

POLYSPECIFIC MONOCLONAL LUPUS AUTOANTIBODIES
REACTIVE WITH BOTH POLYNUCLEOTIDES AND
PHOSPHOLIPIDS*

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The diversity of autoantibodies produced in systemic lupus erythematosus (SLE)¹ (1) differentiates this disease from other autoimmune disorders in which the autoantibodies react with a narrow range of antigenic determinants. In autoimmune thyrotoxicosis (2), myasthenia gravis (3), and autoimmune hemolytic anemia (4), as examples, the diversity of autoantibody reactions is limited by comparison with the serological findings in SLE. A typical lupus serum can react with nucleic acids (native or denatured DNA; RNA) and nucleoproteins (Sm and RNP antigens; ribosomes; nucleohistones), cell surface antigens, cardiolipin, and certain coagulant activities of the blood, but usually not with any organ- or receptor-specific autoantigens (reviewed in [1]). Therefore, there is restricted heterogeneity within the serological polymorphism of SLE. The extent of autoantibody diversity in SLE is of fundamental importance because it pertains to the widely held view that this disease is a result of a generalized disturbance of the immune system (5, 6).

We have undertaken an analysis of the diversity of lupus autoantibodies by means of hybridoma technology (7, 8). The advantage of this method over analysis of serum antibodies is that individual molecular species of autoantibody can be examined so that true diversity can be distinguished from cross-reactivity. The hybridomas under study were prepared by fusion of spleen cells from unimmunized MRL/1 mice with mouse myeloma cells. MRL/1 mice were chosen because they spontaneously develop a severe form of SLE and because they produce high levels of anti-DNA antibodies (9). Some of the hybridoma anti-DNA autoantibodies we prepared have an immunochemical preference for particular purine bases, whereas others react with a wide range of polynucleotides (8). The latter kind of autoantibody might be reactive with the sugar-phosphate backbone of nucleic acids, a structural feature of all polynucleotides (8). The sugar-phosphate backbone of nucleic acids consists of phosphate groups, in phosphodiester linkage, separated by three carbon atoms of adjacent sugar molecules. The phospholipid cardiolipin also contains phosphodiester-linked phos-

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¹ *Abbreviations used in this paper:* dDNA, denatured DNA; H, heavy; pI, isoelectric point; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; SLE, systemic lupus erythematosus.

phate groups that are separated by three carbon atoms. In these experiments we show that monoclonal anti-DNA autoantibodies that bind to a variety of polynucleotides can react with phospholipids, including cardiolipin, and also mimic the in vitro action of a lupus anticoagulant. The serological diversity of certain lupus autoantibodies can therefore be explained by their reactivity with appropriately spaced phosphate residues that are present in a variety of biological molecules.

Materials and Methods

Hybridoma Autoantibodies and Anti-Idiotypes. The fusion of spleen cells of MRL/1 mice with either Sp2/O-Ag-14 or MPC-11 plasmacytomas, selection of cell lines producing anti-DNA antibodies, cloning and subcloning of these lines, purification of their immunoglobulin products, measurement of the nucleic acid-binding specificity, and production and characterization of anti-idiotypic antibodies were all described in detail previously (7, 8). Hybridoma H43 is a fusion with MPC-11, whereas hybridomas H102, H104, and H130 are fusions with Sp2/O-Ag-14.

Preparation of Phospholipid Micelles. Purified phospholipids obtained from Supelco, Inc., Bellefonte, Pa. were supplied in either chloroform or chloroform:methanol (2:1). 12-mg samples were transferred to 15-ml Pyrex conical test tubes and the organic solvents were evaporated under a nitrogen stream. Micelles were created by the slow addition of 6 ml of standard saline citrate (0.15 M NaCl, 0.015 M citrate, pH 7) to the tubes, with frequent vortex mixing, and incubation of the mixtures at 37°C for 15 min.

Competition for Hybridoma Antibody Binding to Denatured DNA (dDNA). Samples (100 µg) of hybridoma immunoglobulin diluted in phosphate-EDTA buffer (60 mM potassium phosphate, 30 mM EDTA, pH 8) were incubated with varying concentrations of either dDNA or phospholipid micelles in 100 µl of the same buffer in 1.5-ml microfuge tubes for 30 min at room temperature. [³H]Thymidine-labeled dDNA (75 ng) in 50 µl of phosphate-EDTA buffer prepared as described elsewhere (8) was added and the incubation continued for another 1 h. Then 50 µl of the gammaglobulin fraction of goat anti-mouse Ig serum (8) was added, and the mixture was kept at room temperature for 1 h. The resulting precipitate was centrifuged, washed twice with 1 ml of phosphate-EDTA buffer, dissolved in 0.2 ml of 0.1 N NaOH, and counted in 3 ml of Beckman Ready-Solv HP (Beckman Instruments, Inc., Fullerton, Calif.) in microvials.

Competition for Hybridoma Antibody Binding to dDNA on a Solid Phase. Samples (100 µl) of hybridoma antibody, diluted in 0.1 M KPO₄, pH 7, buffer with 0.5% fetal calf serum added to prevent nonspecific binding, were incubated with various amounts of either dDNA or phospholipid micelles in 100 µl of the same buffer for 1 h at 37°C. Aliquots (75 µl) of the mixtures were then transferred in duplicate to polystyrene tubes coated with 5 µg of dDNA (7) and incubated for 30 min at 37°C. The solution was aspirated and the tubes washed twice with buffer. ¹²⁵I-labeled affinity-purified goat anti-mouse Ig was added and the tubes were incubated for 45 min at room temperature. The solution was aspirated and the tubes were washed three times with buffer and assayed for ¹²⁵I.

