DNA AND THE DNA IMMUNE COMPLEX IN SYSTEMIC LUPUS ERYTHEMATOSUS

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P R E F A C E

Systemic lupus erythematosus continues to fascinate the clinician, the immunologist, the virologist, the geneticist and the epidemiologist. The etiological agent or agents remain elusive and while much is known about the pathogenesis of the disease there are as yet many unanswered questions. Evidence for a central role of DNA immune complexes in the pathogenesis of SLE has been derived from histological, immunofluorescence and acid elution studies of the kidney and other organs. The presence in the circulation of double-stranded anti-DNA antibodies, considered to be virtually exclusive to SLE, and DNA itself, provide further support for the role of specific DNA immune complexes in the disease. While a number of assays exist for the detection of circulating antigen nonspecific complexes, there are by contrast very few techniques available for measuring specific DNA complexes, and studies correlating their presence with clinical activity are few. Prompted by this, a technique for the measurement of circulating DNA complexes by millipore filtration and deoxyribonuclease digestion, and a physicochemical assay for the detection of double-stranded DNA were developed. Their role in the pathogenesis of SLE and as monitors of disease activity were evaluated in a series of patients during both clinically active and inactive disease.

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ABBREVIATIONS

ADA	Anti-DNA antibody
ANA	Antinuclear antibody
ARA	American Rheumatism Association
CDLE	Chronic discoid lupus erythematosus
CH_{50}	Total haemolytic complement
CIE	Counterimmunoelectrophoresis
C_1q, C_3b, C_4b	Complement components
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds-	double-stranded
EDTA	Ethylenediaminetetraacetic acid
ENA	Extractable nuclear antigen
Fc	Terminal end of heavy chain of antibodies
GFR	Glomerular filtration rate
Hb_sAg	Serum hepatitis-associated antigen
HLA	Human leucocyte antigen
IC	Immune complex
Ig	Immunoglobulin
LCA	Lymphocytotoxic antibodies
LE cell	Lupus erythematosus cell
MCTD	Mixed connective tissue disease
MED	Minimal erythema dose
NZB/W	New Zealand Black/White mouse strain
ONB	oxoid nutrient broth
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
PPD	purified protein derivative
RA	Rheumatoid arthritis
RES	Reticuloendothelial system
RF	Rheumatoid factor
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Nuclear ribonucleoprotein antigen component of extractable nuclear antigen
SLE	Systemic lupus erythematosus
Sm	Non-nucleic acid protein antigen component of extractable nuclear antigen
SS	Systemic sclerosis
ss-	single-stranded
SSC	Saline sodium citrate
UV	Ultraviolet

SUMMARY

DEOXYRIBONUCLEIC ACID

1 A physicochemical technique for the assay of dsDNA in plasma by polyacrylamide gel electrophoresis and ethidium bromide staining was developed.

2 The specificity of the assay was established by the staining and mobility characteristics, DNase and RNase digestion, heat denaturation and gel range analysis.

3 The steps necessary to ensure that plasma used in the assay is free of contamination with cellular material are outlined.

4 dsDNA was found to be stable at temperatures of between -20°C and 37°C when studied over 24 hours and at -20°C over a period of 4 weeks.

5 The % recovery was 97, 84, 89 and 56 for 500, 250, 50 and 25 ng/ml respectively.

6 The within-group variability of plasma dsDNA levels showed a standard deviation of 13.8% for levels of 40 ng/ml and above.

7 Serum was found to be unsuitable for dsDNA estimation by this method.

8 dsDNA was undetectable in 47 of 58 normal subjects, with levels ranging from 10 to 54 ng/ml in the remaining 11 subjects.

9 Plasma dsDNA levels measured in normal subjects over a 24 hour period and after exposure to UV irradiation showed no variation.

10 Plasma dsDNA levels in patients with SLE were no different to those found in normal subjects, irrespective of the system involved or whether the disease was active or inactive. Significant elevation was however recorded in patients after haemodialysis.

dsDNA IMMUNE COMPLEX ASSAY

1 An assay for the detection of dsDNA immune complexes, based on a principle of millipore filtration and DNase digestion was developed.

2 The degree of sensitivity of the assay was not affected by the presence of free anti-DNA antibody in the serum even at high levels.

3 Circulating levels of DNA complexes were taken to be significant at a P value of less than 0.02 for levels of 0.03 units/ml or greater.

4 The possibility that a proportion of the material detected is due to nonspecific aggregates rather than true DNA complex material was investigated by comparing levels in freshly collected serum and in serum exposed to conditions known to favour the formation of aggregates. The data suggests that nonspecific aggregates do not affect the levels obtained for reasons which will be discussed.

5 Complexes were undetectable in 19 (95%) of 20 normal subjects while in the remaining subject a level of only 0.03 units/ml was present. .

6 The diagnostic value of the assay for SLE, particularly during active disease, was demonstrated in a comparative study of a group of patients with active rheumatoid synovitis.

7 Five categories, with respect to the relationship between free anti-DNA antibody and DNA complexes in patients with active SLE, were found. The majority of patients remained"true" to category irrespective of the system involved with each attack, or whether they were tested early or late in the acute episode.

8 The DNA complex assay was compared to the C_1q binding assay in 16 patients with SLE. While the numbers are too small for statistical analysis, this preliminary study suggests that a good correlation between the assays may be shown.

9 A study of the DNA complex assay in 40 patients with SLE showed that:

(i) A good correlation exists between the presence or absence of circulating DNA complexes and active or quiescent disease.

(ii) The assay is superior to the anti-DNA antibody as a parameter of activity, and when evaluated in conjunction with CH_{50} and anti-DNA antibody levels a prediction of the relative risk of active disease being present is enhanced.

(iii) There is a good correlation between actual levels of circulating complexes and the degree of activity.

(iv) The DNA complex assay is of predictive value, as in some cases their appearance in the circulation antedates the onset of activity.

10 The case histories and serial studies of CH_{50}, anti-DNA antibody and DNA complex levels in 9 patients with SLE during active and inactive disease are presented to illustrate the interesting variations which occur.

SECTION I : INTRODUCTION

1 HISTORICAL BACKGROUND

Voltaire in his volume "Letters" (1733) refers to an ancient Chinese custom of inhaling the dried powder of smallpox crusts, rather like taking snuff to prevent the development or recurrence of the disease. From practices such as these the rudiments of immunity to disease and the fundamentals of immunology as we know them today became established, centuries before the concept of the germ theory of infectious disease.

An important contribution and one which assured the future of immunology was made by Edward Jenner who published the results of his experiments in 1798 after 20 years research in which he verified the belief that contact with cowpox afforded protection against smallpox. However, it was largely due to the research of Pasteur, the first great experimental immunologist, that preventive immunisation reached a high degree of sophistication with the development of living, heat-killed and attenuated vaccines. The term "vaccine" was coined by him in acknowledgement of the early work by Jenner in the field (Vacca, cow). Pasteur was also the pioneer in the germ theory of disease, and bacteriology as a science was born (1,2).

