JCI The Journal of Clinical Investigation

Circulating DNA in systemic lupus erythematosus. Isolation and characterization.

C R Steinman

J Clin Invest. 1984;73(3):832-841. https://doi.org/10.1172/JCI111278.

Research Article

Immunoprecipitable double-stranded (dsDNA) was previously shown to persist in the circulation of a clinically recognizable subgroup of patients with systemic lupus erythematosus (SLE). Plasma from 10 such patients was subjected to a DNA isolation procedure that used a combination of proteolysis, phenol extraction, and hydroxylapatite adsorption and elution in the presence of urea. The isolated dsDNA was radiolabeled by nick translation and then characterized by isopyknic ultracentrifugation in CsCl under both neutral and alkaline conditions, as well as after digestion with S1-endonuclease. These experiments demonstrated essential identity in nucleotide base composition between the plasma-derived DNA and human genomic DNA. The presence of specific human base sequences in the plasma DNA was demonstrated by finding that authentic human genomic DNA accelerated the renaturation of plasma DNA when compared with the effect of nonhuman, control DNA. The proportion of such sequences in plasma DNA was estimated by attempting to renature the plasma DNA in the presence of human DNA under conditions shown to result in complete renaturation of human DNA in model experiments. In this way, a minimum of 47% of plasma DNA base sequences could be shown also to be present in human genomic DNA. However, an average of 10-20% of the plasma-derived DNA failed to renature under these conditions, a result that was further confirmed by comparing the renaturation of […]

Find the latest version:

https://jci.me/111278/pdf

Circulating DNA in Systemic Lupus Erythematosus

Isolation and Characterization

Charles R. Steinman
Department of Medicine, Division of Rheumatology, The State University of New York-Downstate Medical Center, Brooklyn, New York 11203

Abstract. Immunoprecipitable double-stranded (dsDNA) was previously shown to persist in the circulation of a clinically recognizable subgroup of patients with systemic lupus erythematosus (SLE). Plasma from 10 such patients was subjected to a DNA isolation procedure that used a combination of proteolysis, phenol extraction, and hydroxylapatite adsorption and elution in the presence of urea. The isolated dsDNA was radiolabeled by nick translation and then characterized by isopyknic ultracentrifugation in CsCl under both neutral and alkaline conditions, as well as after digestion with S_1-endonuclease. These experiments demonstrated essential identity in nucleotide base composition between the plasma-derived DNA and human genomic DNA. The presence of specific human base sequences in the plasma DNA was demonstrated by finding that authentic human genomic DNA accelerated the renaturation of plasma DNA when compared with the effect of nonhuman, control DNA. The proportion of such sequences in plasma DNA was estimated by attempting to renature the plasma DNA in the presence of human DNA under conditions shown to result in complete renaturation of human DNA in model experiments. In this way, a minimum of 47% of plasma DNA base sequences could be shown also to be present in human genomic DNA. However, an average of 10–20% of the plasma-derived DNA failed to renature under these conditions, a result that was further confirmed by comparing the renaturation of the tritium-labeled plasma DNA specimens, in double-label experiments, with internal controls consisting of ^{14}C-labeled authentic human DNA. Attempts to drive the reaction to completion with human DNA led to a similar conclusion. The relative nonrenaturability of this fraction of plasma DNA did not appear to be attributable to extensive chain breakage, although adequate analysis of this DNA subfraction was limited by reagent availability. It was therefore concluded that, in this group of SLE patients, persistently circulating DNA consisted largely of base sequences also found in human genomic DNA. The additional presence in plasma of a DNA subfraction that differed in its renaturation behavior from human genomic DNA was recognized, although its significance could not be established with certainty.

Introduction

Circulating immune complexes composed of DNA and anti-DNA have long been thought to have a central pathogenetic role in lupus nephritis. Although much has been learned in this regard about the antibody component of such complexes, information about circulating DNA has proved elusive. This appears due, in part, to the early use of insensitive or nonspecific assays for DNA as well as to failure to recognize that normal human serum contains immunoprecipitable (IP)[1] double-stranded DNA (dsDNA) not detectable in simultaneously collected plasma, apparently the result of DNA release during blood coagulation in vitro (1–3). In addition, a number of common non-systemic lupus erythematosus (SLE) clinical states have been associated, with varying degrees of certainty, with at least the transitory appearance of circulating dsDNA (2, 4–6) so that failure to appreciate their presence could yield misleading conclusions about the clinical significance of circulating dsDNA. Consequently, there now exist conflicting conclusions, based

This work was presented in preliminary form at the 43rd Annual Meeting of The American Rheumatism Association, May 30, 1979 and published in abstract form (1979. *Arthritis Rheum.* 22:664).

Received for publication 30 August 1982 and in revised form 7 November 1983.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/84/03/0832/10 $1.00
Volume 73, March 1984, 832–841

1. *Abbreviations used in this paper:* CIE, counterimmunoelectrophoresis; Cot, product of the concentration of DNA nucleotides and the time of incubation in units of mol-s/liter (27); Cot ½, the value of Cot required to achieve 50% reassociation; G + C, deoxyguanosine plus deoxycytosine; dsDNA, double-stranded DNA; IP, immunoprecipitable; ssDNA, single-stranded DNA; SLE, systemic lupus erythematosus.

on data derived by a variety of methods, about the occurrence of circulating free dsDNA in both normal individuals and lupus patients.

In contrast, studies in which attempts were made to control these variables have yielded more consistent results (2, 3, 7). Although these studies are largely dependent on the use of counterimmunoelectrophoresis (CIE) for detection, specifically, of IP dsDNA, partial confirmation has been obtained by several independent methods (3, 8–10). These studies have indicated that <5% of randomly collected normal plasma specimens contain IP dsDNA. Similar results were obtained in patients with active SLE, with the notable exception of those patients with clear evidence of active systemic arteritis and/or central nervous system involvement. The persistently circulating IP dsDNA found in this subgroup of patients appeared to be associated with the underlying disease rather than with a coincidental complicating disorder or with an in vitro artifact and was therefore selected for further characterization.

This DNA was of particular interest in view of circumstantial evidence suggesting the existence of an etiologic association between some connective tissue disorders and a microbial agent (11, 12). Although not established for SLE, such an association appears to exist between the hepatitis B virus and a polyarteritis nodosa-like syndrome (13). Further, some patients with circulating hepatitis B antigen have been reported to exhibit evidence of specific viral DNA in their peripheral plasma (14).

Several reports of partial characterization of circulating DNA in SLE have appeared (15–19). In some reports, however, a clear interpretation of the results is hampered by one or more methodologic factors, most commonly the use of serum-derived materials with the consequent difficulty of determining whether the DNA studied had been released in vivo or in vitro, as just discussed.