Inhibition of Idiotypic-Anti-Idiotypic Reactions. ¹²⁵I-labeled hybridoma antibody was incubated with dDNA or phospholipids in glass tubes for 1 h at 37°C. This mixture (100 µl) was then transferred to washed anti-idiotypic-coated tubes and incubated for 1 h at 37°C. The tubes were washed three times with phosphate buffer and counted for ¹²⁵I.

Inhibition of Antinuclear Antibody Reactions. Cardiolipin or phosphatidyl ethanolamine in concentrations indicated in Results were mixed with an equal volume of hybridoma culture fluid for 1 h at 37°C. An aliquot of the mixture was then tested for antinuclear antibody reactivity by a previously described method (7).

Anticoagulant Properties of Monoclonal Antibodies. The anticoagulant properties of the monoclonal antibody preparations added to plasma were assessed using the activated partial thromboplastin time (10). Normal human plasma was obtained after centrifugation of whole blood, anticoagulated with one part 3.8% sodium citrate to nine parts whole blood. Before use, pooled plasma was recentrifuged at 35,000 g. Normal pooled plasma (0.2 ml), monoclonal antibody solution in 0.1 M Tris-HCl, pH 7.6 (0.2 ml), and phospholipid (Thrombofax Reagent;

Ortho Pharmaceutical Corp., Raritan, N. J.) diluted 1:32 or 1:64 in 0.1 M Tris-HCl, pH 7.6; 0.4 ml were incubated at 37°C for 2 h. Kaolin (2 mg/ml in 0.1 M Tris-HCl, pH 7.6; 0.1 ml) was added to 0.2 ml of the above solution and incubated for 15 min at 37°. Clotting was initiated with the addition of 25 mM CaCl₂ (0.1 ml) and the time to clot formation recorded. As a control, tests were performed in which the monoclonal antibody solution was replaced by the Tris buffer. All assays were performed in duplicate.

Isoelectric Focusing. Isoelectric focusing in agarose (Isogel; Marine Colloids, Inc., Rockland, Maine) was performed according to the method of Saravis and Zamcheck (11), in which 0.5% agarose gels that containing 2.5% Ampholytes (Ampholytes or Pharmalytes; LKB Instruments, Inc., Rockville, Md.) of the desired pH range were poured on GelBond (Marine Colloids, Inc.)-covered preheated glass plates and stored 1–24 h at 4°C in a humid chamber before use. Horse ferritin (isoelectric point [pI] 4.1–4.7) and human hemoglobin (pI 6.7), kindly supplied by J. Drysdale, served as marker proteins. Immediately after focusing, gels were sliced for pH measurements. In addition, 0.5-cm slices of gels were eluted into 0.5 ml of 0.1 M KPO₄-0.5% fetal calf serum buffer, and aliquots of these eluates were then tested in the DNA-binding assay and were also analyzed for Ig content in a solid-phase radioimmunoassay (RIA) using goat anti-mouse-Ig-coated tubes (8).

Results

Reactivity of Anti-DNA Autoantibodies with Phospholipids. Hybridoma autoantibodies H130, H104, and H102 react with a variety of polynucleotides, whereas H43 has a marked preference for a guanine -or hypoxanthine-containing determinant (8). The results with the first three autoantibodies suggest that they bind to polynucleotides by reaction with the sugar-phosphate backbone, a structure that contains repeating phosphodiester linkages. The presence of such linkages in phospholipids led us to test the hypothesis that monoclonal autoantibodies that bind to DNA can also bind to phospholipids. Fig. 1 shows the results of RIA in which either dDNA or various phospholipids were used as competitors of the binding of the autoantibodies H130, H104, H102, and H43 to dDNA. In the case of H130 (Fig. 1A), both cardiolipin (diphosphatidyl glycerol) and phosphatidic acid competed as well as or better than dDNA; 50% inhibition was achieved with ~0.12 nmol cardiolipin, 0.16 nmol phosphatidic acid, and 0.20 nmol dDNA (molar equivalents for both phospholipids and dDNA are expressed in terms of phosphorous content). Phosphatidyl glycerol was a weaker inhibitor than either cardiolipin or phosphatidic acid and 50% inhibition required 15 nmol. Phosphatidyl serine, phosphatidyl choline, and phosphatidyl ethanolamine failed to inhibit the reaction in amounts up to 100 nmol.

Similar results were obtained with H104 (Fig. 1B). The amounts (in nmol) of cardiolipin, phosphatidic acid, dDNA, and phosphatidyl glycerol that caused 50% inhibition were: 0.20, 0.45, 0.40, and 9.0, respectively. In the case of H102 (Fig. 1C), dDNA was ~80 times more effective as a competitor than cardiolipin. Nevertheless, cardiolipin, phosphatidic acid, and phosphatidyl glycerol inhibited the binding of this autoantibody to dDNA, but relatively high amounts (16, 220, and ~250 nmol, respectively) were required for 50% inhibition. As with H130 and H104, phosphatidyl serine, phosphatidyl choline, and phosphatidyl ethanolamine failed to compete with H102 for dDNA. By contrast with the preceding results, H43, the base-specific autoantibody, was not inhibited by any of the phospholipids tested (Fig. 1D).