Immunology developed along with bacteriology with the discovery of bacterial toxins by Roux and Yersin in

1888 and antitoxins by Behring and Kitasato in 1890 (1). A major contribution was made by Buchner who was the chief advocate of the theory that body fluids contain a substance considered to be bactericidal which he termed alexine, and which we now know to be complement (1). The agglutination test for bacteria is associated with the names of von Gruber and Durham (3,4) while in 1896 Widal described the test for the diagnosis of typhoid fever (1).

Towards the turn of the century the concept that the immune response depends on two distinct mechanisms, namely cellular (phagocytic theory) and humoral, emerged, the former attributable to Metchnikoff (1) and the latter to Ehrlich (5). At the time the close interrelationship between these two mechanisms was not fully appreciated and it was not until decades later that the importance of cellular immunity was realised. Research in the field of humoral immunity by contrast gathered momentum and it became increasingly clear that immunology had implications far wider than the field of microbiology.

With the elucidation of the mechanisms involved in anaphylaxis, the Arthus reaction and the allergic response, a new perspective was introduced in immunology (6) which was to have a profound effect on future thinking; namely that aberrations in the immune response may occur which are not only harmful, but which

may result in the death of the host.

The concept of "tolerance" and "self" versus "non-self" was developed from Ehrlich's theory of "horror autotoxicus" by Burnet and Fenner in 1949 (7). This was followed by such discoveries as chimerism (8) and the clonal selection theory of Jerne (9) and Burnet (10) while in 1962 the central role of the thymus was established by Miller (11,12). At about the same time Porter (13) and Edelman (14) defined the structure and formation of gamma globulin.

Immunology has thus emerged as a multi-disciplinary science encompassing virtually all the fields of medicine and in particular the autoimmune diseases, cancer and organ transplantation. Furthermore in the past two decades there has been an upsurge of interest in a number of disorders in which a complex interrelationship appears to exist between the immune system and viral infections in genetically predisposed individuals.

Systemic lupus erythematosus (SLE) is a prototype of such a disorder. The term "Lupus" derived from the Latin meaning "wolf" was coined at least seven centuries ago and described any erythematous spreading ulceration of the face - a disease which eats away, bites and destroys. The earliest concept of lupus as a specific entity can be attributed to Cazenave

who in 1851, described a condition he termed "lupus erythemateux" and which almost certainly refers to discoid lupus. In 1872 Kaposi described acute and chronic forms of the disease and hinted at its systemic nature. However it was largely due to Osler in his classic series of communications between 1895 and 1904 (15,16) in which he described the wide clinical spectrum of SLE, that the systemic nature of the disease was fully appreciated. He also proposed an underlying vasculitis as a basis for the disease.

The discovery by Hargraves (17) in 1948 of the lupus erythematosus (LE) cell phenomenon evoked much interest and generated the concept that SLE was an autoimmune disease. Another important milestone was reached when, from four centres, the occurrence of antibodies to DNA was reported (18). Since that time a large number of antibodies directed mainly to other nuclear and cytoplasmic constituents have been described, and there is now ample evidence to support an immune complex basis for the disease. The etiological agent or agents however remain elusive and current research is being directed at the possible role of viral, immunologic, genetic and hormonal factors in the genesis of the disease, and also at further elucidating the resultant immunologic aberrations which occur.

2 THE DIAGNOSIS AND ASSESSMENT OF ACTIVITY IN SLE

(i) CLINICAL

In the majority of cases the diagnosis of SLE can be made clinically with reasonable confidence especially when the presentation is that of a multisystem disorder or when the organ involvement is typical, such as a butterfly skin rash.

The clinical spectrum is very wide and it is not the intention to discuss the presentations in detail. There has been general agreement in the prevalence and spectrum of organ involvement in patients with SLE as borne out in the studies by Dubois (19,20,21), Harvey (22), Haserick (23) and Jessop and Meyers (24).

Many of the features of SLE are not specific to the disease and may occur in a number of related and unrelated conditions such as other connective tissue disorders and viral or bacterial infections. In addition many cases of SLE may masquerade as idiopathic thrombocytopenic purpura, epilepsy, migraine, depression, idiopathic nephrotic syndrome, or seronegative arthritis (25). While the arthropathy of SLE is often of a relatively mild nature with little if any objective evidence of synovitis or deformity, severe involvement, indistinguishable from

rheumatoid arthritis (RA) may occur (26-28) and it is only by performing the appropriate serological tests that the diagnosis of SLE may be established.

The diagnosis may also be complicated by the development in some patients of syndromes in which features of two or more of the connective tissue disorders occur. A recent addition to this list of "overlap" syndromes is that of mixed connective tissue disease (MCTD), a term introduced by Sharp (29) describing a syndrome comprising some of the features of SLE, systemic sclerosis (SS) and polymyositis. A number of reports further elucidating the disease have since appeared (30-35). There is however a divergence of opinion as to the validity of regarding MCTD as a distinct entity. Those in favour of the concept feel that the differentiation from SLE is justified because in general terms patients with MCTD have a better response to corticosteriod therapy, tend not to develop the systemic manifestations of SS, and renal involvement when it occurs is usually of a milder nature.

(ii) ARA CRITERIA FOR THE CLASSIFICATION OF SLE

The American Rheumatism Association (ARA) in 1971

published their preliminary criteria for the classification of SLE (36) based on a retrospective multicentre analysis of 696 cases with SLE. Fourteen criteria were selected from 57 features present in these patients. These are: facial erythema, chronic discoid lupus erythematosus (CDLE), Raynaud's phenomenon, alopecia, photosensitivity, oral or nasal ulceration, non-deforming arthritis, LE cells (2 or more), false positive serological tests for syphilis, proteinuria ($>$ 3.5g/day), cellular casts, pleurisy or pericarditis, psychosis or convulsions and haemolytic anaemia or leucopenia ($<4000/mm^3$) or thrombocytopenia ($<100,000/mm^3$). The presence of 4 or more or these criteria together, or developing subsequently were regarded as compatible with the diagnosis of SLE. While the usefulness of this classification has been established in most epidemiological and clinical studies and in differentiating SLE from other connective tissue disorders such as RA (37-41), it excludes many with milder forms of the disease and those presenting with single organ involvement without the necessary laboratory parameters to fulfil the criteria. A revision of the criteria is awaited and will almost certainly include the anti-DNA antibody (ADA) which has stood the test of time as the single most specific test currently available for the diagnosis of SLE. (See

pp 11-14.)

It is often possible by careful clinical evaluation to assess disease activity. Thus the development of a florid skin rash, polyarthritis, alopecia, constitutional symptoms or visceral involvement such as pericarditis indicate active disease. The clinical impression in most cases will dictate whether or not active therapy should be instituted. However, subclinical disease activity undoubtedly does occur and when this involves an organ such as the kidney with its potentially poor prognosis, the problem has to be considered in conjunction with serological and other testing. Unfortunately, as will be discussed, none of the currently available immunological tests in isolation is sufficiently specific or reliable to either establish the presence of, or to predict the onset of an acute episode.

