Circulating DNA:Anti-DNA Complexes in Systemic Lupus Erythematosus

DETECTION AND CHARACTERIZATION BY ULTRACENTRIFUGATION

CHARLES BRUNEAU and JACQUES BENVENISTE, *Laboratoire de Recherche sur l'Allergie et l'Immunopathologie, Institut National de la Santé et de la Recherche Medicale, U 131, Clamart, France*

ABSTRACT Although it is generally accepted that DNA:anti-DNA immune complexes play a significant role in the pathogenesis of tissue injury in systemic lupus erythematosus, their presence in the circulation is still a matter of controversy. In this study, we detected DNA:anti-DNA complexes by identification of both the antigen and(or) the antibody, the necessary requisites for immune complex definition, in 14 of 24 plasmas (7 of 11 patients). These antibodies were specific for native DNA and could be adsorbed by anti-immunoglobulin (Ig)G antisera. The DNA recovered was, at least in part, of low molecular weight. The presence of DNA: anti-DNA complexes was not related to high molecular weight IgG, cryoprecipitins, positive polyethylene glycol precipitation, or low plasma C3 levels. It was related significantly to low plasma C4 levels and to the presence of diffuse proliferative nephritis. The lack of correlation with other methods of detection of immune complexes and with the presence of heavy IgG (above 13 S) is in favor of the existence of other antigen-antibody systems (or aggregated immunoglobulins) in systemic lupus erythematosus plasmas. From the results, it appears that methods directed towards the demonstration of specific immune complexes are more informative than those detecting heavy or altered immunoglobulins.

INTRODUCTION

Systemic lupus erythematosus (SLE)[1] has been proposed as the prototype of human disease where immune complex (IC) deposition plays an important pathogenic role (1, 2). Many techniques have been developed recently to detect circulating IC, and evidence for their existence in SLE sera has been obtained with most of them (3). However, these methods are not specific for IC (4, 5) and do not permit their characterization.

Although there is evidence that some of the circulating IC are composed of DNA and antibody to DNA (6–9), the subject is still controversial. Recently published articles have disputed the presence of these IC in the circulation in any significant amount (10–12). Moreover, some studies suggest that DNA:anti-DNA IC may be formed *in situ* rather than deposited (13, 14). According to the definition of "immune complexes," the identification of the antigen and the antibody is a desirable goal when attempting to detect the presence of IC. In the last analysis, it does not appear that this condition has been adequately met by most investigators.

In this article, we present the results obtained after ultracentrifugation of SLE plasmas in sucrose density gradients at physiologic and acid pHs. We have shown that this method was adequate for the detection and dissociation of IC formed in vitro, including DNA:anti-DNA IC, and allowed the recovery of the components of these IC (15). We demonstrate here the presence of DNA:anti-DNA IC, i.e., the identification of anti-DNA antibody and of DNA after dissociation of high molecular weight material, in 14 of 24 SLE plasmas (7 of 11 patients). These results are compared with the presence of high molecular weight immunoglobulin (Ig)G, with other tests used for the detection of circulating IC, and with some clinical features.

METHODS

Patients and plasmas. 24 plasmas from 11 patients fulfilling the American Rheumatism Association criteria for SLE (16) were studied. Four samples were obtained from one patient, three from each of five patients, and five from five patients. Heparinized blood was centrifuged at room temperature.

Dr. Bruneau's present address is Department of Rheumatology, Montreal General Hospital, Montreal, Canada.

Received for publication 22 February 1978 and in revised form 13 March 1979.

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DBC, DNA binding capacity; IC, immune complex(es); PEG, polyethylene glycol; SAS, saturated ammonium sulfate; SLE, systemic lupus erythematosus; TBS, Tris-buffered saline.

Plasmas were kept at −20°C in frozen aliquots and thawed only once. They were used in preference to sera because the release of DNA during clotting may have resulted in the formation of DNA:anti-DNA IC (17).

Analytical ultracentrifugation. These plasmas were analyzed by ultracentrifugation at 170,000 *g* for 14 h in sucrose gradients as described previously (15). Briefly, 100 μl of plasma, diluted twofold in 0.14 M saline buffered with 0.01 M Tris, pH 7.4 (Tris-buffered saline [TBS]), was analyzed in 10–37% (wt/vol) sucrose gradients dissolved either in TBS or in 0.12 M glycine-HCl buffer, pH 2.8. All parameters measured in gradients were plotted on millimetric paper against the gradient volume expressed in percentage of total. The total surface and the areas of zones 0–5 S, 5–9 S, 9–13 S, 13–17 S, and above 21 S, delimited by comparison with the position of markers, were measured by planimetry and expressed in centimeters squared. The 7-S peak was found at 70–74% of the gradient (top: 100%), and the 19-S peak was at 36–40%. In some instances, the heavy fractions of SLE plasmas (from 25 to 30 S), obtained from aliquots of an individual plasma fractionated on six sucrose gradients at pH 7.4, were pooled, dialyzed against TBS and concentrated about five times on a PM 30 Amicon membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). They were then ultracentrifuged a second time at pH 7.4 and 2.8. We have described elsewhere the recentrifugation technique (15). In these studies, we have measured the total recovery of several proteins separated on neutral or acid gradients. 90–95% of isolated IgM, IgA, IgG, or bovine serum albumin (BSA), were recovered. When BSA: anti-BSA IC were centrifuged, recovery of the radiolabeled BSA from the whole gradient was at both pHs above 95% for IC at 100:1 and 20:1 antigen excess. On acid gradients, we recovered 91.4 and 85.6% of the ^{125}I-BSA applied to the gradient in the zone 0–9 S for IC at 100:1 and 20:1 antigen excess, respectively (15). A counting of the empty gradient tubes showed the labeled BSA binding to be <2%.

