

Novel DNA-protein complex and a large DNA in SLE cryoprecipitates

M. RIEBER, CARMEN E. CONTRERAS,* MARY S. RIEBER & N. E. BIANCO*
IVIC, Centre of Microbiology and Cell Biology, Apartado 1827, Caracas 1010-A, Venezuela and
**WHO/PAHO, Center for Clinical Immunology, Institute of Pathology (UCV), Apartado 50647,*
Caracas 1051, Venezuela

(Accepted for publication 21 May 1986)

SUMMARY

Agarose gel electrophoresis of cryoprecipitates from systemic lupus erythematosus (SLE) patients revealed the presence of a slowly migrating DNase I-sensitive DNA species at the top of the gel. Upon deproteinization, electrophoretic migration was modified favouring the migration of a 17.5 kb DNA fragment. Mixing experiments adding human serum or plasma to a λ phage DNA digest revealed a DNA-protein interaction shown by an accumulation of high mol. wt polynucleotide at the top of the gels, and a slowed migration of the DNA bands. No comparable effect was observed when serum albumin was added to the λ DNA digest. Dot hybridization analysis showed preferential reactivity of the 17.5 kb DNA to human DNA, implying its human origin. Our data suggests that most of this high molecular weight DNA exists as a DNA-2 protein complex. Our mixing experiments also suggest the occurrence of an excess of free DNA antibodies. We propose that the DNA-protein association may play a role in the stabilization and immunogenicity of the nucleoprotein complex.

Keywords circulating DNA DNA-binding immune complexes SLE

INTRODUCTION

DNA-protein immune complexes are believed to play an important role in the pathogenesis of systemic lupus erythematosus (SLE) (Adu, Dobson & Williams, 1981; Emlen & Mannik, 1984). However, although the level of anti-DNA antibodies has been correlated with disease (Bruneau & Benveniste, 1979; Koffler *et al.*, 1971), the levels of these antibodies do not adequately explain the variations in clinical activity. Presumably, extracellular DNA of a particular size and molecular configuration must be available to form nucleoprotein or immune complexes, which in turn modulate the clearance and organ localization of the DNA (Emlen & Mannik, 1984). It is therefore important to gain some further insight into the molecular properties of the DNA as it exists in cryoprecipitates in which immune complexes are shown to be enriched (Winfield, Koffler & Kunkel, 1975). In this report, we looked for DNA in extracellular nucleoprotein complexes, identified the polynucleotide by its selective staining with ethidium bromide and DNase I susceptibility (Maniatis, Fritsch & Sambrook, 1982), and compared its electrophoretic behaviour to that of DNA in which immune complex interactions were abolished by deproteinization. We report that when DNA degradation and its reaction with protein are prevented, most of the extracellular DNA occurs as part of a nucleoprotein complex with a novel high molecular weight DNA. We also show

Correspondence: Carmen E. Contreras, Unidad de Immunología Clínica, Instituto de Anatomía Patológica, UCV, Apartado 50647, Caracas 1051, Venezuela.

the presence of additional free DNA binding proteins, preferentially in SLE patients and to a lesser extent in normal controls.

MATERIALS AND METHODS

Patients and samples. Patients fulfilling the preliminary criteria of the American Rheumatism Association for SLE (Tan *et al.*, 1982) and control normal individuals (Contreras *et al.*, 1982) were studied. Cryoprecipitates were obtained from plasma containing 0.01% sodium azide after incubation at 4°C for 7 days as previously described (Contreras *et al.*, 1982). Cryoprecipitates were obtained from 10 ml of plasma from clinically inactive SLE patients or normal individuals, or from 5 ml of plasma from active SLE patients. The latter showed higher levels of plasma circulating immune complexes (Orozco *et al.*, 1983), cryoprecipitates with high protein content, and elevated level of anti DNA antibodies (Contreras *et al.*, 1982) measured by a filter radioimmunoassay with ³H-native DNA from *E. coli*. This showed a 40–55% binding of the DNA added to the serum from active SLE patients when assayed on nitrocellulose filters, in contrast with less than 6% binding by sera from normal individuals and inactive SLE patients.

