

# Quantitation and Characterization of Plasma DNA in Normals and Patients with Systemic Lupus Erythematosus

LEDA RAPTIS and HENRI A. MENARD, *Laboratoire d'Immuno-Rhumatologie,  
Unité des Maladies Rhumatismales, Centre Hospitalier Universitaire  
de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4*

**ABSTRACT** Using the *in vitro* DNA labeling technique of nick translation on purified plasma DNA, we have estimated the plasma DNA concentration in three normal individuals to be  $266 \pm 57$  ng/ml (mean  $\pm$  SD). This was not significantly different in three patients with a chronic inflammatory disease ( $209 \pm 14$  ng/ml) or in five patients with steroid-inactivated systemic lupus erythematosus (SLE) ( $293 \pm 57$  ng/ml). In two untreated, newly diagnosed, active SLE patients, however, the plasma DNA concentration was considerably higher (4,024 and 2,437 ng/ml, respectively). Characterization of these *in vitro* labeled DNA preparations by neutral sucrose-gradient sedimentation analysis showed a sedimentation coefficient of 6–8S, corresponding to a molecular weight of  $\sim 0.2\text{--}0.45 \times 10^6$ . No difference was observed between normal subjects or patients. In addition, the relative size uniformity of these DNA molecules might suggest some form of specific protection of the DNA from blood DNAses. Further characterization in terms of buoyant density in cesium chloride did not reveal a difference between normal or SLE plasma and the human (HEp-2 cell) DNA used as marker. Taking into account the limitations of the method, no indication of a possible exogenous origin of the DNA circulating in SLE patients could be found. The physiological or pathophysiological role of this plasma DNA remains to be determined.

## INTRODUCTION

Free DNA was originally detected in 1966 in the serum of systemic lupus erythematosus (SLE)<sup>1</sup> patients (1).

Abstract presented at the American Rheumatism Association, 44th Annual Scientific Meeting, Atlanta, Georgia, May 1980.

Received for publication 17 April 1980 and in revised form 18 August 1980.

<sup>1</sup>Abbreviations used in this paper: BND-cellulose, benzoylated, naphthoylated, DEAE-cellulose; dsDNA, double-

This provided a strong argument implicating immune complexes of the DNA:anti-DNA type in the pathogenesis of SLE. Since then, however, it has been recognized (2, 3) that DNA can be found in normal serum, probably being released from leukocytes during the clotting process. Thus, reports on the existence of free or complexed DNA in SLE serum should be interpreted with caution.

In normal or SLE plasma, the presence of DNA is still a matter of controversy. Early positive results obtained from normal plasma have been questioned by Steinman (3) on the basis of methodological arguments and he suggested that in plasma, amounts of DNA  $> 100$  ng/ml could be considered pathological. Others, however, have found that the frequency of free circulating DNA is similar in SLE and in normal plasma (2). According to a recent article regarding the detection of complexed DNA in plasma (4), negative results were obtained with normals, while DNA could be detected in 7 of 11 SLE patients. Still other reports showed positive findings in normals (5) or largely negative results in SLE (6).

Given the transmissibility of certain features of the disease in animal models of SLE (7, 8), the characterization of the DNA circulating in human SLE could be important in the elucidation of its origin, whether endogenous or exogenous. We have therefore attempted the quantitation and characterization of normal and SLE plasma DNA after purification and *in vitro* labeling by the technique of nick translation. In this procedure, single-stranded nicks are introduced into DNA and they constitute binding sites for the DNA polymerase I of *Escherichia coli*. This enzyme, in the presence of the four deoxyribonucleoside

stranded DNA; dXTP, deoxyribonucleoside 5'-triphosphate; G + C, guanine + cytosine;  $\phi$ X174 (RFII), replicating form II of the bacteriophage  $\phi$ X174; SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA.

5'-triphosphates (dXTP), will extend the 3'OH primer terminus with a simultaneous hydrolysis of the pre-existing strand by the 5' to 3' exonuclease activity, residing in a separate active site of the same enzyme (9). Thus, when radioactive dXTP are used as substrates, one can label a DNA molecule at specific activities in excess of  $10^8$  cpm/ $\mu$ g.

Based on this technique, we have found that DNA is normally present in human plasma and exists in much higher quantities in some untreated active SLE patients. Furthermore, we report a preliminary characterization of the properties of the nick-translated plasma DNA regarding its size and buoyant density in cesium chloride (CsCl). As far as these properties are concerned, no difference could be found between normal or SLE plasma DNA.

## METHODS

**Nucleic acid and enzyme preparations.** Lambda-phage DNA,  $\phi$ X174 replicating form II (RFII) DNA, and *E. coli* ribosomal RNA were purchased from New England Biolabs, Beverly, Mass. Pancreatic DNAase I was purchased from Worthington Biochemical Corp., Freehold, N. J., pancreatic RNAase from Miles Laboratories, Inc., Elkhart, Ind., pronase from Sigma Chemical Co., St. Louis, Mo., and *E. coli* DNA polymerase I from Boehringer Mannheim Biochemicals, Indianapolis, Ind. (catalogue no. 104485, lot no. 1158124).

