Measurement of plasma DNA by a physicochemical method: relevance in SLE

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SUMMARY Plasma DNA has been measured by a new physicochemical approach based on fluorimetric analysis of phenol extracted nucleic acids electrophoresed on polyacrylamide gels. This method is specific for native double-stranded DNA of molecular weight greater than 5×10^6 . The use of plasma rigorously freed of leucocytes is essential to prevent falsely high values. When such precautions are taken levels seldom exceed 50 ng/ml in normal individuals. Plasma DNA was assayed in 107 samples from 47 patients with both active and inactive systemic lupus erythematosus. In direct contrast to several previous reports no significant increase of plasma DNA was found.

Circulating native, double-stranded deoxyribonucleic acid (dsDNA) has been reported in a wide variety of unrelated conditions such as systemic lupus erythematosus (SLE), rheumatoid arthritis, malignancy, liver disease, and in infective endocarditis, pulmonary embolism, and after surgery.¹⁻⁷ In addition raised levels have also been reported after cardiac surgery involving periods of extracorporeal circulation,⁷ after haemodialysis,⁸ in patients on high-dose corticosteroid therapy,⁹ and in response to the injection of bacterial lipopolysaccharides.¹⁰

In some of the above conditions it is claimed that the levels of circulating DNA found are pathologically raised. However, there is a wide divergence of opinion as to what constitutes the normal level of circulating DNA, levels ranging from less than 50 ng/ml to as high as 13.8 μ g/ml having been reported.^{1 2 4-7 11-14} Confirmation of raised levels of circulating DNA in pathological states therefore requires resolution of this problem of the normal range. This wide variation in reported levels is probably partly due to lack of specificity in the assay method and also depends on whether the assay is performed on plasma or serum.

The relevance of circulating DNA has been a subject of much debate, particularly with regard to its possible role in immune complex formation in SLE. It has been suggested that the DNA in the majority of cases is of endogenous origin from tissue injury

(traumatic or inflammatory) and therefore represents a nonspecific phenomenon.^{3 9} An alternative view is that in at least some cases the DNA may be of exogenous (for example, viral) origin.^{15 16}

Methods for the detection of circulating DNA include: (1) the diphenylamine reaction¹⁷; (2) immune precipitation by double diffusion in agarose¹⁸; (3) complement fixation²; (4) fluorimetry using ethidium bromide¹¹; (5) counterimmunoelectrophoresis (CIE)⁷; (6) RNA-DNA hybridisation¹⁴; and (7) radioimmunoassay (RIA).^{12 15}

Many of these assays are indirect measurements of DNA and lack either specificity or sensitivity or both. Furthermore, nonspecific protein binding¹² or the presence of interfering substances such as serum chromogens has been found to cause falsely high values, as, for example, in the diphenylamine method. The RIA and modified CIE techniques are the most sensitive methods, the lower limit of detection being about 50 ng/ml of circulating dsDNA.

We have developed a physicochemical assay for circulating dsDNA which is specific, reproducible, and sensitive down to 50 ng/ml.

By this method a normal range for dsDNA was established and levels were studied with particular emphasis on SLE, covering a wide spectrum of presentations and monitored through periods of exacerbation and remission. Circulating DNA levels were also investigated after exposure to ultraviolet light, since sunlight is a known precipitating factor in SLE. The possibility that entry of DNA into plasma is an episodic phenomenon

Accepted for publication 16 January 1981

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secondary to physiological factors such as diet, exercise, or emotion was studied by frequent measurements of plasma DNA over a 24-hour period.

Materials and methods

CHEMICALS

Acrylamide and N, N-methylene bisacrylamide (British Drug Houses, Poole, UK) were recrystallised as described by Loening.¹⁹ Ribonuclease 3'-pyrimidino-oligonucleotidohydrolase (RNase), EC No. 2.7.7.16, and deoxyribonuclease 5'-oligonucleotidohydrolase (DNase), EC No. 3.1.4.5, were chromatographically purified preparations (Miles Laboratories, Indiana). Ethidium bromide was purchased from Sigma Chemical Company. All other reagents were of Analar or equivalent quality.