Measurement of DNA binding capacity (DBC). Measurement of DBC was performed by the Farr assay as modified by Pincus (18). 50 ng of *Escherichia coli* [^{14}C]DNA (Amersham Corp., Arlington Heights, Ill.) or of a circular viral [^{3}H]DNA from SV-40 was added to 25 μl of the gradient fractions. Human IgG, 125 μg in 150 μl TBS, was added to all tubes. The percentage of precipitated DNA over total labeled DNA was calculated. Binding in excess of 10% was considered significant.

Detection of DNA. DNA was detected by inhibition of the Farr assay. A 50% ammonium sulfate precipitate of a SLE plasma was diluted to bind 50–60% of 50 ng of *E. coli* [^{14}C]-DNA, and this binding was measured in the presence of standard amounts of native and heat-denatured calf-thymus DNA. Inhibitions of 46.7±3.6 and 5% of DBC were obtained with 80 ng of native and denatured DNA, respectively (Fig. 1). To eliminate the anti-DNA antibody activity in some of the gradient fractions, the globulins were precipitated with 50% ammonium sulfate, and 100-μl samples of the dialyzed supernates were used in the inhibition assay.

DNase digestion of gradient fractions. 10 μg of DNase I (Worthington Biochemical Corp., Freehold, N. J.) in 10 μl of TBS containing 0.3 μM of $MgCl_2$ was reacted with 100-μl fractions for 1 h at 37°C; 100 μl of 0.01 M EDTA was then added. In the control samples, the EDTA was added to the DNase-$MgCl_2$ mixture before incubation. DBC was then measured as above.

Other technical parameters. The solid phase radioimmunoassay for IgG, the preparation of immunoadsorbants, and the ^{125}I-labeling of proteins have been used as described already (15). From previous experiments, we determined that the IgG distribution of fresh plasmas or of plasmas stored at −20°C for <1 mo could be reliably studied at both pHs. Those stored at −20°C for 1–6 mo were suitable for analysis at pH 7.4 only. Decomplementation by heating at 56°C for 30 min also altered this IgG distribution.

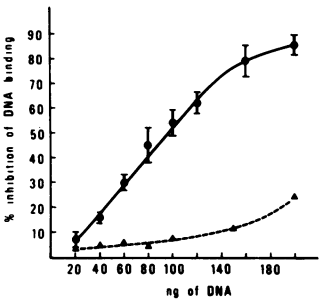

FIGURE 1 Calibration of radioimmunoassay for DNA. Native calf thymus DNA (mean of six measurements ±SD) (●) and of heat-denatured DNA (mean of three measurements) (▲) were added to a mixture of labeled DNA and a fixed amount of anti-DNA antibody.

Precipitation with 3.5% polyethylene glycol (PEG) was performed as described by Creighton et al. (19), and OD readings at 280 nm exceeding 0.120 were considered abnormal. Cryoprecipitation was read after 4 d at 4°C. The amount of precipitate that could be dissolved at 37°C was graded from 1+ to 3+. C3 and C4 levels were determined by radial immunodiffusion in agar gel (Partigen, Behring, Marburg/Lahn, West Germany). Values below 60 mg/100 ml for C3 and 16 mg/100 ml for C4 were considered abnormal.

RESULTS

DBC of normal plasmas

Altogether, 298 individual determinations of DBC were performed on fractions obtained from 10 normal plasmas, after ultracentrifugation at pH 7.4 and 2.8, out of which one reached 12.5%, two were between 10 and 12.5%, and 10 were between 5 and 10%. All others were below 5%. These results were plotted on millimetric paper, and the DBC in zones and total gradients were expressed in square centimeters (Table I). For the total gradient, this upper limit was 14.33 cm² at pH 7.4 and 18.87 cm² at pH 2.8. The mean +2 SD of DBC in individual zones or in the total gradients was taken as the upper normal limit, above which we considered the activity to be significant.