**Preparation of in vivo  $^{32}$ P-labeled HEp-2 cell DNA.** HEp-2 cells were grown in monolayer cultures in Dulbecco modified Eagle's medium containing 10% calf serum. When the cells were 50% confluent, this medium was replaced by the same medium lacking phosphate ions and supplemented with 50  $\mu$ Ci/ml of [ $^{32}$ P]orthophosphate (ICN Pharmaceuticals, Irvine, Calif., carrier free, specific activity 285 Ci/mg). 12 h later, the medium was discarded and the cells lysed by the addition of 20 mM Tris-HCl buffer, pH 8.5, 20 mM EDTA containing 1% sodium dodecyl sulfate per petri dish ( $\sim 2 \times 10^6$  cells). The lysate was incubated with 2 mg/ml of pronase for 3 h at 37°C. Phenol extraction followed using redistilled phenol. Lipids were removed by chloroform-isoamyl alcohol (24:1) extraction, and the preparation was dialyzed against 10 mM Tris-HCl buffer, pH 8.5, 1 mM EDTA. The purified  $^{32}$ P-labeled HEp-2 cell DNA was mixed with the blood sample and subjected to the same extraction procedure as plasma DNA, described below.

**Preparation of in vivo  $^3$ H-labeled adenovirus-2 DNA.** The method described by Raptis et al. (10) was followed. HEp-2 cells were infected with a multiplicity of 10 plaque-forming units of adenovirus-2 per cell. The viral DNA was labeled by an 8-h pulse of [ $^3$ H]thymidine (New England Nuclear, Boston, Mass., specific activity 50–60 Ci/mmol) and purified by CsCl equilibrium centrifugation and benzoylated, naphthoylated, DEAE-cellulose (BND-cellulose, Serva, Heidelberg, West Germany) chromatography. This material adsorbs DNA molecules at a NaCl concentration of 0.3 M. Double-stranded DNA (dsDNA) can be subsequently eluted with a solution of 1 M NaCl in 20 mM Tris-HCl buffer, 2 mM EDTA, pH 8.1, while DNA molecules containing single-stranded regions (ssDNA) can be eluted with the same solution supplemented with 2% caffeine (saline and caffeine fraction, respectively). The saline fraction, consisting of mature adenovirus-2 DNA (10), was used in preliminary experiments to establish the optimal conditions for the purification of plasma DNA.

**Plasma collection.** Blood was obtained atraumatically using heparinized syringes, and plasma was immediately separated by a double centrifugation for 20 min at 2,000 rpm and 4°C. From each individual subject, two blood samples of 10 ml each were collected. One was mixed directly in the syringe with a tracer amount ( $0.5 \times 10^6$   $^{32}$ P cpm) of in vivo labeled HEp-2 (human) cell DNA in 0.5 ml of saline, and the other blood sample was mixed with the saline alone. After plasma separation, the DNA from the latter sample was purified and labeled in vitro by nick translation as described below. The DNA from the former sample was purified in an identical manner and the [ $^{32}$ P]HEp-2-cell DNA in this sample served as a marker for human DNA buoyant-density profile in CsCl equilibrium gradients.

**Purification of plasma DNA.** The last supernatant plasma ( $\sim 5$  ml) was diluted four times in 50 mM Tris-HCl buffer, pH 8.5, 20 mM EDTA containing 1% sodium dodecyl sulfate. 20 ml of the diluted plasma was mixed with 50 mg of self-digested pronase and incubated for 4 h at 37°C. This was followed by sequential extractions with phenol, chloroform-isoamyl alcohol (24:1), and ether. After extensive dialysis against 20 mM Tris-HCl buffer, pH 7.6, 10 mM NaCl, 5 mM EDTA, the extract was digested with 10  $\mu$ g/ml of pancreatic RNAase at 37°C for 30 min. The RNAase stock had been previously heated at 80°C for 10 min in 1 mM acetate buffer, pH 4.5, in order to destroy any contaminating DNAases. Further purification followed by adsorption of the material onto a BND-cellulose column at a pH of 8.1 and a salt concentration of 300 mM NaCl, and elution in one step as a caffeine fraction (10). After ethanol precipitation, the DNA pellet was resuspended in 100  $\mu$ l of 10 mM Tris-HCl buffer, pH 7.6, 1 mM EDTA, and stored at  $-20^\circ\text{C}$  until used. Similar results were obtained when the BND-cellulose step was omitted.

**In vitro labeling of plasma DNA by nick translation.** In preliminary trials, using  $\lambda$ -phage DNA, nicks were introduced into the DNA by the addition of  $10^{-6}$   $\mu$ g/ml of DNAase I, but this step was later proven to be unnecessary. Presumably, the commercial *E. coli* DNA polymerase I preparation used contained some contaminant nicking activity. Thus, the conditions used for the nick translation reaction were (11): 50 mM Tris-HCl buffer, pH 7.6, 10 mM 2-mercaptoethanol, 5 mM MgCl<sub>2</sub>, and 50  $\mu$ g/ml bovine serum albumin (J. T. Baker Chemical Co., Phillipsburg, N. J.). The reaction was performed in a total volume of 20–40  $\mu$ l in siliconized polyethylene tubes containing 0.25 nmol of each of the four  $^3$ H-labeled dXTP per reaction. Depending on the exact specific activities of each, these quantities would correspond to  $\sim 2.5$ , 5, 7, and 25  $\mu$ Ci of dGTP, dATP, dCTP and dTTP, respectively (New England Nuclear). The quantities of the enzyme used were generally 1 U/reaction or 0.1  $\mu$ l of the commercial preparation. Incubation followed for 3 h at 15°C. The reaction was stopped by the addition of 100  $\mu$ g/ml pronase, followed by a 1-h incubation at 37°C. The sample was subsequently extracted twice with redistilled phenol and the unincorporated dXTP were removed by Sephadex G-50 (fine) chromatography.

