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Human repetitive and unique sequences coexist in a large circulating DNA species found in cryoprecipitates from SLE patients

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SUMMARY

Cryoprecipitates from systemic lupus erythematosus (SLE) patients with high levels of anti DNA antibodies show a sharply migrating large circulating DNA species of about 17–20 kb (M. Rieber *et al.*, *Clin. exp. Immunol.* (1986) **66,** 61). We have now used Southern blot analysis of circulating DNA from different individuals to analyse the relative cross-hybridization of circulating DNA from different individuals, as well as their homology with genomic DNA from different species. Molecular hybridization showed significant homology of the various circulating DNA examined, only with human genomic DNA, but limited cross-reactivity among circulating DNA from different individuals. This suggests that the circulating DNA is composed of sequences repeated in human genomic DNA and by specific sequences unique to circulating DNA from some individuals. Our data suggests the possibility of using probes derived from the specific sequences now reported in the circulating DNA, in gene typing and in the analysis of susceptibility to disease.

Keywords systemic lupus erythematosus DNA anti DNA antibodies

INTRODUCTION

Patients with systemic lupus erythematosus (SLE) manifest a variety of immunological abnormalities which include an over production of autoantibodies to DNA. However, since this polynucleotide is a poor immunogen, it is possible that the formation of anti-DNA antibodies found in SLE patients is directed against an unusual DNA species to be found in the circulation (Van Helden, 1985; Sano & Morimoto, 1982; Ikebe *et al.*, 1983). Although these studies have reported the presence of low molecular weight DNA in plasma or serum of both normal and SLE patients (Van Helden, 1985; Ikebe *et al.*, 1983), the size of this DNA makes it likely that it may represent a degradation product of a possibly 'immunogenic' DNA during processing (Sano *et al.*, 1983). We recently described conditions to show a novel DNA-protein complex and a large DNA in SLE cryoprecipitates (Rieber *et al.*, 1986). We have now investigated whether the reason for absence of high molecular weight circulating DNA in plasma or serum are due to the absence of such DNA from the fluids of specific individuals. For this, we have compared the levels of large circulating DNA in plasma, serum and cryoprecipitates of the same SLE patients. Evidence is presented to support the preferential detection of this large DNA in cryoprecipitates. Southern blots and molecular hybridization data suggest that this novel DNA species is of human origin but with limited cross hybridization with similar circulating DNA from different individuals.

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MATERIALS AND METHODS

Circulating DNA

Patients fulfilling the criteria of the American Rheumatism Association for SLE (Tan *et al.*, 1982) were studied. Sera and plasma were obtained from blood and from EDTA-anticoagulated blood, respectively after centrifugation for 15 min at 2000 *g*. The cryoprecipitates were obtained from plasma containing 0·01% sodium azide after incubation at 4°C for 7 days (Winfield *et al.*, 1975; Contreras *et al.*, 1982). In the case of patients with active SLE, cryoprecipitates were obtained from 5 ml of plasma, in contrast with the use of 10 ml of plasma for the preparation of cryoprecipitates from individuals with inactive SLE, diseases other than SLE, and normal controls. Plasma considered within the active SLE group were characterized by clinical parameters (Tan *et al.*, 1982) and high levels of serum circulating immune complexes (Orozco *et al.*, 1983), anti-DNA antibodies (Contreras *et al.*, 1982) and a high protein content in the cryoprecipitates (Contreras *et al.*, 1982). For comparison with active SLE patients, we used clinically inactive SLE patients, and individuals with Sjögren's syndrome, paracoccidioidomycosis and healthy controls. From these individuals cryoprecipitates were obtained from 10 ml of plasma (Contreras *et al.*, 1982).