DBC of SLE plasmas (24 plasmas from 11 patients)

Reproducibility. To assess the reproducibility of gradient analysis, the distribution of DBC of an IgG fraction of one SLE plasma was fractionated on three separate occasions at pH 7.4 and 2.8. There was no difference in distribution between pH 7.4 and 2.8 or between the different experiments (Table II). Plasmas that exhibited increase in DBC in the 7-S fraction after acid dissociation (see below) were also repeatedly frac-

TABLE I

*Distribution of DBC of Normal Plasmas after Fractionation in Sucrose Gradient**

	0–5 S	5–9 S	9–13 S	13–17 S	17–21 S	>21 S	Total
				cm²±1 SD			
pH 7.4	0.57 (±0.51)	1.29 (±0.42)	1.55 (±0.95)	1.09 (±0.47)	1.35 (±0.62)	2.64 (±1.16)	8.39 (±2.97)
pH 2.8	0.49 (±0.24)	1.79 (±0.95)	1.82 (±1.48)	1.49 (±1.46)	1.01 (±0.69)	1.96 (±0.55)	9.37 (±4.75)
Difference‡ (7.4–2.8)	0.36 (±0.26)	1.00 (±0.78)	1.07 (±1.03)	1.14 (±1.33)	0.92 (±0.49)	1.74 (±1.72)	4.93 (±4.00)

* Mean of 10 plasmas.

‡ Mean of differences between DBC of individual plasmas.

tionated. This was done three times with one sample and twice with two. No significant differences in DBC were detected between the different experiments (results not shown).

Fractionation at pH 7.4. A total DBC, above the upper limit of normal (14.33 cm²), was found in 19 plasmas (Table III); a 5- to 9-S zone of peak activity, defined as a zone containing >35% of the total DBC of the gradient, was identified in 13 of these 19 plasmas; and a peak above 21 S was found in four. There was no zone of peak activity in two plasmas with a low total DBC of 19 and 32.3 cm². Altogether, significant activity was found above 13 S in 17 plasmas.

Fractionation at pH 2.8. A total DBC, above the upper limit of normal (18.87 cm²), was found in 23 of the 24 plasmas (Table III). A zone of peak activity between 5 and 9 S was identified in 19 plasmas. Four of the five plasmas which were within normal range at pH 7.4 showed significant antibody activity after fractionation at pH 2.8, their total DBC increasing from 14.3 to 28.3, 14 to 24.9, 13.4 to 29.2, and 8.5 to 28.9 cm², respectively. Altogether, 14 plasmas retained significant DBC above 13 S.

TABLE II

*Distribution of DBC after Three Separate Fractionations at pH 7.4 and 2.8 of an IgG Fraction from a SLE Plasma**

pH	0–5 S	5–9 S	9–13 S	13–17 S	17–21 S	21 S	Total
				cm²			
7.4	2.1	11.3	5.0	2.1	1.0	3.3	24.8
	2.5	11.6	6.2	1.9	1.3	2.7	26.2
	2.4	11.7	5.8	2.2	2.1	2.9	26.1
2.8	2.5	11.4	6.1	1.8	1.1	3.8	26.8
	2.3	11.2	6.6	2.2	0.8	3.6	27.2
	2.3	11.5	6.4	2.2	1.5	2.5	26.4

* IgG peak of a Sephadex G-200 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.).

Differences: pH 7.4 vs. pH 2.8. Individual results are shown in Table IV where only the significant DBC changes in the 5- to 9-S zone, above 21 S, and in the whole gradient, are presented. The DBC in the total gradient was increased in 6 of 24 plasmas by a mean of 45%, and in the 5- to 9-S zone in 11 plasmas by a mean of 108% (Table V). 5 of these last 11 were included in the six exhibiting an increase in total activity. The DBC in the zone above 21 S was always less at pH 2.8 than at pH 7.4, this decrease reached significance in six plasmas. There was no significant difference in the distribution and titer of DBC in 8 of the 24 plasmas (not shown in Table V). In four of these, no significant DBC has been found above 13 S at pH 7.4; in the others, there had been significant, but moderate, DBC above 13 S (<18% of the total DBC). In six patients serial samples were analyzed. In two patients, there was no DNA:anti-DNA IC; in two patients, they were present in all three of the serial samples; in two other patients, in one or two of the three samples. The data are insufficient to establish clinical correlates in these individual cases.

The 7-S gradient fractions of seven plasmas showing increases in DBC after fractionation at pH 2.8 were further analyzed (Table VI). The DBC of the fractions recovered at pH 2.8 and then brought to pH 7.4 was precipitated by 50% saturated ammonium sulfate (SAS)

TABLE III

Distribution of DBC after Fractionation of 24 SLE Plasmas

pH	Significant* total activity	Peak activity‡ 5–9 S	Peak activity >21 S	Significant activity >13 S
7.4	19§	13	4	17
2.8	23	19	0	14

* Activity above mean of normal plasmas +2 SD (see Table I).

‡ A zone of peak activity was defined as a zone containing more than 35% of the total DBC of the gradient.

§ Number of plasmas.