**Neutral sucrose gradient sedimentation analysis.** Simian virus forty (SV40) DNA was used as a marker for neutral sucrose gradient sedimentation analysis of the plasma DNA, which was performed as described (12). Supercoiled (form I) SV40 DNA was purified by CsCl-ethidium bromide equilibrium centrifugation (13), labeled in vitro by nick translation (11) using [ $^{32}$ P]dXTP (New England Nuclear, specific activity 300–400 Ci/mmol), and linearized by cleavage with the EcoRI restriction endonuclease. The sedimentation coefficient of this DNA is 15S (14). The plasma DNA, in vitro  $^3$ H-labeled by nick translation, was mixed with this

marker and layered onto linear 5–20% sucrose gradients, made in 20 mM Tris-HCl buffer, pH 8.5, 2 mM EDTA, 0.5 M NaCl. The gradients were made in SB 405 tubes of a B-60 ultracentrifuge (IEC, Needham Hts., Mass.) and spun at 20°C for 2.5 h at 60,000 rpm. After the run, fractions were collected from the bottom of every tube and acid-precipitable  $^3\text{H}$  and  $^{32}\text{P}$  radioactivity determined on each.

*CsCl equilibrium centrifugation analysis.* The buoyant density in neutral CsCl of the in vitro  $^3\text{H}$ -labeled plasma DNA was verified by equilibrium centrifugation analysis as described (10). The density of the sample was adjusted to 1.699 g/ml, i.e., the density of mammalian DNA (15), by the addition of solid CsCl (BDH Chemicals, Montréal, Canada). The preparation was subsequently centrifuged to equilibrium at 50,000 rpm for 50 h in the A321 rotor of a B-60 ultracentrifuge (IEC). Gradient volumes were usually 2 ml. Fractions were collected from the bottom of every tube and acid-precipitable radioactivity was determined on each.

## RESULTS

*Purification of plasma DNA.* One of the important steps in the purification of free circulating DNA is the inactivation of the existing blood DNAases (16). Therefore, the separation of blood cells from plasma was conducted immediately after bleeding, at 4°C. Inhibition of plasma DNAases was achieved by dilution of the plasma sample with a solution containing EDTA, sodium dodecyl sulfate, and self-digested pronase. In preliminary experiments, in vivo  $^3\text{H}$ -labeled adenovirus-2 DNA was added to the blood sample and subsequently extracted following the procedure for plasma DNA purification described in Methods. After purification, >80% of the initial radioactivity was recovered in an acid-precipitable form. Moreover, this DNA remained intact after the whole process, as evidenced by sucrose gradient sedimentation analysis.

Another important point in the extraction of circulating DNA is the prevention of cellular DNA liberation which may occur during clotting (2). In agreement with the results from other laboratories (2, 3), two to three times more DNA was found in normal serum, than in plasma (not shown).

*Labeling of plasma DNA by nick translation.* In preliminary experiments, cold  $\lambda$ -phage DNA was used to establish the optimal conditions for the reaction. The addition of  $10^{-6}$   $\mu\text{g}/\text{ml}$  of DNAase I in the nick translation mixture resulted in a slight increase of the acid-precipitable counts obtained (2.2 vs.  $1.9 \times 10^6$  cpm for 0.1  $\mu\text{g}$  of DNA substrate). However, the addition of DNAase caused a considerable degradation of the final product, as revealed by sucrose gradient sedimentation analysis. When the DNAase was omitted, and the reaction conducted under the conditions described in Methods, using 0.1  $\mu\text{g}$  of  $\lambda$  DNA, the sedimentation profile denoted >90% intact  $\lambda$ -phage DNA molecules.

The following control experiments were considered necessary: 1  $\mu\text{l}$  (10 U) of the DNA polymerase I prepara-

tion was incubated with all four  $^3\text{H}$ dXTP in the absence of added DNA under the nick translation reaction conditions described in Methods. After Sephadex chromatography and acid precipitation,  $\sim 30,000$   $^3\text{H}$  cpm were obtained at the position of DNA. The density of this DNA in CsCl was identical to the density of *E. coli* DNA, i.e., 1.700 g/ml. It thus seems likely that the commercial DNA polymerase I preparation used did contain trace amounts of *E. coli* DNA. The amount of enzyme we used in the standard nick translation reactions was one tenth the quantity used in the above experiment (0.1 vs. 1  $\mu\text{l}$ ). This background radioactivity incorporated into the endogenous *E. coli* DNA would therefore be negligible compared with the counts per minute incorporated into the plasma DNA sample. No counts per minute were obtained when  $^3\text{H}$ dATP and  $^3\text{H}$ dTTP alone (or  $^3\text{H}$ dGTP and  $^3\text{H}$ dCTP alone) were incubated with the enzyme under the same conditions. This would indicate that the incorporation of  $^3\text{H}$ dXTP into an acid-insoluble product in the absence of added DNA was not because of the *de novo* DNA synthesizing activity of the DNA polymerase (17).

It is well known that the DNA polymerase I may also act as a reverse transcriptase, using RNA molecules as template and primer (17), although at a much lower efficiency. Thus, when 5  $\mu\text{g}$  of *E. coli* ribosomal RNA were incubated with 10 U of DNA polymerase I and standard amounts of  $^3\text{H}$ dXTP,  $\sim 300,000$  cpm were obtained in acid-precipitable form. Because the plasma DNA preparations had been extensively treated with RNAase during purification and before the nick translation reaction was carried out, the possibility of labeling plasma RNA with this enzyme may be excluded. Moreover, an RNA molecule which has been labeled by means of the DNA polymerase I would most probably present the structure of a RNA-DNA hybrid, whose buoyant density in CsCl (or  $\text{Cs}_2\text{SO}_4$ ) would be considerably higher than the one actually observed (see below) for our nick-translated plasma DNA preparations (15).