Methods

Patients and plasma specimens. Specimens were obtained from patients with active SLE whose selection has been described in detail (7). All met preliminary American Rheumatism Association diagnostic criteria for SLE (20) and had exhibited persistently circulating dsDNA in association with manifestations of systemic arteritis and/or central nervous system involvement as defined previously. Patients with clinical states other than SLE, suspected or shown to be associated with circulating DNA, were prospectively excluded.

Only plasma specimens from patients who could be shown, by examination of multiple, separately collected specimens, to exhibit persistently circulating IP dsDNA, as contrasted with the more common transitory phenomenon, were included, with one specimen per patient being studied. Specimens were collected and separated promptly, as previously described, using methods that result in <5% false positives (5). Specimens were thus obtained from a total of 10 different patients, although the small amounts available from some patients necessitated using fewer specimens in some experiments as indicated.

CIE. DNA was detected in plasma by a modified CIE assay using previously defined antiserums capable of detecting 0.02–0.05 μg dsDNA/ml of plasma in control specimens. This assay, including the antiserums used and the relevant controls, have all been described in detail (7, 21). Positive specimens were confirmed by examination with dsDNA-absorbed antiserum as well as by demonstrating abolition of positive results by pretreatment of plasma with DNase.

Reagents. DNA from human placenta and salmon sperm were obtained from Calbiochem-Behring, La Jolla, CA. In addition DNA was purified from human liver obtained at autopsy. Calf thymus DNA and DNA polymerase I were purchased from Worthington Biochemical Corp., Freehold, NJ. Tritiated DNA from *Escherichia coli* and from KB cells was purchased from Amersham Corp., Arlington Heights, IL or Electronucleonics, Bethesda, MD. Their specific activities ranged from 0.2 to 0.6 μCi/μg. Human DNA labeled with ^{14}C, with specific activities of 0.01 to 0.05 μCi/μg was either from Electronucleonics or was the gift of Dr. Paul Phillips (SUNY-Upstate Medical Center, Syracuse, NY). Where indicated, high molecular weight DNA was sonicated by a procedure shown to result in chain lengths < 2,000 base pairs (21). Tritium-labeled deoxynucleoside triphosphates were from New England Nuclear, Boston, MA or Amersham Corp. and had specific activities of 18–70 Ci/mM. Deoxynucleoside triphosphates, deoxyribonuclease I, and Pronase were the purest grades available from Sigma Chemical Co., St. Louis, MO. S_1-endonuclease was obtained from Miles Laboratories, Inc., Elkhart, IN. Hydroxylapatite was the "DNA grade" from Bio-rad Laboratories, Richmond, CA. Sucrose, CsCl, and urea were from Schwarz-Mann, Spring Valley, NY. Other reagents were of the highest commercial grades available.

Plasma DNA purification. The following were added to a final volume of 6.7 ml: 1.2% sodium dodecyl sulfate, 1 M sodium perchlorate, 1 mg/ml of Pronase and 4 ml (or 2 ml, using half the indicated volumes) of plasma, that had been heated for 30 min at 56°C and clarified by centrifugation. The mixture was incubated at 60°C for 3 h after which 0.74 ml of 1 M Tris HCl, pH 9.1, was added, followed by three extractions with equal volumes of phenol that had been equilibrated with 0.1 M Tris HCl, pH 9.1. After dialysis against 0.1 M sodium phosphate, pH 6.8, plus 1% sodium azide for at least 24 h at 4°C, solid urea was added to a final concentration of 8 M. The phosphate concentration was then adjusted to 0.14 M. Next, 4 mg of hydroxylapatite was added with mixing and allowed to stand at 20°C for 15–30 min with gentle agitation. This was repeated four more times after which the hydroxylapatite was removed by centrifugation and washed four times with 0.5-ml portions of 8 M urea containing 0.14 M sodium phosphate, pH 6.8. The adsorbed DNA was then eluted by washing with five 50-μl portions of 1 M sodium phosphate, pH 6.8. The eluates were then pooled. Specimens were concentrated by lyophilization after dialysis against ammonium acetate, 0.01 M. Each specimen was then redissolved in 40 μl of water. Recovery ranged from 30 to 80% as approximated by reference to specimens, examined in parallel, to which sonicated, tritium-labeled DNA had been added. This recovery was consistent with that estimated by the less precise method of directly examining serial dilutions of the recovered material by CIE (3).

Radiolabeling of DNA was performed by nick translation as described (22, 23) except that tritium-labeled precursors were used and that plasma DNA, as isolated, was found to be sufficiently nicked not to require additional treatment with DNase. With the method used, incorporation of radioactivity was dependent on added DNA as well as on DNase nicking when intact DNA was used. Incorporated radioactivity was DNase sensitive and alkali resistant. Specific activities could not be directly determined because of the small quantities of DNA available but were estimated to be $\sim 10^5$–10^6 cpm/μg.

Isopyknic ultracentrifugation in CsCl (24) was performed using a Beckman model L ultracentrifuge (Beckman Instruments, Fullerton, CA), a type 50 fixed angle rotor with polyallomer tubes, and centrifuging

at 40,000 rpm for 42 h at 16°C. Neutral gradients contained, in addition to CsCl, 0.3 M NaCl and 0.03 M sodium citrate, whereas alkaline gradients contained 0.1 M NaOH. Each gradient also contained at least one density marker, generally ^{14}C-labeled human DNA. The initial density was adjusted with a refractometer (Bausch & Lomb, Inc., Rochester, NY) at 25°C to an initial refractive index of 1.3992, corresponding to a density of 1.6995 g/ml, for neutral gradients, and 1.4035 (d = 1.7425 g/ml) for alkaline gradients. Fractions were collected dropwise from the bottom. For neutral gradients refractometry was performed on alternate fractions.

Alkaline sucrose gradients contained 5–25% sucrose in 0.1 M NaOH and 0.9 M NaCl. Centrifugation was carried out in an SW 50.1 rotor at 16°C for 16 h at 40,000 rpm. Because appropriately sized alkali-stable standards were not available, no attempt was made to accurately determine absolute sedimentation rates.