TABLE IV

*DBC after Fractionation at pH 7.4 and 2.8 of the SLE Plasmas where Significant Differences were Observed**

Patient	In zone 5–9 S		In zone 21 S		In whole gradient	
	pH 7.4	pH 2.8	pH 7.4	pH 2.8	pH 7.4	pH 2.8
	cm²		*cm²*		*cm²*	
	(2.1)	(3.4)	(5.0)	(2.0)	(14.3)	(18.9)
M.R.‡	1.1	10.3	—‖	—	—	—
B.X.‡	30.0	54.0	27.4	16.2	115.4	144.4
7§	33.3	55.1	35.3	19.0	125.5	162.6
78	16.9	54.3	32.5	16.4	80.1	141.5
S.M.‡	3.2	11.9	18.8	7.9	—	—
17	1.7	9.7	—	—	—	—
28	1.9	8.6	—	—	—	—
P.R.‡	21.6	44.4	10.9	4.7	—	—
7	—	—	16.2	4.1	—	—
C.G.	14.9	36.4	—	—	44.0	72.1
D.R.	—	—	—	—	49.4	85.9
A.L.	1.2	22.5	—	—	9.1	35.4
R.D.	5.3	11.4	—	—	—	—

* Changes were considered significant when differences in the whole gradient or in individual zones between pH 7.4 and 2.8 were in excess of the mean +2 SD of those found when normal plasmas were compared (see Table I). In parenthesis are indicated the values above which DBC are significant as defined in Table III.

‡ Indicates that three serial samples were analyzed from the same patient within 2 mo.

§ The interval in days between the collection of the analyzed sample and that of the first sample tested.

‖ Indicates that the results were omitted because of insufficient differences between 7.4 and 2.8.

and disappeared after adsorption to an antiserum specific for human γ-chains, but was unchanged by a control anti-IgA gel. The binding capacities of these fractions to [³H]SV-40 DNA were always greater or equal to that obtained with *E. coli* [¹⁴C]DNA.

Altogether, a significant increase in DBC in the total gradient and(or) in the 5- to 9-S zone, usually associated with a decrease in DBC above 21 S, was seen in 14 of the 24 plasmas and was taken as evidence for the presence of DNA:anti-DNA IC.

TABLE V

*Changes in DBC of Plasmas after Fractionation at pH 2.8 vs. 7.4**

	Whole gradient	Zone of gradient	
		5–9 S	>21 S
Higher binding at pH 2.8	6‡	11	0
Lower binding at pH 2.8	1	1	6

No significant change in titer or distribution in eight plasmas.

* Changes were considered significant when differences in the whole gradient or in individual zones were in excess of the mean +2 SD of differences found in normal plasmas.

‡ Number of plasmas.

TABLE VI

DBC of 7-S Fractions from SLE Plasmas with Higher Antibody Activity after Fractionation at pH 2.8. The Effects of 50% SAS and of Immunoadsorption on Fractions Recovered at pH 2.8.

Patient	DBC to [¹⁴C]*E. coli* DNA*	DBC in 50% SAS precipitates	DBC after adsorption to anti-IgG	DBC after adsorption to anti-IgA
P.R.	59.5§ (33)‖	49.5	4.5	44.5
B.X.				
1	57.0 (20.5)	52.2	3.1	46.8
2‡	82.5 (42)	73.5	4.0	52.5
A.M.	82.0 (53.5)	74.0	3.0	55.0
B.K.	86.0 (34.5)	81.5	8.0	67.0
B.M.	94.5 (56.5)	90.0	11.0	72.5
M.M.	26.0 (11.5)	22.4	9.4	18.5

* Values obtained with [³H]SV-40 DNA were equal or greater.

‡ Plasma from the same patient, collected 45 d later.

§ Results expressed as percentage of bound DNA over total DNA added. Each value represents the mean of two measurements.

‖ In parenthesis is shown the DBC of the same fraction at pH 7.4.

Refractionation of previously isolated heavy components

The 25- to 30-S, pH 7.4 fractions of plasmas from three patients with DNA:anti-DNA complexes, as defined above, were ultracentrifuged a second time (Table VII and Fig. 2). There was no significant DBC at pH 7.4 except above 21 S in plasma 2. At pH 2.8, significant binding activity, localized mainly to the 5- to 9-S zone, appeared in the three experiments. This activity was found in the 50% SAS precipitates and bound [³H]SV-40 DNA as well as *E. coli* [¹⁴C]DNA. In plasmas 2 and 3, the binding in the 5- to 9-S zone was sufficient to demonstrate its adsorption by an anti-γ-chain antiserum. Only in experiment 2 was there sufficient IgG to be measured by our radioimmunoassay. About 120 ng/ml were found above 21 S at pH 7.4. This peak disappeared at pH 2.8, and 150 ng/ml were then detected in the zone 5- to 9-S. In the two other experiments, DBC was present at pH 2.8 in the absence of detectable IgG by our radioimmunoassay.