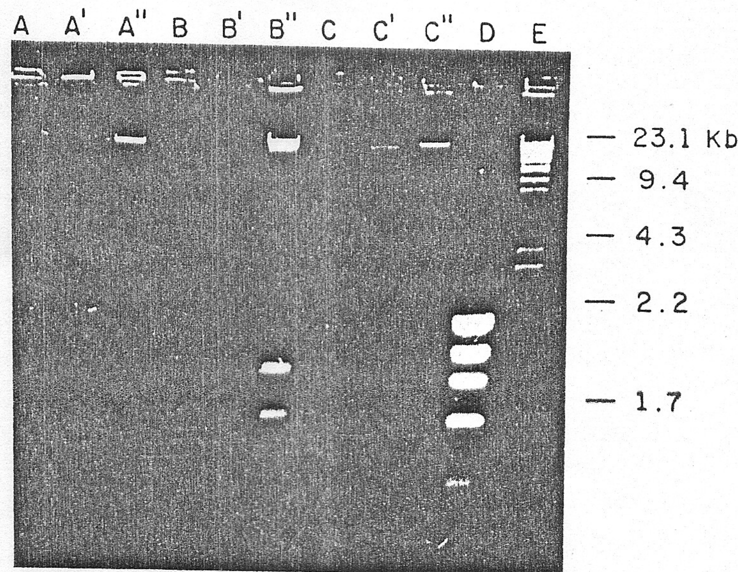

Fig. 1. High molecular weight circulating DNA is increased in cryoprecipitates. Comparable amounts of serum, plasma and cyroprecipitates were obtained from patients with active SLE for nucleic acid isolation and gel electrophoresis on 1% agarose gels. A,B,C, the nucleic acid detected in serum. A′,B′,C′, the nucleic acid detected in plasma. A″,B″,C″, the nucleic acid detected in cryoprecipitates. D and E, molecular weight markers.

DNA isolation

This was done by treating one volume of the test sample with proteinase K in the presence of 20 mM EDTA pH 8·0 and SDS 1% for 30 min at 65°C. Protein was removed three times with 1/2 vol of phenol and 1/2 vol of chloroform (Maniatis *et al.*, 1982). 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 was then washed with 70% ethanol, lyophilized and resuspended in 0·01 M Tris, 0·001 M EDTA pH 7·4, and kept at 4°C.

Nuclease treatment and agarose gel electrophoresis

Whenever indicated, circulating DNA was treated with 1 μg of DNase I per μg DNA in 0·01 M $MgCl_2$ for 60 min at 37°C or with 2 units of Eco R_1 per μg DNA in 0·01 M $MgCl_2$, NaCl 0·1 M, 0·05 M Tris pH 7·4 for a similar interval. Samples were then analysed in 1% agarose gels in Tris-Acetate-EDTA buffer (Maniatis *et al.*, 1982).

Southern blots nick translation and hybridization

Following electrophoresis, agarose mini-gels were treated with 0·5 M NaOH, 1·5 M NaCl for 60 min and then neutralized with 1 M Tris 1·5 M NaCl pH 7·5 for subsequent transfer to nitrocellulose membranes (Southern, 1975). Hybridization was carried out using the same mixture containing 0·75 μg denatured nick-translated DNA (Rigby *et al.*, 1975) labelled with ^{35}S-deoxyadenosine 5′-α thiotriphosphate.

RESULTS

Differential expression of the large circulating DNA in serum plasma and cryoprecipitates

Since serum or plasma are usually used as sources of extracellular DNA (Van Helden, 1985; Sano & Morimoto, 1981; 1982; Morimoto *et al.*, 1982; Ikebe *et al.*, 1983), and cryoprecipitates of patients with SLE are known to be enriched in anti-DNA antibodies, we carried out a preliminary comparison of comparable amounts of serum, plasma and cryoprecipitates from SLE patients for the presence of high molecular weight extracellular DNA. An analysis of samples from three different individuals with active SLE in 1% agarose gels which permits the resolution of DNA of more than 1 kb, showed that serum (Fig. 1, A, B, C) and plasma (Fig. 1, A′,B′,C′) have lower levels of high molecular