DNA reassociation. Reassociation of alkali-denatured DNA was examined by the phenol emulsion technique described by Kohne et al. (25). Preliminary studies using routine methods (26) had yielded similar but more limited data. The methods used were exactly as described (25) and, briefly, consisted of allowing DNA renaturation to occur, at 20°C, in a two-phase system of 9% phenol and either the thiocyanate or phosphate buffer described, with the emulsion being maintained by constant agitation using the recommended apparatus. Corrections were made for zero-time foldback. It should be noted that the specificity of the renaturation reaction is fully maintained, although this technique results in greatly accelerated reaction rates, thereby allowing the use of lower DNA concentrations and/or shorter incubation times than are required in the routine method to achieve comparable degrees of renaturation. Before inclusion in renaturation reaction mixtures, all unlabeled, high molecular weight DNA preparations were fragmented by ultrasonication as described. Plasma DNA specimens were not subjected to this treatment. Timed aliquots were removed from the reaction mixture, diluted, and examined, generally in duplicate or triplicate, for their extent of renaturation by routine hydroxylapatite chromatography (26, 27), in water-jacketed columns at 60°C. Results are expressed as the percent of unreassociated single-stranded DNA (ssDNA) remaining.

These experiments were designed to answer two questions. The first was whether any human genomic DNA base sequences are present in plasma DNA. The second was whether such sequences, assuming some were found, could account for all or only for part of the plasma DNA. To answer the first of these questions the ability of authentic human DNA to accelerate the renaturation of plasma DNA over that observed in the presence of nonhuman eukaryotic DNA was studied. To answer the second question, assuming that some degree of acceleration was observed, it was planned to compare the extent of renaturation of plasma DNA with that of authentic human DNA under conditions that allow the latter to renature essentially to completion.

For convenience in describing these experiments, the term driving DNA is used to describe the DNA (not radiolabeled) present in relatively high concentration (5 or 50 μg/ml) and which therefore controls the rate of renaturation of DNA sequences homologous to it, including those contained in the driven DNA, present in trace amounts, but whose renaturation is directly measured by virtue of its radiolabel. Also, the term driven renaturation will be used to mean that degree of reassociation that can be attributed to the presence of the driving DNA as compared with that due to the self-renaturation of the radiolabeled test DNA as seen in the presence of unlabeled heterologous control DNA (undriven renaturation).

Statistical analysis of the data was by use of standard techniques (28).

Results

Density gradient analysis. Fig. 1 summarizes the peak locations of several DNA standards in neutral isopyknic density gradients identical to those used to analyze plasma-derived DNA specimens and demonstrates the ability of this gradient to band DNA's ranging from 31 to 71% deoxyguanosine plus deoxycytosine (G + C) content. Also shown is a gradient demonstrating the separation achieved using control human and lambda DNAs.

The possibility that behavior of plasma DNA, in these gradients, might have been altered by the extraction and labeling procedures was examined by adding human DNA from KB cells, previously labeled with ^{14}C in tissue culture, to normal plasma, subjecting it to the routine extraction and tritium-labeling procedure and then co-centrifuging this DNA, containing both tritium and ^{14}C, with the ^{14}C-labeled starting material. By adjusting the relative specific activities of the two DNAs, each could be detected by routine double-label methods. As seen in Fig. 2, the DNA peak location was unaltered by the extraction procedures, but the distribution is skewed toward the dense end of the gradient.

DNA from a total of 10 patients was examined in such CsCl gradients, most on more than one occasion. All exhibited a similar pattern with the peak at that of the human dsDNA marker, or between it and the human ssDNA marker, but with varying degrees of skewing toward the left, similar to that seen in the control gradient just described, but generally more marked. A typical gradient is shown in Fig. 3 with additional examples in Figs. 4 *A* and 5 *A*. The simplest interpretation appeared to be that the skewness (both here and with the control DNAs just described) resulted, not from base composition heterogeneity, but rather from the presence of partially denatured DNA. To explore this further, plasma DNAs from eight patients were completely denatured and examined, along with denatured human marker DNA, by centrifugation in alkaline CsCl gradients.

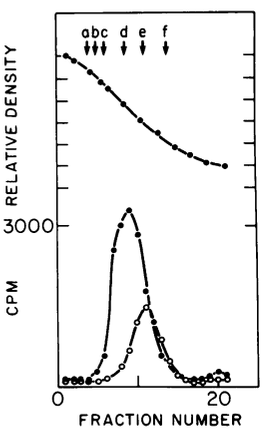

Figure 1. Separation, by neutral isopyknic ultracentrifugation, of dsDNA from lambda bacteriophage (●), labeled with tritium, with (G + C) mole % = 50, from that of human KB cells (○) labeled with ^{14}C with (G + C) mole % = 39.5. Also shown are the peak positions, determined on separately run gradients, of DNA from organisms selected to represent a range of base compositions that includes most biologically relevant DNA species. The arrows correspond to the positions of the following markers with their (G + C) mole % shown in parentheses: (*a*) *Micrococcus leisodeikticus* dsDNA (71); (*b*) *E. coli* ssDNA (50); (*c*) human ssDNA (39.5); (*d*) *E. coli* dsDNA (50); (*e*) human dsDNA (39.5); and (*f*) *Clostridium perfringens* dsDNA (31).

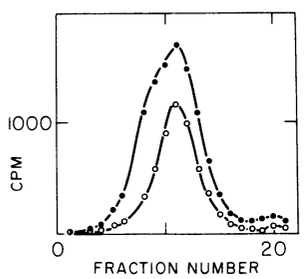

Figure 2. Effect of isolation and labeling procedure on human DNA. o, ^{14}C-counts from untreated human DNA labeled in tissue culture. •, tritium counts from that same DNA, after having been added to plasma and subjected to the routinely used DNA isolation and nick-translation procedure. Both specimens were examined in the same CsCl gradient.

In all cases, a broadening of the peak over that seen in neutral gradients was noted. This is assumed to have resulted from increased diffusion due to shortened chain lengths upon denaturation as would be expected if the original native DNA contained many single-strand breaks or gaps as would be consistent with the ability of the nick-translation reaction to proceed without predigestion with DNase, as discussed. In two cases this peak broadening was sufficient to prevent its localization. For the remainder, however, as shown in Fig. 4, the peak appeared to coincide approximately with that of the human ssDNA marker as would be consistent with the above interpretation.

The converse experiment was performed on specimens from nine patients which were subjected to digestion with S_1-endonuclease in an attempt to remove single-stranded regions. The remaining material, presumably all double stranded, was then examined in neutral CsCl gradients and compared with the undigested plasma DNA. As illustrated in Fig. 5, broadening of the peaks over those of the undigested DNA was again observed, along with a shift to or toward the human dsDNA marker, findings that were again consistent with the above interpretation.