DNA EXTRACTION

Blood was collected into 10 ml heparinised Vacutainer tubes (Becton-Dickinson, Rutherford, New Jersey) and centrifuged within 4 hours of collection at 3000 rpm in a Sorvall RC-3 centrifuge at 15°C. Plasma was removed carefully with a clear 1 ml (equivalent to about 7 mm) being left above the cell layer. The plasma was then again centrifuged at 3000 rpm for 10 minutes and the top 3 ml of plasma again pipetted off, care being taken not to disturb the remaining plasma (about 1 ml) and any pelleted material. The plasma was stored at this stage by freezing at -15°C. DNA was extracted from 1.5 ml plasma by mixing with an equal volume of phenol saturated with water. Water-saturated chloroform, 1.5 ml, containing 1% (v/v) isoamylalcohol was then added. After vortex mixing for 40 seconds the two phases were separated by centrifugation for 15 minutes at 6000 rpm and 4°C in the SS-34 rotor of a Sorvall RC-2B centrifuge. After a further extraction with chloroform, nucleic acids in 1.0 ml of the aqueous phase were precipitated by the addition of 2 ml of absolute ethanol, and after standing at -15°C overnight, centrifuged for 30 minutes at 10 000 rpm and 0°C in the SS-34 Sorvall rotor. The tubes were inverted and left to drain until almost dry. The precipitated nucleic acids were dissolved in 120 µl of electrophoresis buffer containing 5% v/v glycerol (layering buffer).

PREPARATION OF DNA STANDARDS

DNA was prepared from rat hepatoma cells as described previously.²⁰ Three or 4 standard concentrations were prepared by serial dilution of this DNA in electrophoresis buffer containing 5% glycerol so as to contain between 12.5 and 250 ng of DNA in the 100 µl aliquots to be applied to the gel.

POLYACRYLAMIDE GEL ELECTROPHORESIS

This was performed in Tris-phosphate EDTA buffer essentially as described previously.²¹ An electrophoresis apparatus was designed to hold 20 cylindrical Perspex tubes (4 × 84 mm) into which the gels were cast. A concentration of 3.6% acrylamide was prepared by dilution from a stock 12% (w/v) acrylamide solution containing 0.3% (w/v) N, N'-methylene bisacrylamide (2.5% cross-linked). The gels were stored at 4°C for 18 hours before use. After the gels were cleared of any residual polymerisation products by passing current for 30 minutes at 100 volts, 100 µl of the test samples and the standards were layered on and electrophoresed for 2 hours at 100 volts. The gels were extruded into electrophoresis buffer containing 0.5 µg/ml ethidium bromide and left overnight to equilibrate with the stain.

QUANTITATION OF FLUORESCENCE

AND CALCULATION OF DNA CONCENTRATION

Fluorescent bands were quantitated with a Vitatron

Fig. 1 (a) A typical fluorimetric scan of plasma DNA electrophoresed in a 3.6% polyacrylamide gel; direction of electrophoresis is from left to right. (b) Calibration curve constructed from 4 different concentrations of a standard rat hepatoma DNA preparation electrophoresed in polyacrylamide gels and scanned as illustrated in (a) quantitating the band with an electronic integrator.

TLD 100 fluorescent scanner and integrator using UVB excitation and UV4 emission filters. The aperture was a 0.25 mm spot. A calibration curve was drawn using the 3 standard DNA concentrations and the quantity of DNA in each unknown read off this linear plot. Plasma DNA could be quantitated in this way at levels of 10 ng/ml or greater. A typical calibration curve, and an electropherogram of a plasma DNA sample are illustrated in Fig. 1.