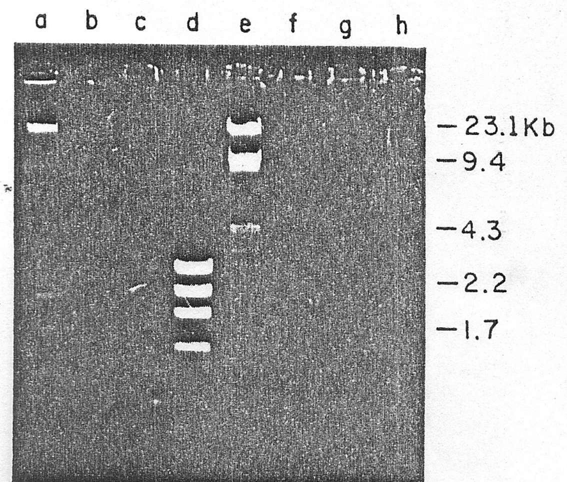

Fig. 2. Circulating DNA is preferentially detected in SLE patients. DNA obtained from 10 ml of cryoprecipitates was used for electrophoretic analysis in all cases, except for the active SLE patient. For this, the DNA assayed represents half the nucleic acid obtained from the cryoprecipitate resulting from 5 ml of plasma. All DNA samples were subjected to electrophoretic analysis in 1% agarose gels. a, an active SLE patient; b and c, SLE patient in remission; d, Hae III digest of $\phi\chi$174 marker; e, Hind III digest of λDNA; f, a patient with Sjögren's syndrome; g, a paracoccidioidomycosis patient; h, a normal control.

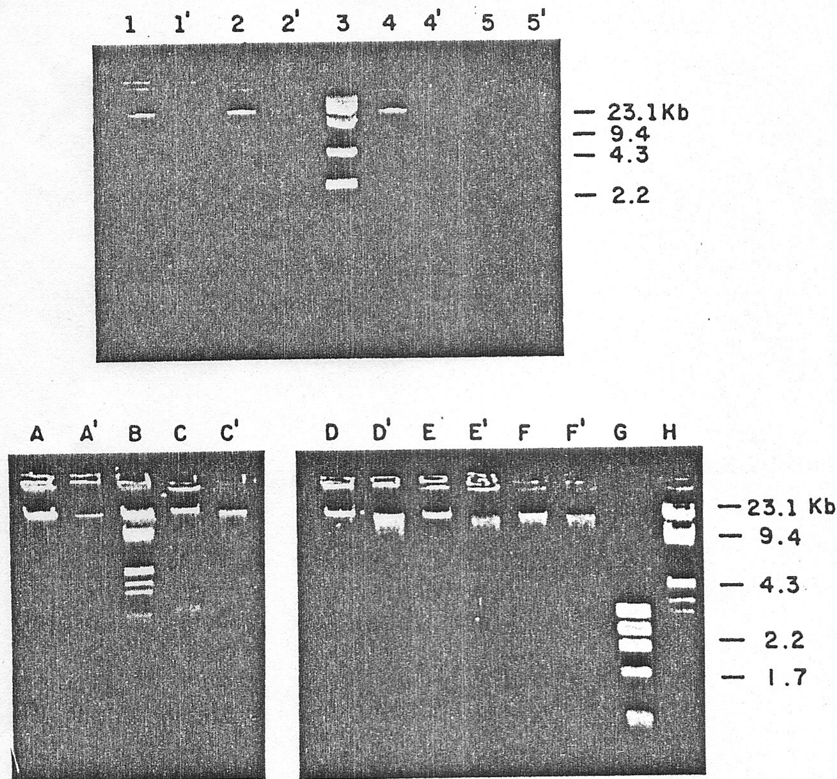

Fig. 3. Enzymatic susceptibility of the circulating DNA. Lanes 1,2,4, show control circulating DNA from three active SLE patients, and lane 5, the circulating DNA from a healthy individual, and the adjacent lanes (1′ to 5′) show the effect of DNase I on the corresponding samples. Lanes A′,C′,D′,E′,F′, show the effect of Eco R_I treatment on the corresponding untreated samples (A,C,D,E,F) prepared from active SLE patients whereas lanes 3 B,G, and H are molecular weight markers.