The possibility remained that a minor subfraction of plasma DNA, differing significantly in G + C content from that of human DNA, might have been obscured by the broad DNA banding observed. To evaluate this, each of two specimens was subjected to routine separation on an alkaline CsCl gradient with the broadened peak collected in three separate fractions each of which was separately recentrifuged in identical, simultaneously run gradients. The results were similar for both specimens examined, one of which is shown in Fig. 6. No significant

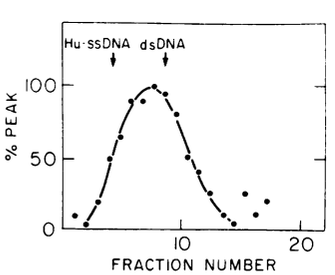

Figure 3. Typical behavior of plasma-derived DNA preparations in neutral CsCl gradients performed as described in the text. The specimen illustrated was from patient La. The positions of density markers consisting of human dsDNA and ssDNA are indicated. Additional examples of such gradients with plasma DNA from other patients are shown in Figs. 4 *A* and 5 *A*.

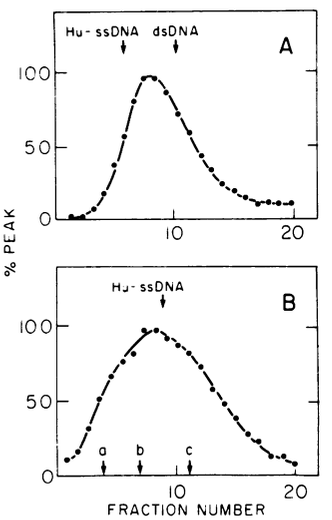

Figure 4. Behavior of a plasma-derived DNA specimen from patient Ro in (*A*) neutral and (*B*) alkaline CsCl gradients. The neutral gradient was identical to that in Fig. 3. For added resolution, the initial density of the alkaline gradient was greater than that of the neutral one, as noted in the text. The locations of separately run DNA density markers are indicated at the bottom, with the internal human DNA marker indicated at the top. The indicated DNA markers were: (*a*) *M. leisodeikticus;* (*b*) bacteriophage lambda (50% G + C); (*c*) *C. perfringens.*

differences in peak locations or distributions were discernible. The only suggestion of a second peak appeared at the extreme light end of the least dense fraction, designated "pool C." However, the density of this material would imply a G + C content so low that it seems more likely to have resulted from the binding of a trace contaminant, probably protein, to the DNA, although this was not directly demonstrated.

Thus, it is concluded that, on the basis of density measurements alone, plasma DNA is indistinguishable from authentic human DNA, which suggests an identical base composition. Further, no evidence for a significant subfraction differing in this parameter could be found. The skewing of the peaks observed in neutral CsCl appears best explained as being due to partial denaturation of otherwise double-stranded molecules. Whether this denaturation occurred partially or entirely in vitro cannot be determined from these data, although the

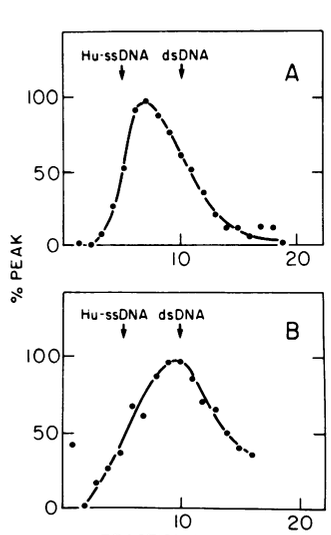

Figure 5. Behavior of plasma-derived DNA from patient Fg in neutral CsCl gradients before (*A*) and after (*B*) treatment with S_1-endonuclease as described in the text.

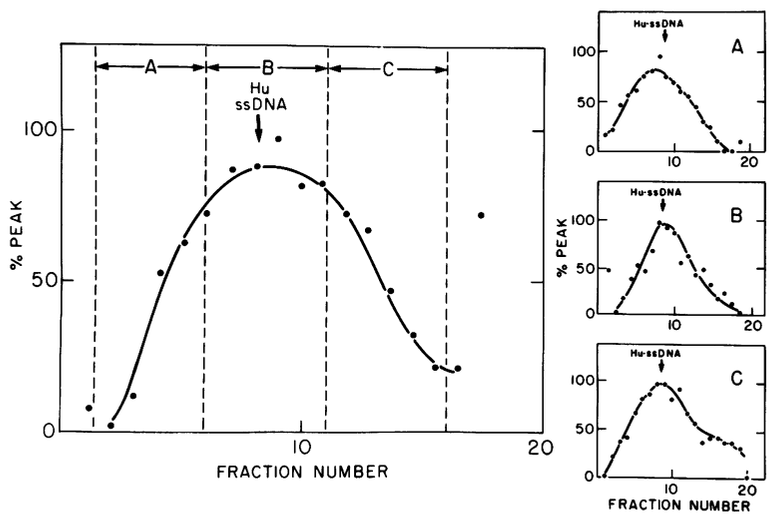

Figure 6. Behavior of plasma-derived DNA from patient Av in an alkaline CsCl gradient, on the left, along with the behavior, shown on the right, of the separately run pooled subfractions of that gradient, labeled *A*, *B*, and *C*, as indicated. Of note are the similar distributions of all the fractions as well as the somewhat narrower distribution of the central fraction, (*B*), as would be expected if the broadness of the unfractionated peak were due entirely to diffusion rather than to heterogeneity of base composition.

effect of the isolation and labeling procedures on exogenously added dsDNA suggests that at least some denaturation occurred in vitro.

DNA reassociation studies. As a more critical test of the identity of circulating DNA, a series of DNA-DNA renaturation studies was carried out to explore the base sequences represented with examination initially of model DNA mixtures to attempt to establish the validity of this approach directly.

Model experiments. First, it was shown that, as expected, the renaturation of authentic human DNA was accelerated by addition of homologous driving DNA, as compared with the effect of heterologous control DNA (Fig. 7). Also of note in this experiment is the achievement of almost complete renaturation of the labeled human DNA under these experimental conditions, implying that single-copy base sequences, which compose a large proportion of human genomic DNA, as well as repetitive sequences would completely and rapidly renature under the conditions of the phenol emulsion method, confirming the findings of Kohne et al. (25). The specificity of this reaction is further demonstrated using prokaryotic DNA (from *E. coli*) where addition of the homologous DNA again results in a much greater renaturation rate than occurred in the presence of either human or calf thymus DNA, although, even without added homologous DNA, the (undriven) self-renaturation rate is relatively high, as would be expected for the less complex prokaryotic DNA, as compared with human DNA (Fig. 8).

