

## Analysis of the Complement-Fixing Potential of dsDNA/Anti-dsDNA Immune Complexes

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**Summary.** Prepared antibody/<sup>3</sup>H-dsDNA immune complexes have been analyzed with respect to both primary binding (the Farr assay) and complement fixation (the RBC-CF assay). In SLE sera an average of only one-third of the anti-dsDNA antibodies fix complement upon binding dsDNA. However, this proportion can vary considerably for different sera. In the case of bacteriophage PM2 dsDNA, 1 antibody molecule per molecule of dsDNA is sufficient for binding in the Farr assay. However, an average of  $4 \pm 1$  complement-fixing antibodies must be bound to give 50% binding in the RBC-CF assay. Through an independent proximity analysis it was calculated that *two* adjacent antibodies bound to the DNA are required to form a complement-fixing unit.

**Key words:** Systemic lupus erythematosus – Antibody – Complement – DNA – Immune complexes

### Introduction

Investigative efforts into the pathogenic mechanisms of systemic lupus erythematosus (SLE) have revealed that the complement-fixing abilities of anti-dsDNA antibodies in the immune complexes they form with native DNA [1–6] may play a significant role in SLE pathogenesis. A method is now available for quantitating the potential of these complexes to fix complement – the red blood cell-linked complement-fixation (RBC-CF) assay [7]. In this method the cell-surface complement receptors of erythrocytes are utilized to separate complement-fixing complexes from complexes that cannot fix complement.

Previous studies have demonstrated the potential for nonequilibrium distribution in the DNA/anti-DNA system because both the size [8] and complement-fixing ability [7] of DNA/anti-DNA immune complexes depend upon the time course of addition of the DNA to an SLE serum. In fact, a direct relationship exists between size and complement fixation for antibody/DNA complexes in general [9] for dsDNA of a given molecular weight. These properties are dictated by the relative concentrations of serum and DNA for a system in equilibrium and thus

appear to depend upon the antibody:DNA ratio of the actual complexes. For example, multiple antibodies bound to DNA are required for these complexes to fix complement, as evidenced by the observation that 8–20 times as much SLE serum is usually needed to achieve the same binding level of DNA in the RBC-CF assay as in the Farr assay [7].

The determination of the exact conditions that must be fulfilled in order for a DNA/anti-DNA immune complex to fix complement may be considerably more complicated than merely the number of antibodies bound per DNA. Other considerations of importance are: a) the proportion of complement-fixing to non-complement-fixing antibodies [10]; b) the arrangement of the antibodies bound to the DNA [9]; c) the molecular weight of the DNA [9, 11].

This paper describes an analysis of the antibody/dsDNA system based on the assumption that complement-fixing antibodies are randomly distributed on the DNA. This analysis has allowed the determination of some of the requirements for complement fixation by DNA/anti-DNA immune complexes.

### Materials and Methods

**Sera, DNA, and Radioimmunoassays.** Procedures for obtaining sera, <sup>3</sup>H-DNA, and reduced and alkylated 7S antibodies have been reported in earlier publications [9, 11–14]. The Farr assay [15] and RBC-CF assay were also performed following previously published protocols [7, 9, 13].

**Computer Analyses.** The binding curves from both the Farr and RBC-CF assays were fitted to a Poisson distribution:

$$P(x) = \sum_{y=x}^{\infty} \frac{e^{-r} r^y}{y!}, \text{ where } P(x) \text{ is the fraction of DNA molecules}$$

having  $x$  or more antibodies bound, and  $r$  is the average ratio of bound anti-DNA antibodies to DNA molecules in the system. Non-integer values for  $x$  were obtained by linear interpolation between integer values. The least-squares minimization procedure employed for this fitting process has been described elsewhere [16]. In this analysis, it is assumed that for a given assay, if  $x$  (to be determined) or more antibodies are bound per dsDNA, then the DNA will register as *bound* in that assay. If fewer than  $x$  antibodies are bound, then the DNA will be *unbound*. The analysis generates both the requisite number of antibodies needed

to bind DNA ( $x$ ), and  $r$  (which at constant DNA is linearly proportional to the concentration of serum or 7S subfraction).