DNA was measured after 50% ammonium sulfate precipitation in the globulin-free dialyzates of these fractions by inhibition of the Farr assay as described in Methods. After fractionation at pH 7.4, DNA was detected in the heaviest fractions in the three experiments and, in small amounts, in the 4- to 6-S zone in one

TABLE VII

DBC after Refractionation at pH 7.4 and 2.8 of Previously Isolated 25- to 30-S Fractions from SLE Plasmas

Plasma	pH	0–5 S	5–9 S	9–13 S	13–17 S	17–21 S	>21 S	Total
				*cm²**				
1	7.4	0.8	0.6	0.1	0.6	0.8	2.8	5.7
	2.8	3.3‡	7.8	4.4	1.1	2.7	3.4	22.7
2	7.4	1.0	1.8	1.3	1.0	0.8	12.0	16.9
	2.8	0	23.3	13.8	6.0	1.0	3.2	47.3
3	7.4	0.5	1.2	1.8	1.1	0.9	3.1	8.6
	2.8	2.0	12.0	8.2	3.0	0.1	4.2	29.2

* Calculated by plotting the DBC of each fraction against the gradient volume.
‡ Significant DBC as defined in Table III are underlined. Significant differences as defined in Table IV are underlined twice.

(Table VIII and Fig. 3). At pH 2.8, more free DNA was present throughout the gradient, most notably in the 4- to 6- and 21- to 25-S zones. There was little DNA in the bottom fractions. This inhibitory activity was destroyed by DNase, but not by RNase.

Distribution of IgG

Only 16 and 12 of the 24 plasmas could be adequately studied for the distribution of IgG at pH 7.4 and 2.8, respectively, because of the technical limitations in relation to storage. At pH 7.4, there was an abnormal concentration of heavy IgG above 13 S in 12 of 16 plasmas by comparison with the values calculated from 22 normals +2 SD. 9 of these 12 plasmas were also fractionated at pH 2.8: the IgG distribution returned to normal in seven when heavy IgG persisted in two. To assess the influence of IgG concentrations, we studied the distribution of IgG in six normal plasmas either undiluted or diluted 1:2 in TBS. There were no significant differences. Moreover, in seven of nine plasmas where heavy IgG were present at pH 7.4, the distribution returned to normal at pH 2.8; there was no correlation between the level of IgG and the presence of heavy IgG, and whereas the mean IgG of the SLE samples exceeded that of normals (9±2.7 vs. 14±3.0 g/liter) in only one instance, the IgG exceeded the upper limit of normal of 18 g/liter (i.e., 22 g/liter). The plasmas that showed higher amounts of IgG above 13 S were also positive for cryoglobulinemia and PEG precipitation (Fig. 4). However, some increase in IgG above 13 S

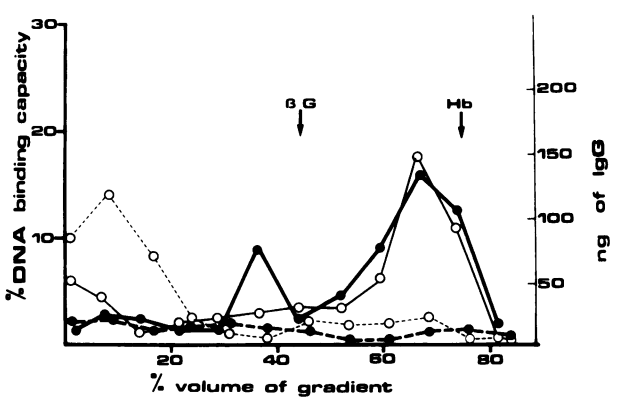

FIGURE 2 DBC after a second fractionation of heavy components of a SLE plasma at pH 7.4 (●– – –●) and 2.8 (●——●). In this figure and in Fig. 3, the heavy part of the gradient is shown on the left of the abscissa. On the ordinate are plotted the DNA bound over the labeled DNA added. The IgG distribution at pH 7.4 (○– – –○) and 2.8 (○——○) is also plotted. βG, β-galactosidase.

TABLE VIII

Concentration of DNA in Various Fractions after Recentrifugation at pH 7.4 and 2.8 of Previously Isolated 25 to 30S Fractions from SLE Plasmas

Plasma	pH	4- to 6-S fractions	21- to 25-S fractions	Bottom fractions
		%*	%	%
1	7.4	42	6	91
	2.8	88 (4)‡	92 (33)	16
2	7.4	16	10	90
	2.8	79 (5)	84 (8)	3
3	7.4	14	11	86
	2.8	92 (2)	91 (21)	23

* Results are expressed in percent inhibition of the Farr assay (see Methods). Each result represents the mean of two measurements.
‡ In parenthesis are shown the values after DNase digestion.