Inhibition of Idiotypic Reactions by Phospholipids. Anti-idiotypic antibodies against H102 and H43, prepared in rabbits, have been shown to be site-specific in that their ability to bind to corresponding hybridoma autoantibody is completely inhibited by antigen (8). With H102, the order of inhibition by various polynucleotides

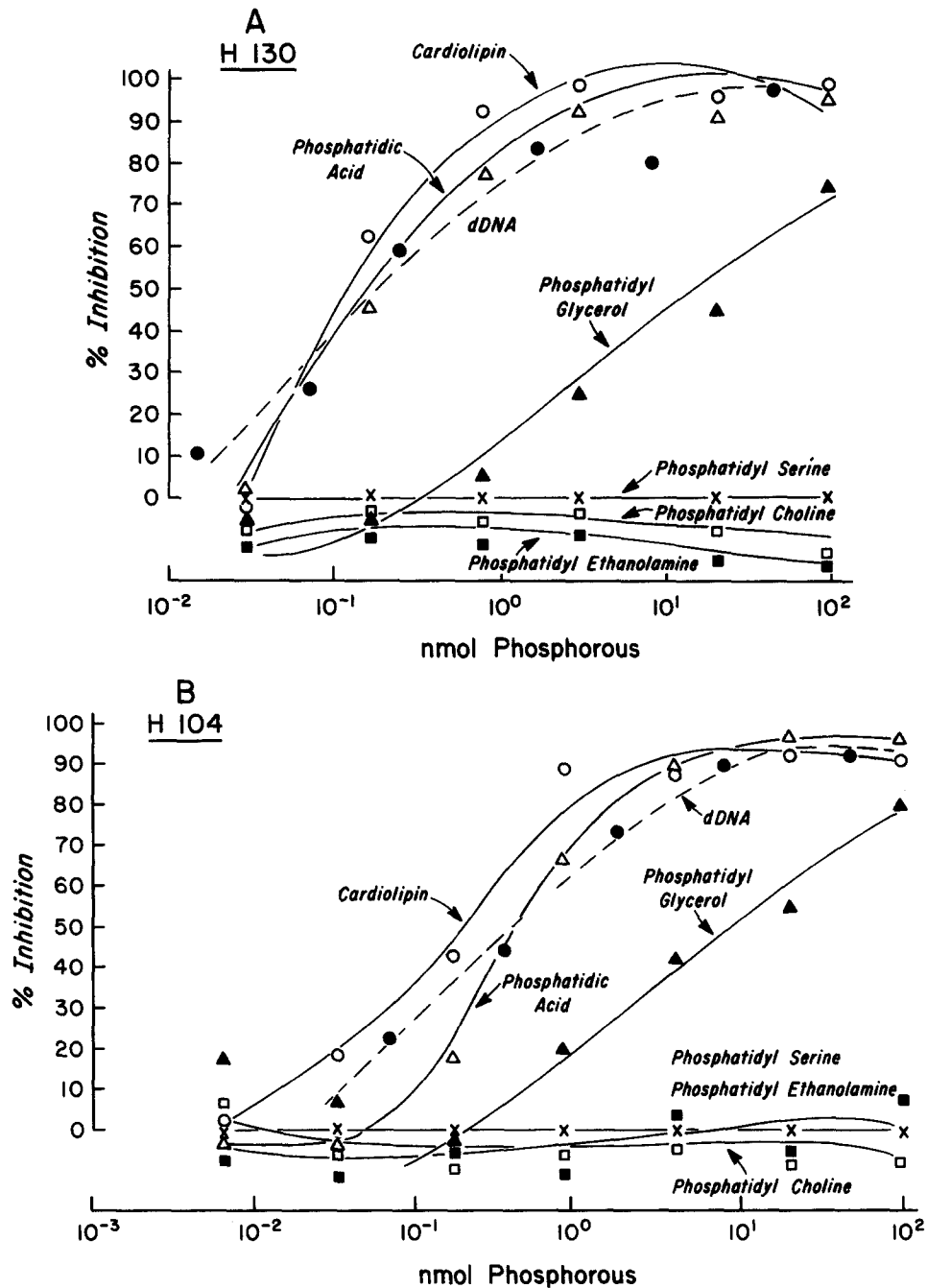


FIG. 1. A and B.

FIG. 1. Competitive RIA with hybridoma antibodies that bind to dDNA. H130, H104, and H43 were bound to dDNA on a solid phase. ^{125}I -labeled, affinity-purified goat anti-mouse Ig (40,000 cpm) was added to detect bound hybridoma. The counts bound in the absence of inhibitor were: H130, 667 cpm; H104, 622 cpm; H43, 2,304 cpm; normal Ig (MPC-11), 0 cpm. The amount of hybridoma antibody used was chosen so that the counts per minute bound increased linearly with increasing amount of the antibody. Purified MPC-11 Ig was used at identical protein concentrations. H102 was bound to ^3H dDNA in solution phase. In the absence of inhibitor, H102 bound 28.2% of the ^3H dDNA and normal Ig bound 2.0%. Values represent the mean percent inhibition of dDNA binding for duplicate samples in the presence of indicated competitors.