To examine the rationale of measuring the extent of renaturation as an estimate of the proportion of base sequences held in common between the labeled DNA and the unlabeled driving DNA, a mixture of equal amounts (in terms of radioactivity) of tritium-labeled human and *E. coli* DNA was examined. As shown in Fig. 9, approximately half the labeled DNA had renatured under conditions under which the 'driven' fraction, from *E. coli* in this case, was completely renatured (as shown above). It should be noted that in this model experiment because of the use of the rapidly self-renaturing prokaryotic DNA, it was necessary to correct for undriven renaturation in order to determine the renaturation attributable specifically to

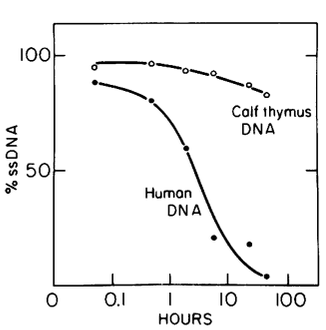

Figure 7. Renaturation, as described in the text, of tritiated human DNA in the presence of 5 μg/ml of either human DNA or of calf thymus DNA. Specific acceleration of renaturation is seen in the presence of homologous DNA as compared with the heterologous DNA. Also of note is that virtually complete renaturation of the human DNA is seen in ~100 h, with a Cot ½ (27) of ~0.25, in confirmation of Kohne et al.'s results (26).

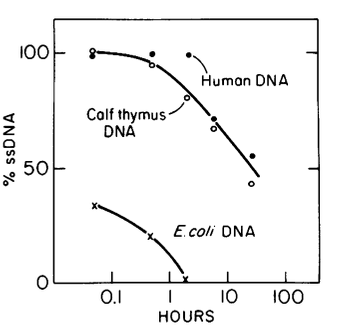

Figure 8. Renaturation of tritium-labeled DNA from *E. coli* in the presence of two heterologous DNAs (human and calf thymus) and the homologous DNA from *E. coli* as indicated. No difference was observed between the undriven renaturation rates seen in the presence of the two heterologous DNAs, as expected. However, in both cases, the undriven renaturation rate of *E. coli* DNA is greater than that seen for the more complex eukaryotic human DNA shown in Fig. 7. Renaturation in the presence of homologous driving DNA from *E. coli* is almost unmeasurably rapid in this system.

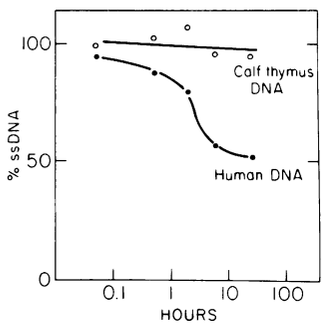

Figure 9. Renaturation of a model system consisting of a mixture of equal amounts of tritium-labeled prokaryotic (*E. coli*) and human DNAs driven by either heterologous (calf thymus) or by partially homologous (human) DNA. After correcting for undriven renaturation of the labeled DNA, as described in the text, the reaction driven by the partially homologous human DNA appears to have reached a plateau at ~50% completion at a time when the completely homologous human DNA system (as seen in Fig. 7) has completely renatured.

the presence of driving DNA. For human DNA the undriven self-renaturation seen in the presence of heterologous DNA was, as expected, much less than with *E. coli*, although with DNA of relatively low specific activity (as with the material labeled in tissue culture with ^{14}C) it still occurred to a significant extent.

Specificity of reassociation acceleration by human DNA. Having confirmed the validity of the underlying rationale with model experiments, renaturation studies of plasma DNAs were carried out initially to determine whether their rates of reassociation would be accelerated by authentic human DNA as compared with controls containing heterologous DNA. All of nine plasma DNA specimens were found to have their rates of reassociation specifically accelerated by human DNA when examined in this way. The results of one such experiment, in which five plasma DNA specimens were examined at two time points are summarized in Table I. For each specimen, at both time points, the percentage reassociated in the presence of human DNA exceeded that reassociated in the presence of heterologous control DNA (in this case from calf thymus). The results were statistically significant with $P < 0.01$ when examined either as paired or unpaired data. Indeed, in this experiment, the radiolabeled human DNA examined as a positive control did not appear to behave differently from the plasma DNA specimens, although, when more carefully examined, this was not uniformly observed, as will be shown. It was concluded that, since a mean of 47% (=86 − 39%) of plasma DNA specifically renatured in the presence of human DNA, this represents the minimum proportion of plasma DNA sequences held in common with those of human genomic DNA. A similar conclusion was reached when individual renaturation curves were studied in more detail as illustrated, for one such plasma DNA, in Fig. 10.

Table I. Plasma DNA Reassociation Rates in Presence of Driver Consisting of Normal Human DNA or Heterologous Nonhuman DNA (% ssDNA)

	16-h reaction		120-h reaction	
	Driver DNA		Driver DNA	
Patient No.	Human	Nonhuman	Human	Nonhuman
5	58	96	32	92
6	66	95	43	91
4	52	94	40	83
7	67	91	42	84
1	62	89	36	81
Mean±SD*	61±6	93±3	39±5	86±5
Human DNA	58	93	33	88

* Reassociation driven by human DNA is significantly greater (<0.01) than that driven by nonhuman DNA (from calf thymus in this experiment). It should be noted in this and subsequent tables that the data shown refer to ssDNA remaining in the reaction mixture (i.e., not reassociated).

Extent of specific human DNA-driven reassociation. To attempt to estimate the proportion, if any, of plasma-derived DNA base sequences that were not represented in human genomic DNA, additional studies to compare the extent of plasma DNA renaturation with authentic human DNA, in the presence of human driver DNA, were carried out.

For these studies, each tritium-labeled plasma DNA specimen was allowed to renature in the presence of an internal control consisting of authentic human DNA labeled with ^{14}C as well as unlabeled human driver DNA. In Fig. 11 is illustrated one such experiment in which six plasma DNA specimens were studied. Both the rate and extent of reassociation of the internal control human DNA exceeded that of the plasma DNA with the difference achieving statistical significance ($P < 0.05$) at the last two time points, both when evaluated as paired and unpaired data.

In a similar experiment where four plasma DNA specimens were examined in this way at two time points, again the plasma and human DNA reassociation rates were significantly different at each time point studied (Table II). Also included in this was a control consisting of authentic human DNA that had been added to normal plasma, and then reextracted and radiolabeled in parallel with the SLE plasma DNA specimens. In contrast to the results with the lupus-derived plasma DNA specimens, no difference was seen between the reassociation rate of this

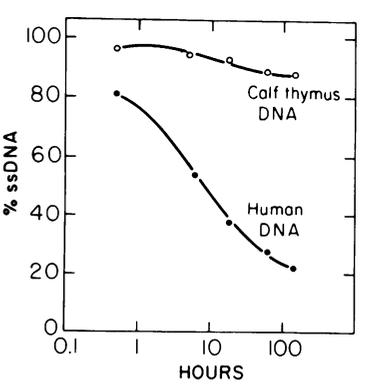

Figure 10. Renaturation of an SLE plasma-derived DNA specimen in the presence of either calf thymus or of human driver DNA illustrating the specific acceleration of renaturation by human driver DNA as described in the text.