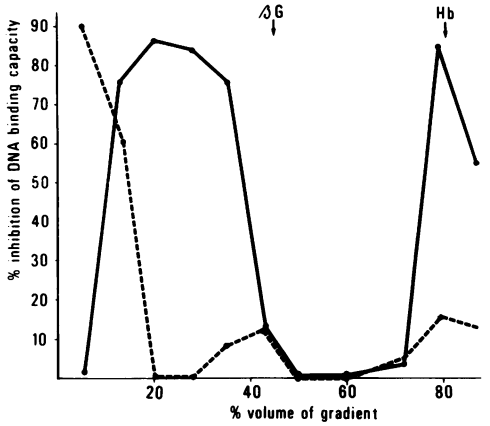

FIGURE 3 Distribution of DNA after a second fractionation of heavy components of a SLE plasma at pH 7.4 (● - - - ●) and 2.8 (● —— ●). DNA was measured by an inhibition of the Farr assay. βG, β-galactosidase.

was found in six plasmas in the absence of cryoglobulinemia or positive PEG precipitation. These tests were also positive, one each in two different plasmas in the absence of heavy IgG. There was no relationship between the presence of heavy IgG and the levels of plasma C3 or C4.

Relationship between DNA:anti-DNA and other parameters

The amounts of heavy IgG (above 13 S) were not different in the specific IC-positive plasmas, as compared with the negative ones (Fig. 4): IC were present in 6 of 12 plasmas with heavy IgG and in three of four with a normal IgG distribution. Sera positive for cryoglobulinemia and PEG precipitation had significantly

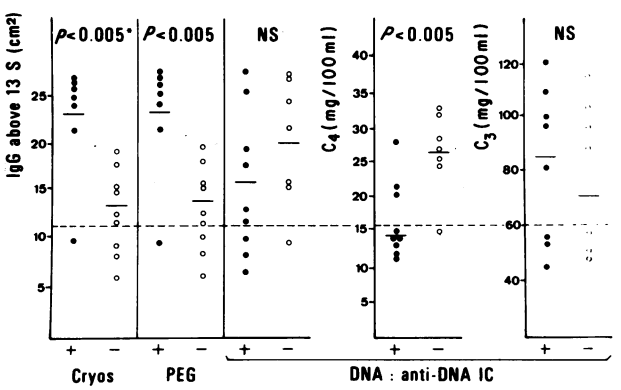

FIGURE 4 Correlations between IgG above 13 S, DNA:anti-DNA IC, cryoglobulins, PEG precipitins, and C3 and C4 plasma levels. The dotted line represents the means of normals −2 SD. NS: not significant.

more heavy IgG than the negative ones; no differences were found between the IC-positive and the IC-negative sera in their cryoglobulin and PEG-precipitable protein content. Conversely, C4 levels were significantly lower in the presence of DNA:anti-DNA IC: values below 16 mg/100 ml were found in six of nine plasmas containing IC vs. one of seven without IC. There was no difference in C3 concentration between IC-positive and IC-negative plasmas.

DNA:anti-DNA IC were present at some time in 7 of the 11 patients studied. Five of these had a proliferative glomerulonephritis proven by biopsy; one had an abnormal urinary sediment but a biopsy was not performed; the other had a severe Coombs' positive hemolytic anemia and extensive skin involvement. Of the four patients without circulating DNA:anti-DNA IC, one had a proliferative nephritis on a first renal biopsy but a repeat biopsy performed 2 yr later, and 2 mo before the studies on his plasma, was normal on light microscopy. IgG, IgA, and IgM deposits were detected by immunofluorescence in the first biopsy, whereas IgA and IgM were present in the second one. No renal involvement was found in three, two of whom presented with predominantly articular symptoms and one with purpura, thrombocytopenia, and an acquired von Willebrand syndrome.

DISCUSSION

Recovery of anti-DNA antibodies and DNA after dissociation as evidence for DNA:anti-DNA IC. Significant increases in total DBC and, more frequently and importantly, in DBC titers of the 5- to 9-S zone were seen in 14 of the 24 SLE plasmas after dissociation at pH 2.8. There remained a significant DBC above 13 S in 14 of the 24 plasmas, which suggests that either DNA:anti-DNA IC were not completely dissociated or that storage caused irreversible aggregation of anti-DNA antibodies or IC. We demonstrated that this DBC was directed against a native, circular, viral DNA, and was IgG-dependent, as shown by its precipitation in 50% SAS and its immunoadsorption to an anti-IgG gel. A decrease in total DBC and in the activity of the 5- to 9-S zone at pH 2.8 was found only once. This may be a result of the liberation from IC of DNA with a 7 S and the subsequent reformation of IC upon neutralization of the gradient fractions. This effect may have also impeded the recovery of anti-DNA antibody after fractionation at pH 2.8 in the 7-S zone in the other plasmas studied.

On second fractionation of heavy components of SLE plasmas, significant 7-S DBC against *E. coli* DNA and native, circular, viral DNA was unmasked after the acid pH centrifugation. This activity was also precipitated in 50% SAS and adsorbed to an anti-IgG gel. In these

refractionation experiments, DNA was detected by inhibition of the Farr assay in the 4- to 6-S zone after dissociation at pH 2.8. This inhibitory activity was digested by DNase. DNA was also found in the 20- to 25-S sedimentation range but not in the heaviest fractions of the gradients, whereas it was strictly confined to those heaviest fractions at pH 7.4. It was impossible to determine if 7-S DNA was present, because we had to bring the fractions to pH 7.4 before testing, therefore probably inducing recombination of DNA:anti-DNA IC. We have not yet found a reliable way of separating DNA and IgG or of eliminating IgG at pH 2.8. At this pH, unbound DNA precipitated with immunoglobulins in 33% SAS and pronase and trypsin did not reliably destroy antibody activity.