Aarden [17] used a similar analysis to demonstrate that in the Farr assay  $x=1$  if the dsDNA molecular weight is less than or equal to about  $10 \cdot 10^6$ . The present results (see below) confirm his findings.

These simplifying assumptions were made to allow for a first order analysis of the results of the RBC-CF assay. However, in fact, the system is more complicated. For example, for some SLE sera (see below)  $x=4$ ; i.e., 4 or more complement-fixing anti-dsDNA antibodies must be bound to the DNA in order for it to register as bound in the RBC-CF assay. It is assumed that if only 3 such antibodies are bound, then the probability that these complexes fix complement is zero. Although this 'all or none' simplification cannot rigorously be correct, the symmetry of the Poisson distribution suggests that it does allow for a reasonable first approximation. The error made in assigning a probability of 1 for binding in the RBC-CF assay to a complex of stoichiometry 5 antibodies per DNA, when  $x=4$ , will be approximately cancelled by the error made in assigning a probability of 0 to a complex of stoichiometry 3 antibodies per DNA. Alternatively, a consequence of the symmetry of the Poisson distribution and an integral part of the analysis is that when the average ratio of bound antibodies per DNA ( $r$ ) is equal to  $x$ , the required number for complement fixation, then roughly 50% of the DNA will be bound in the RBC-CF assay. In fact, the results of this computer analysis of antibody/dsDNA stoichiometries are in rather good agreement with *completely independent* stoichiometric determinations (see below) and therefore the analysis does offer a reasonable approach to this complex problem.

**Proximity Analyses.** The assay binding curves were also subjected to a microscopic analysis of the packing requirements for complement fixation in antibody/DNA immune complexes. This involved equating the probability of a DNA molecule precipitating in the RBC-CF assay with its probability of having  $M$  complement-fixing antibodies bound *closely enough together* to form an active site. The value of  $M$  is given by the slope of the binding data plotted as:  $\ln[-\ln(1-f_B)]$  against  $\ln v$ , (see Appendix), where  $f_B$  is the fraction of DNA bound, and  $v$  is the input volume of serum. It should be noted that while this analysis allows for an easy determination of  $M$  it does not allow for the determination of the size of the domain in which they are bound. The slope and correlation coefficient were determined by linear regression.

Finally, RBC-CF binding data used in the computer analyses were normalized by dividing the fraction of DNA bound by the maximum fraction able to be bound in that assay; e.g., this maximum value was 94% for PM2 DNA [7].

**Determinations of Complex Size and Stoichiometry.** The sedimentation coefficients ('sizes') of the  $^3\text{H}$ -DNA/anti-DNA immune complexes were measured by ultracentrifugation in sucrose gradients isokinetic for DNA [8, 9, 18]. The antibody:DNA ratios of the isolated complexes were determined in the following manner. The antibody was released from the original DNA by direct DNase digestion (3 h at 37 °C, final concentration of 0.5 mg/ml deoxyribonuclease I and 0.01 M  $\text{MgCl}_2$ ) of the gradient fractions. Additional  $^3\text{H}$ -PM2 DNA was then incubated (in a final concentration of 0.05 M EDTA) with the DNased fractions, and Farr assays were performed to obtain the antibody concentration by the Poisson analysis. (In a few cases control experiments indicated that for certain SLE sera EDTA partially inhibited their DNA binding activity. Sera that displayed this effect were excluded from these studies.) The number of antibodies thus obtained was related back to the number of originally bound  $^3\text{H}$ -DNA molecules to obtain the stoichiometry of the isolated complexes. Control assays with a final concentration of 5% trichloroacetic acid instead of SAS showed that in all cases >95% of the original  $^3\text{H}$ -DNA in the complexes was digested, and that >95% of the  $^3\text{H}$ -DNA added after digestion remained undigested.

## Results

### Size-Stoichiometry Relationships

The number of antibodies bound per DNA increases steadily as a function of the sedimentation coefficient,  $S$ , for soluble complexes (Table 1). This observation is in accord with previous reports [9, 17] that as the formal ratio of antibodies to DNA in the system is increased, the  $S$  value of the complexes also increases. Also, Table 1 indicates that in the presence of a large excess of an SLE serum the immune complex stoichiometry levels off at about 50 antibodies per PM2 DNA, or approximately 5.6 antibodies per kilobase pair. Similarly, the maximum number of antibodies that can pack onto PM2 DNA is about 50 for a serum that spontaneously precipitates dsDNA [11, 14].