DNA isolation from cryoprecipitates. One volume of the test sample was added with constant stirring to one volume of protease K (100 µg/ml; P-0390 Sigma-Chemical Company, Inc.) in the presence of 20 mM EDTA pH 8 and SDS 1% for 30 min at 65°C. Subsequently, protein was extracted three times from nucleic acids with 0.5 volume phenol – 0.5 vol chloroform. The aqueous phase was then treated with 2.2 vol of cold ethanol in the presence of 0.3 M sodium acetate for 24 h at –20°C. The DNA pellet was washed with 0.5 ml of 70% cold ethanol and incubated at –70° for 30 min, and then centrifuged, lyophilized, resuspended in 0.01 M Tris, 10 mM EDTA pH 7.4 and stored at 4°C until used.

DNA-protein interaction. λ DNA fragments were obtained by exposing the DNA to complete digestion with the Hind III enzyme, which produces restriction cuts which include fragments of 17.5, 9.7, 4.3, 2.2 and 1.7 kilobases (calibrated with plasmid DNA mol. wt markers from New England Nuclear Lot No 1594-042) and detected in 1% agarose gels. This DNA was incubated with serum albumin fraction V or with comparable protein concentration of complete serum or plasma at 37°C for 60 min in Eppendorf microcentrifuge tubes.

DNA dot hybridization. DNA from human placenta and mouse liver was prepared by the method described above for DNA in cryoprecipitates. Pure nucleic acids were denatured for 3 min in 0.5 M NaOH at 90°C, the cooled DNA samples were neutralized with 0.5 M HCl–1.5 M NaCl and placed as dots in a BRL Hybridot on nitrocellulose membranes (BRL No 1051 MM) previously washed with H₂O and with 10X SSC. Each well received a 50 µl aliquot of denatured DNA of 1:1 dilutions of the preceding applications. Subsequently, the nitrocellulose membranes were washed with 8X SSC, baked at 80°C for 3 h and used for prehybridization and hybridization treatments. Prehybridization was carried out with 50% formamide, 5X Denhardt solution, 5X SSC and 0.1% SDS using as a carrier 100 µg/ml denatured salmon sperm DNA (Maniatis, Fritsch & Sambrook, 1982). Hybridization was carried out using the same mixture, to which was added 1.5 µg denatured nick-translated DNA (Rigby *et al.*, 1977) in the presence of deoxyadenosine 5'-(α-thio)triphosphate [³⁵S] (³⁵S-dATPαS) (NEN), and in the presence 10 mM DTT to prevent isotope hybridization background.

Agarose gel electrophoresis. This was carried out in 1% agarose gels (BRL, electrophoresis grade) prepared in 0.04 M Tris-acetate and 0.002 M EDTA containing 0.5 µg/ml ethidium bromide. Samples were usually applied in 9 µl volumes to which 1 µl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol 15% Ficoll 400) was added. Electrophoresis was allowed to proceed for 16 h at 0.5 V/cm to permit good resolution of high molecular weight DNA (Maniatis *et al.*, 1982).

RESULTS

Extracellular DNA exists as a nucleoprotein complex in SLE patients. Since ethidium bromide forms fluorescent complexes between base pairs (Le Pecq & Paoletti, 1967) in nucleic acids, we took