Results

SPECIFICITY OF THE ASSAY FOR DS DNA

The staining and mobility characteristics of various preparations of DNA electrophoresed in polyacrylamide gels are illustrated in Fig. 2 (upper). The standard rat hepatoma DNA preparation shows a single well-defined fluorescent band on the gel. An extract from a human plasma sample shows a

Fig. 2 (Upper) PAGE for 2 h at 100 volts of 100 ng standard rat hepatoma DNA (1) or of nucleic acid preparations from either plasma (2, 4–10) or serum (3) from 1 individual, the serum and plasma samples being taken at the same time. (2) Untreated plasma extract. (4) Plasma treated with 30 µg/ml DNase for 60 min at 37° prior to extraction. (5) Plasma treated with 10 µg/ml RNase for 30 min at 37° prior to extraction. (6) Plasma extract heated for 5 min at 100° followed by rapid cooling. (7–10) Plasma extract electrophoresed on 2.1, 2.7, 3.3, and 4.0% polyacrylamide gels respectively. (Lower) Gel concentration range analysis of ○—○ plasma nucleic acid extract, and ●—● rat hepatoma DNA. Samples were run at the same time on 2.1, 2.7, 3.3, and 4.0% gels (plasma samples illustrated in upper panel 7–10). The distance migrated was determined from scans of the gels, correcting for differential stretching, hence the migration values do not correspond exactly with the photographed gels in upper panel. Quadratic functions were fitted to the 2 data sets by the method of least squares.

fluorescent band migrating with similar mobility to the standard, and except for minor quantities of fluorescence at the top of the gel no other bands are visible. The disappearance of this band after DNase treatment, and its persistence after RNase treatment confirm that the band seen in plasma extracts is DNA. After heat denaturation fluorescent material is demonstrable only as a diffuse smear in the top few millimetres of the gel. The slope of a plot of electrophoretic mobility versus gel concentration is characteristic for different classes of nucleic acid structure, namely, single stranded, double-stranded linear, and double-stranded circular.²¹ Plots of this type are illustrated in Fig. 2 (lower) for the plasma DNA and the standard DNA. Quadratic equations were fitted to the 2 data sets and standard deviations on the quadratic coefficients were determined. Statistical evaluation of the 2 sets by the *t* test showed that they arise from the same population ($p > 95\%$). This confirms that the structure and conformation of the fluorescing material in the band is double-stranded linear DNA of molecular weight greater than about 5×10^6 .

NATURE AND COLLECTION OF BLOOD SAMPLES

The effect on plasma DNA concentrations of using needles of different gauges was tested by drawing blood into heparinised syringes with either a 19 or a 25 gauge needle, and into a heparinised Vacutainer tube with a 21 gauge needle. The sample taken through the 25 gauge needle was traumatised by forcibly expressing the blood through the needle several times to the point of red cell lysis. The sample analysed in duplicate by the Vacutainer method gave a mean DNA level of 56 ng/ml ($SD \pm 3$ ng/ml) which was not significantly different ($p > 0.1$) from the level produced when using the heparinised syringe with a 19 gauge needle (63 ± 4 ng/ml). However, DNA levels were significantly higher in the traumatised sample (126 ± 33 ng/ml), $p < 0.02$, implying that damage to leucocytes can release DNA into the plasma. Two centrifugation steps were found to be necessary to remove all cells from the plasma sample. Cytocentrifugation (Cytospin, Shandon Elliot) and the use of May-Grünwald and Giemsa stains confirmed that no cellular material could be detected in plasma subjected to the standard 2 cycles of centrifugation, whereas leucocytes were still visible after only 1 centrifugation. The effect of residual cells on DNA levels were shown by measuring DNA in the top 2 ml of plasma after a single cycle of centrifugation and comparing with DNA levels in the 2 ml below this, with 1 ml of plasma left undisturbed above the buffy layer. In 7 plasma

samples analysed in this way the top layer of plasma gave a mean value of 114 ± 75 ng DNA/ml, whereas the lower 2 ml gave a value of 718 ± 366 ng DNA/ml ($p < 0.001$).

STABILITY OF DNA IN STORED SAMPLES

In a patient selected with a relatively high concentration of plasma DNA the levels were measured in freshly separated plasma, and over a period of 24 hours, in plasma samples stored at 37°C, 20°C, 4°C, and -20°C (Table 1). Similarly samples stored at -20°C were analysed at weekly intervals over a period of 4 weeks. No significant change in DNA concentration was found under any of the storage conditions described.