When the molecular weight of the DNA is decreased, the maximum stoichiometry of the complexes decreases concomitantly, as seen in Fig. 1. The slope in this figure again indicates that when maximally packed, there is an average of approximately 5.6 antibodies bound per kilobase pair of DNA, regardless of the length of the DNA.

### Requirements for Complement Fixation

Since about half of antibody/PM2 DNA complexes peaking at 200–270S fix complement [9], it would seem from Table 1 that an average of about  $11 \pm 3$  antibodies are required per PM2 DNA for a 50% probability of complement fixation to occur. A more precise answer for the average required number of bound antibodies per DNA

**Table 1.** Relationship between sedimentation coefficient and stoichiometry for antibody/DNA immune complexes<sup>a</sup>

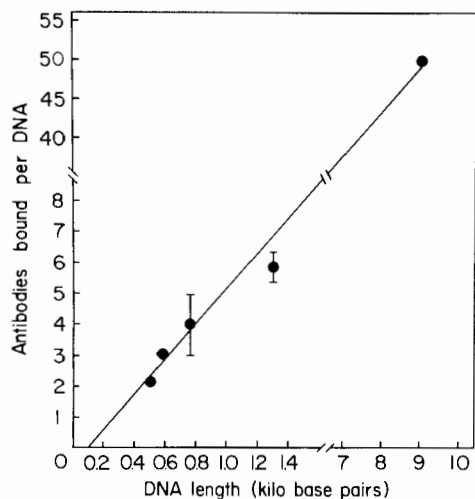
Input plasma or serum	PM2 dsDNA (μl/ng)	Peak S value of immune complexes	Stoichiometry of isolated complexes (Antibodies per DNA) <sup>b</sup>	Percentage binding in the RBC-CF assay <sup>c</sup>
Plasma Mu	0.10	200	$8.2 \pm 0.7$	45
	0.21	270	$12.6 \pm 0.9$	70
	0.47	325	$27 \pm 3$	85
	0.85	465	$47 \pm 8$	95
	5.0	580	$52 \pm 2$	95
	7.4	516	$50 \pm 5$	95
Serum Hi	6.7	Precipitate <sup>d</sup>	$51 \pm 5$	95

<sup>a</sup> For SLE plasma or sera able to form complement-fixing complexes with DNA [9]

<sup>b</sup> Mean  $\pm$  range of multiple determinations, determined by measuring the DNA binding capacity of the isolated complexes after the initial input DNA was enzyme digested (see experimental section)

<sup>c</sup> The RBC-CF assay was not run in parallel with the stoichiometry experiments, but was estimated from detailed studies previously published [9]

<sup>d</sup> Insoluble complexes [11]

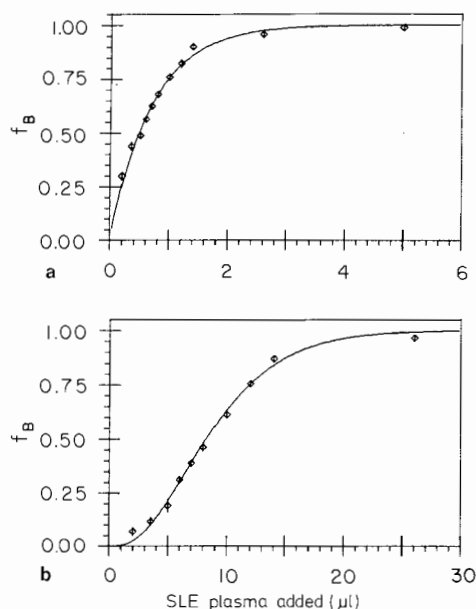


**Fig. 1.** Maximum antibody: DNA stoichiometries of isolated immune complexes formed from DNA of various molecular weights [11] and SLE plasma Mu at extreme antibody excess. The maximum packing density, as given from the slope, is 5.6 antibodies bound per kilo-base pair of DNA (correlation coefficient 0.9998). This value thus appears to be independent of DNA molecular weight or conformation (i.e., the supercoiled whole PM2 DNA is not an exception). *Breaks* in the graph indicate changes in scale by a factor of 4 on each axis.