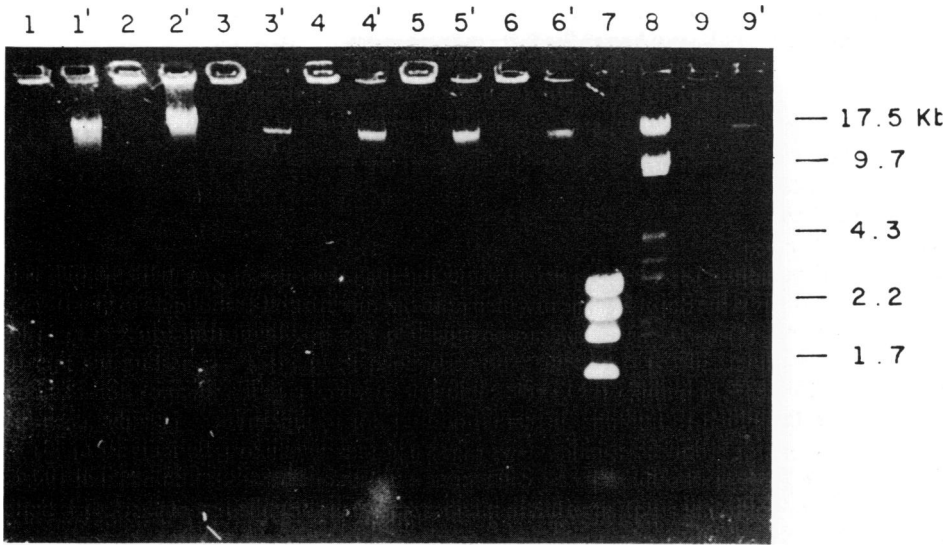


Fig. 1. Occurrence of DNA protein complexes in cryoprecipitates from SLE patients. Cryoprecipitates were run as such or after deproteinization as described in Materials and Methods, in 1% agarose gels for staining with ethidium bromide and fluorescent detection of the DNA under ultraviolet light. Lanes 1 to 6, cryoprecipitates from different SLE patients. Lanes 1' to 6', similar aliquots of cryoprecipitates after deproteinization and nucleic acid isolation. Lane 7, Hae III digest of ϕ X 174 DNA as a mol. wt marker. Lane 8, Hind III digest of λ DNA as a mol. wt. marker. Lane 9, cryoprecipitate from SLE patient in remission. Lane 9', same as 9 after deproteinization and nucleic acid isolation. Approximate molecular weights were estimated by calibration with plasmid DNA mol. wt markers from New England Nuclear Cat No NEE-117, lot 1594-042.

advantage of this to compare electrophoretic properties of DNA in deproteinized and untreated cryoprecipitates from SLE patients. This revealed the presence of a very slow migrating band, stainable with ethidium bromide, at the top of the gel in six patients with active SLE, and a similar but lesser band in an SLE patient in remission (Fig. 1, lanes 1–6 and lane 9). However, when a similar sample of cryoprecipitate from the above individuals was deproteinized with proteinase K and phenol in the presence of EDTA and SDS, we detected a faster migrating 17.5 kb component in active SLE patients (Fig. 1, lanes 1'–6') and to a lesser extent in the clinically inactive patient (Fig. 1, lane 9'). The low levels of 17.5 kb DNA reported for the clinically inactive SLE patient (Fig. 1, lane 9') compared to that in active patients (lanes 1'–6') was also observed in normal individuals with low levels of circulating immune complexes and anti-DNA antibodies (data not shown), suggesting that SLE patients in remission and normal individuals have lower levels of the 17.5 kb circulating DNA in their cryoprecipitates. When two of the deproteinized samples were treated with DNase I before electrophoretic analysis, we observed the disappearance of both the slow-migrating band and the 17.5 kb component, implying that it was DNA (Fig. 2).

Free DNA binding proteins in human serum and plasma. The results presented in Fig. 1 suggested that the extracellular DNA in cryoprecipitates from SLE patients exists mainly as a nucleoprotein complex, in which protein prevents electrophoretic migration. We examined the ability of plasma and serum of different SLE patients to interact specifically with native DNA prepared from Hind III digests of λ DNA. We used as a control, serum albumin fraction V. No change in migration was observed when the λ DNA digest was incubated with 20, 50 or 100 μ g of the albumin fraction. In contrast, when 70 μ g of a complete serum from an SLE patient was added to the λ DNA digest, we noticed an increase in a slow migrating band at the top of the gel, suggesting an interaction of serum proteins with DNA (data not shown). We then investigated whether this interaction was differentially manifested in active and inactive SLE patients as well as in normal individuals. When the λ DNA Hind III digest was incubated with 150 μ g of total serum or plasma protein, we noticed