weight circulating DNA compared to cryoprecipitates (Fig. 1, A″,B″,C″), which showed a common band in the 17–20 kb region. Although larger components appeared at the top origin of the gel in sample B″, we usually observed a DNA band of about 20 kb in all cases examined. This confirmed our recent findings of a large circulating DNA in cryoprecipitates from SLE patients (Rieber *et al.*, 1986) and contrasted with recent findings in which DNA up to 150 bases (Sano & Morimoto, 1981; 1982) or up to 350 bp (Van Halden, 1985) which were detected in human serum. We then investigated whether comparable amounts of the large circulating DNA were present in individuals other than SLE patients. This revealed that the high molecular weight DNA is increased in cryoprecipitates from active SLE individuals (Fig. 2, lane a) compared to that of individuals in remission with an inactive SLE (Fig. 2, lanes b,c), a patient with Sjögren's syndrome (Fig. 2, lane f) and individual with paracoccidioidomicosis (Fig. 2, lane g) or a healthy individual (Fig. 2, lane h).

These data suggest that the circulating DNA is preferentially detected in cryoprecipitates from active SLE patients, particularly when considering that these were obtained from 5 ml of plasma in contrast with the use of 10 ml of plasma as source of cryoprecipitate DNA from the other patients examined in Fig. 2. However, one should emphasize that the levels of protein and DNA per cryoprecipitate from subjects with inactive SLE and other individuals, were usually lower and the corresponding cryoprecipitates were also smaller. Nevertheless, as shown Fig. 1, cryoprecipitates indeed appear to be a good source for the detection of the large circulating DNA (Rieber *et al.*, 1986).

Enzymatic susceptibility of the large circulating nucleic acid

Smaller DNA fragments detected in some of the cryoprecipitates may represent degradation products of the large DNA since they were not detected in all samples. To gain some insight into the enzymatic susceptibility of the circulating nucleic acids, we examined their behaviour after incubation with DNase I and Eco R_I. Samples obtained from three different cryoprecipitates of individuals with SLE (Fig. 3, lanes 1,2,4) and from one control individual (Fig. 3, lane 5) all revealed clear susceptibility to DNase I (Fig. 3, 1′,2′,4′,5′). Addition of Eco R_I to other extracellular DNA samples from active SLE patients also led to electrophoretic alterations which implied that the susceptible DNA are double stranded and possess some G↓ AATTC sequences, known to be cleaved by Eco R_I (Maniatis *et al.*, 1982) (see Fig. 3 bottom).

Hybridization of extracellular DNA to human DNA and to circulating DNAs from different individuals

Since it was important to determine the origin of the circulating high molecular weight DNA reported above, we carried out bidirectional blots (Southern, 1975) to obtain parallel replicas of the DNAs from the same agarose gels for hybridization to