molecule for the complex to fix complement was sought through the use of an analysis based on the Poisson distribution. Aarden et al. [17] previously used a Poisson analysis of the binding curves obtained in the Farr assay to determine the number of antibodies required per DNA for precipitation in ammonium sulfate. The data of Fig. 2a and Table 2 confirm Aarden's result that one antibody will precipitate DNA of  $6 \cdot 10^6$  Daltons or less. Although the total antibody concentration varies considerably for the different SLE sera and 7S subfractions (Table 2), generally the data analysis indicates that one antibody per DNA does register in the Farr assay. We have no immediate explanation for the few cases in which higher values of  $x$  (2.2 for serum An and 2.8 for 7S Ri) are obtained in the Farr assay.

When the Poisson analysis is applied to the binding curves obtained from the RBC-CF assay, the results suggest that about 4 antibodies must be bound per PM2 DNA for complement fixation to occur (Fig. 2b). However, a comparison of the antibody concentrations given by parallel analyses of the Farr and RBC-CF assays reveals that only a fraction of the antibodies that cause DNA precipitation in ammonium sulfate can also contribute to the fixation of complement. Typically, about one-third of the total antibodies detected are complement-fixing antibodies. This proportion varies from 0% to more than 100% for certain sera. The average number of bound antibodies required for 50% complement fixation is 4 for a variety of SLE sera and 7S subfractions and is independent of the proportion of complement-fixing to total antibodies (Table 2).

The RBC-CF binding curves were also analyzed for the requisite number of antibodies bound to the DNA in



**Fig. 2a, b.** Representative binding curves: experimental data from the Farr and RBC-CF assays run in parallel, with theoretical computer-generated fits based on the Poisson distribution. Ninety-five ng  $^3\text{H}$ -PM2 dsDNA was incubated with varying amounts of SLE plasma Mu. **a** Results of the Farr assay. Parameters generated: total antibody concentration =  $133 \cdot 10^8$  molecules per  $\mu\text{l}$  (67% confidence limits, 124 to  $218 \cdot 10^8$ );  $x = 0.98$  = required number of bound antibodies per DNA to cause precipitation in ammonium sulfate (67% confidence limits, 0.9–1.5). **b** Results of the RBC-CF assay. Parameters generated: complement-fixing antibody concentration =  $36 \cdot 10^8$  molecules per  $\mu\text{l}$  (67% confidence limits, 31 to  $42 \cdot 10^8$ );  $x = 3.5$  = required number of bound antibodies per DNA to cause complement fixation (67% confidence limits, 3.05 to 3.96). Note that the antibody concentration of the plasma as given by analysis of the RBC-CF data is only 27% of the concentration in the Farr assay. Also note the different scales on the  $x$ -axis in **a** (Farr assay) and **b** (RBC-CF assay). The  $y$ -axis is the fraction of  $^3\text{H}$ -DNA bound in either assay.

sufficiently close proximity that they formed a complement-fixing unit. It is evident from the slopes of plots such as Fig. 3 that, as anticipated, 2 adjacent antibodies (presumably IgG) are required for the formation of an active complement-fixing site, regardless of the DNA molecular weight or even in the case when low avidity antibodies are dissociated from the DNA (Table 3). This result focuses simply on the requisite number of bound anti-DNA antibodies required to form a complement-fixing unit. It does not provide information with respect to the *total* number of antibodies bound, and a priori this number would not necessarily correlate with the results of the Poisson analysis (see above).

