Lupus Nephritis: Varying Complement-Fixing Properties of Immunoglobulin G Antibodies to Antigens of Cell Nuclei

Abstract. *Relative complement-fixing activity of antibodies to nuclear antigens, including DNA, were determined in serums of 15 patients with, and 65 patients without, active lupus nephritis, by comparing titers obtained in two methods. High complement-fixing activity of antibody was found in the nephritis group and low activity in the others. Results with immunoglobulin G fractions were similar.*

The consistent occurrence of (i) antibodies to nuclear antigens and of lowered concentrations of complement in serum (*1*) and (ii) the deposition of such antibodies and a component of complement in the kidney lesions (*2*) are accepted as evidence that antigen-antibody complexes composed of antigenic nuclear substances and antibodies to them may be involved in pathogenesis of the glomerulonephritis of systemic lupus erythematosus (SLE). However, although these antibodies are apparently always present in SLE and most often in high titer, nephritis is not. In addition, although in other diseases antibodies to components of cell nuclei are usually of low titer, equally high titers (*3*) may occur without associated glomerulonephritis.

We now report that high complement-fixing activity (serum antibody to nuclear antigens) is associated with nephritis, whereas low activity is associated with lack of nephritis. Even when the titer of antibody which fixes complement is high in the absence of nephritis, the relative complement-fixing activity of the antibody is low, since the total amount of antibody is even higher, as determined by a method not involving complement fixation. Similar differences in properties of the antibodies to nuclear antigens were observed when pure IgG (immunoglobulin G) fractions were used. To relate our observations to other evidence linking these antibodies and lupus nephritis we propose that the occurrence of glomerulonephritis in some patients and the absence in others with equally high antibody titers may result from differing biological activity of these antibodies. Our findings suggest that pathogenic significance of antibody of the IgG class in tissue damage due to antigen-antibody complexes may in general be determined by varying complement-fixing activity of the antibody, possibly resulting from varying mixtures of molecules with different complement-fixing capacity.

Eighty patients with high titers of antibodies to nuclear material (1 : 16 or greater) were studied. Groups tested were 15 SLE patients with active nephritis and reduced activity of complement in serum; 26 other SLE patients, including those with nephritis but normal activity of complement in serum; 20 patients with rheumatoid arthritis; 7 patients with chronic active hepatitis; and 12 patients with antibodies to nuclear antigens which appeared during administration of procainamide for cardiac arrhythmias. No patients in the last three groups gave evidence of nephritis. Antibodies to nuclear antigens were demonstrated by the indirect immunofluorescent technique with rat liver sections as a source of nuclei, and with antiserum to human gamma globulin as the conjugate. Complement-fixing antibodies to nuclear antigens were determined by the complement fluorescent technique (*2*) with rat liver sections, fresh normal human serum as a source of complement, and goat antiserum to the B_1C globulin fraction of human serum conjugated with fluorescein isothiocyanate (*4*).

Table 1. Comparison of antibodies to nuclear antigens (ANA) and complement-fixing antibodies to nuclear antigens (CFANA) in IgG fractions; SLE, systemic lupus erythematosus.

Ratio of ANA: CFANA in serum*	Minimum concentration of IgG giving positive reaction		Ratio of CFANA: ANA in IgG
	IgG-ANA ($\times 10^{-7}M$)	CFANA ($\times 10^{-7}M$)	
SLE with active nephritis			
1	2.8	2.8	1
1	1.1	1.1	1
1	3.3	3.3	1
1	1.6	1.6	1
1	2.3	2.3	1
Other SLE			
8	1.7	13.4	8
16	9.1	290.0	32
64	2.8	45.0	16
Drug-induced ANA			
32	6.7	$>$ 213.3	$>$ 32
32	7.3	58.0	8
64	15.9	$>$ 127.2	$>$ 8
64	34.6	$>$ 276.8	$>$ 8
Rheumatoid arthritis			
32	87.1	$>$ 697.1	$>$ 8
32	535.7	$>$ 1071.6	$>$ 2
64	90.4	$>$ 180.8	$>$ 2
128	83.7	$>$ 334.8	$>$ 4
256	$>$ 415.1	$>$ 415.1	

* From data in Fig. 1. † A molecular weight of 160,000 was used.