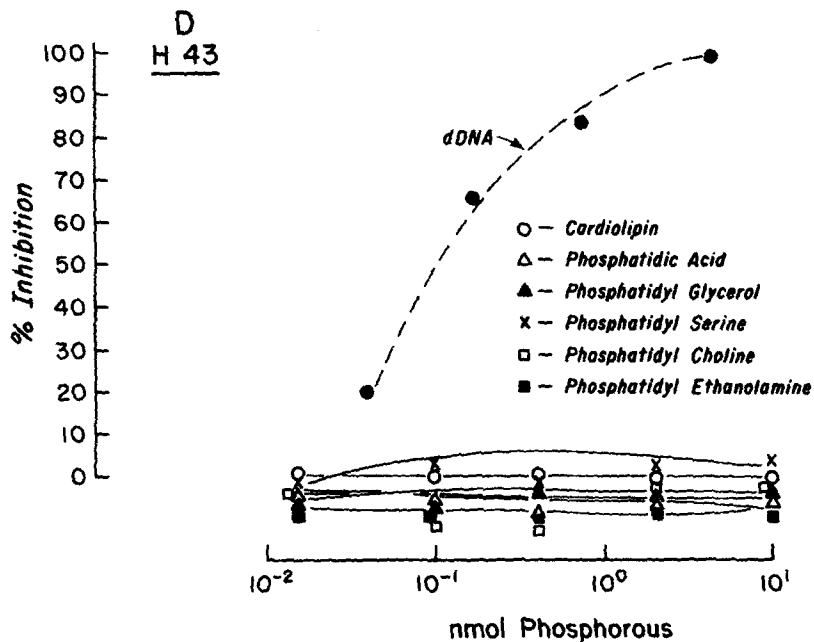
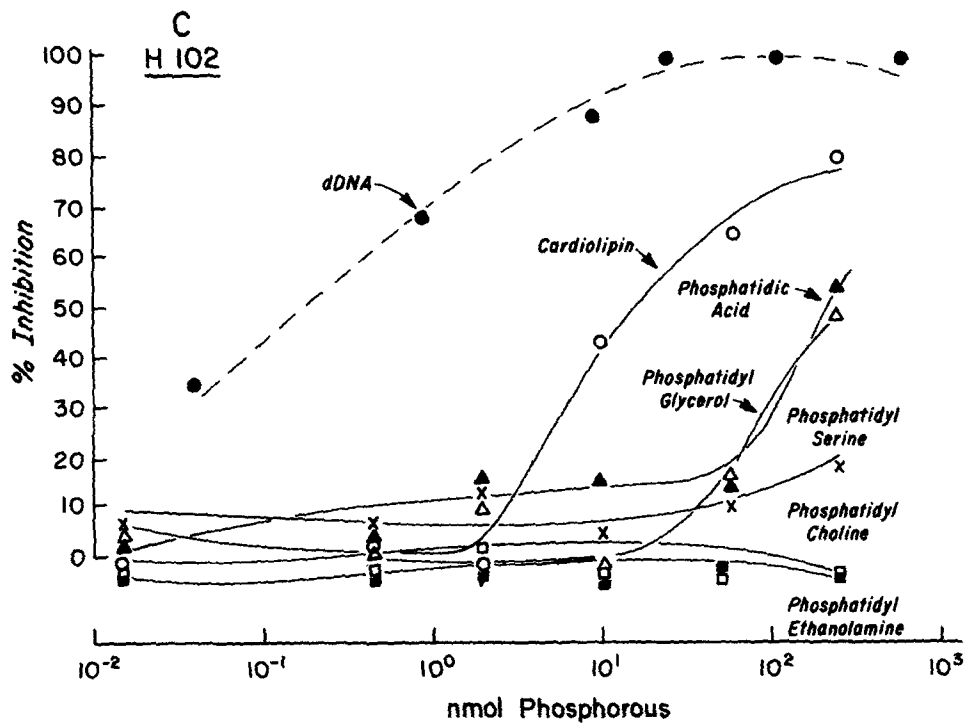


FIG. 1. C and D.

is the same as the order of the binding preference of this autoantibody for the polynucleotides (8). Fig. 2 A shows the results when phospholipids were used to inhibit the reaction between H102 and its anti-idiotypic antibody (anti-H102). Both cardiolipin and phosphatidic acid effectively inhibited the reaction. Cardiolipin was 160 times, and phosphatidic acid ~ 15 times, more effective than dDNA as competitors in the H102-anti-H102 assay. It is also noteworthy that weak, but consistently reproducible inhibition of the binding of H102 to its anti-idiotypic antibody was obtained with phosphatidyl ethanolamine, phosphatidyl glycerol, and phosphatidyl serine.