(iii) LABORATORY

a) LE Cell Phenomenon

The discovery of the LE cell phenomenon by Hargraves in 1948 (18) was of great historical significance as the first diagnostic test for SLE. Not only could milder forms of the disease be recognised but it also paved the

way for further research in the field of antinuclear antibodies. While still performed as a routine test in most laboratories, it is time consuming, lacks specificity and sensitivity and is not quantitative (42). It may be positive in a number of other conditions such as CDLE (43), Sjogren's syndrome (44), RA (45), and in chronic active hepatitis (46). It is of no assistance in the assessment of disease activity and its inclusion as one of the ARA classification criteria for SLE is likely to be reviewed.

b) Antinuclear Antibodies

The immunofluorescence test for antinuclear antibody (ANA) is the standard screening test for SLE. It is semi-quantitative and is more sensitive that the LE cell test. However it also lacks specificity and a positive test for ANA (usually low titre) may be found in some patients with RA (47,48), SS (49), Sjogren's syndrome (50), ulcerative colitis (51), myasthenia gravis (52) and in burns (53,54) amongst others. It may also be found in relatives of patients with SLE (55) and in the elderly (56). However ANA in high titre is strong evidence in favour of SLE. Conversely a negative test usually but not

always excludes the diagnosis especially if active disease is present, and for some cases of cerebral lupus (57), late stage inactive lupus nephritis (58) and patients with lupus-like syndromes associated with complement component deficiency states (59).

Four major staining patterns of nuclear fluorescence are recognised (60). These patterns are not as specific for certain antibodies as previously thought although the rim pattern (also called ring, peripheral, membranous or shaggy) correlates well with ADA (61). The homogenous pattern, due mainly to antibody to DNA-histone complex is frequently seen in SLE (62). The speckled pattern is due to antibody to saline-soluble nuclear components and is found in SLE, but recently a good correlation has been shown between this pattern, antibodies reacting specifically with ribonucleoprotein and MCTD (29). The nucleolar pattern is seen most frequently in SS and in a proportion of patients with Sjogren's syndrome (63). It is not uncommon to find mixed patterns in any one serum.

The ANA test is therefore useful diagnostically but not as a parameter for monitoring disease activity.

c) Anti-DNA Antibodies

The Farr ammonium sulphate precipitation (64-66) and the Ginsberg and Keiser millipore filter techniques (67) are sensitive and quantitative for the measurement of antibodies to double-stranded DNA (dsADA). A good correlation between the two techniques has been demonstrated except at high levels of activity (67). Antibodies to DNA have been regarded for some years as the mainstay in the diagnosis of, and as a measure of activity in SLE because of their specificity (68). Antibodies to single-stranded DNA (ssADA) on the other hand lack specificity for SLE and occur in a number of other conditions (69,70).

Binding levels of more than 30% for dsDNA by the Farr technique or levels in excess of 10 µg DNA bound per ml serum by the Ginsberg and Keiser technique were considered virtually diagnostic for SLE.

Apart from its diagnostic value dsADA levels in general correlate well with disease activity (65,66,71-76). Thus binding levels of less than 30% (or less than 10 µg DNA bound per ml serum) are usually associated with inactive disease while higher levels, especial-

ly if binding is greater than 70%, (or greater than 20 µg DNA bound per ml serum) strongly suggest active disease. Unfortunately this relationship is not absolute and it is not unusual to find that persistently high levels of dsADA may exist with little or no evidence of clinical activity (77-79).

Nevertheless Lightfoot and Hughes (80) are of the opinion that in such cases severe exacerbations are more likely to occur eventually. It is also not possible to predict the nature of the acute attack which might follow rising levels of ADA. This poses a problem in treating on ADA levels alone, as corticosteroid or immunosuppressive therapy are not necessarily indicated where the exacerbation takes the form of a mild flare of arthritis or skin lesions, whereas more aggressive treatment would be justified if active renal involvement ensued.

It is well known that apart from antibodies which react exclusively with either dsDNA or ssDNA others, which crossreact with both dsDNA and ssDNA also exist. For this reason it was believed that in order to ensure the specificity of the assay for dsDNA (and therefore for SLE) care was necessary to

ensure that the DNA used in the assay was free of single-stranded regions.

It has been shown however that it is technically difficult to prepare and to maintain preparations containing pure dsDNA (81,82). Even millipore filtration just prior to assay does not remove all single-stranded regions (83). In addition the assay method used to detect the antibodies may influence the results obtained. Claims of elevated dsADA occurring in some cases of Sjogren's syndrome (72), chronic active hepatitis (84), CDLE (85), uveitis (86) and RA (87) must therefore be viewed in the light of these observations (88).

In view of these difficulties the use of alternate forms of DNA has been proposed and include heat-denatured sonicated ssDNA of known molecular weight (89), a more stable circular DNA (90) or synthetic dsDNA (91).

The inconsistency between disease activity and ADA levels has raised a further possibility namely that the pathogenicity of the ADA may be related to the qualitative properties of the ADA (namely the immunoglobulin class (92), the avidity (93,94) or the

precipitating or nonprecipitating ability (93)) rather than the actual circulating levels. These properties are discussed in greater detail elsewhere. Another factor that must be considered is that if disease activity is associated with the formation and deposition of DNA-ADA immune complexes (IC's), then episodes of activity should be associated with decreasing rather than high or rising levels of ADA. Many autoantibodies other than ADA are found in SLE sera (95). It is therefore possible that additional non-ADA-containing IC systems may participate in the pathogenesis of the disease.

d) Crithidia Luciliae Immunofluorescent Assay for dsADA

This assay has been developed recently and is based on the detection of dsADA using the kinetoplast of the haemoflaggelate Crithidia luciliae which consists almost entirely of dsDNA (96-98). The assay is thus specific for dsADA, and the antibody class and titre can in addition be determined. It is, however, less sensitive than the Farr technique.

e) Complement

Many studies have demonstrated an association between complement depletion and clinical

disease activity, particularly when renal involvement is present (66,71,99-102). There is evidence that complement activation in SLE occurs by both the classical (103) and alternate (104) pathways and that fixation by the IC's of complement results in the development of hypocomplementaemia. While total haemolytic complement (CH_{50}), C_1q, C_3 and C_4 may all be depressed at some stage, C_4 levels are of particular value as they are the first to fall with activity and the last to return to normal levels with remission (101). Hypocomplementaemia and high levels of ADA imply smouldering, subclinical IC disease and are regarded by some as an indication for therapy (79). There is as yet no uniformity of opinion on this matter since periods of hypocomplementaemia, sometimes of long duration, may revert to normal levels spontaneously without apparent clinical deterioration. A conservative approach to therapy, based on the clinical picture and guided by histology in the case of the kidney, rather than serologic abnormalities seems appropriate at present.

f) Lymphocytotoxic Antibodies

An overall prevalence of lymphocytotoxic antibodies (LCA) in up to 80% of patients

with SLE has been reported (105-107). In the study by Bortolotti and his colleagues (106) LCA were found in 100% of patients with active disease and in 52% of those in remission. These antibodies are however not specific to SLE and have been reported in other connective tissue disorders, in viral infections such as infectious mononucleosis and measles, and in tuberculosis (reviewed by Dawkins et al (108)).

LCA has also been documented in between 39% and 60% of consanguineous and nonconsanguineous relatives of patients with SLE, in their household contacts and amongst laboratory personnel handling SLE blood (105,107, 109), as compared to a prevalence of 15% in normal subjects (107).