Serum complement was determined on fresh serum by an adaptation of the method of Nelson *et al.* (*5*). After inactivation, antibody titers of both sorts were determined simultaneously. In other immunofluorescent tests, calf thymus DNA spots (*6*) or deoxyribonucleoprotein were used as the antigens (*7*). In conventional complement-fixation tests suspensions of deoxyribonucleoprotein or solutions of DNA were used. Relative complement-fixing activity of antibody was determined by comparison of the titer obtained in the fluorescent complement technique or conventional complement fixation with the titer obtained by an indirect immunofluorescent technique not involving complement.

In Fig. 1, the data which were obtained with whole serum and tissue sections are presented. Group 1 is definitely different from groups 3, 4, and 5. All serums from patients with chronic active hepatitis and those with antibodies to nuclear antigens induced by procainamide, as well as serum from 18 of 20 patients with rheumatoid arthritis, showed complement-fixing antibody titers two to seven tubes lower than antibody to nuclear antigens. The results imply that a distinctive feature related to the absence of nephritis in patients of groups 3, 4, and 5 is the occurrence of antibody low in complement-fixing activity. This is indicated by antibody titers substantially higher by the technique not involving complement than by the complement-fixation technique, even though the titer of antibody which fixes complement may itself be relatively high. Results in SLE patients with normal activity of serum complement (group 2) were variable, as expected from the modification of disease in many by the anti-inflammatory effect of corticosteroid therapy. Consideration of titers of antibody to nuclear antigens alone reveals marked overlap between all groups. There was no apparent correlation between the presence of rheumatoid factor and low complement-fixing antibody to nuclear antigens.

Results with DNA and deoxyribonucleoprotein as antigens (Fig. 2) were similar to those obtained with whole nuclei. Titers obtained in conventional complement-fixation tests were compared with those obtained in immunofluorescent spot tests with the two antigens and an antiserum to human gamma globulin conjugated with fluorescein isothiocyanate. Serums were titrated from group 1 where both types of antibody

were equal (from Fig. 1) and from groups 2 to 5 having the total antibody greater than the complement-fixing antibody. For technical reasons only serums with titers of 1:4 or higher were useful for the desired comparison.

Studies were made with pure IgG isolated from 17 serums by column chromatography (*8*) and the conjugate specific for IgG (Table 1). Purified samples gave a single line against rabbit antiserum to whole human serum in immunoelectrophoresis. All were negative at maximum concentration using conjugated specific antibody to IgA and IgM. Minimum concentrations of IgG giving positive tests with both types of antibody (rat liver sections) were the same in five different IgG samples from patients whose whole serum also showed that titers to both types of antibody were the same. Immunoglobulin G from 12 patients whose serum showed a lower titer of complement-fixing antibody required a 2 to 32 times higher concentration of IgG for positive complement fluorescent tests. This group included three patients with SLE and normal serum complement level, five with rheumatoid arthritis, and four with antibodies to nuclear antigens induced by procainamide.

Our results indicate that serum antibody to nuclear antigens in patients with active lupus nephritis is very high in complement-fixing activity, whereas in patients without nephritis comparable titers of antibody are accompanied by lower complement-fixing activity. Antibodies to nuclei are directed at several nuclear antigens, and serums vary in content of antibodies to the various antigens. We therefore studied activity of serum antibodies to DNA and found that results were similar to those obtained when we used rat liver nuclei (*9*, *10*).

In view of the results obtained with antibody to DNA, it is clear that there are differences between the groups which cannot be attributed to varying content of antibody to different nuclear antigens. Antibody (other than to DNA) to nuclear and cytoplasmic antigens have been eluted along with antibody to DNA from kidneys of patients dying of SLE (*11*). In addition, we have observed that, in some of the serums in the nephritis group, at high titer the complement-fixing antibody was of the "speckled" pattern of fluorescent nuclear staining. This pattern usually indicates antibody to a nuclear antigen which does not contain DNA (*12*). It is possible, therefore, that although com-