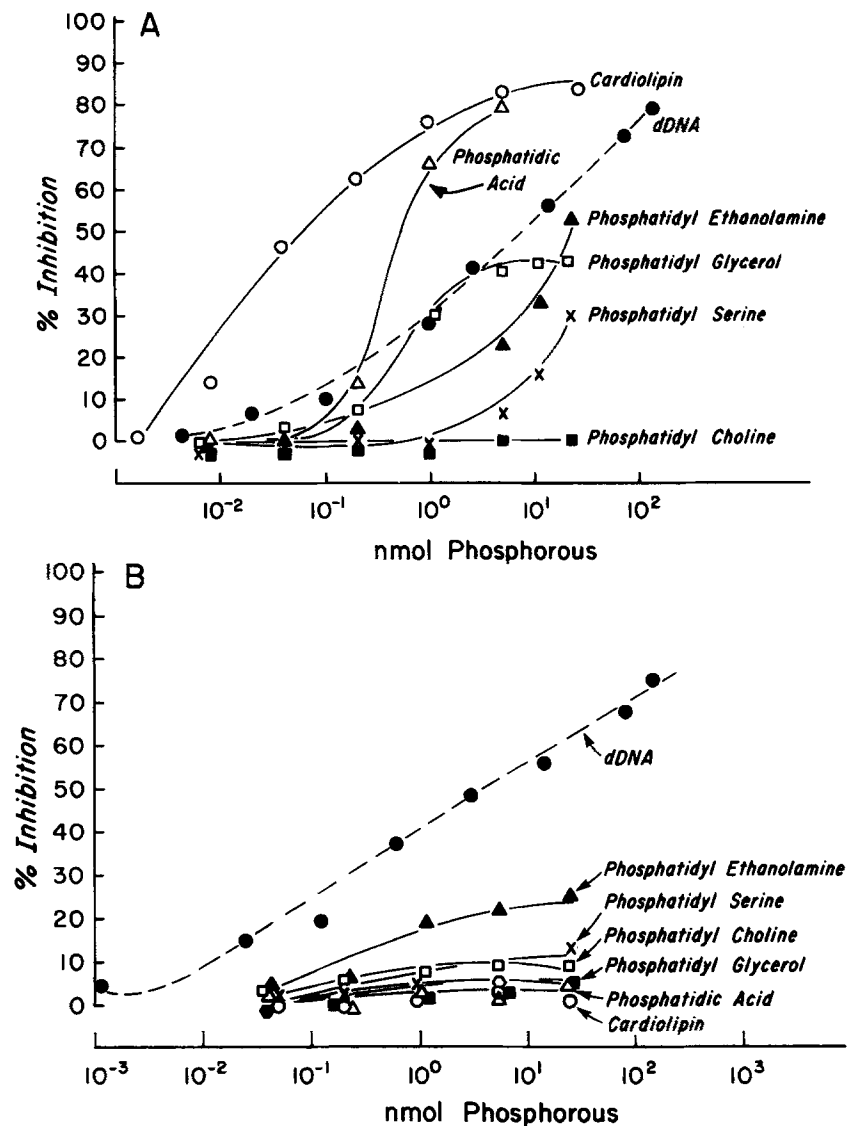


FIG. 2. Inhibition of idiotype-anti-idiotype binding by phospholipids. In these solid-phase radioimmunoassays, various concentrations of phospholipids or dDNA were incubated with ^{125}I -hybridoma antibody (idiotype) and the mixture was transferred to anti-idiotype-coated tubes. Values represent means of duplicate samples. (A) H102/anti-H102; (B) H43/anti-H43.

Phosphatidyl choline, by contrast, failed to inhibit the reaction. None of the six phospholipids were effective inhibitors of the reaction between anti-H43 with H43, the autoantibody with a preference for purine bases. A weak reaction, which never exceeded 20% inhibition, was found with phosphatidyl ethanolamine (Fig. 2B).

Inhibition of Antinuclear Reactions by Cardiolipin. Monoclonal autoantibody H102 was found to produce a strongly positive fluorescent antinuclear antibody reaction when tested on a substrate of rat liver sections (Fig. 3). The ability of cardiolipin and phosphatidyl ethanolamine to inhibit this serological reaction was tested by incubation of H102 with the phospholipids before the test. The results in Table I show that the binding of H102 to nuclei was completely inhibited by 50 $\mu\text{g}/\text{ml}$ of cardiolipin. Phosphatidyl ethanolamine, however, failed to inhibit the reaction. Nine other hybridoma autoantibodies that produce positive antinuclear reactions were also tested in this manner. Cardiolipin failed to inhibit the reaction in each of these cases.

Anticoagulant Activity of Anti-DNA Autoantibody. The anticoagulant properties of monoclonal anti-DNA antibodies were evaluated using the activated partial thromboplastin time assay. In this clotting assay, the kaolin-activated intrinsic pathway of blood coagulation is tested with a phospholipid (Thrombofax) which substitutes for the *in vivo* role of platelets. To increase the sensitivity of the assay, relatively low concentrations of phospholipid were used. In experiment A (Table II), phospholipid stock solutions were diluted 1:64. As shown in Table II, H43, H104, and H130 had no effect on the clotting time. However, H102 significantly prolonged the clotting time.