Finally, when the plasma sample was treated with pancreatic DNAase I (20  $\mu\text{g}/\text{ml}$ , in the presence of 10 mM  $\text{MgCl}_2$  for 60 min at 37°C) before the purification process, virtually no counts per minute were obtained in acid-precipitable form after nick translation. Therefore, there is little doubt that the material labeled by this method is sensitive to DNAase.

*Quantitation of plasma DNA.* The technique described above was used for DNA quantitation in a purified plasma DNA preparation, based on a standard curve of the counts per minute obtained as a function of the amount of  $\lambda$ -phage DNA used in each nick translation reaction (Fig. 1). There is an almost linear relationship from 0.01 to 0.1  $\mu\text{g}$  of  $\lambda$  phage DNA, whereas at higher levels of this substrate, a plateau is reached. This is presumably because the amounts

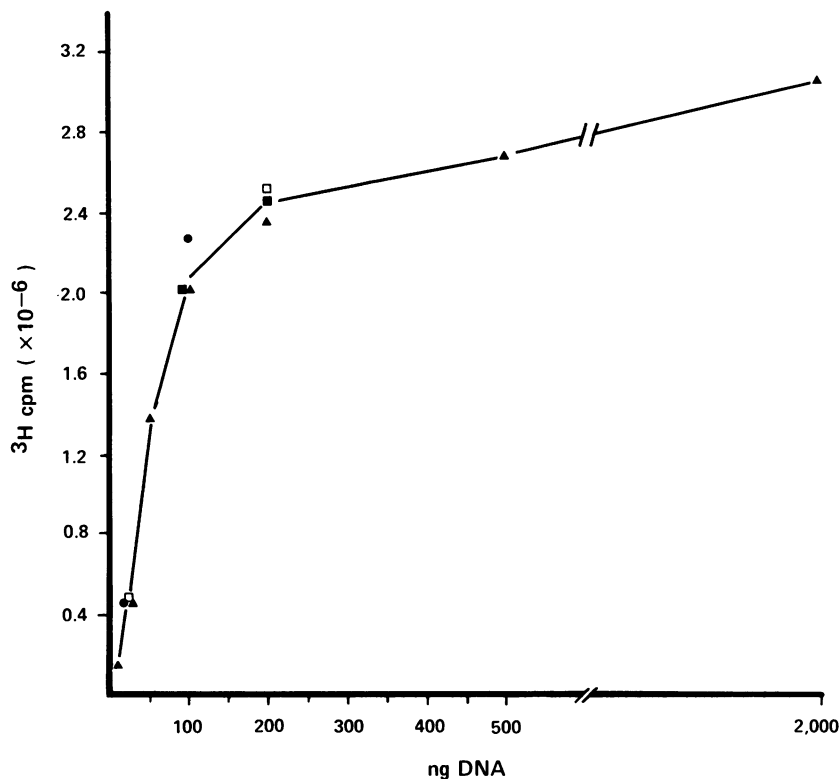


FIGURE 1 Nick translation reaction reference curve. Acid-precipitable counts per minute obtained after nick translation labeling of increasing quantities of  $\lambda$  phage ( $\blacktriangle$ ),  $\phi$ X174 RFII ( $\square$ ), SV40 form I ( $\blacksquare$ ), and calf thymus ( $\bullet$ ) DNA under the standard reaction conditions described in Methods. The radioactivity was determined on a pool of the DNA-containing fractions after removal of the unincorporated dXTP by Sephadex G-50 chromatography. The line is joining the means of the counts per minute obtained using a given quantity of DNA.

of [ $^3$ H]dXTP used (0.25 nmol) are limiting. Similar results were obtained when  $\phi$ X174 RFII, SV40 form I, or calf thymus DNA were used as substrates. Based on this standard curve, we could estimate the amount of DNA in a preparation labeled at the same time, using strictly identical conditions, especially when this amount lies in the range of 0.01 to 0.1  $\mu$ g. Therefore, if the counts per minute obtained after labeling a plasma DNA sample exceeded  $2.4 \times 10^6$ , the experiment was repeated using a lower amount of DNA.

The concentrations of DNA found in the plasma samples of 13 individuals are given in Table I. The following observations could be made: (a) normal plasmas do contain DNA; (b) patients with non-SLE chronic inflammatory diseases with antinuclear antibodies have a similar amount of DNA in their plasma; (c) patients with clinically inactive SLE on prednisone therapy have amounts of DNA similar to those of normals; (d) only the two untreated active SLE patients have a clear elevation of their plasma DNA.

*Characterization of nick-translated plasma DNA.* When the nick translation reaction has been completed,

the DNA obtained is mostly double stranded, any existing "gaps" having been repaired by the enzyme (17). This property was actually verified in our case: when normal or SLE plasma DNA, labeled by nick translation, was adsorbed onto a BND-cellulose column, >95% of the  $^3$ H-radioactivity could subsequently be eluted as the saline fraction, i.e., as dsDNA.

The size of the plasma DNA, after purification and nick translation labeling was estimated by neutral sucrose sedimentation analysis. As shown in Fig. 2, the sedimentation profile of this DNA is similar, in both the normal and the SLE individuals, ranging in sedimentation coefficient from 6 to 8S. This would correspond to a molecular weight of  $0.2-0.45 \times 10^6$  (18). Similar profiles were obtained whether or not the SLE patients were clinically active and untreated.