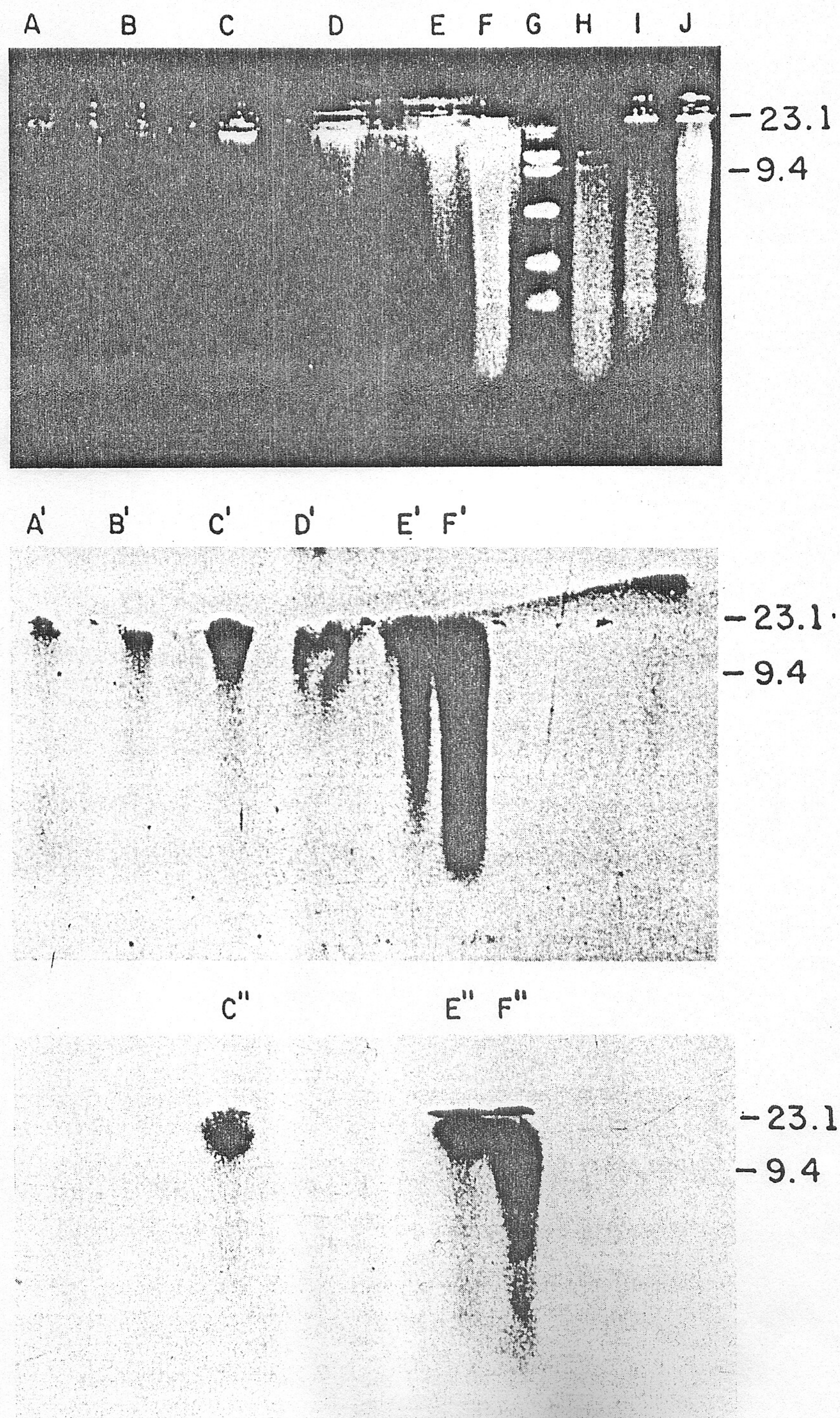

Fig. 4. Human repeated and unique sequences in extracellular DNA. Extracellular DNA from different SLE patients (lanes A,B,C,D), human placental DNA (lane F), a Hind III digest of λ-DNA (lane G), salmon sperm DNA (lane H), calf thymus DNA (lane I) and mouse liver DNA (lane J) were run on a 1·1% agarose gel, denatured and subjected to bidirectional blotting onto nitrocellulose membranes. The upper figure shows the ultraviolet pattern of ethidium bromide stained nucleic acids; the middle pattern shows the nucleic acids that hybridized to nick translated ^{35}S-labelled human placental DNA and the lower pattern shows the nucleic acids that revealed hybridization with the large nick-translated ^{35}S-labelled extracellular DNA run in lane C.

human placental DNA and extracellular DNA obtained from cryoprecipitates. When nick-translated labelled human placental DNA was used as a probe, we detected hybridization to several extracellular DNAs from different individuals (Fig. 4, lanes A′, B′, C′, D′) and to human lymphocyte and human placental DNA (Fig. 4, E′,F′, respectively). However, use of isolated 17–20 kb circulating extracellular DNA from patient C as a probe, showed hybridization only to itself (Fig. 4, lane C″) and to human lymphocyte and placental DNA (Fig. 4, lanes E″, F″, respectively), and low cross-hybridization to the other