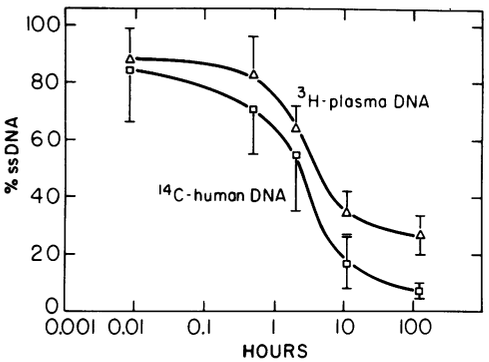

Figure 11. Renaturation, with human driver DNA, of plasma-derived DNA and of authentic human DNA present in the same reaction mixture. Each point represents the mean of six different plasma specimens, each from a different patient, with the bar indicating 1 SEM. The data achieve statistical significance at the last two time points as discussed in the text.

tritium-labeled, human plasma DNA and the ^{14}C-labeled internal control DNA. The reassociation rates of 10 plasma DNA specimens were examined by comparison with those of individual internal human DNA controls with similar results in each case. It was therefore concluded that there existed a significant difference of 10–20% in the apparent reassociation endpoints between the plasma DNA specimens and authentic human DNA. However, it remained to be established that this was attributable to base sequence differences.

One alternate explanation might be the existence, in the plasma DNA preparations, of a population of DNA chain lengths too short to form stable duplexes under the conditions used.

Table II. Reassociation of Plasma DNA Compared with That of an Internal Human DNA Control in the Presence of Human Driver DNA (% ssDNA)

	16 h reaction		160 h reaction	
Patient	Plasma DNA	Human DNA	Plasma DNA	Human DNA
1	61	36	50	27
2	48	33	31	18
3	55	33	37	25
4	50	40	35	22
Mean±SD*	54±6	35±3	38±8	23±4
Human plasma DNA control‡	49	43	26	20

* Plasma DNA reassociation is significantly less (i.e., the percentage ssDNA remaining is greater) than that of the corresponding human DNA at both time points ($P < 0.01$).

‡ Human DNA added to and reextracted from normal plasma and radiolabeled in parallel with plasma DNA specimens.

To explore this, two plasma DNA specimens were subjected to extensive renaturation in the presence of human driver DNA as before. The renatured and unrenatured fractions were then separated by hydroxylapatite chromatography, and then examined in parallel, after reconcentration, first, for precipitability in trichloracetic acid and, second, for chain length by polyacrylamide gel electrophoresis after again subjecting them to denaturation. No significant differences between the two fractions were observed for either specimen, suggesting that this explanation was inadequate. However, the limited amounts of plasma DNA available prevented larger numbers of specimens from being examined in this way so that this explanation cannot be considered to have been adequately eliminated. In particular, it remained possible that minor differences in chain length distribution, not readily detectable by gel electrophoresis could have a significant effect on reassociation rates, thereby resulting in apparent, but not real, differences in the reaction endpoints.

To try to establish these endpoints with more certainty, the following experiment was performed in an effort to drive the reaction to completion, thereby minimizing any effect that reassociation rate differences might have on the final extent of reassociation observed. Two plasma DNA specimens, plus an authentic human DNA specimen (which had been added to normal plasma and then reextracted and radiolabeled as just described) were each studied in the following way. Each specimen was allowed to renature in the presence of 50 μg/ml of human driver DNA for 66 h after which an additional 50 μg/ml of freshly denatured human ssDNA was added to each reaction mixture. Renaturation was then allowed to continue for an additional 120 h. The resulting renaturation is shown in Table III. Neither plasma DNA specimen renatured to an extent >80%. In contrast, the human DNA control, under the same conditions, renatured to 93%. This difference is consistent with the presence, in the plasma DNA preparations, in contrast to the human DNA control, of a small, relatively nonrenaturable fraction, possibly resulting from base sequence differences between it and the driver human DNA. Again, however, because this experiment could be performed on so few specimens and because the difference observed was small, this conclusion cannot as yet be considered secure. Hence, the question of the extent of plasma DNA renaturability in the presence of human driver DNA remains incompletely resolved.

Table III. Plasma DNA Reassociation Intensively Driven by Human DNA (% ssDNA)

Patient	1 h	186 h*
8	67	23
9	60	24
Human DNA	57	7

* More human ssDNA (50 μg/ml) added at 66 h.

Discussion

The present data characterizing circulating dsDNA in some SLE patients are best interpreted in the context of the present uncertainty regarding the prevalence of such circulating DNA. Thus, it is relevant to note that there currently exists no agreement in the literature on whether the majority of lupus patients or, for that matter, normal individuals, exhibits extracellular circulating DNA. This appears likely to be due, at least in part, to major methodologic differences among studies as well as to the use of indirect assay techniques, some of which are not readily controlled. As a consequence, it is difficult to directly compare studies to resolve such conflicts.

With regard to normal individuals, however, data obtained by carefully controlled CIE assays have been more consistent and have, in two different laboratories, including our own, generally failed to demonstrate plasma DNA by methods capable of detecting as little as 20–50 ng/ml of IP dsDNA (2, 3). Moreover, several independent assays not dependent on immunoprecipitability have given similar negative results (3, 9), but only when carefully controlled for specificity. These results are in striking contrast to reports of finding large amounts of plasma DNA using some of these same methods when such specificity controls were omitted (29, 30). In a more recent study, by using quantitation of plasma dsDNA by direct electron micrography, it was concluded that no more than 10 ng/ml of dsDNA was present in normal plasma, an amount corresponding to the DNA content of ~0.02% of the leukocytic DNA in whole blood and thus possibly resulting from undetected traumatic release during specimen collection (8). As noted, similar results have not been uniformly obtained (18, 31). It is nonetheless suggested that, with the possible exception of very short DNA molecules which might be difficult to detect (21), the best current evidence appears consistent with there being no more than 10 ng/ml of free dsDNA in normal plasma. However, because the data remain sparse, this conclusion must be considered tentative, pending additional information.