The existence of a DNA molecular species, possessing a guanine + cytosine (G + C) content different from the one of human DNA, would be of particular interest. Therefore, in an attempt to obtain some information on the origin of the plasma DNA extracted, neutral CsCl equilibrium centrifugation analysis was

TABLE I  
Quantitation of Purified Plasma DNA by Nick Translation

Patient	Plasma	cpm/reaction	DNA ng/ml	Mean $\pm$ SD*
1	Normal	430,560	233	
2	Normal	613,200	332	266 $\pm$ 57
3	Normal	428,000	232	
4	Rheumatoid	390,000	211	
5	Rheumatoid	358,000	194	209 $\pm$ 14
6	Dermatomyositis†	410,000	222	
7	Inactive SLE	534,750	290	
8	Inactive SLE	520,000	282	
9	Inactive SLE	620,000	336	293 $\pm$ 57
10	Inactive SLE	320,560	206	
11	Inactive SLE	650,000	352	
12	Active SLE§	1,858,000	4,024	3,231 $\pm$ 1,122
13	Active SLE	1,125,000	2,437	

\* Except for the active SLE ( $P < 0.007$ ), the means are not different from those of the normals by the unpaired  $t$  test.

† Patients 6, 9, 10, and 11 were treated with 30–50 mg prednisone daily. Patient 6 also received 50 mg azathioprine daily. Their mean  $\pm$  SD plasma DNA concentration was 279  $\pm$  76 ng/ml. This is not significantly different from the normal individuals.

§ Patients 12 and 13 were newly diagnosed, active, untreated SLE. Plasmas 12 (pretreatment) and 11 (6 mo posttreatment) are from the same patient. All other plasmas are from different patients.

performed. In vivo,  $^{32}\text{P}$ -labeled HEP-2 (human) cell DNA, mixed with the blood and purified in exactly the same manner as the plasma DNA, served as a density marker ( $\rho = 1.699$ ). As shown in Fig. 3, the buoyant-density profile of either the normal or SLE individuals could not be distinguished from the density of the marker. This may be taken as an indication that most of the circulating plasma DNA is not of exogenous origin (see Discussion).

## DISCUSSION

The purpose of this study was the quantitation and characterization of circulating DNA in the blood of normal or SLE individuals. Our results indicate that DNA is normally present in plasma and that in some clinically active SLE patients, this amount is considerably higher (Table I). Furthermore, characterization of this DNA in terms of buoyant density did not suggest an exogenous origin.

Among the various techniques of in vitro labeling of DNA, nick translation was chosen because of its extreme sensitivity for the detection of DNA. As already mentioned, high specific activities were obtained under our conditions ( $2 \times 10^7$  cpm/ $\mu\text{g}$  when 0.1  $\mu\text{g}$  of DNA was used as substrate) and these values

were apparently the same, regardless of the source of DNA being labeled ( $\lambda$  phage,  $\phi\text{X174}$  RFII, SV40 form I, or calf thymus DNA). Because of the reproducibility of the reference curve (Fig. 1) when DNA preparations of widely different origins are employed, this technique was considered appropriate for the quantitation of plasma DNA, provided a reference curve is constructed simultaneously and the reaction conditions remain strictly identical.

Our data regarding the quantitation of DNA in normal plasma are at variance with those of Steinman (3). According to this author, DNA was undetectable in normal plasma by using four different methods. The most sensitive of these (counterimmunoelectrophoresis) could detect 50 ng/ml of native DNA or 100 ng/ml of ssDNA, while our finding for normal plasma DNA is over 200 ng/ml. In agreement with his results, however, considerably higher amounts were found in the corresponding serum, possibly because of lysis of leukocytes during blood clotting (2, 3).

In patients with chronic inflammatory diseases (4–11 in Table I), the plasma DNA concentration was similar to that of normals. These patients had been selected because they had positive antinuclear antibody tests and a low serum dsDNA binding activity as determined by the Farr assay, using a DNA-antigen