#### *The Effect of Non-Complement-Fixing Antibody*

In addition to complement-fixing antibodies, typical SLE sera contain anti-DNA antibodies that cannot fix complement but do bind to DNA and thus contribute to Farr binding [1–4]. In fact, a few high titer SLE sera are

**Table 2.** Summarized results of theoretical computer fits (based on the Poisson distribution), to experimental data of DNA binding by SLE sera<sup>a</sup>. *np* not performed

## a) Experiments with SLE plasma Mu

Plasma Mu	Farr assay		RBC-CF assay		Percentage complement-fixing antibody <sup>c</sup>
	$x^b$	Antibody concentration ( $\times 10^8$ molecules/ $\mu$ l)	$x^b$	Antibody concentration ( $\times 10^8$ molecules/ $\mu$ l)	
Plasma <sup>d</sup>	0.98	133	3.5	36	27
Plasma	1.0	79	1.9	18	23
Plasma	1.0	140	2.9	45	32
Plasma	<i>np</i>	<i>np</i>	5.7	34	<i>np</i>
Plasma	<i>np</i>	<i>np</i>	5.7	43	<i>np</i>
Plasma <sup>e</sup> (high avidity antibody only)	1.0	94	5.8	60	64
7S <sup>f</sup>	0.96	0.81	<i>np</i>	<i>np</i>	<i>np</i>
7S	<i>np</i>	<i>np</i>	4.0	1.3	<i>np</i>
7S	<i>np</i>	<i>np</i>	3.0	1.2	<i>np</i>
Plasma <sup>g</sup>	0.88	194	2.5	94	48
7S <sup>h</sup>	1.0	5.5	3.0	2.3	42

## b) Experiments with other SLE patients

Plasma/Serum	Farr assay		RBC-CF assay		Percentage complement-fixing antibody <sup>c</sup>
	$x^b$	Antibody concentration ( $\times 10^8$ molecules/ $\mu$ l)	$x^b$	Antibody concentration ( $\times 10^8$ molecules/ $\mu$ l)	
Re plasma	0.95	2.5	7.8	4.6	177
Rs serum	1.65	4.3	4.0	3.2	74
An serum	2.2	7.6	3.6	3.0	39
Fi serum	<i>np</i>	<i>np</i>	6.8	18	<i>np</i>
7S <sup>f</sup> Se	1.15	1.35	3.0	0.39	29
7S Wc	1.0	7.2	2.8	3.6	50
7S Ri	2.8	0.9	2.7	0.06	7
7S Wd	1.1	5.7	4.8	4.3	75

<sup>a</sup> See Fig. 2 for a detailed explanation for a representative experiment. PM2 dsDNA ( $6 \cdot 10^6$  Daltons) was used in all cases except those denoted otherwise

<sup>b</sup>  $x$  = required number of bound antibodies per DNA to cause precipitation in the indicated assay

<sup>c</sup> Percentage complement-fixing antibody = antibody concentration as given by the analysis of the RBC-CF assay, divided by the antibody concentration given by analysis of the Farr assay. Not all anti-dsDNA antibodies register in the Farr assay (see Discussion)

<sup>d</sup> The quantity of plasma Mu available allowed multiple determinations over a time span of several months. The uncertainties within an individual determination (e. g.,  $x$  for the RBC-CF assay =  $3.5 \pm 0.5$ ) are represented in Fig. 2, which depicts the line of data referenced by this footnote

<sup>e</sup> This particular assay was performed after incubating the complexes with excess, unlabeled dsDNA to dissociate the low avidity antibodies ('reversal' protocol [8, 14]). The maximum binding achieved by the RBC-CF assay was ca. 70%; thus this plateau was assigned a value of 100% prior to computer analysis

<sup>f</sup> 7S subfractions of the plasma or serum samples [9]

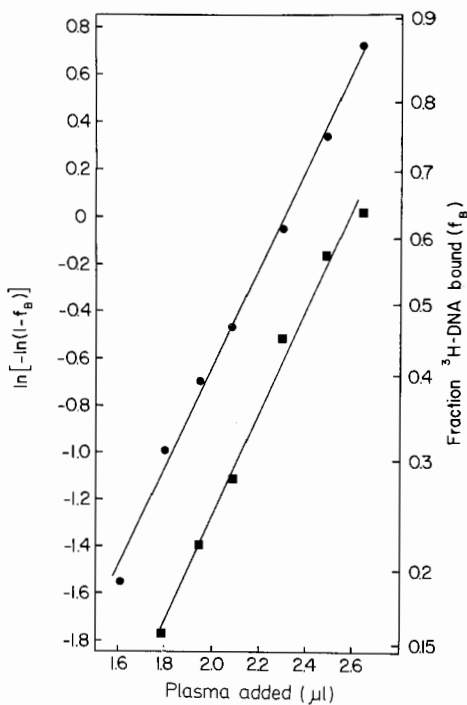
<sup>g</sup> Incubated with sonicated DNA, ca.  $5 \cdot 10^5$  Daltons [14]. This dsDNA exhibits approximately a 50% maximum binding level in the RBC-CF assay [9], and the data have been normalized for the computer fitting such that the plateau is assigned a value of 100%