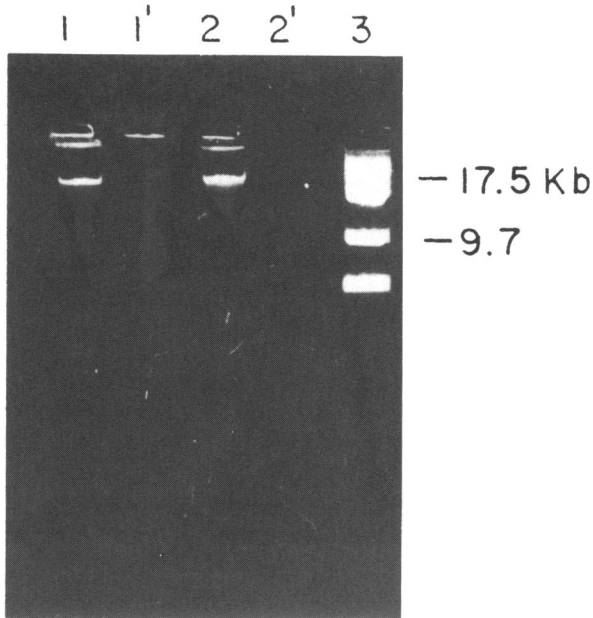


Fig. 2. Enzymic susceptibility of the high molecular weight DNA in cryoprecipitates. Lanes 1 and 2 represent control DNA samples from patients with active SLE exposed to 0.01 M MgCl₂ for 30 min at 37°C. Lanes 1' and 2' are the corresponding samples to which 5 µg DNase I was added during the incubation. Lane 3 is a Hind III λ DNA digest used as a molecular weight marker, calibrated as shown in Fig. 1.

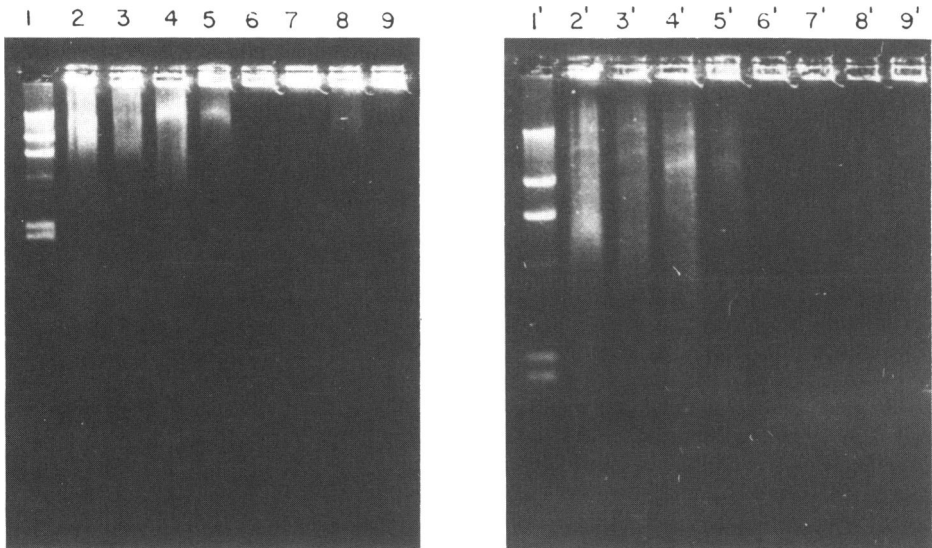


Fig. 3. Differential effect of serum proteins from active and inactive SLE patients and normal controls on the electrophoretic migration of DNA. A Hind III digest of λ DNA was incubated with 120 µg of serum protein from: Lanes 2-4, normal individuals. Lane 5, SLE patient in remission. Lanes 6-9, active SLE patients. Lane 1 represents a control λ DNA digest. Lanes 1'-9' are the same samples after a longer electrophoresis interval.