Utsinger (110) found a correlation between lymphopenia and SLE in a study of 40 patients. Lymphopenia was greater during clinically active disease and showed a strong correlation with LCA, hypocomplementaemia and ADA. Bortolotti (106) on the other hand found that lymphocytotoxins tend to persist in the circulation for several months after episodes of activity and he does not regard their presence as a suitable marker of activity.

Zvaifler and Bluestein (111) were unable to demonstrate a correlation between LCA in cryoprecipitates and either the severity of SLE or the presence or absence of active lupus nephritis.

Goldberg et al (112) made the interesting observation that LCA have specificity for the i antigen which is present on lymphocytes and cord erythrocytes. It is known that cold haemagglutinins with i specificity are capable of inducing haemolytic anaemia. Thus this crossreactivity between LCA and cord erythrocytes might explain the observation by Butler et al (113) that LCA are found more commonly and in greater titre in patients with haematologic abnormalities.

Cerebral lupus is notoriously difficult to diagnose serologically. It is hoped that the suggested relationship between LCA and neurological involvement (113,114) will be borne out in further studies.

The role of LCA in SLE has not been resolved. Family studies showing an association of LCA in patients with SLE, their relatives, including nonconsanguineous relatives and other household contacts suggest that an

environmental factor apart from genetic influences may be relevant to their development. The practical value of determining LCA remains to be established. They appear to be too nonspecific to be of use diagnostically and their value as a parameter of activity needs to be confirmed in further studies. Their role in the pathogenesis of the disease also requires further study. It is possible that they reflect an antiviral response and that by their action on cell membranes may lead to complement-mediated cell injury with the release of nuclear antigens (113).

g) Extractable Nuclear Antigen

Extractable nuclear antigen (ENA) is a mixture of soluble nuclear antigens, two of which have been well characterised namely ribonucleoprotein (RNP) and a non-nucleic acid protein antigen (Sm) (115). The value of measuring these antigens in general terms lies not in the diagnosis of SLE or as an index of disease activity but rather to identify MCTD, a subset of SLE (29,116).

h) Immune Complexes

Measurement of circulating levels of IC's would appear to be the most rational approach

in the search of a marker of disease activity. A number of studies investigating this avenue have appeared in the literature recently with encouraging results (117-126). There are however a number of problems, methodological and other, which have to be solved before any meaningful conclusions can be drawn. These are discussed on pages 51-62.

In summary, a number of immunological tests are available for the diagnosis of SLE. Some of these tests are also useful adjuncts in the assessment of disease activity. Recognising their limitations, investigations directed at the particular organ involved should always be carried out. In the case of the kidney assessment of renal function, the degree of proteinuria and the examination of the urinary sediment are still of major importance, while the decision of whether or not to treat depends almost entirely on the histological appearance (127,128).

In cerebral lupus the problem of diagnosis and the assessment of activity are particularly thorny as there is very poor correlation between cerebral involvement and the usual monitors (129-132). Even serological changes in the cerebrospinal fluid have yielded conflicting

results. This may be due to the fact that different pathogenetic mechanisms to the rest of the lupus syndrome are probably involved. Of interest therefore are two recent papers describing the presence of an antineuronal IgG antibody in cerebral lupus (133,134). It is hoped that this will provide a new serological guide in the diagnosis and assessment of activity in cerebral lupus.

3 ETIOLOGY OF SLE

A large body of clinical and experimental evidence is currently available which favours four major factors in the etiology of SLE.

(i) GENETIC

A genetic predisposition to the development of SLE is suggested by the higher prevalence of SLE itself, other connective tissue disorders and of "silent" serological abnormalities such as ANA, in close relatives of lupus patients (135-137). A few instances of SLE occurring in monozygous twins (138) and the development of lupus-like syndromes in subjects with inherited complement component deficiency states (59,139) have been documented.

Early studies attempting to link SLE with specific

genetic markers of the human leucocyte antigen (HLA) system have yielded interesting but discordant data. Nies et al (140) reported an increased incidence of HLA-A5 amongst black Americans with SLE while in other series (141,142) an association between HLA-B8 and SLE was found.

It has recently been shown (143) that in families with multiple cases of SLE, either HLA-A or -B haplotypes may be present in the individual family units. This suggests that a closely-linked genetic factor rather than HLA-A or -B may be associated with the disease.

Against this background the findings of the 7th International Histocompatibility Workshop in relation to the HLA-D region and SLE are of particular interest (144,145). HLA-DRw2 was present in 51% of patients with SLE and 26% of controls, while HLA-DRw3 was present in 49% of patients with SLE and 22% of controls - a striking increase of these antigens in SLE. No significant increase in HLA-A, -B or -C was found in 39 patients with SLE studied. In view of these findings it has been suggested that since HLA-B8 and HLA-DRw3 are commonly co-inherited, the association between HLA-B8 and SLE is apparent rather than real and that SLE should more cor-

rectly be linked with HLA-DRw3. Further support for the importance of the HLA-D region as a genetic marker in SLE has been derived from family studies by the same group and also provide further evidence that co-inheritance of two or more genes in linkage disequilibrium with genes determining HLA-DRw2 or -DRw3 may be necessary for clinical disease to be expressed.

It must however be pointed out that while these genetic markers may provide the soil for the development of SLE it is likely that additional genetic or environmental factors are necessary for full expression of clinical disease.

The latter concept is illustrated in the study by Lowenstein and Rothfield (137) in which Ig, C_3, C_4 and properdin deposits at the dermo-epidermal junction were found in close contacts of patients with SLE, even in nonconsanguineous relatives, and in the study by De Horatius et al (109) of lymphocytotoxic antibodies.

(ii) VIRAL

A large volume of literature has accumulated over the last decade or more on the possible role of viruses in the etiology of SLE. While there is no firm evidence to implicate viruses either as

initiating or as perpetuating agents in the disease, several important contributions in the field have been made.

It has been shown in animal studies that chronic viral infection can lead to immune complex disease while NZB/W F_1 hybrid mice all of which carry a Gross type leukaemia virus develop an autoimmune disorder with many similarities to human SLE (146,147). The fact that the complexes in NZB/W mice are DNA-ADA in nature rather than viral antigen-containing suggests that additional factors, possibly genetic, are necessary for the development of autoimmunity. The discovery of a disease in dogs similar to human lupus, and transmissible by cell free extracts (148) further supports a viral etiology for SLE.

Tubuloreticular and paramyxovirus-like structures have been demonstrated in the renal glomerular basement membrane of patients with SLE (149). It remains to be established whether these inclusion bodies represent viral material or whether they are products of chronic tissue damage, since similar inclusions have been demonstrated in a number of other conditions and in normal subjects. These inclusions are however found more frequently and in greater quantities in SLE.

A number of studies have shown an increase in viral antibody titres in patients with SLE as compared to controls. These include antibodies to reovirus double-stranded ribonucleic acid (150), type C RNA (151), paramyxo (152), Ebstein Barr (153) and measles viruses (154-155).