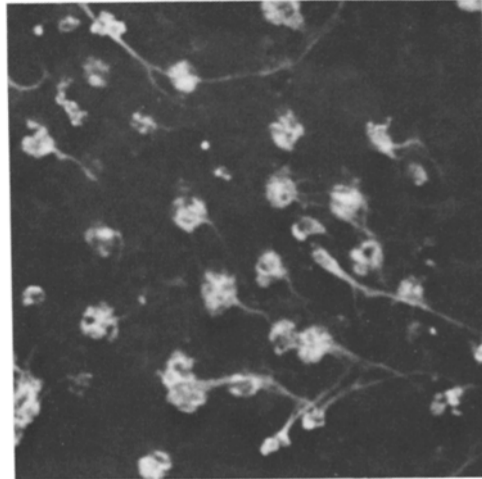


FIG. 3. Antinuclear antibody reaction of H102.

TABLE I
Effect of Cardiolipin on the Antinuclear Antibody Reactivity of H102 Antibody

	Concentration of cardiolipin (mg/ml)							
	0	0.00016	0.003	0.006	0.0125	0.025	0.05	0.1
Fluorescence reaction	4 ⁺ *	3 ⁺	3 ⁺	2 ⁺	1 ⁺	2 ⁺	0	0

* 4⁺, 3⁺, 2⁺, and 1⁺ represent a qualitative estimate of the intensity of fluorescent staining from the most intense positive reaction (4⁺) to least intense positive antinuclear antibody (1⁺). 0 is no reaction.

TABLE II
*Anticoagulant Properties of Monoclonal Antibodies: Effect on the Partial
 Thromboplastin Time*

	Antibody concentration	Clotting time	
		Experiment A	Experiment B
	$\mu\text{g/ml}$	<i>s</i>	
Buffer	0	71.0, 71.8	52.2, 52.0
H43	17	72.4, 72.8	53.0, 52.9
	170	71.8, 72.0	52.2, 53.0
H102	14	75.0, 75.2	55.2, 54.8
	140	78.8, 78.6	56.0, 56.8
Buffer	0		47.8, 48.2
H104	27		47.8, 47.6
	267		42.4, 42.0
H130	28		48.0, 48.0
	280		41.0, 41.2

For experiments in column A, the stock Thromboplastin solution was used in a dilution of 1:64 and for experiments in column B, in a dilution of 1:32. The clotting time, in seconds, was measured from the initiation of clotting with kaolin and calcium to clot formation. H102 prolonged the partial thromboplastin time; H43, H104, and H130 had no effect.

At final concentrations of H102 in the incubation mixture of 14 $\mu\text{g/ml}$ and 140 $\mu\text{g/ml}$, the clotting time was increased by ~ 4 s and 7 s, respectively. In experiment B (Table II), when the phospholipid stock solutions were diluted 1:32, H43, H104 and H130 again had no effect on the clotting time. H102 at concentrations of 14 $\mu\text{g/ml}$ and 140 $\mu\text{g/ml}$ again prolonged the clotting time by 3 s and 4.5 s. ^{125}I -labeled $\text{F}(\text{ab}')_2$ fragments of H102 failed to bind to platelets, whether they were activated or not (data not shown). This result resembles that obtained with a human monoclonal IgM paraprotein that binds to phospholipids and prolongs the partial thromboplastin time, but does not bind to platelets (12). The finding that Thromboplastin, a commercial phospholipid reagent used in the partial thromboplastin time assay, inhibited the reaction of H102 with dDNA supports the interpretation that H102 prolonged the partial thromboplastin time by binding to a phospholipid required for activation of the intrinsic blood coagulation pathway. Undiluted and fivefold-diluted Thromboplastin inhibited the H102-dDNA reaction by 60 and 30%, respectively; neither amount inhibited the H43-dDNA interaction.

Isoelectric Focusing of Hybridoma Autoantibodies. After the isoelectric focusing procedure, fractions eluted from the sliced gels were assayed for both immunoglobulin content and binding to dDNA. The latter was done in the presence of either buffer or buffer containing 0.2 mg/ml cardiolipin. H43 gave a sharp dDNA-binding peak at pI 8.05 (Fig. 4A). The pattern of Ig content of the gel fractions of this protein had a broader peak than the DNA-binding peak. This result probably represents the presence of MPC-11-derived hybrid molecules that do not bind to dDNA. H102