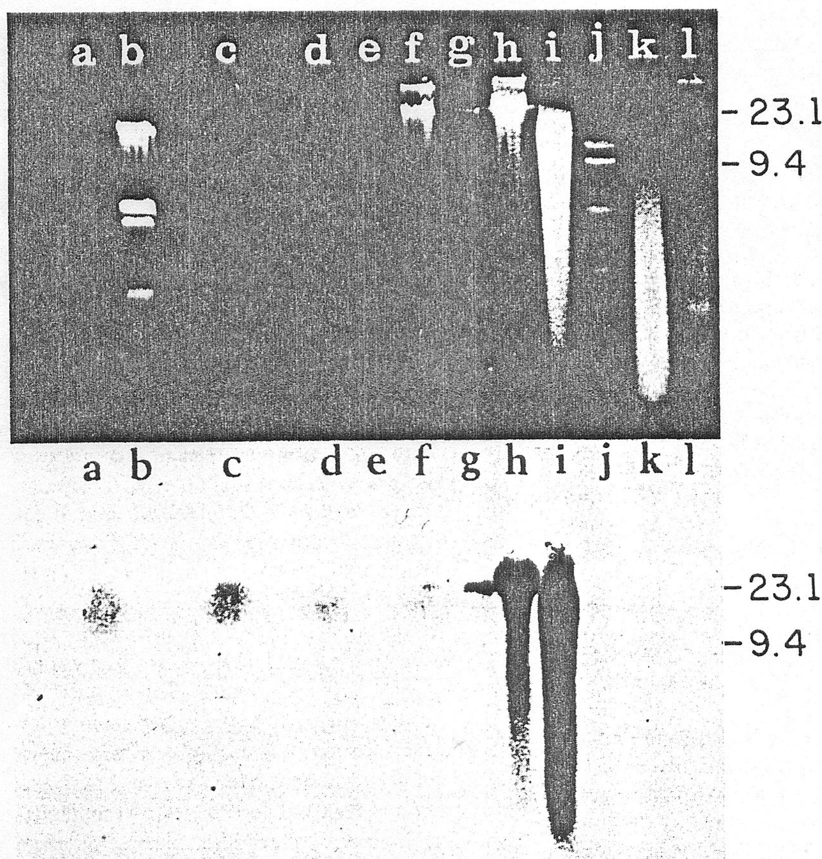

Fig. 5. Differential cross hybridization of circulating DNA. Circulating DNA from different individuals lanes a,c,d,e,f,g, were run in a 1% agarose gel, in parallel with plasmid DNA mol. wt markers (lanes b and j), human lymphocyte DNA (lane h), human placental DNA (lane i), mouse liver DNA (lane k) and calf thymus DNA (lane l), for subsequent Southern blotting onto nitrocellulose membranes and hybridization with circulating DNA from patient g. The upper figure shows the ultraviolet pattern of ethidium-bromide stained nucleic acids and the lower pattern shows the nucleic acids and the lower pattern shows the nucleic acids that hybridized to the large nick-translated ^{35}S-labelled extracellular DNA from patient g.

extracellular DNA samples that hybridized to human placental DNA. A similar lack of hybridization was observed between the human probe described and DNA samples from λ phage, salmon sperm, calf thymus or mouse liver (Fig. 4, lanes G,H,I,J, respectively). Examination of the hybridization properties of other circulating DNA-species, was carried out with cryoprecipitates from different individuals to test whether the previous findings were not restricted to one patient. This showed (Fig. 5) that when circulating DNA from patient g is labelled by nick translation and used to examine its homology with other DNA species, it revealed significant hybridization with itself and with lymphocyte and placental DNA of human origin (lower lanes g, h, i) but limited cross hybridization with circulating DNA from patient f, which was about 20 times as concentrated as DNA from patient g. In spite of the much lower levels of circulating DNA in patients a, c, and d compared to those in patient f (Fig. 5 upper lane), the former circulating DNAs showed more evident hybridization signals with probe g (Fig. 5, lower lane). The hybridization data presented in Figs 4 and 5, suggest the human origin of the extracellular DNAs used as probes, as well as the occurrence of nucleic acid sequences specific to some of these DNAs.