With regard to the presence of circulating dsDNA in SLE patients, still less agreement exists. Most studies have been based on examination of serum or serum-derived material (32–38) where, as noted, DNA may appear in vitro in widely varying amounts so that its presence, in most cases, cannot be unambiguously interpreted. Moreover, this difficulty is compounded by the general failure to exclude non-SLE clinical states that have been shown, with varying degrees of certainty, to be associated with at least transitory circulating DNA, and that, in some instances, occur frequently in patients with active SLE as, for example, recent institution of corticosteroid therapy (4), pulmonary embolism (6), and hemodialysis (5). Even those few studies that have used plasma rather than serum specimens to prevent DNA release in vitro have generally failed to control for this potential source of irrelevant circulating DNA (18, 39, 40).

Perhaps because of these factors, major discrepancies remain among these studies as to the prevalence and concentration, if any, of dsDNA in the circulation of SLE patients, whether free or complexed with antibody.

In contrast, as with the studies of normal plasmas, those using CIE have been consistent in indicating that the majority of SLE patients fail to exhibit IP dsDNA and thus do not differ in this regard from normals (2, 3). A relatively small subgroup of lupus patients, however, do exhibit CIE-detectable IP dsDNA in their plasma in association with clinically recognizable systemic vasculitis (7). In these patients, IP DNA can be identified in sequential plasma specimens collected on different days and disappears after the clinical activity subsides. Its presence could not be attributed to non-specific systemic inflammation or to drug administration and thus appears to be associated with the underlying disease itself. Further, this circulating DNA has been well characterized as such, within the limits of the immunologic method used to detect it. In addition, its detection in plasma seems likely not to have resulted from in vitro release during plasma separation (5), and therefore is likely to reflect its presence in the circulation. Accordingly, the present study of circulating DNA in SLE was confined to material derived from this specific subgroup of patients.

The major conclusion of the present study was that this DNA appeared to consist largely or entirely of human DNA base sequences. The possibility that a minor fraction differs in sequence from human genomic DNA could not be entirely excluded, however, because characterization of a small subfraction of plasma-derived DNA that differed in its renaturability with human DNA was limited by reagent availability.

It should be emphasized that, because the finding of persistently (in contrast to transiently) circulating IP dsDNA is distinctly infrequent, even among patients with SLE (2, 3), it would be hazardous to apply these results to less rigorously selected patient populations. In this regard it is relevant to note that the present findings are generally consistent with those reported by Sano and co-workers (16, 19, 41) on circulating protein-bound DNA in apparently unselected SLE serums. However, these studies were not controlled for the sources of interpretational ambiguity just discussed (42) and thus cannot be considered to support the generality of the present findings. Moreover, these findings provide no information on the possible occurrence in plasma of ssDNA, free of double-stranded regions, since the methods used would be expected neither to have isolated nor to have detected it.

The present data also do not allow conclusions about whether the circulating plasma IP dsDNA contains all, or only a fraction of human genomic sequences. Further, the distribution of repeated and unique sequences remains undefined. Were this DNA to contain all the sequences of the human genome, with a similar distribution of repetition frequencies, it would suggest that it originated by nonspecific release from cells as might be expected to accompany cell lysis. However, more specific extracellular release of dsDNA has been described in conjunction with several immunologically relevant systems (43–47). It currently is unclear what significance, if any, such DNA has for either the immune response or for this group of diseases. If it should, in fact, prove

to have such significance, as suggested by recent, still unconfirmed data (46, 47), it would seem particularly important to further characterize circulating DNA in these disorders. Moreover, regardless of any functional significance it may have, its further characterization may, as already discussed, shed light on its origin, a point of interest at least to the extent of its pathogenic importance in SLE. Finally, the possibility has not been eliminated that a subfraction of circulating DNA, presumably represented by that portion appearing relatively nonrenaturable in the presence of human driver DNA, could be of nonhuman origin, an unlikely but obviously important consideration.

Acknowledgments

The advice and help of Drs. David Kingsbury, David Kohne, and Steven Tracy are gratefully acknowledged as is the technical help of Mrs. Nana Chan and assistance with the manuscript by Mrs. Anita Sardo.

This study was supported in part by grant AM 33278 from the National Institutes of Health and by grants from the Lupus Foundation and the Gina Finzi Lupus Research Fund.

References

1. Yamashita, T., and M. Yamada. 1952. Nucleic acid in serum. *Chem. Abstr.* 46:10362b. (Abstr.)

2. Davis, G. L., Jr., and J. S. Davis IV. 1973. Detection of circulating DNA by counterimmunoelectrophoresis (CIE). *Arthritis Rheum.* 16:52–58.

3. Steinman, C. R. 1975. Free DNA in serum and plasma from normal adults. *J. Clin. Invest.* 56:512–515.

4. Hughes, G. R. V., S. A. Cohen, R. W. Lightfoot, Jr., J. I. Meltzer, and C. L. Christian. 1971. The release of DNA into serum and synovial fluid. *Arthritis Rheum.* 14:259–266.

5. Steinman, C. R., and A. Ackad. 1977. Appearance of circulating DNA during hemodialysis. *Am. J. Med.* 62:693–697.

6. Sipes, J. N., P. N. Surątt, C. D. Teates, F. A. Barada, J. S. Davis, and C. J. Tegtmeyer. 1978. A prospective study of plasma DNA in the diagnosis of pulmonary embolism. *Am. Rev. Respir. Dis.* 118:475–478.

7. Steinman, C. R. 1979. Circulating DNA in systemic lupus erythematosus. Association with central nervous system involvement and systemic vasculitis. *Am. J. Med.* 67:429–435.

8. Dennin, R. H. 1979. DNA of free and complexed origin in human plasma. Concentration and length distribution. *Klin. Wochenschr.* 57:451–456.

9. Steinman, C. 1975. Use of nucleic acid hybridization for specific detection of submicrogram quantities of DNA and its application to human plasma. *Clin. Chem.* 21:407–411.

10. Steinman, C. 1979. Quantitation of submicrogram concentrations of DNA by a modified Laurell electrophoresis method. *J. Immunol. Methods.* 31:373–378.

11. Phillips, P. E. 1975. The virus hypothesis in systemic lupus erythematosus. *Ann. Int. Med.* 83:709–715.

12. Tan, E. M. 1979. Systemic lupus erythematosus—immunologic aspects. *In* Arthritis and Allied Conditions. D. J. McCarty, editor. Lea & Febiger, Philadelphia. 19th ed. 715–722.

13. Gocke, D. J., C. Morgan, M. Lockshin, K. Hsu, S. Bombardieri, and C. Christian. 1970. Association between polyarteritis and Australia antigen. *Lancet.* II:1149–1153.