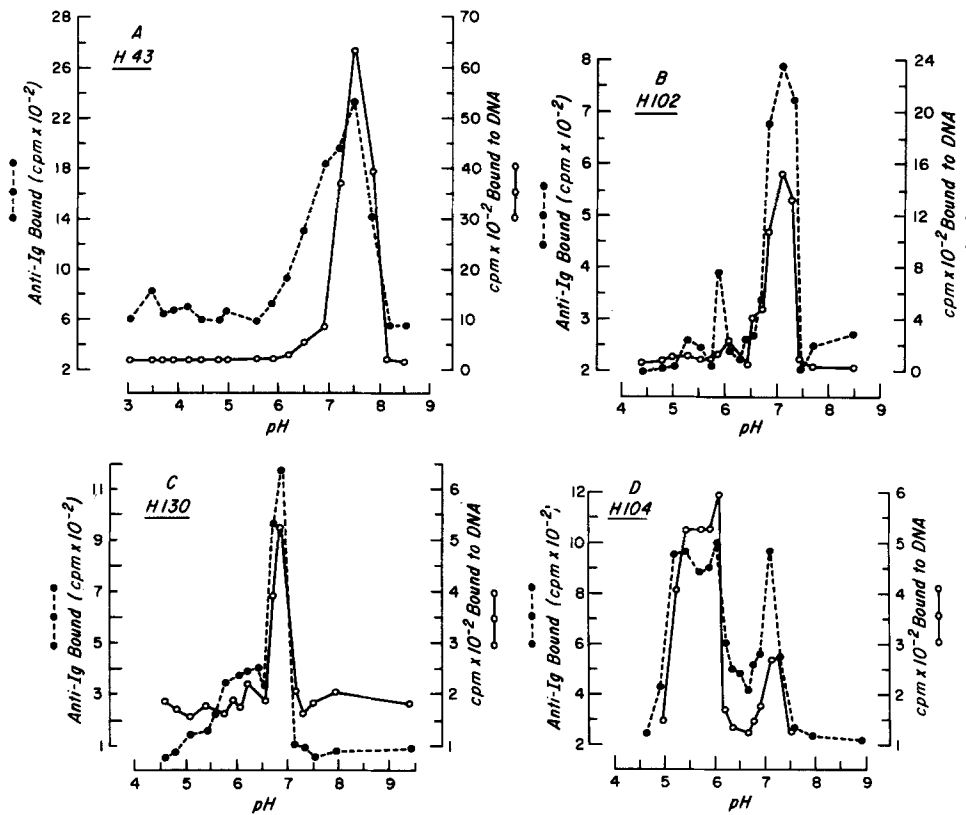


FIG. 4. Isoelectric focusing of hybridoma autoantibodies. The patterns represent results of assays on eluates of gel slices for Ig concentration (—) or capacity to bind to dDNA (---). (A) H43; (B) H102; (C) H130; (D) H104.

showed a single peak of Ig at pI 7.05 which corresponded exactly to the peak of dDNA binding activity (Fig. 4B). The latter was completely inhibited in the presence of cardiolipin. H130 gave single, concordant peaks of Ig content and dDNA binding, both with pI of 6.85 (Fig. 4C). The peak dDNA binding was inhibited by 40% at the tested concentration of cardiolipin.

H104 has reproducibly shown two superimposable Ig and dDNA-binding peaks (Fig. 4D). The first, and largest, peak covers a pI range from 5.2–6.2. This peak has been found to increase during storage and with repeated freezing and thawing of the antibody. The second peak has a sharp pI of 7.1. Cardiolipin inhibited dDNA binding of the first peak by 50%, but enhanced (twofold) the dDNA binding of the second peak. H104 is unusual because it seems to consist mainly of κ chains. Analyses of H104 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 5) demonstrated a strongly staining band with an $\sim 30,000$ mol wt, but little staining in the position expected of heavy (H) chains. H chains could not be detected in H104 by immunodiffusion with antisera directed against all classes of Ig, but κ chains were readily found. This hybridoma product has a strong tendency to aggregate, and most of the dDNA-binding activity is removed by centrifugation at 80,000–100,000 *g*. These results are consistent with the interpretation that the first broad peak that

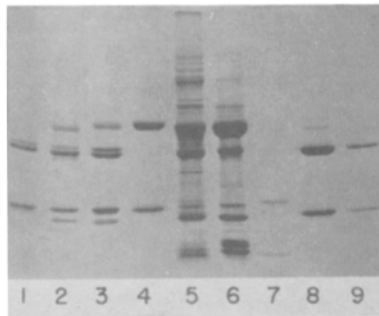


FIG. 5. SDS-polyacrylamide gel electrophoresis. Lane 1: MPC-11 Ig; lane 4: human IgM; lane 7: H104 Ig; lane 9: H102 Ig.

H104 shows on isoelectric focusing represents aggregates that vary in size and charge, whereas the second peak consists of nonaggregated material. The increased dDNA-binding activity brought about by cardiolipin may be the result of the aggregation of single light chains into a favorable binding configuration.

Discussion

Our results with monoclonal autoantibodies demonstrate that certain anti-DNA antibodies are serologically polymorphic, with the capacity to react with a diversity of molecules. A single molecular species of autoantibody (e.g., H102) has been shown to produce many of the usual serological findings of SLE: a positive antinuclear antibody test, antibody reactions with DNA and other polynucleotides, antibody reactions with cardiolipin, and prolongation of the activated partial thromboplastin test that is characteristic of a lupus anticoagulant (13). These diverse reactions are apparently a result of the ability of the autoantibody to bind to structures that contain phosphate esters. The identification of such lupus autoantibodies as anti-DNA may thus be a fortuitous result of the choice of DNA as a test antigen. As noted previously, some of the monoclonal autoantibodies obtained from MRL/1 mice react strongly with polynucleotides other than DNA (8) as do some of the anti-DNA antibodies in human SLE sera (14). Therefore, both diversity of antibody populations (8, 14) and cross-reactions of individual antibodies appear to contribute to the serological heterogeneity observed in SLE.

The chemical structure of cardiolipin resembles that of a DNA backbone (Fig. 6), with phosphodiester-linked phosphate groups separated by an equal spacing of carbon atoms. This similarity may explain why anti-cardiolipin antibodies that were produced by rabbits after injection of micelles with cardiolipin determinants of their surface cross-reacted with micelles into which DNA was incorporated (15). Stollar (16) has suggested that the polynucleotide backbone may form the determinant for many lupus autoantibodies, and that this property could account for the false-positive serological test for syphilis, which depends on a reaction with cardiolipin.