RECOVERY AND REPRODUCIBILITY

DNA was added in 25, 50, 250, and 500 ng quantities per ml to aliquots of plasma having levels of endogenous DNA less than 10 ng/ml. The samples were then extracted and analysed in duplicate. Recoveries of 56%, 89%, 84%, and 97% respectively were obtained. Serum treated in similar manner produced no clear bands on the gels. Only a slight diffuse fluorescence was noted in the top few centimetres of the gels (compare with Fig. 2 (upper), gel 3). In 11 subjects DNA levels were measured in both plasma and serum drawn at the same time. In the 5 subjects having detectable levels of DNA in plasma extracts the serum extracts showed a similar diffuse fluorescence to that described above for DNA added to serum. This diffuse fluorescence precluded accurate quantitation. Because of this marked difference between DNA levels in heparinised plasma and serum, DNA was assayed in plasma with both heparin and EDTA as anticoagulants and the levels compared with those in serum. The plasma levels were the same irrespective of whether heparin or EDTA was used. All subsequent determinations were therefore routinely performed on plasma.

The within-group variability of plasma DNA levels performed in duplicate on 42 samples showed a standard deviation of 13.8%. This degree of variability was maintained down to levels of about 40 ng/ml plasma.

Table 1 Effect on plasma DNA levels of incubation of plasma at various temperatures. Figures give plasma DNA in ng/ml (mean of duplicate measurement ± 1 SD)

	1	3	6	24
Plasma incubated at 37°C	93 $\pm$ 4	96 $\pm$ 8	123 $\pm$ 21	111 $\pm$ 12
" " 20°	102 $\pm$ 2		57 $\pm$ 21	96 $\pm$ 8
" " 4°				90 $\pm$ 25
" " -20°				86 $\pm$ 6

NORMAL RANGE OF PLASMA DNA

Plasma DNA was measured in 58 normal subjects (Fig. 3) comprising 29 males and 29 females aged 15 to 68 years (mean 34 years). In 47 subjects (81%) DNA levels were <10 ng/ml. In the remaining 11 subjects levels ranged from 10 to 54 ng/ml. There were no significant age or sex differences.

PHYSIOLOGICAL VARIATION IN DNA LEVELS

The effect on circulating DNA levels of exposure to the sunburn spectrum of ultraviolet light was tested in two normal Caucasian volunteers as follows: the minimal erythema dose (MED) was established for each subject, whereupon a whole-body exposure of 4 MED was administered to each subject by means of a Theraktin sunlamp fitted with a TL/1240 W fluorescent tube with a spectrum peaking at 306 nm.

Blood was drawn before and 6, 24, 48, and 72 hours after exposure and assayed for DNA. No DNA was detected in any of the samples.

Five blood samples were drawn from each of 4 male and female subjects aged 15–68 years (mean 37 years) over a 24-hour period and assayed for DNA. Plasma DNA levels remained undetectable (less than 10 ng/ml) in all subjects throughout the 24 hours.

PLASMA DNA IN PATHOLOGICAL CONDITIONS

Plasma DNA levels were measured in a series of patients with SLE. Forty-seven patients, all of whom fulfilled the preliminary American Rheumatism

Association criteria for the classification of SLE,²² had 107 assays performed over a period of 8 months (Fig. 3). None of these patients was on high-dose corticosteroid therapy at the time measurements were made. The majority had levels below 10 ng/ml, and in the few cases where higher levels were recorded no association could be established with respect to the type of organ involvement, nor were there significant differences in levels between clinically active and quiescent phases of the disease (Table 2). Since high levels of circulating DNA have been reported after haemodialysis,⁸ plasma DNA levels were also measured in 4 patients with chronic renal failure (not associated with SLE) immediately on termination of 6 hours of haemodialysis. Two were found to have considerably elevated levels (Fig. 3).

Table 2 Breakdown of plasma DNA levels in SLE with respect to the predominant organ or system involved at the time of measurement

Organ or system	No. of samples with levels <10 ng/ml 10 ng/ml Total or more		
<i>Clinically active</i>			
Skin	7	1	8
Musculoskeletal system	18	2	20
Kidney	5	0	5
Nervous system	3	0	3
Cardiovascular system	0	1	1
Nonspecific (constitutional symptoms)	5	1	6
<i>Clinically inactive</i>	53	11	64

Fig. 3 Distribution of plasma DNA levels in active and inactive SLE (differentiation made on clinical criteria) in patients immediately on termination of 6 hours of haemodialysis, and in controls, the latter consisting of normal blood donors not matched for age or sex.