14. Hung, P. P., J. C-Mao, C. M. Ling, and L. R. Overby. 1975. Hybridization of Dane particle DNA with the free plasma DNA of hepatitis carriers. *Nature (Lond.).* 253:571–572.

15. Harbeck, R., A. Hoffmann, and R. Carr. 1975. Studies on the nature of circulating DNA in systemic lupus erythematosus (SLE). *J. Rheumatol.* 2:194–203.

16. Sano, H., and C. Morimoto. 1981. Isolation of DNA from DNA/anti-DNA antibody immune complexes in systemic lupus erythematosus. *J. Immunol.* 126:538–539.

17. Ikebe, K., C. G. Ramesh, and E. M. Tan. 1982. Characterization of DNA in polyethylene glycol precipitated circulating immune complexes from SLE. *Arthritis Rheum.* 25:S20.

18. Raptis, L., and H. A. Menard. 1980. Quantitation and characterization of plasma DNA in normals and patients with systemic lupus erythematosus. *J. Clin. Invest.* 66:1391–1399.

19. Sano, H., and C. Morimoto. 1982. DNA isolated from DNA/antiDNA antibody immune complexes in systemic lupus erythematosus is rich in guanine-cytosine content. *J. Immunol.* 128:1341–1345.

20. Cohen, A. S., W. E. Reynolds, E. C. Franklin, J. P. Kulka, M. W. Ropes, L. E. Shulman, and S. L. Wallace. 1971. Preliminary criteria for the classification of systemic lupus erythematosus. *Bull. Rheum. Dis.* 21:643–648.

21. Steinman, C. R. 1982. Detection and semiquantitation of DNA by counterimmunoelectrophoresis (CIE). *Methods Enzymol.* 84:187–193.

22. Maniatis, T., A. Jeffrey, and D. Kleid. 1975. Nucleotide sequence of the rightward operator of phage lambda. *Proc. Natl. Acad. Sci. USA.* 72:1184–1188.

23. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237–251.

24. Szybalski, W. 1968. Cesium salt density gradient centrifugation. *Methods Enzymol.* 12B:330–360.

25. Kohne, D. E., S. A. Levison, and M. J. Byers. 1977. Room temperature method for increasing the rate of DNA reassociation by many thousandfold: the phenol emulsion reassociation technique. *Biochemistry.* 16:5329–5341.

26. Britten, R. J., D. E. Graham, and B. R. Neufeld. 1974. Analysis of repeating DNA sequences by reassociation. *Methods Enzymol.* 29:363–418.

27. Bernardi, G. 1971. Chromatography of nucleic acids on hydroxyapatite columns. *Methods Enzymol.* 21:95–139.

28. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa. Sixth ed. 32–116.

29. Kamm, R. C., and A. G. Smith. 1972. Nucleic acid concentrations in normal human plasma. *Clin. Chem.* 18:519–522.

30. Kaznacheev, V. P., and I. V. Poliakov. 1967. A study of some indices of nuclein metabolism in patients with atherosclerosis [in Russian]. *Kardiologiya.* 7:22–25.

31. Lippmann, M. L., L. Morgan, A. Fein, B. Shapiro, and S. A. Leon. 1982. Plasma and serum concentrations of DNA in pulmonary embolism. *Am. Rev. Respir. Dis.* 125:416–419.

32. Harbeck, R. J., A. A. Hoffman, R. I. Carr, and E. J. Bardana. 1975. Measurement of DNA/Anti-DNA complexes. *Scand. J. Rheum.* Suppl. II:26–34.

33. Lee, S. L., and I. Rivero. 1964. Cryoglobulins in systemic lupus erythematosus (SLE) as circulating immune complexes. *Arthritis Rheum.* 7:321.

34. Harbeck, R. J., E. J. Bardana, P. F. Kohler, and R. I. Carr. 1973. DNA:Anti-DNA complexes. Their detection in systemic lupus erythematosus sera. *J. Clin. Invest.* 52:789–795.

35. 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.

36. Stingl, G., G. J. Meingassner, P. Swelty, and W. Knapp. 1976. An immunofluorescence procedure for the demonstration of antibodies to native double-stranded DNA and of circulating DNA-anti-DNA complexes. *Clin. Immunol. Immunopathol.* 6:131–140.

37. Adu, J., and D. Williams. 1981. DNA-anti-DNA circulating complexes in the nephritis of systemic lupus erythematosus. *Clin. Exp. Immunol.* 43:605–614.

38. Davis, J. S., S. M. Godfrey, and J. B. Winfield. 1978. Direct evidence for circulating DNA/anti-DNA complexes in systemic lupus erythematosus. *Arthritis Rheum.* 21:117–122.

39. Bruneau, C., and J. Benveniste. 1979. Circulating DNA: Anti-DNA complexes in sytemic lupus erythematosus. Detection and characterization by ultracentrifugation. *J. Clin. Invest.* 64:191–198.

40. Roberts, L. R., M. F. Robinson, and E. J. Lewis. 1981. Low-molecular-weight plasma cryoprecipitable antinative DNA. Polynucleotide complexes in lupus glomerulonephritis. *Clin. Immunol. Immunopath.* 19:79–90.

41. Morimoto, C., H. Sano, T. Abe, M. Homma, and A. Steinberg. 1982. Correlation between clinical activity of systemic lupus erythematosus and the amounts of DNA in DNA/antiDNA antibody immune complexes. *J. Immunol.* 139:1960–1965.

42. Steinman, C. R. 1983. Letter to the Editor. *J. Immunol.* 130:1990.

43. Rogers, J. C., D. Boldt, S. Kornfeld, S. A. Skinner, and C. R. Valeri. 1972. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohaemagglutinin or antigen. *Proc. Natl. Acad. Sci. USA.* 69:1685–1689.

44. Olsen, I., and G. Harris. 1974. Uptake and release of DNA by lymphoid tissue and cells. *Immunology.* 27:973–987.

45. Jachertz, D. 1979. Gene activation during immune reaction. *Mol. Cell. Biochem.* 24:93–126.

46. Anker, P., D. Jachertz, M. Stroun, R. Brogger, C. Lederrey, L. Henry, and P. Maurice. 1980. The role of extracellular DNA in the transfer of information from T to B human lymphocytes in the course of an immune response. *J. Immunogenet. (Oxf.).* 7:475–481.

47. Pancer, L. B., M. F. Milazzo, V. L. Morris, S. K. Singhal, and D. A. Bell. 1981. Immunogenicity and characterization of supernatant DNA released by murine spleen cells. *J. Immunol.* 127:98–104.