In another study antiviral antibody titres were significantly increased in relatives of patients with SLE when compared to controls (156). No association between hepatitis B virus and SLE was demonstrated in a study by Shorey et al (157). An interesting observation was made by Johanssen and colleagues (158) that while warts were shown to occur more commonly in a group of patients with SLE (25/26) than in a control group (19/160), wart virus antibodies were found significantly less often in patients with SLE. They postulate that some immune deficiency in the lupus subjects may account for these findings.

It is not clear how to interpret the relationship between SLE and viral antibodies from these studies. A direct causal relationship may be implied while on the other hand the increased incidence of these viral antibodies may simply be a reflection of the underlying immunological hyperreactivity common to many autoimmune diseases. No etiological relationship between SLE and other infectious

agents, such as bacteria, has been established.

(iii) ENDOCRINE

There is a marked predilection of SLE and other connective tissue disorders for females. In adults the sex ratio is 9 females to 1 male (21) while the sex difference for prepubertal SLE is less striking being in the order of 2-3 to 1 in favour of females (159). This association together with experimental evidence that females are more resistant to certain bacterial infections (160) led many investigators to study the effect of sex on the immune system. A large volume of data has accumulated confirming a profound effect of sex on both humoral and cellular immunity and on the development of autoimmunity. This data with particular reference to SLE will be reviewed.

a) Humoral Immunity

A hyperreactive humoral response based on antibody titres has been observed in females on exposure to E coli (161),brucella (162) and measles (163). In an earlier study immunoglobulin (Ig) levels and in particular IgM were found to be higher in females than in males (162), while family studies showed that the genes for IgM production are present

in the X chromosome (164). Higher levels of IgM have also been demonstrated in women on oral contraceptive agents (165) and in younger rather than older women (162). Furthermore oestrogens administered to mice result in an augmented antibody response to sheep red blood cells (RBC's) (166) while females on dialysis who become infected with hepatitis B virus tend to develop antibodies to Hb_sAg and become antigen-negative whereas males on dialysis tend to become chronic Hb_sAg carriers (167).

b) Cellular Immunity

Cellular immunity by contrast appears to be depressed in females compared to males. It has for example been shown in cytotoxicity studies that lymphocytes from normal females are half as effective as from males (168). Depressed phytohaemagglutinin-induced (PHA) lymphocyte transformation has also been demonstrated in females taking oral contraceptives (169), while oestrogen and progesterone have been shown to suppress the stimulatory effect of PHA and purified protein derivative (PPD) on human lymphocytes (170).

c) Autoimmunity and SLE

Not only is there a higher prevalence in women of SLE and other connective tissue disorders, but also of a variety of auto-antibodies (171). Acute attacks of SLE precipitated by oral contraceptive agents have been reported (172). Murine lupus, a disorder of NZB/NZW F_1 mice which bears a close resemblance to the human counterpart runs a more aggressive course with earlier onset of glomerulonephritis, renal failure and death in female mice. In a number of experiments designed to alter the sex hormone status of both male and female mice either by castration or by the administration of oestrogens or androgens to males and females respectively it was shown that severity of disease was related to the presence of oestrogens (173-175). It has not been established whether SLE is commoner in Klinefelter's sydrome (176) but in a study on the hormonal status of two patients with the condition (177) augmented oestrogenic effects were shown. It was suggested that this might influence the development of SLE in these individuals.

There is thus ample evidence that females have hyperactive humoral immunity, depressed

cellular immunity and a greater predilection to the development of autoimmune disorders than males.

(iv) IMMUNOLOGICAL

The mechanisms responsible for the abnormalities of T and B cell function in SLE and the inter-relationship between viral, genetic and hormonal factors in bringing about these aberrations are incompletely understood. In two excellent recent reviews Steinberg (178) and Williams and Bankhurst (179) discuss the subject in the light of more recent developments.

The absolute numbers of T and B cells are reduced in SLE especially during periods of disease activity (180). Anti-T cell antibodies may partly account for the reduced numbers of T cells. However, the major T cell abnormality in SLE appears to involve specifically the suppressor T cells (181,182). The defect may not simply be a reduction in the generation of T cells but rather a defect in suppressor signal production.

Since suppressor T cells are thought to play an important role in immunologic regulation and in the prevention of autoantibody production by

their effect on B lymphocytes, their absence or a defect in their ability to respond to B cell signals would result in uncontrolled proliferation of B lymphocytes.

There are many unanswered questions but from the evidence presented there is little doubt that genetic, viral and hormonal factors have a marked influence on the immune system in SLE. The end result is that of a semiautonomous situation of uncontrolled B cell proliferation, autoantibody production and a state of autoimmunity.

4 EVIDENCE THAT SLE IS AN IMMUNE COMPLEX DISEASE

There is considerable evidence that SLE is an IC disorder. This is derived from many detailed immunofluorescence and ultrastructural studies of renal tissue, analysis of glomerular eluates and from complement, antigen, antibody, and IC studies in serum.

(i) THE KIDNEY

The kidney is ideal for the study of SLE. Apart from the fact that biopsy material for sequential studies can be obtained with relative ease it has been shown by immunofluorescence studies that evidence of renal involvement in SLE can be found in almost 100% of cases (183).

a) Immunofluorescence

Deposits localised along the glomerular basement membrane contain IgG, IgM, C_1q, C_3, C_4 and DNA (184,185). The granular distribution of these deposits is typical of an IC disorder and while fine in the initial stages of the disease usually become coarser as the disease progresses (69).

b) Electronmicroscopy

Ultrastructural studies provide indirect evidence that these glomerular structures are IC in nature since they are electron dense and therefore unlikely to be fibrin (186,187).

c) Acid Elution/DNase Digestion

Acid elution and DNase digestion studies of glomerular material have isolated immunoglobulins with antibody activity to ds- and ssDNA, ENA and nucleoprotein (188,189). Whether the presence of ssDNA in the eluates is merely due to denaturation of dsDNA after IC formation or whether de novo ssDNA complexes are formed in the circulation has not been resolved. Koffler et al (190) believe that ssDNA IC's are formed in the circulation and constitute a second important IC system in SLE.

(ii) THE BRAIN

By contrast the role of the IC in the genesis of cerebral lupus is less clear. The lack of correlation between the neurological manifestations and disease activity with the usual serological parameters, unless another organ is involved concomitantly, has led to the view that cerebral lupus is pathogenetically a distinct entity.

There is however some evidence that IC deposition does occur. Atkins et al (191) demonstrated the presence of gamma globulin deposits in the choroid plexus of some patients with cerebral lupus. The staining pattern of these deposits diminished markedly after DNase digestion suggesting that the complexes involved were specifically DNA-ADA in nature. IgG deposits were demonstrated by Sher and Pertschuk (192) in the choroid plexus of a child with SLE and in NZB/W mice by Lambert and Oldstone (193). Carr et al (120) demonstrated DNA-ADA complexes in the serum and cerebrospinal fluid of a patient with lupus meningitis. Renal involvement in the absence of histological evidence could not be entirely excluded in this case and, while of interest, no conclusions can be drawn from this single case report.

It is hoped that the recently described association

between antineuronal and lymphocytotoxic antibodies with cerebral lupus will shed more light on its pathogenesis (113,114,133,134).

(iii) THE SKIN

While skin manifestations are common in SLE the histologic features in the majority of cases are nonspecific.