Discussion

The specificity of this physicochemical assay for ds DNA is determined by 3 features. Firstly, the extraction procedure quantitatively removes proteins, lipids, and low-molecular weight compounds soluble in 70% alcohol. Polysaccharides are the only components which regularly contaminate nucleic acids extracted in this way. Secondly, only polynucleotides form well-defined bands which fluoresce significantly when stained under these conditions with ethidium bromide. Thirdly, nucleic acids have well-defined electrophoretic mobility characteristics in polyacrylamide gels, which are dependent on size, strandedness (i.e., double or single), conformation (i.e., circularity or linearity of the molecule), and nature of the ribose sugar (i.e., DNA or RNA).^{21 23} The combination of the selective extraction procedures and the demonstration of a single band on gel electrophoresis with characteristic dye binding properties, nuclease sensitivity, and electrophoretic mobility at different acrylamide concentrations confirms unequivocally that we are quantitating native ds DNA.

A useful feature of the gel electrophoresis method is that the electrophoretic mobility of ds DNA is virtually independent of molecular weight above a value of about 5×10^6 daltons.²¹ This property is also shown by ds RNA, though its electrophoretic mobility at higher molecular weight values is lower than that of DNA²³ and therefore readily distinguishable by comparison with DNA markers. Single-stranded polynucleotides may electrophorese either faster or slower than high-molecular weight dsDNA depending both on their size and on acrylamide concentration, and can be readily distinguished by this latter property and by the less well defined bands which they form. In these studies the variable presence of small quantities of ill-defined fluorescence near the top of the gel represented the only evidence for single-stranded or extensively denatured species detectable by this method in plasma.

The diffuse fluorescence seen in gel analysis of extracts of serum to which DNA had been added and the lack of any discernible dsDNA band is probably due to nuclease activity from the DNase known to be released from platelets during clotting.²⁴

In plasma DNA appears to be quite stable. Even at temperatures as high as 37°C there is no significant change in DNA concentration over 24 hours.

The recovery studies have shown that dsDNA in plasma can be measured accurately at 50 ng/ml and above; below 50 ng/ml recovery falls appreciably.

Levels of plasma DNA found in normal subjects were considerably lower than those of some reported series,^{4 5 11} but similar to those reported by others.

^{1 6 7 13} It is most unlikely that this can be explained by differences in the selection of normal subjects and the reasons are presumably methodological. The importance of ensuring that plasma is completely free of white blood cells needs emphasis. Since 1 human leucocyte contains about 6 pg of DNA, it would require a contamination level of only 10^4 cells/ml plasma to give a DNA level of 60 ng/ml. It is therefore necessary to remove more than 99.9% leucocytes from the plasma. This is especially relevant when an extraction procedure which disrupts membranes is used prior to assay.

In our small series of posthaemodialysis patients we found raised levels of plasma dsDNA as previously reported.⁸ However, in contrast to other reports^{1 2 4 5} raised plasma DNA levels were not observed in our patients with SLE, and in no instance was there any demonstrable relationship between plasma dsDNA levels and the type of organ involved. Neither did plasma dsDNA levels vary with disease activity. In some studies reporting raised DNA levels in SLE (e.g., Tan *et al.*¹) patients were included who were receiving high-dose corticosteroid therapy, which is itself capable of increasing the level of DNA in blood.⁹ In other studies the predominant form of DNA measured was ssDNA,⁴ and these are not therefore, strictly comparable with our findings. Unless increased entry of DNA into the circulation is a feature of SLE, it would in fact be logical to expect that the complexing of DNA with anti-DNA antibody would result in more rapid clearance of DNA from the circulation and therefore low circulating DNA levels.

We thank the Isaac Albow Fund and the South African Medical Research Council for financial support.