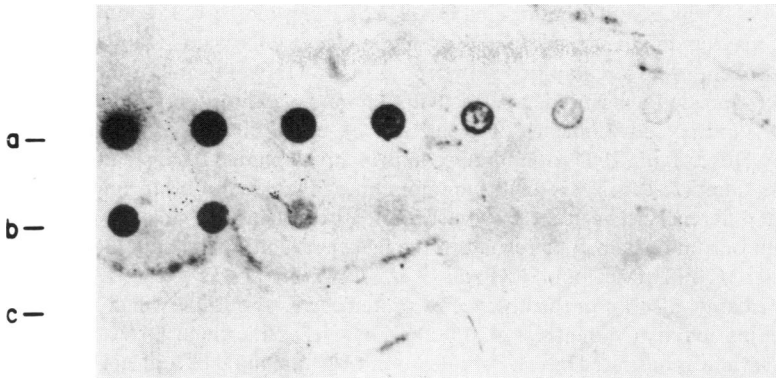


Fig. 4. Dot hybridization analysis of extracellular DNA in cryoprecipitates. DNA samples were prepared from human placenta, cryoprecipitates from an SLE patient and mouse liver as described in Materials and Methods. Denatured nucleic acids were immobilized in 50 μ l dots in BRL nitrocellulose membrane followed by baking at 80°C for 3 h for hybridization with nick-translated 1.5 μ g human placental DNA labelled with 35 S-dATP α S. The first dot to the left of lane a contained 2.0 μ g of human placental DNA. The first dot to the left of lane b contained 0.75 μ g of DNA from a cryoprecipitate obtained from a SLE patient. The first dot to the left of lane c contained 8 μ g mouse liver DNA. Dots to the right of the first application contained a 1:1 dilution of the preceding applications.

that samples from active SLE patients exhibited a greater ability to interact with this DNA as evidenced by a preferential accumulation of ethidium bromide-stained polynucleotide at the top of the gel (Fig. 3, lanes 6–9). However, whereas a detectable interaction was evident in plasma or serum from normal individuals (Fig. 3, lanes 2–4) and in a serum from an SLE patient in remission (Fig. 3, lane 5), all these samples allowed some migration of the λ DNA digest suggesting either a less tenacious interaction or a lower titre of DNA binding activity (Fig. 3).

17.5 kb extracellular DNA hybridizes with human placental DNA. In order to investigate the origin of the extracellular DNA reported above, some preliminary experiments using dot hybridization with human placental DNA labelled by nick-translation were done (Rigby *et al.*, 1977). A comparative analysis using mouse liver DNA human placental DNA, and the 17.5 kb extracellular DNA revealed detectable hybridization with human placental and extracellular DNA (Fig. 4a,b) but not with a 4-fold greater amount of mouse DNA (Fig. 4c), suggesting its human origin. Separate experiments revealed that nick-translated circulating DNA hybridized with human placental DNA and human lymphocyte DNA but did not hybridize with DNA from mouse lymphocytes, salmon sperm, *E. coli* or calf thymus (data not shown).

DISCUSSION

Since we were interested in defining the size of cryoprecipitable native DNA in SLE patients when bound as a circulating complex (Burdick & Emlen, 1985), we took advantage of the ability of ethidium bromide (Le Pecq & Paoletti, 1971) to intercalate within nucleic acid bases to compare the size of the DNA in nucleoprotein complexes before and after deproteinization using phenol, sodium dodecyl sulphate and chelating agents like EDTA that remove divalent ions required for nuclease degradation of the DNA (Maniatis, 1982). We found a DNase I-sensitive band stained with ethidium bromide in cryoprecipitates at the top of the gel whereas removal of protein from the same samples showed a faster migrating DNA species. These observations strongly suggested that most of the extracellular DNA in SLE patients exists as a nucleoprotein complex in cryoprecipitates. Our data also imply that the DNA moiety of this complex is of high molecular weight as indicated by an electrophoretic migration resembling that of a double stranded 17.5 kb DNA. Our findings of an extracellular high molecular weight DNA differ from a recent report (McCoubrey-Hoyer, Okarma