Immunopathological studies similarly have failed to demonstrate any features specific to SLE, but have nevertheless provided evidence to support an IC basis for the lesions by the demonstration of Ig and complement deposition at the dermo-epidermal junction in 80-100% of patients (194-196). Of interest is the fact that similar deposits may be present in nonlesional skin particularly during periods of disease activity, in relatives (including spouses) of patients with SLE and in technicians handling lupus sera. Electron dense deposits similar to those seen in the glomeruli are present at the dermo-epidermal junction.

Immune complex deposition has also been found in blood vessels (197) and in the spleen (198).

(iv) SERUM

Further evidence that SLE is an IC disorder is

derived from the fact that all the components of the IC can be demonstrated in the serum of most patients with SLE at some stage during the course of the disease. These include a variety of auto-antibodies directed against nuclear and cyto-plasmic constituents, notably antibodies to DNA and a reduction in serum total haemolytic comple-ment and the C_3 and C_4 components of complement, especially during episodes of disease activity. It is also possible to detect soluble IC's and free DNA itself in the serum of patients with SLE. These are discussed in greater detail elsewhere.

Interesting data has emerged from studies of cryoprecipitates found in about 35% of patients with SLE. Winfield et al (199) have shown that cryoprecipitates contain a high concentration of ADA with respect to that of the serum in SLE. Antibodies to ribonucleoprotein were also isolated from cryoprecipitates but less frequently and not in the concentrated form. C_1q has been found in the precipitates and appears essential for cryo-precipitation to occur. Thus cryoprecipitates provide a convenient source of antibody for the study of IC's in SLE.

Davis et al (200) in a subsequent study confirmed the findings of Winfield and in addition reported

the presence of significant quantities of DNA in cryoprecipitates. This latter observation however has not been borne out by other studies.

In a recent review of the subject Lightfoot (201) makes the point that while the presence of IC's in lupus cryoprecipitates is not in dispute, the lack of demonstrable cryoproteinaemia in most patients with lupus nephritis raises some doubt as to their relevance in the pathogenesis of the disease.

5 ANTI-DNA ANTIBODIES

While little is known of the role of DNA-ADA complexes in SLE, numerous investigations suggest that the qualitative properties of the antibodies which participate in the formation of IC's are of considerable importance in determining the pathogenicity of complexes.

(i) SPECIFICITY

The clinical significance of ds- and ssADA and the role of these and other autoantibodies in IC formation has been discussed.

(ii) IMMUNOGLOBULIN CLASS

An association exists between the class of antibody in the circulation and the presence of renal

disease. IgG - 1 and IgG - 3 (185) the main complement fixing antibody classes tend to produce nephritis, while IgM antibodies which are not complement fixing do not.

(iii) AVIDITY AND PRECIPITABILITY

The interaction between antibody and antigen is of fundamental importance in the humoral immune response and has been intensively studied in SLE, particularly in relationship to the development of renal lesions.

It was generally accepted that low avidity non-precipitating antibodies are associated with the development of renal lupus due to their persistence in the circulation, while high avidity precipitating antibodies are rapidly cleared in the form of complexes. Conflicting results, however, have been reported from various studies.

Steward et al (202) found that non-precipitating low avidity antibodies were associated with glomerulonephritis in NZB/W mice and Kuriyama (203) related low avidity antibody to the development of membranous and high avidity antibody to membranoproliferative lesions in rabbits.

Koyama and his colleagues (204) found antibody

with higher avidity in the sera of a group of patients with active renal lupus than in those with inactive disease and in a group without nephritis. In those with high avidity antibody IC deposition was predominantly subendothelial and subepithelial in those with low avidity antibody. The latter findings are in agreement with a subsequent study of 10 female patients with SLE by Asano and Nakamoto (205).

Transformation in renal lupus from one histological pattern to another, although uncommon, is well documented (127,128). An interesting correlation between histological transformation and changing antibody avidity was reported by Asano and Nakamoto (205). A patient with mesangial lupus nephritis with high avidity antibodies in the serum subsequently developed membranous changes preceded by the appearance of low avidity antibodies in the circulation. No relationship between antibody avidity and renal morphology was demonstrated in the study by Tron and Bach (75).

However in two studies (93,206) an association between high rather than low avidity dsADA and the presence of lupus nephritis was shown, while in another study Winfield et al (94) were unable to show any difference in the avidity of ssADA in

patients with lupus nephritis or active SLE without nephritis as opposed to patients with active nephritis. They postulate that the discrepant results in various studies may be due to the more rapid clearance from the circulation of high avidity antibody in the form of complexes. These may therefore escape detection while lower avidity antibodies which persist for longer periods are more easily detectable. Thus the reported association of low avidity antibody with renal lupus may be apparent rather than real.

The theory that lupus nephritis develops in the presence of non-precipitating antibodies must also be challenged following a report by Cameron et al (78) in which they found precipitating antibodies in one third of sera from 53 patients with lupus nephritis. Some of the patients with severe renal involvement showed the greatest precipitation. They concluded that the presence of precipitating antibodies does not necessarily protect against the development of nephritis.

A strong association between cutaneous vasculitis and the presence of precipitating antibodies is suggested since the latter were found in 11 out of 12 patients in two series (77,78).

Cameron suggests that avidity studies on material eluted from biopsies or from the kidneys of patients dying from nephritis may clarify the question of antibody avidity and precipitability in SLE.

6 DEOXYRIBONUCLEIC ACID

(i) CIRCULATING DNA IN HEALTH AND DISEASE

Circulating DNA has been reported in a wide variety of unrelated conditions such as SLE (207-211), RA (208,210,211,212), malignancy (207,211), liver disease (207,213) and in infective endocarditis, pulmonary embolism and after surgery (213). In addition elevated levels have also been reported after cardiac surgery involving periods of extracorporeal circulation (213), after haemodialysis (214), in patients on high dose corticosteroid therapy (215) and in response to the injection of bacterial lipopolysaccharides (216).

In some of the above conditions it is claimed that the levels of circulating DNA found are pathologically elevated; however, there is a wide divergence of opinion as to what constitutes the normal levels of circulating DNA, levels ranging from less than 50 ng/ml to as high as 13,8 µg/ml having been reported (207,208,210-213, 217-219).

Confirmation of elevated 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 due to lack of specificity in the assay method, and also depends on whether the assay is performed on plasma or serum (213,219,220). Davis and Davis (213) showed that DNA may be released into serum during the process of coagulation or during separation of the clot from the serum. Similarly Steinman (219) in comparing four assays for the measurement of DNA in plasma and serum found that DNA was undetectable in plasma while a mean level of 1,9 µg/ml was detectable in serum. On the basis of these findings it was concluded that serum is unsuitable for the determination of circulating DNA levels.

(ii) SOURCE OF DNA

It has been suggested that while the DNA may be of exogenous (for example viral) origin (221-223) in the majority of cases it is probably of endogenous origin from tissue injury (traumatic or inflammatory) and as such represents a nonspecific phenomenon (207,209,215)

It is interesting to speculate on the role of leucocytes as a source of DNA in the circulation.