References

- 1 Tan E M, Schur P H, Carr R I, Kunkel H G. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J Clin Invest* 1966; **45**: 1732-40.
- 2 Barnett E V. Detection of nuclear antigens (DNA) in normal and pathologic human fluids by quantitative complement fixation. *Arthritis Rheum* 1968; **11**: 407-17.
- 3 Harbeck R J, Hoffman A A, Carr R I. Studies on the nature of circulating DNA in systemic lupus erythematosus (SLE). *J Rheumatol* 1975; **2**: 194-203.
- 4 Koffler D, Agnello V, Winchester R, Kunkel H G. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. *J Clin Invest* 1973; **52**: 198-204.
- 5 Cox R A, Gokcen M. Circulating DNA levels in man. *Biochem Med* 1976; **15**: 126-37.
- 6 Leon S A, Ehrlich G E, Shapiro B, Labbat A. Free DNA in the serum of rheumatoid arthritis patients. *J Rheumatol* 1977; **4**: 139-42.
- 7 Davis G L, Davis J S. Detection of circulating DNA by counterimmunoelectrophoresis (CIE). *Arthritis Rheum* 1973; **16**: 52-8.

- ⁸ Steinman C R, Ackad A. Appearance of circulating DNA during haemodialysis. *Am J Med* 1977; **62**: 693-7.
- ⁹ Hughes G R V, Cohen S A, Lightfoot R W, Meltzer J I, Christian C L. The release of DNA into serum and synovial fluid. *Arthritis Rheum* 1971; **14**: 259-66.
- ¹⁰ Fournie G J, Lambert P H, Miescher P A. Release of DNA in circulating blood after induction of anti-DNA antibodies after injection of bacterial lipopolysaccharides. *J Exp Med* 1974; **140**: 1189-204.
- ¹¹ Kamm R C, Smith A G. Nucleic acid concentrations in normal human plasma. *Clin Chem* 1972; **18**: 519-22.
- ¹² Leon S A, Green A, Yaros M J. Radioimmunoassay for nanogram quantities of DNA. *J Immunol Methods* 1975; **9**: 157-64.
- ¹³ Steinman C R. Free DNA in serum and plasma from normal adults. *J Clin Invest* 1975; **56**: 512-5.
- ¹⁴ Steinman C R. Use of nucleic acid hybridization for specific detection of submicrogram quantities of DNA and its application to human plasma. *Clin Chem* 1975; **21**: 407-11.
- ¹⁵ Barnett E V, Torikai K, Mayor H D. Immunologic detection of single-stranded DNA in adeno-associated satellite virions. *J Immunol* 1970; **105**: 824-8.
- ¹⁶ Lewis R M, Tannenber W, Smith C, Schwartz R S. C-type viruses in systemic lupus erythematosus. *Nature* 1974; **252**: 178-9.
- ¹⁷ Giles K W, Myers A. An improved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* 1965; **206**: 93.
- ¹⁸ Tan E M, Kunkel H G. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. *J Immunol* 1966; **96**: 464-71.
- ¹⁹ Loening V E. The fractionation of high-molecular weight ribonucleic acid by polyacrylamide gel electrophoresis. *Biochem J* 1967; **102**: 251-7.
- ²⁰ Harley E H, White J S. The identification of the circular forms of SV40 DNA in whole infected cell preparations. *Virology* 1973; **52**: 395-407.
- ²¹ Harley E H, White J S, Rees K R. The identification of different structural classes of nucleic acids by electrophoresis in polyacrylamide gels of different concentration. *Biochim Biophys Acta* 1973; **299**: 253-63.
- ²² Cohen A S, Reynolds W E, Franklin E C, et al. Preliminary criteria for the classification of SLE. *Bull Rheum Dis* 1971; **21**: 643-8.
- ²³ Bozarth R F, Harley E H. The electrophoretic mobility of double-stranded RNA in polyacrylamide gels as a function of molecular weight. *Biochim Biophys Acta* 1976; **432**: 329-35.
- ²⁴ Connolly J H, Herriott R M, and Gupta S. Deoxyribonuclease in human blood and platelets. *Br J Exp Pathol* 1962; **43**: 392-401.