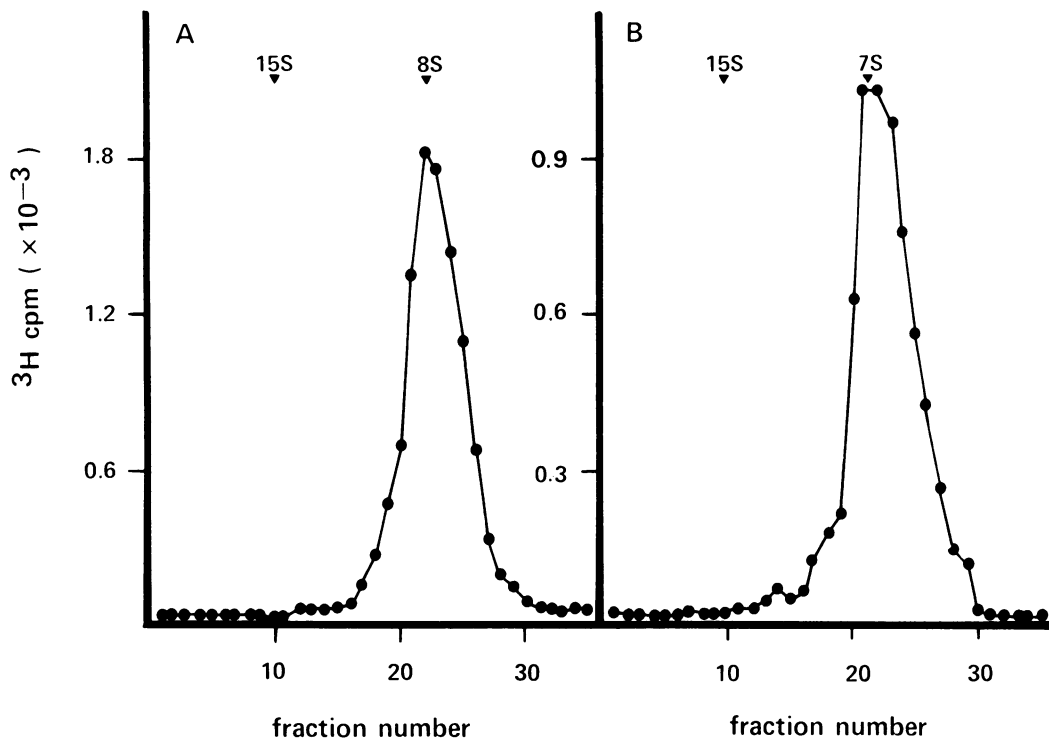


FIGURE 2 Neutral sucrose gradient sedimentation analysis of plasma DNA. This DNA, after purification and  $^3\text{H}$ -nick translation labeling, was mixed with labeled SV40 DNA linearized by EcoRI treatment. The latter DNA served as a 15 S marker, and the mixture was loaded onto linear 5–20% sucrose gradients. After centrifugation for 150 min at 60,000 rpm and 20°C in the SB 405 rotor of a IEC B-60 ultracentrifuge, fractions were collected from the bottom of every tube and acid-precipitable radioactivity was determined on each. Sedimentation is from right to left. (A) Plasma DNA from patient 12 (untreated active SLE). (B) Plasma DNA from normal 3.

of a molecular weight comparable to that of the plasma DNA (12). Furthermore, their disease had been therapeutically stabilized with gold salts for the rheumatoid patients, with antimalarial drugs and/or prednisone for the SLE patients, and with prednisone and azathioprine for the dermatomyositic patient. Thus, chronic administration of these drugs did not increase the amount of circulating plasma DNA.

Only two newly diagnosed, active and untreated SLE patients (12 and 13 in Table I) have been studied and both had a much higher concentration of plasma DNA. When studied, both had decreased C3 levels and elevated serum dsDNA binding activity (12). Neither had central nervous system involvement, features suggestive of polyarteritis (19), or any of the conditions reported to be associated with circulating plasma DNA (20–22). One patient was studied sequentially. At the onset, the plasma DNA was 4,024 ng/ml and the serum dsDNA binding activity >90%. When stabilized 6 mo later, the values were 352 ng/ml and <20%, respectively.

The second part of our work deals with the characterization of the circulating plasma DNA. One of the

intrinsic properties of a given DNA molecule is the G + C content. This may yield some information about its origin and can be determined from the molecule's buoyant density in CsCl (23). The buoyant density of a nucleic acid molecule in a neutral CsCl solution depends mainly on three factors: the G + C content, the secondary structure, and the nature of the nucleotides present. The third factor would be excluded from consideration in this case, since the existence of abnormal bases would be unlikely and because of the RNAase treatment of the preparation before the labeling process, polyribonucleotides should not be present. Therefore, a further advantage of the nick translation labeling is the fact that the resulting DNA molecules are mostly double stranded, any existing gaps having been repaired by the enzyme (17). This would permit a direct calculation of the G + C content, based on the buoyant density. Only single-stranded "tails" with a 3' end projecting cannot be "filled in" by this enzyme (or by any other known pro- or eucaryotic DNA polymerase). Given that such structures, if present, are not prominent enough to alter substantially the behavior of the molecule on

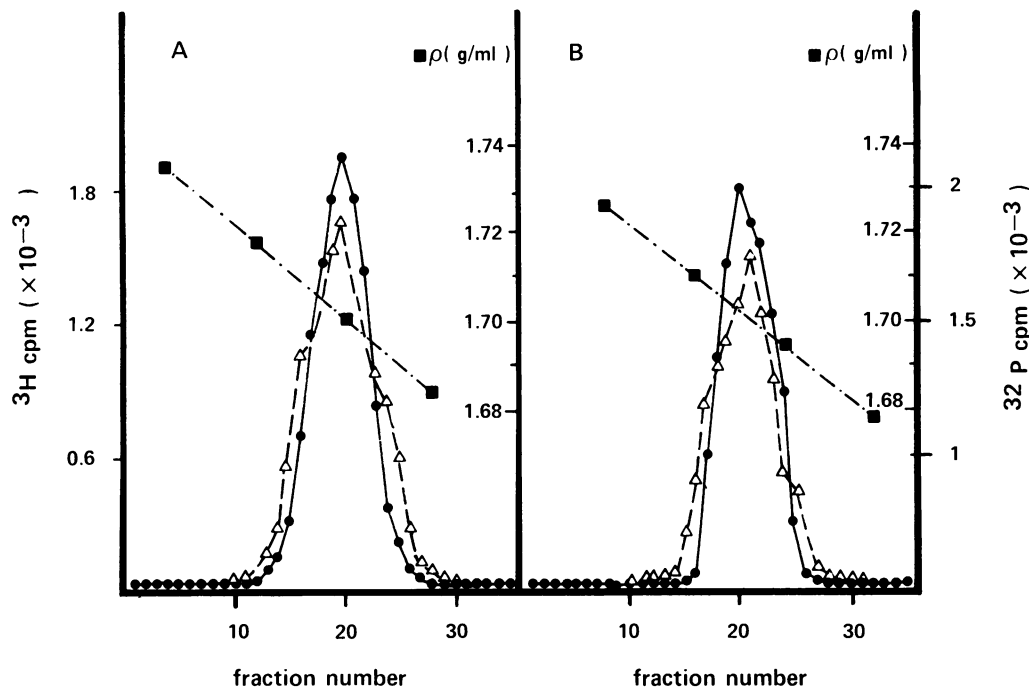


FIGURE 3 Neutral CsCl equilibrium centrifugation analysis of plasma DNA. This DNA, after purification and  $^3\text{H}$  labeling by nick translation was mixed with *in vivo*  $^{32}\text{P}$ -labeled HEP-2 (human) cell DNA which had been subjected to the same extraction scheme as the plasma DNA (see Methods). After the addition of solid CsCl to  $\rho$  1.699, the preparation was centrifuged to equilibrium. Fractions were collected from the bottom of each tube and acid-precipitable  $^3\text{H}$  ( $\Delta$ ) and  $^{32}\text{P}$  ( $\bullet$ ) radioactivity was measured.  $\blacksquare$ , actual density of the fractions as determined by refractive index. (A) plasma DNA from normal 2. (B) plasma DNA from patient 12 (untreated, active SLE).

