). Furthermore, if our assumption that these antibodies are more generally directed against a variety of epitopes with repeating negative structures is right, then capillary basement membranes and cell membranes may be other targets for these antibodies. We would predict that their deposition in, for instance, the basement membrane of the glomerulus would show not a linear but a granular pattern, because the charge distribution in this membrane is not homogeneous but is characterized by discrete anionic sites (Kanwar & Farquhar, 1979). The granular deposition of immunoglobulins found in renal biopsies of patients with SLE nephritis is therefore in accordance with hypothesis. A similar explanation may hold for the granular deposits in the skin biopsies of patients with this disease. This would imply that the damage of tissues and organs in SLE is not initiated by the deposition of circulating immune complexes but by the direct binding of antibodies to fixed antigenic structures in the target organs.

The authors wish to thank Drs T. Westgeest and L. Aarden for providing the anti-DNA MoAb.

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