These results, notably the recovery of anti-DNA antibody after acid dissociation and fractionation, agree with those obtained by Bardana et al. (20) who compared the DBC of sera before and after DNase digestion and found an increase in DBC in 25 of 32 patients with overt lupus nephritis, a result not confirmed by others. This may be explained by our observation that increases in DBC were usually greater and often only significant when the activities of the 7-S peaks rather than those of the whole gradient were studied at acid vs. physiologic pHs. Significant differences in whole plasmas would be more difficult to demonstrate, and our technique, which allows us to focus on the 7-S peaks, may be more sensitive. Stingl et al. (21) also found a rise in titer of anti-DNA antibody after DNase treatment of 5 to 13 SLE sera, detected by immunofluorescence on *Crithidia luciliae* kinetoplasts. Winfield et al. (7), in a study of cryoprecipitates from SLE patients, demonstrated that DNA antibodies were highly enriched in the precipitates relative to serum levels. In many of them, DNA was detected by a diphenylamine assay but not by hemagglutination inhibition, even after sucrose gradient ultracentrifugation at pH 3.5 (7). DNA has also been found by the diphenylamine assay in the cryoglobulins of patients with Waldenström's macroglobulinemia and with essential mixed cryoglobulinemia (22). Other workers could not find evidence for the presence of DNA:anti-DNA IC in the circulation in a mouse experimental model (23) and in man (12) by methods such as the measurement of C1q binding activity before and after DNase digestion or the measurement of human IgG after fixation of IgG: DNA complexes to methylated BSA coupled to gel. In the face of these discrepancies, our results strongly support the existence of DNA:anti-DNA IC in SLE plasmas. In effect we fulfilled the essential criteria for their presence by recovering anti-DNA antibody after acid dissociation in most SLE plasmas and DNA in some of these.

Acid dissociation has the further advantage of allowing the isolation of DNA for further characterization. Our results revealed this DNA to be, at least in part, of a small molecular weight, possibly because of partial digestion by serum DNase. Winfield et al. (7) also identified a 7-S DNA in four SLE plasmas containing DNA detected by hemagglutination inhibition. Similarly, Izui et al. (23) demonstrated the presence of a circulating 4- to 6-S DNA in mice injected with lipopolysaccharide. It is unknown if the acid conditions used by us caused denaturation, and if the DNA is of endogenous or exogenous origin.

Relationships between the presence of DNA:anti-DNA IC and other parameters measured. In two of the three refractionation experiments, anti-DNA antibodies were found in the absence of detectable IgG (<50 ng/ml). This may be explained by the observation of Aarden et al. (24), who demonstrated that one IgG molecule was sufficient to precipitate one labeled DNA molecule in the Farr assay. These specific IC may then be few in number despite the important increases in DBC observed after dissociation. This may explain our failure to correlate the presence of IC with heavy IgG. This lack of correlation could also be related to the small size of these IC, a possibility in view of the low molecular weight DNA involved and of the great antibody excess in SLE plasmas. It further demonstrates the need to search for specific IC rather than heavy or altered IgG, which are the basis for most of the methods of IC detection currently used; it underlines the importance of antigen-antibody systems other than DNA:anti-DNA (or possibly of aggregated immunoglobulins) in SLE plasmas. It may also explain the failure of others who searched for specific IC by the amount of immunoglobulins bound with the antigen or released by its enzymatic digestion rather than by the recovery of specific antibody activity.

The role of the DNA:anti-DNA IC that we have found in SLE plasmas is unknown. From this small series of patients, we did not attempt to correlate their presence with the course of the disease. However, DNA:anti-DNA IC were correlated with proliferative glomerulonephritis and low plasmatic C4. This does not indicate whether or not these circulating IC deposit in tissues. There is indeed evidence for the local formation of the IC found in pathological lesions (12, 21). Further analysis of more SLE plasmas is needed to establish the value of these measurements in following disease course, but it already appears that these circulating IC are frequently present in SLE plasmas, and that by acid dissociation the antibody and the DNA involved may be isolated for further study.

This technique is already no more cumbersome than many others used for IC detection, and appears worthwhile in that it allows the isolation of specific IC and the recovery of antigen and antibody. It is restricted to pathological situations where the nature of the antigen is suspected, which already represents a large number

of IC diseases. It is, however, conceivable that, when fully automatized, a panel of antigens could be tested for more precise identification of the pathological agents responsible for these diseases.