<sup>h</sup> Incubated with a ca.  $10^6$  Daltons dsDNA generated by a restriction endonuclease [11]; data normalized from a 75% maximum binding in the RBC-CF

completely unable to form complement-fixing complexes with DNA [7, 9]. Attempts to prevent the formation of complement-fixing antibody/DNA complexes by using an excess of these non-complement-fixing sera to block the binding of complement-fixing anti-DNA antibodies have thus far proved unsuccessful [7, and unpublished data].

Anti-dsDNA antibody was reduced and alkylated (R/A) to produce high avidity, non-complement-fixing antibody [14, 19, 20] to facilitate additional studies of this

potential blocking effect. The unexpected result is that the formation of complement-fixing immune complexes from DNA and anti-DNA antibodies is actually *enhanced* in the presence of R/A anti-DNA antibodies (Table 4). This result is quite reproducible and approximately identical enhancements are obtained when the excess of R/A antibodies are incubated with the DNA before or after the non-R/A antibodies are added. This enhancement is definitely saturable. For example, for one particular ex-



**Fig. 3.** Typical proximity analysis. These RBC-CF assay binding data were analyzed as described in the Appendix. The probability that a complex will fix complement is equated with the chance that the complex will have  $M$  antibodies bound in close enough proximity to form a complement activation site.  $M$  as given from the slope of the plots above is 2.09 (correlation coefficient, 0.997) for plasma Mu (●), and 2.18 (correlation coefficient, 0.994) for the same plasma after the low avidity antibodies had been dissociated (■)

**Table 3.** Summarized results of proximity analysis. Variation of SLE sera, subfractions, and DNA molecular weight. Mean = 2.1; SD =  $\pm 0.2$

Antibody source	DNA <sup>a</sup>	Slope <sup>b</sup>	Correlation coefficient
Plasma Mu	PM2	2.09	0.997
Serum Ri	PM2	2.14	0.98
Serum An	PM2	2.00	0.992
Serum Fi	PM2	2.53	0.991
Serum Rk	PM2	2.07	0.98
7S Wc	PM2	1.87	0.9992
7S Wr	PM2	2.10	0.992
7S Ri	PM2	1.98	0.98
7S Mu	PM2	1.95	0.996
7S Mu	PM2	1.71	0.98
7S Mu	PM2	2.44	0.9998
Plasma Mu	Sonicated	2.18	0.996
Plasma Mu	Fragment	2.03	0.992

<sup>a</sup> PM2 =  $6 \cdot 10^6$  mol. wt.; sonicated = ca.  $5 \cdot 10^5$  mol. wt.; fragment =  $4.6 \cdot 10^5$  mol. wt., isolated after restriction endonuclease digestion [11]

<sup>b</sup> Slope of RBC-CF assay binding data plotted as in Fig. 4

**Table 4.** Effect of the presence of reduced and alkylated antibody on complement fixation

Volume of 7S Mu <sup>a</sup> (µl)	Percentage <sup>3</sup> H-DNA bound in the RBC-CF assay <sup>b</sup>	
	No R/A	+ R/A
0	0	0
5	0	6
10	10	28
20	41	70
30	74	86
45	90	91

<sup>a</sup> 7S Mu is the 7S subfraction of the complement-fixing plasma Mu

<sup>b</sup> R/A = the reduced and alkylated 7S subfraction of plasma Mu. The protocol was to mix aliquots of 7S Mu with either buffer (under no R/A), or an excess (100 µl) of R/A (under + R/A). These mixtures were then incubated with <sup>3</sup>H-DNA, followed by the RBC-CF assay. Under the conditions of the assay there was sufficient R/A anti-dsDNA antibody present in the assay for a 20-fold dilution to give more than 90% binding of the input DNA in the Farr assay

periment the binding of <sup>3</sup>H-dsPM2 by a constant amount of 7S anti-dsDNA antibodies was measured as a function of the input of R/A antibodies. The percentage bound for a given input (in µl) of R/A antibodies was as follows: 27%, no addition; 32%, 12.5 µl; 38%, 25 µl; 51%, 50 µl; 52%, 100 µl. Finally, this enhancement cannot be merely an additive one, since the R/A antibodies by themselves cannot form complement-fixing complexes with DNA (Table 4).