It is well known that their turnover is increased in pathological states such as inflammation or injury, but in addition a number of "physiological" factors are responsible for the normal diurnal variation in the leucocyte count (224). These include physical and emotional stress, pregnancy and exposure to sunlight. These factors may also precipitate acute attacks of SLE. It is possible that this phenomenon may be related to the increased turnover of leucocytes during episodes of physiological (or other) leucocytosis with the release of increased quantities of DNA into the circulation of patients with SLE, with IC formation. Whatever the source of the DNA, in SLE patients with circulating ADA, the presence of DNA in the circulation provides an opportunity for IC formation.

(iii) ANTIGENICITY OF DNA

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.

Why DNA should become antigenic in patients with SLE is unexplained. It is possible that antibodies produced to viral nucleic acids merely crossreact with human DNA since it has been shown

that ADA in lupus sera will crossreact with DNA from many sources (225).

Alternatively interference with DNA breakdown may render it immunogenic. It has for example been shown that in 50% of patients with ANA a heat-liable inhibitor to deoxyribonuclease (DNase) is present (226).

ssDNA on the other hand is a more potent immunogen than dsDNA. Levine et al (227) found that rabbits immunised with ultraviolet-irradiated (UV) DNA produced antibodies to unaltered purines and pyrimidines and to thymine dimers. The immunogenicity of UV-irradiated DNA was confirmed by Tan (228) in a subsequent study.

It has also been shown that thymine dimers are resistant to DNase digestion (229). It is also possible to induce thymine dimer formation in the cell nuclei of skin (230), and in a most interesting experiment Natali and Tan (231) having induced high circulating levels of antibodies to thymine dimers in mice, subjected them to whole body UV-irradiation. Skin biopsies taken subsequently revealed changes virtually identical to those at the dermo-epidermal junction of human SLE.

There is also some evidence that irradiated DNA induces the formation of antibodies, usually of low titre, which crossreact with dsDNA (230,232).

The important fact which emerges from these and other experiments is that the UV spectrum producing alterations in DNA structure with increased antigenicity encompasses the sunburn wavelength. The relevance of this finding is important in relation to SLE where exposure to sunlight may in some cases precipitate acute attacks, and needs to be further explored.

(iv) CLEARANCE OF DNA FROM THE CIRCULATION

Natali and Tan (233) showed that the clearance of UV-irradiated and native ^{3H}DNA administered intravenously to normal rabbits was similar in that less than 10% of the radiolabel was detectable after 60 minutes. After the injection of large amounts of UV-DNA to hyperimmunised rabbits some radioactivity was still detectable after 60 minutes. No comparison however was made of the disappearance characteristics of the two forms of DNA in these animals.

Tsumita and Iwonga (234) showed that DNA is rapidly cleared from the circulation due mainly to excretion by the kidney but that a small amount

is trapped in the liver.

In another study (235) a more rapid clearance of nucleic acids (dsDNA, ssDNA and poly I-C) was demonstrated in mice with circulating antibodies than in those without. In addition uptake by the liver and spleen was noted.

Barnett (236) demonstrated that most of an administered dose of ^{125}I ss- or dsDNA to normal and immunised rabbits was cleared by approximately 10 minutes but that the clearance was somewhat slower in the immunised rabbits. In addition there were also qualitative differences in the clearance of ss- and dsDNA. A secondary rise of radioactivity in the circulation was shown which was attributed to the release of DNA which had been taken up by some area of the body. Autopsy studies of the animals showed that this uptake was confined to the liver and spleen.

These studies therefore indicate that DNA is rapidly cleared from the circulation by renal excretion but that some is trapped, at least temporarily, in the liver and spleen. A difference in clearance rates of nucleic acids from normal and immunised animals has also been demonstrated. The difference in clearance in ss- versus dsDNA may indicate immunogenicity of the

former and non-immunogenicity of the latter.

(v) METHODS FOR THE DETECTION OF CIRCULATING DNA

These include:

1 The Diphenylamine reaction (237,238)

2 Immune precipitation by double diffusion in agarose (239)

3 Complement fixation (208)

4 Fluorimetry using ethidium bromide (217)

5 Counterimmunoelectrophoresis (CIE) (213)

6 RNA-DNA hybridisation (220)

7 Radioimmunoassay (RIA) (211,218)

Some of these assays, such as complement fixation are indirect measurements of DNA. Furthermore many of the assays lack either specificity or sensitivity or both. Thus nonspecific protein binding (218) or the presence in biological fluids of substances which interfere with assays based on colorimetry or fluorimetry, such as serum chromogens in the diphenylamine method have resulted in falsely high DNA values. In most cases the sensitivity of these assays is limited to the μg range. The RIA and modified CIE techniques are currently the most sensitive methods, the lower limit of detection being about 50 ng/ml of circulating dsDNA. Immunological methods such as these, while sensitive have

the disadvantage that only sterically unhindered antigenic sites are detected, so that in crude nucleoprotein preparations only 1% to 5% of the total DNA may be measured (240). The RNA-DNA hybridisation assay overcomes this problem and in addition provides information about the base sequence (and therefore the origin) of the DNA assayed. Steinman, however, notes that the high specificity of this technique may impose limitations on its usefulness in that it may not be applicable where the base sequence of the DNA is unknown. It is therefore useful where a qualitative and highly specific assay of DNA is required but, due to its complexity is unsuitable as a routine laboratory procedure.

7 THE IMMUNE COMPLEX

While dsDNA-ADA complexes are virtually exclusive to SLE the likelihood that ssDNA-ADA and other IC systems also participate in the pathogenesis of the disease has been alluded to. Apart from the antigen responsible for the formation of the IC, other factors including the properties of the IC formed, their rate of clearance from the circulation and various tissue factors are equally if not more important in the initiation of tissue damage.

(i) SITE OF FORMATION

The fundamental issue of whether immune complexes are formed primarily in the circulation and subsequently filtered out in tissues or whether de novo formation in tissues occurs has not been fully resolved. The fact that soluble IC's can be measured in serum suggests that intravascular formation occurs. On the other hand a number of interesting observations have been made to support the theory that local formation of immune complexes also occurs. Izui et al (241,242) recently demonstrated that free DNA binds readily to collagen in the glomerular basement membrane raising the possibility that DNA binding may occur locally as a primary event followed by antibody deposition and IC formation. They showed in addition that ssDNA has a greater affinity for collagen than dsDNA so that complexes of the former tend to occur locally while the latter forms complexes predominantly in the circulation. Should this hypothesis be correct it follows that the nature of the circulating DNA is of importance. Davis et al (200) in a discussion on the mechanisms in DNA-ADA mediated nephritis in SLE suggest that in a situation of antigenic excess DNA readily binds to collagen. Should an anamnestic response ensue ADA becomes bound to DNA with

resultant local IC formation in tissues. Alternatively where elevated levels of circulating ADA already exist, intravascular formation of IC's occurs followed by secondary entrapment along the glomerular basement membrane or by clearance from the circulation by the reticulo-endothelial system (RES).

It is likely therefore from the available evidence that both intravascular and local formation of IC's occur. The extent to which intravascular formation occurs and the nature of the immune complex formed are of importance in determining the therapeutic efficacy of manoeuvres such as plasmaphoresis or extracorporeal circulation (243). These forms of treatment would theoretically only be rational if significant intravascular formation of immune complexes occurs, unless tissue-bound complexes are released into the circulation.