BND-cellulose, one may assume that the reaction product is mostly dsDNA. In our work, the nick-translated plasma DNA presented a prominent peak at a  $\rho$  of 1.699 g/ml, the predominant density of human DNA. This would correspond to a G + C content of 50% (15). Because the molecular weight of the DNA extracted was much smaller than that of mammalian cell DNA, causing a considerable broadening of the peak, an extraneous DNA with a density similar to human DNA would not have been detected by this method. Similar conclusions regarding the provenance of SLE plasma DNA have been reported by Steinman (24), using equilibrium centrifugation and hybridization kinetics.

The size of the plasma DNA, as estimated by sucrose gradient sedimentation analysis, presents a rather uniform profile. Again, the fact that the DNA is double stranded permits an estimation of the molecular weight based on the sedimentation coefficient (18). Our data suggesting an average size of 500 base pairs compare well with those obtained recently with a similar method (4) but give a smaller figure than the data obtained by electron microscopy (5).

As far as the actual source of this DNA is concerned, there is suggestive evidence that it originates from

lysis of circulating nucleated cells (2, 3, 20) or of endothelial cells (19). There are also several reports on DNA excreted *in vitro* by lymphocytes especially during mitogen stimulation (25). Possible intracellular origins of the plasma DNA are nuclear, cytoplasmic, or membrane associated (26). Once liberated, DNA molecules of various molecular weights are present in the blood (5). Most of these (the larger ones?) are cleared rapidly from the circulation to the reticulo-endothelial system (27, 28) and/or cleaved by blood DNAases (16) to smaller molecular weight pieces which can be purified and labeled by our method. DNA is normally associated in the circulation with proteins (29, 30) that could possibly protect it from DNAase degradation (31, 32) or modify its clearance. These proteins appear to be either phase reactants, as suggested by the increased binding activity against ssDNA commonly observed in a variety of inflammatory states (12) or, under special circumstances, specific antibodies (32). The uniformity in DNA size found here suggests a degree of protection from blood DNAases. In any case, the presence of circulating DNA in normal individuals would appear to be the result of a physiological process, the role of which (if any) is not clear.

Further elucidation of the properties of circulating DNA and the binding activity of the corresponding plasma could provide some information on their physiological and pathogenic roles. As far as the characterization of this DNA is concerned, other *in vitro* labeling techniques could be used in conjunction with nick translation. Indeed, one of the disadvantages of this method is the fact that the relative length or distribution of the single-stranded regions cannot be studied. Nevertheless, the relative proportion of those, with an available 3'OH primer terminus, can be estimated by use of the Klenow subfraction of the DNA polymerase I (33). This enzyme has the 5' to 3' DNA polymerase activity but not the 5' to 3' exonucleolytic one and is thus capable of performing a "gap filling" reaction exclusively. Other techniques that may prove useful involve the enzymes terminal transferase (34) or polynucleotide kinase (35). Either of these may yield DNA molecules of unaltered properties although of much lower specific activities.

In conclusion, DNA circulates normally in human plasma. This DNA is of low molecular weight, possibly DNAase resistant to a certain extent and appears to be predominantly, if not essentially, of endogenous origin. In SLE, it behaves as an acute phase reactant. Whether this material has a role in the abnormal immune regulation mostly found in the active phases of SLE and whether it is primarily or secondarily related to the tissue injury, are unanswered questions.

#### ACKNOWLEDGMENTS

We are grateful to Mr. Jean-Claude Demers for his technical assistance and to Mrs. Ginette Hébert and Diane Maréchal who typed the manuscript.

This investigation was supported by the Societé d'Arthrite, the Conseil de Recherches Médicales du Canada, and le Centre de la Recherche Médicale de l'Université de Sherbrooke.