Poisson analyses of R/A antibody-enhanced RBC-CF DNA binding curves reveal that the apparent number of required complement-fixing antibodies is lowered from typical values of 4 to about 2. Typical proximity slopes are also decreased from 2.0 to approximately 1.3, in the presence of R/A antibody (data not shown).

## Discussion

Earlier studies [7, 8] established that the distribution of anti-dsDNA antibodies binding to dsDNA is controlled by kinetic considerations. For example, if a given amount of DNA is titrated slowly into an SLE serum, the properties of the immune complexes formed are quite different than if the DNA is added all at once. These studies were clear demonstrations of the potential for a stable, nonrandom distribution in an anti-dsDNA/dsDNA system, due to the fact that high avidity antibodies do not re-equilibrate rapidly.

The present study confirms the conclusion of Aarden et al. [17] that the binding of antibodies onto dsDNA can be governed by random distribution, one added proviso being that the components of the system must all be incubated simultaneously (or, theoretically, allowed to equilibrate for a time infinite in relation to the slowest

antibody dissociation rate). Earlier work [11] and the present studies indicate that when this condition is fulfilled, the formal antibody : DNA ratio of the system determines the size, stoichiometry, and complement-fixing potential of the immune complexes (for DNA of a given mol. wt.).

The least-squares analysis of binding data based on the Poisson distribution generates two parameters. One of these parameters is the anti-DNA antibody concentration. The difference in anti-DNA concentrations given in the Farr assay compared with the RBC-CF assay should directly reflect the difference in the concentration of total as opposed to complement-fixing anti-dsDNA antibodies. Some anti-dsDNA complement-fixing antibodies may be of such low avidity that they dissociate in ammonium sulfate [21]. This may explain the very rare cases in which more antibodies are detected in the RBC-CF assay.

The analysis described for directly quantitating complement-fixing anti-DNA antibodies does involve approximations. For example, binding levels in the RBC-CF assay were represented as the fraction of the maximum binding obtainable for a given DNA molecular weight and SLE serum. Also, the number of bound antibodies per DNA required for complement fixation is treated as a sharp transition, whereas the probability for complement fixation increases continuously with increasing immune complex size (i.e., with increasing number of antibodies per DNA [9]). This probability of complement fixation vs antibody/DNA immune complex stoichiometry is approximated as symmetrical about the 50% complement fixation point. Within the context of these approximations, this analysis does provide a means to directly quantitate the serum concentration of anti-DNA antibodies that contribute to complement fixation.

The other parameter given by the Poisson analysis is the average number of bound antibodies per DNA required to give a 50% probability of binding in the RBC-CF assay (see Materials and Methods); this number is typically found to be about 4. If this is taken to mean 4 complement-fixing antibodies, this represents a total of ca. 13 DNA-binding antibodies in the case of a typical serum having ca. 30% complement-fixing antibodies. This value of 13 correlates well with previous determinations that there are  $11 \pm 3$  antibodies bound per PM2 DNA in 200–270S complexes, half of which fix complement [9]. It also correlates with the observation that usually 8–20 times as much of a serum is needed to give 50% binding in the RBC-CF assay as in the Farr assay where only 1 antibody bound per DNA is needed for the DNA to register as bound [7]. Thus, there is internal consistency in the different parts of this study, which lends credibility to the overall approach.