REFERENCES

1. Koffler, D., V. Agnello, R. Thoburn, and H. G. Kunkel. 1971. Systemic lupus erythematosus: prototype of immune complex nephritis in man. *J. Exp. Med.* **134:** 169s–179s.
2. Koffler, D., V. Agnello, and H. G. Kunkel. 1974. Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. *Am. J. Pathol.* **74:** 109–122.
3. Theophilopoulos, A. N., C. B. Wilson, and F. J. Dixon. 1976. The Raji cell radioimmune assay for detecting immune complexes in human sera. *J. Clin. Invest.* **59:** 169–182.
4. Agnello, V., D. Koffler, J. W. Eisenberg, R. S. Winchester, and H. G. Kunkel. 1971. C1q precipitins in the sera of patients with systemic lupus erythematosus and other hypocomplementemic states: characterization of high and low molecular weight types. *J. Exp. Med.* **134:** 228s–241s.
5. Sobel, A. T., V. A. Bokisch, and H. J. Müller-Eberhard. 1975. C1q deviation test for the detection of immune complexes, aggregates of IgG, and bacterial products in human serum. *J. Exp. Med.* **142:** 139–151.
6. Tan, E. M., P. H. Schur, R. I. Carr, and H. G. Kunkel. 1966. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J. Clin. Invest.* **45:** 1732–1740.
7. Winfield, J. B., D. Koffler, and H. G. Kunkel. 1975. Specific concentration of polynucleotide immune complexes in the cryoprecipitates of patients with systemic lupus erythematosus. *J. Clin. Invest.* **56:** 563–570.
8. Forsen, N. R., and E. V. Barnett. 1968. Antinuclear antibodies and nuclear antigens in cryoprecipitates. *Arthritis Rheum.* **11:** 479. (Abstr.)
9. Harbeck, R. J., A. A. Hoffman, R. I. Carr, and E. J. Bardana. 1975. Measurement of DNA:anti-DNA complexes. *Scand. J. Rheumatol. Suppl.* **11:** 26–34.
10. Bruneau, C. D., J. P. Edmonds, G. R. V. Hughes, and L. Aarden. 1977. Detection and characterization of DNA: anti-DNA complexes in a patient with specific lupus erythematosus. *Clin. Exp. Immunol.* **28:** 433–436.
11. Feltkamp, T. E. W. 1975. In discussion. *Scand. J. Rheumatol. Suppl.* **11:** 33.
12. Izui, S., P. H. Lambert, and P. A. Miescher. 1977. Failure to detect circulating DNA:anti-DNA complexes in systemic lupus erythematosus. *Clin. Exp. Immunol.* **30:** 384–392.
13. Izui, S., P. H. Lambert, and P. A. Miescher. 1976. In vitro demonstration of a particular affinity of glomerular basement membrane and collagen for DNA. A possible basis for a local formation of DNA:anti-DNA complexes in systemic lupus erythematosus. *J. Exp. Med.* **144:** 428–436.
14. Gillam, J. N. 1977. DNA binding to normal skin connective tissue as a localizing factor for DNA:anti-DNA complexes. *Arthritis Rheum.* **20:** 117–118. (Abstr.)
15. Benveniste, J., and C. Bruneau. 1979. Detection and characterization of circulating immune complexes by ultracentrifugation. I. Technical aspects. *J. Immunol. Methods.* **26:** 99–112.
16. Cohen, A. S., W. E. Reynolds, and E. C. Franklin. 1971. Preliminary criteria for the classification of systemic lupus erythematosus. *Bull. Rheum. Dis.* **21:** 643–648.
17. Davis, J. S. In discussion. 1975. *Scand. J. Rheumatol. Suppl.* **11:** 34.
18. Pincus, T. 1971. Immunochemical conditions affecting the measurement of DNA antibodies using ammonium sulfate precipitation. *Arthritis Rheum.* **14:** 623–628.
19. Creighton, W. D., P. H. Lambert, and P. A. Miescher. 1973. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethyleneglycol. *J. Immunol.* **111:** 1219–1227.
20. Bardana, E. J., R. J. Harbeck, A. A. Hoffman, B. Pirofsky, and R. I. Carr. 1975. The prognostic and therapeutic implications of DNA:anti-DNA immune complexes in systemic lupus erythematosus. *Am. J. Med.* **59:** 515–522.
21. Stingl, G., J. G. Meingassner, P. Swelty, and W. Knapp. 1976. An immunofluorescence procedure for the demonstration of antibody to native double-stranded DNA and of circulating DNA:anti-DNA complexes. *Clin. Immunol. Immunopathol.* **6:** 131–140.
22. Barnett, E. V., R. Bluestone, and A. Cracchiolo, III. 1970. Cryoglobulinemia and disease. *Ann. Intern. Med.* **75:** 131–140.
23. Izui, S., P. H. Lambert, G. J. Fournié, H. Türler, and P. A. Miescher. 1977. Feature of systemic lupus erythematosus in mice injected with bacterial polysaccharides. Identification of circulating DNA and renal localization of DNA:anti-DNA complexes. *J. Exp. Med.* **145:** 1115–1130.
24. Aarden, L. A., F. Lakmaker, and E. R. De Groot. 1976. Immunology of DNA. IV. Quantitative aspects of the Farr assay. *J. Immunol. Methods.* **11:** 153–163.