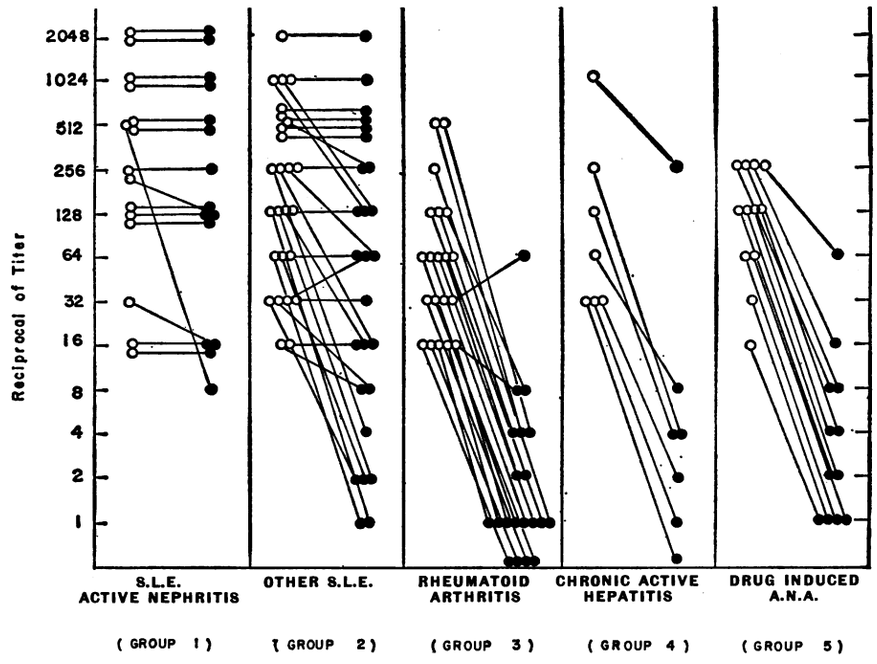

Fig. 1. Titers of antibody to nuclear antigens, as judged by conventional indirect immunofluorescence (total antibody to nuclear antigens, ANA, open circles) and by complement-fixation test (complement fluorescent technique, CFANA, solid circles) in various disease categories. The two titers from individual patients are joined.

plexes of DNA and its specific antibody may be most commonly involved in lupus nephritis, other nuclear and cytoplasmic antigens are also important. The occurrence of immunologic damage in the kidney may be due more to the complement-fixing activity of the antibody than to the nature of the specific antigen.

Different immunoglobulin classes vary in their capacity to fix complement, but it has not been possible to relate immunoglobulin class of antibodies (IgG, IgM, or IgA) to nuclear antigens to the presence or absence of nephritis. We have shown here that the high complement-fixing activity of these antibodies in nephritis serums is also observed with pure IgG fractions. The subclasses of immunoglobulin G also vary in their ability to fix complement (*13*); IgG subclasses may also vary in activity in producing tissue damage after they react with antigens, since this phenomenon has been shown to be dependent on complement activation.

It has been reported that two adjacent IgG molecules are required for complement binding by antigen-antibody complexes. Rabbit gamma G anti-

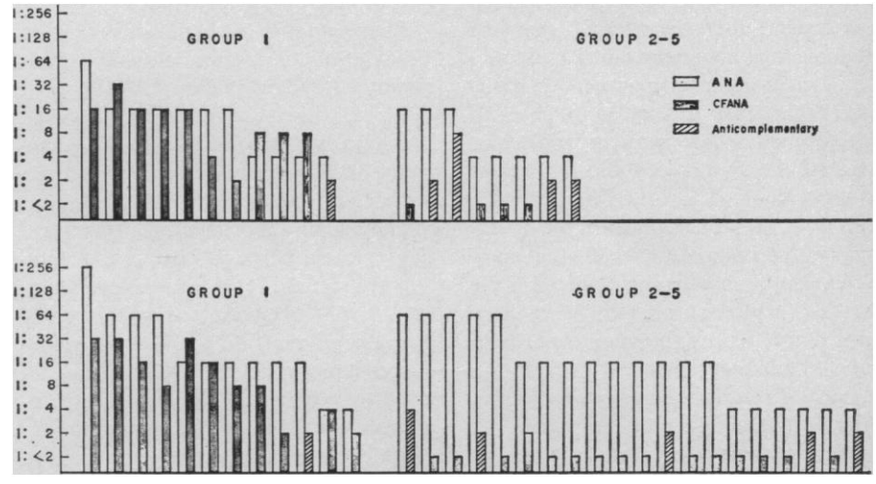

Fig. 2. Comparison of titers of antibody to DNA (top) and antibody to deoxyribonucleoprotein (bottom), as determined by immunofluorescent spot test (ANA) and conventional complement-fixation test (CFANA).

body, treated to eliminate complement-fixing capacity, inhibits complement binding when mixed with untreated complement-fixing antibody (*14*). Mixtures in vivo of specific antibody of variable IgG subclass could affect the extent of tissue damage by determining the probability of the occurrence of two adjacent complement reactive molecules bound to antigen. Our observations would appear consistent with such a concept as applied to the occurrence of glomerulonephritis in patients with antibodies to nuclear antigens. Genetic or other factors might determine the subclass of IgG molecules which comprise the population of a given antibody present at any particular time.