& Holman, 1984) which found that plasma DNA in SLE was mostly of low molecular weight. This discrepancy may be due to the greater DNase resistance of DNA anti-DNA immune complexes (Burdick & Emlen, 1985) as compared with deproteinized DNA. It is important to emphasize that we studied cryoprecipitates enriched in circulating immune complexes (Kalovidouris & Johnson, 1978; Davis Godfrey & Winfield, 1978) instead of using direct deproteinization of plasma (McCoubrey-Hoyer *et al.*, 1984) or immune complexes precipitated from serum with polyethylene glycol (Ikebe, Gupta & Tan, 1983) or 40% ammonium sulphate (Sano & Morimoto, 1981). Another experimental difference between the previously quoted reports of low molecular weight extracellular DNA and our findings may be related to the fact that in all such studies (Sano & Morimoto, 1981; Ikebe *et al.*, 1983; McCoubrey-Hoyer *et al.*, 1984), DNA was phenolized and treated with chloroform isoamyl alcohol in the absence of proteinase K and SDS, which we used to prevent DNA degrading activity (Maniatis *et al.*, 1982). The novel extracellular DNA reported appears to be of human origin as indicated by dot hybridization with human DNA and lack of hybridization to mouse DNA. Since this DNA appears to occur as a nucleoprotein complex, we also investigated whether there was any additional extracellular free DNA complexing activity (Hoch & McVey, 1977; Burdick & Emlen, 1985). When plasma or serum from SLE patients and control individuals was added to a Hind III-digest of λ DNA which exhibits well-defined bands by agarose gel electrophoresis, we observed that these samples modified the electrophoretic migration of the DNA. In particular, in samples from SLE patients we detected a nearly complete inhibition of migration of the DNA producing an electrophoretic pattern like that given by DNA in cryoprecipitates in which deproteinization was not carried out. Moreover, addition of comparable concentrations of serum albumin fraction V to the DNA did not affect electrophoretic migration, suggesting that an active protein interaction with DNA slowed nucleic acid electrophoretic migration. Another potentially interesting observation was the fact that serum from normal individuals also possesses a slowing effect on DNA although not as marked as that found in SLE patients. Further studies should be undertaken to clarify whether the differential behaviour of normal and SLE fluids on DNA migration depends on lower affinities or lower levels of activity in normal individuals and whether this approach may serve as basis for an assay to differentiate different extracellular DNA binding proteins in normal individuals and SLE patients. We have carried out a very preliminary characterization of the DNA binding proteins asking whether they belong only to polypeptides that interact with protein A-Sepharose as expected of immune complexes. However, although our studies indicate DNA binding activity both in fractions that bind to protein A-Sepharose and in unbound fractions, it may well be that the latter result from globulins with low affinity for Protein A (Langone, 1982) or from other DNA binding proteins (Hoch & McVey, 1977) other than immunoglobulins.

In summary, we have now shown that cryoprecipitates from SLE patients can be used to demonstrate the presence of DNA-protein complexes which contain a novel human high molecular weight DNA. We have also demonstrated that serum and plasma contain an excess of free DNA binding components some of which are IgG as shown by their ability to bind to protein A. Our studies suggest the possibility of using some of the approaches now described in the isolation of macromolecules useful in improving the diagnosis of SLE.

The authors thank Lic. Yofre Cabezas and Félix Toro for the preparation of cryoprecipitates, Dr Martín Rodríguez and the graduates from the Clinical Immunology Postgraduate Course at the National Cancer of Immunology, for providing patients described in this research. The financial support of CONICIT through Grants S1-1310 and ICC-1-Fortalecimiento de Centros is gratefully acknowledged.