The reactions of H130, H104, and H102 with various phospholipids (Fig. 1) suggest that regularly spaced phosphate groups constitute the epitope for these antibodies. Presumably, cardiolipin and dDNA react the best because they contain two such groups separated by three carbon atoms. Phosphatidic acid may react equally well because it presents a series of repeating phosphate determinants on a micellar surface.

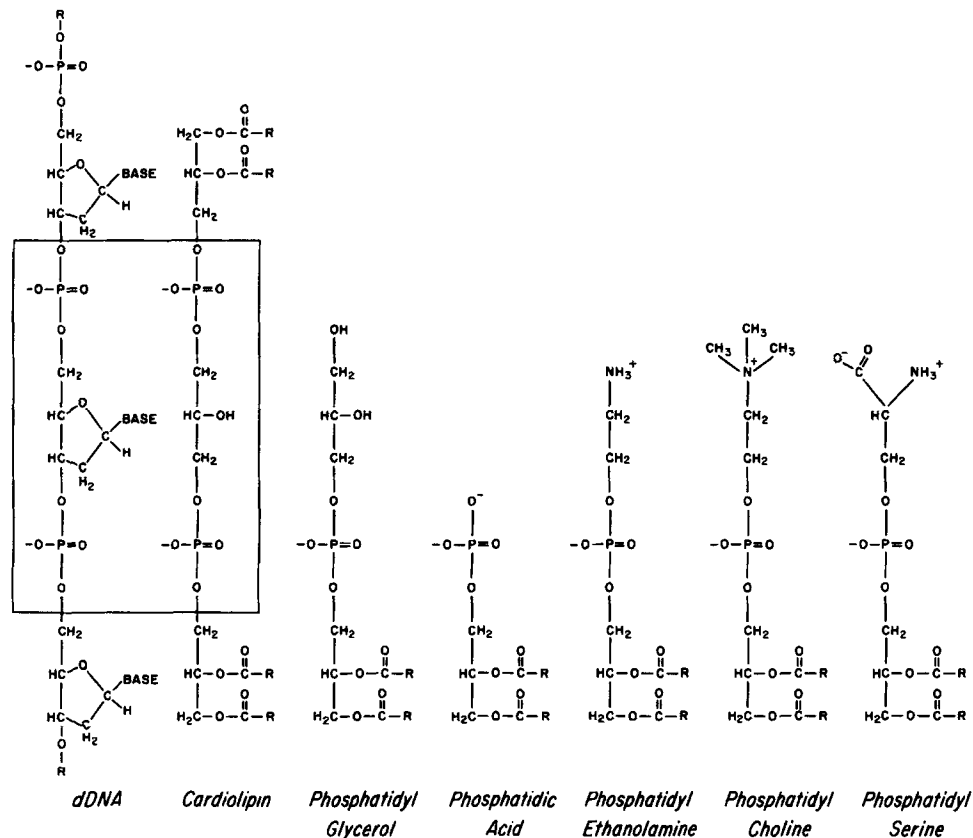


FIG. 6. Comparison of chemical structures of DNA and phospholipids.

Phosphatidyl glycerol also contains a large part of the determinant present in cardiolipin and dDNA. By contrast, when phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine are presented on a micelle, the positively charged groups of these molecules also exposed to the aqueous solvent may interfere with the binding of the phosphate groups with the antibody.

The results obtained with inhibition of the idiotype-anti-idiotype reaction (Fig. 2) support the interpretation that certain phospholipids can occupy the antigen-binding site of H102 because anti-H102 has been shown to be directed against a determinant(s) in the hypervariable region of H102 (8).

The multitude of serological reactions found in SLE has been an important element in the development of theories about this autoimmune disease (5, 6). Our results suggest that some of the diversity of autoantibody reactions in this disease may be a result, in part, of the presence of a relatively simple antigenic structure in a variety of biological molecules. We are currently analyzing a large number of hybridoma products to determine the frequency of polyspecific lupus autoantibodies. The presence of readily detectable cardiolipin-binding antibodies in serum of MRL/1 mice (J. Rauch, unpublished observation) suggests that such antibodies may constitute an important fraction of the autoantibody population in these animals.

Summary

Hybridomas that produce anti-DNA autoantibodies were prepared from spleen cells of unimmunized MRL/1 mice, a strain that spontaneously develops severe systemic lupus erythematosus (SLE). Reactivities of these monoclonal antibodies with a wide range of polynucleotides prompted tests of their reactions with phospholipids which, like polynucleotides, contain diester-linked phosphate groups in their backbones. In competitive radioimmunoassays, cardiolipin, phosphatidic acid, and phosphatidyl glycerol blocked the binding of these hybridoma antibodies to denatured DNA. These phospholipids also specifically inhibited the reaction between a hybridoma antibody and a site-specific anti-idiotypic antibody. The antinuclear reaction of one of these antibodies was specifically inhibited by cardiolipin. This same antibody prolonged the activated partial thromboplastin time in a manner characteristic of a lupus anticoagulant, presumably by binding to phospholipid in the test system. The polyspecific reactivity of a single molecular species of lupus autoantibody suggests that some of the diverse serological abnormalities of SLE may be a result of the binding of certain autoantibodies to a phosphodiester-containing epitope that is present in diverse biological molecules.

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