DISCUSSION

We have now shown that cryoprecipitates from patients with SLE are a better source of circulating DNA than serum or plasma. This observation is compatible with earlier findings (Winfield *et al.*, 1975; Roberts *et al.*, 1981) showing that these cryoprecipitates are selectively enriched in anti-DNA antibodies and DNA antigen, compared to serum. In addition to the greater occurrence of high molecular weight circulating DNA in cryoprecipitates compared to plasma or serum, we have also shown that SLE patients exhibit an increased level of this DNA compared to the corresponding fractions from non-SLE patients or normal controls. An intriguing aspect of this study was the almost invariant size and homogeneity of the circulating DNA band isolated from the cryoprecipitable material. Earlier studies showed that injection of experimental animals with large DNA led to rapid elimination of this nucleic acid from the circulation, either because of liver-mediated clearance of single stranded DNA or because of degradation by serum and/or tissue nucleases (Emlen & Mannik, 1978; Emlen & Mannik, 1984). Nevertheless, our recent studies on a large circulating DNA in SLE cryoprecipitates have shown that this DNA is not

present like an exogenous 'naked' DNA injected into the circulation (Emlen & Mannik, 1984) but rather exists as a DNA-protein complexed with IgG or with serum DNA-binding proteins in cryoprecipitates enriched in immune complexes (Rieber *et al.*, 1986). It is likely that this nucleoprotein complex may be protected from DNase degradation in a manner analogous to that in which DNA-anti DNA immune complexes confer DNase resistance on DNA (Burdick & Emlen, 1985). Another parameter which may influence circulating DNA resistance is a particular base composition (Van Helden, 1985) since it has been shown that the binding of anti-DNA antibodies to DNA is strongly dependent on base composition and specific configuration (Impraim *et al.*, 1985). Hence, an explanation for our findings of a large circulating DNA in SLE may be the greater level of cryoprecipitates and circulating anti-DNA immune complexes in SLE patients (Roberts *et al.*, 1981; Orozco *et al.*, 1983) which may protect a large DNA from degradation (Burdick & Emlen, 1985). Also, it may well be that large DNA complexed to protein prevails in association with cryoprecipitates which are preferentially formed in SLE, and that smaller DNA fragments (Van Helden, 1985; Sano & Morimoto, 1983) occur preferentially in more soluble fractions of serum or plasma.

Hybridization experiments suggest that the extracellular DNA now reported is of human origin. This was concluded since a probe prepared from human placental DNA failed to hybridize with calf thymus, mouse, λ phage and salmon sperm DNA, but clearly hybridized with DNAs from human lymphocytes, human placenta and with circulating DNAs from various individuals. An interesting and intriguing finding was the ability of the circulating 23·1 kb from SLE patients to show self-hybridization and homology with human lymphocyte and placental DNAs but limited cross-hybridization to other human circulating DNAs. This suggests that some circulating 17–20 kb DNA species have specific sequences which may have a potential in human DNA genetic typing and in the analysis of susceptibility to SLE and related diseases. Although we do not know yet the nature of the specific sequences in the circulating DNA, these sequences may correspond to hypervariable regions of tandem minisatellite repeats of human DNA which are highly polymorphic in different individuals (Jeffreys *et al.*, 1985). It has also been shown that human neuroblastoma tumors possess amplified DNA sequences exhibited by some but not other neuroblastoma cells from different patients (Montgomery *et al.*, 1983). It may well be that the sequences found to a greater extent in some circulating DNA samples correspond to a differential amplification of particular DNA sequences interspersed with tandem repetitive sequences, which may provide polymorphism due to variations in repeat copy number (Jeffreys *et al.*, 1985). Finally, it should also be of interest to determine whether the specific sequences detected within the large circulating DNA are more antigenic than the bulk of the chromosomal DNA, and also whether these sequences play a role in the pathology of diseases that promote the formation of anti DNA antibodies.

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