REFERENCES

- ADU, D., DOBSON, J. & WILLIAMS, D.G. (1981) DNA-anti-DNA circulating complexes in the nephritis of systemic lupus erythematosus. *Clin. exp. Immunol.* **43**, 605.
- BRUNEAU, C.H. & BENVENISTE, J. (1979) Circulating DNA: anti-DNA complexes in systemic lupus erythematosus. *J. clin. Invest.* **64**, 191.
- BURDICK, G. & EMLEN, W. (1985) Effect of antibody

- excess on the size stoichiometry and DNase resistance of DNA anti-DNA immune complexes. *J. Immunol.* **135**, 2593.
- CONTRERAS, C.E., OROZCO, A., SANCHEZ, P., ORTEGA, G. & BIANCO, N.E. (1982) Physiological aspects of circulating immune complexes in the normal population. *Clin. exp. Immunol.* **48**, 693.
- DAVIS, J., GODFREY, S. & WINFIELD, J. (1973) Direct evidence for circulating DNA- anti-DNA complexes in systemic lupus erythematosus. *Arthritis Rheum.* **21**, 17.
- EMLÉN, W. & MANNIK, M. (1984) Effect of DNA size and strandedness on the *in vivo* clearance and organ localization of DNA. *Clin. exp. Immunol.* **56**, 185.
- HOCH, S. & MCVHEY, E. (1977) Purification and characterization of two major DNA-binding proteins in human serum. *J. biol. Chem.* **252**, 1881.
- IKEBE, K., GUPTA, R. & TAN, E. (1983) Characterization of DNA in polyethylene glycol precipitated immune complexes from sera of patients with systemic lupus erythematosus. *Clin. exp. Immunol.* **53**, 169.
- KALOVIDOURIUS, A. & JOHNSON L. (1978) Rapid cryoglobulin screening: an aid to the clinician. *Ann. rheum. Dis.* **37**, 444.
- KOFFLER, D., CARR, R., AGNELLO, V., THOBURN, R. & KUNKEL, H.G. (1971) Antibodies to polynucleotides in human sera: antigenic specificity and relation to disease. *J. exp. Med.* **134**, 294.
- LANGONE, J.J. (1982) Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumococci. *Adv. in Immunol.* **32**, 157.
- LE PECQ, J. & PAOLETTI, C. (1967) A fluorescent complex between ethidium bromide and nucleic acid. *J. mol. Biol.* **27**, 87.
- MANIATIS, T., FRITSCH, E.F. & SAMBROOK, J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory.
- MCCOUBREY-HOYER, A., OKARMA, T. & HOLMAN, H. (1984) Partial purification and characterization of plasma DNA and its relation to disease activity in systemic lupus erythematosus. *Am. J. Med.* **77**, 23.
- OROZCO, A., CONTRERAS, C.E., SANCHEZ, P., MEILLISON, O. & BIANCO, N.E. (1983) Solid phase C1q microassay for the rapid detection of circulating immune complexes. *J. immun. Methods.* **59**, 237.
- RIGBY, P.W., DIECKMAN, M., RHODES, C. & BERG, P. (1977) Labeling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237.
- SANO, H. & MORIMOTO, C. (1981) Isolation of DNA/anti DNA antibody immune complexes in systemic lupus erythematosus. *J. Immunol.* **126**, 538.
- TAN, E.G., COHEN, A.S., FRIES, E.F., MASI, A.T., MCSHANE, D.J., ROTHFIELD, N.F., SHALLER, J.G., TALAL, N. & WINCHESTER, R.J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* **25**, 1271.
- WINFIELD, J., KOFFLER, D. & KUNKEL, H.G. (1975) Specific concentration of polynucleotide immune complexes in the cryoprecipitates of patients with systemic lupus erythematosus. *J. Clin. Invest.* **56**, 563.