The observation that reduced and alkylated 7S anti-dsDNA antibodies actually *increase* binding in the RBC-CF was quite surprising and the explanation for this observation remains speculative. Aarden et al. [19] previously reported that this procedure *increases* the titer of IgG anti-dsDNA antibodies in the Farr assay, and in fact

this finding was confirmed by this laboratory [14]. It is possible that the additional conformational flexibility in these particular antibodies causes conformational changes in the dsDNA that allow for the more efficient binding of C3b to the antibody/dsDNA immune complexes. The results also indicate that the required number of complement-fixing antibodies for binding in the RBC-CF assay decreases in the presence of the R/A antibodies (see above). *By themselves* the R/A antibodies cannot fix complement, but perhaps in the presence of a few complement-fixing antibodies on the negatively charged dsDNA antigen (known to have an affinity for Clq) the binding and activation of complement is enhanced due to a cooperative interaction.

In summary, the combined results of the Poisson analyses and the proximity analyses may be interpreted as follows. When an average of 13 anti-dsDNA antibodies (from SLE plasma Mu) are bound to PM2 dsDNA, typically 4 of these antibodies are *capable* of fixing complement. Under these conditions approximately half (i.e., 50%) of the DNA molecules have the complement-fixing anti-dsDNA antibodies bound sufficiently close together that at least one complement-fixing pair of IgG molecules is generated. Thus, under these conditions roughly 50% of the DNA will be bound in the RBC-CF assay, while effectively all of the DNA will be bound in the Farr assay. The requirement of two IgG in a complement-fixing unit agrees with the results found in other antigen:antibody systems [10], and serves to validate further the ability of the RBC-CF assay to distinguish DNA/anti-DNA immune complexes that fix complement from those that do not.

The described mathematical analysis of DNA binding curves in the RBC-CF assay serves to define the nature of complement fixing DNA/anti-DNA immune complexes. Longitudinal studies of the serum concentration of complement fixing anti-dsDNA antibodies in SLE patients using the methods described in this paper may be useful clinically, as it is generally accepted that the titer of these antibodies often correlates with SLE disease activity [1–4].

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## Appendix

The proximity analysis was derived starting with the supposition that 2 antibodies are needed in close proximity for complement fixation to occur [10].

Given a DNA molecule with an average of  $n$  antibodies bound to a total of  $L$  equally spaced binding sites and that  $n$  is small compared with  $L$ , the probability that a given binding site has an antibody bound is  $n/L$ . The probability that in addition to this any other site has an antibody bound is  $(n-1)/L$ . Therefore the probability that a given binding site has an antibody bound and that in addition another antibody is bound within  $d$  sites is:

$$d \binom{n}{L} \binom{n-1}{L}.$$

By summing over all of the binding sites, with care not to count antibodies in groups multiple times, we find that the mean number of complement-fixing groups per DNA is:

$$\frac{d}{L} \frac{n(n-1)}{2}$$

For large values of  $n$  this can then be approximated as:

$$\frac{d}{L} \frac{n^2}{2}$$

This is a reasonable approximation for  $n$  of the order of 4 and excellent for larger  $n$ , as in Fig. 3.

By assuming that the distribution of antibodies is random we can calculate the probability of a DNA molecule with no complement-fixing groups from a Poisson distribution as

$$\exp\left(-\frac{d}{L} \frac{n^2}{2}\right)$$

and therefore the probability that the DNA has at least one complement-fixing pair is

$$f_B = 1 - \exp\left(-\frac{d}{L} \frac{n^2}{2}\right)$$

Thus, after rearranging and taking natural logarithms twice:

$$\ln[-\ln(1-f_B)] = 2 \ln n + \ln\left(\frac{d}{2L}\right)$$

Since  $n$  is the average number of antibodies bound per DNA, it is linearly proportional to the input volume of SLE serum or plasma, and  $(k \cdot \mu l)$  can be substituted for  $n$  in the above equation. The  $\ln$  of  $k$  can be incorporated with  $\ln \frac{d}{2L}$  as a constant,  $j$ :

$$\ln[-\ln(1-f_B)] = 2 \ln(\mu l) + j$$

The left portion of this equation can be plotted against  $\ln(\mu l)$  to give a slope of 2, if the requirement for complement fixation (and binding in the RBC-CF assay) for the DNA:anti-DNA system is indeed 2 adjacently bound antibodies.

An analogous derivation assuming that  $M$  antibodies, instead of two, are required yields a very similar result except that the slope would be  $M$ , the number of antibodies per group.

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