#### REFERENCES

1. Tan, E. M., P. H. Schur, R. H. 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.
2. Davis, G. L., and J. S. Davis. 1973. Detection of circulating DNA by counter-immunoelectrophoresis. *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. Bruneau, C., and J. Benveniste. 1979. Circulating DNA: anti-DNA complexes in systemic lupus erythematosus. Detection and characterization by ultra-centrifugation. *J. Clin. Invest.* **64**: 191-198.
5. Dennin, R. H. 1979. DNA of free and complexed origin in human plasma: concentration and length distribution. *Klin. Wochenschr.* **57**: 451-456.
6. Izui, S., P. H. Lambert, and P. A. Miescher. 1977. Failure to detect circulating DNA:anti-DNA complexes by four radioimmunological methods in patients with systemic lupus erythematosus. *Clin. Exp. Immunol.* **30**: 384-392.
7. Mellors, R. C., and C. X. Huang. 1966. Immunopathology of NZB/BL mice. V. Virus-like (filterable) agent separable from lymphoma cells and identifiable by electron microscopy. *J. Exp. Med.* **124**: 1031-1039.
8. Lewis, R. M., J. André-Schwartz, G. S. Harris, M. S. Hirsch, P. H. Black, and R. S. Schwartz. 1973. Canine systemic lupus erythematosus. Transmission of serologic abnormalities by cell-free filtrates. *J. Clin. Invest.* **52**: 1893-1907.
9. Kelly, R. B., N. R. Cozzarelli, M. P. Deutscher, I. R. Lehman, and A. Kornberg. 1970. Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single-strand break. *J. Biol. Chem.* **245**: 39-45.
10. Raptis, L., L. Delbecchi, and P. Bourgaux. 1978. Further studies on the replication of adenovirus type 2 DNA. *Can. J. Biochem.* **56**: 857-865.
11. Rigby, P. W. J., M. Dieckman, C. Rhodes, and P. Berg. 1977. Labeling of deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**: 237-251.
12. Couture, F., A. Beaulieu, L. Raptis, and H. A. Ménard. 1979. Operationally defined single and double stranded DNA in the Farr assay: diagnostic value. *Eur. J. Clin. Invest.* **9**: 243-251.
13. Bourgaux, P., and D. Bourgaux-Ramoisy. 1972. Unwinding of replicating polyomavirus DNA. *J. Mol. Biol.* **70**: 399-413.
14. Bourgaux, P. 1974. Comparison of genome replication mechanisms of oncogenic DNA viruses. In *Viruses, Evolution and Cancer*. E. Kurstak and K. Maramorosch, editors. Academic Press Inc., New York. 119-149.
15. Szybalski, W. 1968. Use of cesium sulphate for equilibrium density gradient centrifugation. In *Methods in Enzymology*. Grossman and Moldave, editors. Academic Press Inc., New York, **12B**: 330-360.
16. Herriot, R. M., J. H. Connolly, and S. Gupta. 1961. Blood nucleases and infectious nucleic acids. *Nature (Lond.)* **189**: 817-820.
17. Kornberg, A. 1974. DNA Synthesis. W. H. Freeman & Company Publishers, San Francisco. 67-121.
18. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**: 373-390.
19. Steinman, C. R. 1979. Circulating DNA in systemic lupus erythematosus. Association with central nervous system involvement and systemic vasculitides. *Am. J. Med.* **67**: 429-435.
20. Steinman, C. R., and A. Ackad. 1977. Appearance of circulating DNA during hemodialysis. *Am. J. Med.* **62**: 693-697.
21. Sipes, J. N., P. M. Surrat, C. D. Teates, F. A. Barada, J. S. Davis, and C. J. Tegtmeier. 1978. A prospective study of plasma DNA in the diagnosis of pulmonary embolism. *Am. Rev. Respir. Dis.* **118**: 475-478.
22. Hughes, G. R. V., S. A. Cohen, R. W. Lightfoot, J. I. Meltzer, and C. L. Christian. 1971. The release of DNA into serum and synovial fluid. *Arthritis Rheum.* **14**: 259-266.
23. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of DNA from its buoyant density in cesium chloride. *J. Mol. Biol.* **4**: 430-443.
24. Steinman, C. R. 1979. Characterization of circulating DNA in SLE: human or non-human origin. 43rd Annual Meeting of the American Rheumatism Association, June 1979, Denver, Colorado. Abstr. no. 164.
25. Distelhorst, C. W., K. Cramer, and J. C. Rogers. 1978. Selective release of excreted DNA sequences from phytohemagglutinin-stimulated human peripheral blood



- lymphocytes: effects of trypsin and divalent cations. *J. Clin. Invest.* **61**: 1204–1217.
26. Charlson, A. J., and B. L. Reid. 1978. A review of cytoplasmic and cell surface DNA with consideration of their origin. *J. Carbohydr. Nucleosides Nucleotides.* **5**: 381–442.
  27. Tsumita, T., and M. Iwabaga. 1963. Fate of injected deoxyribonucleic acid in mice. *Nature (Lond.)*. **198**: 1088–1089.
  28. Chused, T. M., A. D. Steinberg, and N. Talal. 1973. The clearance and localization of nucleic acids by New Zealand and normal mice. *Clin. Exp. Immunol.* **12**: 465–476.
  29. Brehm, S. P., S. P. Hoch, and J. A. Hoch. 1975. DNA binding proteins in human serum. *Biochem. Biophys. Res. Commun.* **63**: 24–31.
  30. Rubin, R. L., and R. I. Carr. 1979. Anti-DNA activity of IgG F(ab')<sub>2</sub> from normal human serum. *J. Immunol.* **122**: 1604–1607.
  31. Hadjiyannaki, K., and P. J. Lachmann. 1972. The relation of deoxyribonuclease inhibitor levels to the occurrence of antinuclear antibodies in NZB/NZW mice. *Clin. Exp. Immunol.* **11**: 291–295.
  32. Liebling, M. R., and E. V. Barnett. 1977. Substrate competition between DNAase I and anti-DNA antibody. *Clin. Immunol. Immunopathol.* **8**: 80–89.
  33. Klenow, H., and I. Henningsen. 1970. Selective elimination of the exonuclease activity of the DNA polymerase from *E. coli* B by limited proteolysis. *Proc. Natl. Acad. Sci. U. S. A.* **65**: 168–175.
  34. Chang, L. M. S., and F. Bollum. 1971. Deoxynucleotide-polymerizing enzymes of calf thymus gland. V. Homogeneous terminal deoxynucleotidyl transferase. *J. Biol. Chem.* **246**: 909–916.
  35. Bernardi, A. 1974. A fast method of analysis of the 5' terminal nucleotides of deoxyribooligonucleotides. *Anal. Biochem.* **59**: 501–507.