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Proteins in Denaturing Solvents: Gel Exclusion Studies

Abstract. *Many proteins are fully denatured and separated into component polypeptide chains in 6M guanidine hydrochloride containing mercaptoethanol and a chelating agent. In this and similar denaturing solvents, polypeptide chains of molecular weights from 2,000 to 100,000 can be eluted from 6 percent agarose columns and separated according to molecular weight. By this procedure molecular weights may be assigned to proteins (including many normally insoluble in nondenaturing solvents) without the uncertainties that arise when native molecules of unknown shape are studied.*

The volumes accessible to macromolecules in columns of molecular-sieving media such as Sephadex (Pharmacia), polyacrylamide gels (Bio-Rad), or agarose depend upon the size and shape of the molecule; hence the elution volumes for a series of homologous macromolecules reflect their molecular weights (*1*). For estimation of protein molecular weights, the shapes and the hydrations of the sample and of calibrating proteins must be similar. This source of ambiguity in instances in which the shape is unknown can often be obviated if the proteins are denatured; it has been shown that many polypeptide chains show homologous physical behavior in 5*M* or 6*M* solutions of guanidine hydrochloride containing reducing agents (*2*, *3*); under these conditions the chains adopt a conformation close to a random coil (*2*). Therefore the relative weights of peptide chains (and, if the numbers of constituent chains are known, the molecular weights of the parent proteins) can be deduced from gel filtration in denaturing solvents.

I have studied the behavior of insulin (Sigma), bovine serum albumin, ovalbumin, β-lactoglobulin (all Pentex), aldolase, chymotrypsinogen, trypsin, lysozyme, and ribonuclease (all Worthington) on a variety of gel media. Each sample was checked for purity by ultracentrifugal and molecular-sieve analysis. Each protein was dissolved in 6*M* solution of guanidine hydrochloride containing 0.05*M* lithium chloride, 0.1*M* mercaptoethanol, and 0.01*M* ethylenediaminetetraacetic acid (EDTA), adjusted to *p*H 6.5 to 7.3, and chromatographed with 5*M* or 6*M* guanidine hydrochloride or 8*M* urea; each mixture contained these proportions of mercaptoethanol, EDTA, and lithium chloride. The *p*H of the eluting solution was adjusted to between 6.5 and 8. Lithium chloride was used for an irrelevant purpose and can be replaced by other electrolytes.

These experiments were performed at 23°C in Pharmacia 2 by 100 cm columns of siliconized glass or, more commonly, in 0.9 by 60 cm acrylic columns. Usually 0.2 to 3 mg of protein was applied in not more than 0.4 ml—that is, 4 percent of the exclusion volume of the smaller columns. The eluting solutions were pumped through the columns at a constant rate (not exceeding 2.5 ml/hour per square centimeter of column section) and collected in 40 to 60 fractions. On the shorter columns an analysis could be completed within 24 hours. The protein in the eluate samples was estimated turbidimetrically with trichloroacetic acid or sulfosalicylic acid. The procedure can be made more sensitive if the protein can be coupled to a dye (such as rhodamine isothiocyanate) or radioactively labeled, in vivo, or with a suitable reagent (reaction with $H^{14}CHO$, for example).

A large increase in intrinsic viscosity accompanies protein denaturation (*2*), and, in correlation with their greater physical dimensions, the denatured molecules are excluded from gel media that normally admit the native. Sephadex G-100 or beads of 10 percent agarose are suitable only for the characterization of denatured polypeptide chains up to 20,000 daltons in weight. The gel encompassing resolution of proteins over the most useful range of molecular weights proved to be Bio-Gel A-5M with a nominal 6 percent content of agarose. Figure 1 shows a series of elution curves of different polypeptide chains run on this material. The exclusion and internal volumes of the columns were marked by cochromatography of dextran blue (Pharmacia) and dinitrophenyl-alanine with the protein. The exclusion limit of the 6 percent agarose is about 100,000 molecular weight; for longer chains (for example, the α- and β-chains of collagen) 4 percent agarose columns may be employed; however, the peaks—at least on Sepharose 4B—were broader than those in Fig. 1.

The eluted peaks showed a considerable spread that precluded facile discrimination of chains of similar size; nevertheless, the maxima of the peaks (by interpolation between successive tubes) showed good consistency in all experiments. The distribution coefficient (*1*) for a solute is $K = (V_e - V_o)/(V_i - V_o)$, where V_e is the elution volume of