(ii) SIZE

The size of an IC is dependant on the nature and properties of its constituent antigen and antibody and also on the relative concentration of antigen to antibody. Thus complexes are generally larger at equivalence, that is, where an equal number of antigen and antibody mole-

cules are present in the circulation at the time of IC formation. The valency of the antigen and antibody will also determine the size of the IC, the higher the valency, the larger the complex formed. If the antibody is rendered univalent by enzyme digestion, or if the antigen intrinsically contains a small number of determinants, or is relatively tolerogenic, smaller complexes will result (201).

There is evidence to suggest that the size of the IC has clinical relevance. It has for example been shown in mice and in humans that small IgG complexes ie $1\text{-}1.5\times10^6$ daltons are frequently associated with lupus nephritis while larger complexes, $2.5\text{-}5.0\times10^6$ daltons, as might occur if only IgM is present do not produce renal disease (244).

It is important to appreciate in studies of this nature that most IC assays are capable of detecting large more readily than smaller complexes (245). Thus failure to detect IC's in patients with SLE does not necessarily imply their absence.

The importance of the size of IC's in determining their clearance for the circulation is discussed below.

(iii) CLEARANCE

The RES and mononuclear phagocytic systems serve an important function in eliminating IC's from the circulation (246). The facility with which this occurs depends not only on the integrity of the RES (which may be ineffective due to "fatigue" (247), ageing (248) or a genetic factor (249)) but also on the nature of the IC to be cleared. Large complexes are more efficiently eliminated from the circulation by the RES than smaller complexes. Smaller complexes thus tend to remain in the circulation for longer periods of time and are more liable to be deposited in tissues and to produce tissue injury.

A recent contribution by Miller and Nussenzweig (250) proposes a role for C_3b and possibly C_4b in splitting membrane-bound IC's. This is thought to occur via the alternate pathway by the insertion of these components into the antigen-antibody lattice resulting in the formation and release of smaller complexes. The biological properties of these solubilised complexes are altered in such a way that they have a lower binding affinity for platelet and leucocyte membranes. As a result they are less likely to mediate an inflammatory response through the release of vasoactive amines and lysosomal enzymes from these cells. Further

evidence for the role of complement in solubilising membrane-bound complexes is derived from studies which demonstrate a delayed clearance of complexes from the glomeruli of hypocomplementaemic rabbits with acute serum sickness (251).

(iv) TISSUE FACTORS

The vessel wall acts as a filter which retains large complexes and allows smaller complexes to pass through. This is especially true of the glomerulus which may be regarded as a sieve for molecules of varying sizes (252). Depending on the severity of the glomerular involvement the characteristics of the sieve may be altered, but in general terms relatively large complexes are trapped in the mesangial and subendothelial areas, intermediate complexes in the glomerular basement membrane and small complexes under the epithelial foot processes. It has also been postulated that the mesangium may be the initial site for IC deposition and that overflow along the glomerular basement membrane occurs only after this barrier becomes saturated.

The situation regarding DNA-ADA complexes in SLE has by no means been resolved and is due in part to the paucity of studies investigating specifically their role in the disease. This

again can be attributed to the lack of a currently available sensitive method for the detection of these specific complexes.

(v) IMMUNE COMPLEX MEASUREMENT

As a result of interest in the possible clinical significance of circulating IC's a large number of techniques are currently available for their detection (245,253,254). The methods are based on the physical or chemical properties of the IC and vary markedly in their specificity, sensitivity and reproducibility. Some assays serve merely as screening tests for the presence of IC's while others are unsuitable for routine clinical use due to their complexity and are reserved as research tools.

IC assays may be subdivided according to those which detect specific complexes, that is, where the antigen is known, and those which detect the presence of non-specific circulating IC's.

a) Antigen Specific Techniques

Since it is likely that a multiplicity of antigen-antibody systems may be involved in the formation of IC's, methods which detect specific IC's have a limited application in the routine investigation of a suspected IC

disorder, but have the decided advantage of permitting study in individual disorders, such as SLE.

Detection of Specific IC's include:-

1 Electronmicroscopy

Direct visualisation by electron microscopy has been used to detect aggregated particles of HBs-antigen within IC's in patients with HBs-antigen associated infective hepatitis (255).

2 Physico-chemical

These include:

2.1. the detection of neuroblastoma-specific IC's by radio-counter-electrophoresis (256).

2.2. measurement of the actual complex-bound-antigen as opposed to the free antigen, by measuring the amount of the former removed from the circulation after specific precipitation or absorption of the host Ig's (257).

2.3. Harbeck et al (117) claim to detect specific DNA-ADA IC's by demonstrating an increase in free ADA titres following removal of DNA from the

IC's by DNase I digestion. This phenomenon could not be reproduced by others.

There are, therefore, very few methods available for measuring specific IC's and of particular relevance to this study is the lack at the present time of a generally accepted assay for the detection of DNA-ADA IC's in SLE.

b) Antigen Nonspecific Techniques

1 Tests based on the Physical Properties

The physical properties of an IC differ from those of the free antibody and antigen which constitute it. These differences include an increase in molecular size and alterations in the surface properties, solubility and electric charge. Based on these principles a number of assays for IC's have been developed. These include ultracentrifugation (258), gel filtration (259) and affinity chromatography (260). As a group they lack specificity and are not suitable for routine use. Polyethylene glycol (PEG) precipitation which can be used to separate IC's and aggregated IgG from monometric IgG may provide a

simple screening test (261).

2 Tests Based on Biological Properties

The majority of the tests currently in use are based on the biological properties of IC's. These assays depend on the recognition by the IC of either certain humoral factors or cellular receptors.

2.1. Humoral Factors

The humoral factors on which these assays are based include binding of the IC to:-

2.1.1. the first component of C' (C_1, notably C_1q) which triggers activation of the complement system (262). Measurement of the anticomplementary activity (263) has the disadvantage of poor inter-assay reproducibility and is difficult to quantitate. The test is positive in the majority of patients with active SLE and also in many patients with RA. The C_1q deviation (264) and C_1q latex agglutination (265) assays are limited by their high sensitivity to substances other than

IC material, a problem not encountered to the same extent in the direct binding assays, namely C_1q binding (266) and C_1q solid phase (267) assays. The C_1q binding assay is positive in a significant proportion of SLE and RA sera. The C_1q solid phase assay is claimed to improve sensitivity.

2.1.2. antiglobulins, such as rheumatoid factor. These include the RF agarose precipitation test, radio assays using insolubilised monoclonal RF or soluble polyclonal RF, or inhibition of latex agglutination by RF (268,269). In these assays the presence of rheumatoid factor in the test sample may interfere with the assay.

2.2. Cellular Receptors

A number of functional assays have been devised which utilise the principle that the IC will interact

with cells bearing on their surface receptors for the Fc fragment and C_3b of the complex (270,271). These assays depend on the integrity of the cells used and may consequently be influenced by the presence of cytotoxic factors or by rheumatoid factor, in the test samples. These assays include the platelet aggregation test, inhibition of antibody-dependent cell-mediated cytotoxicity and the Raji cell radioimmune assay, inhibition of complement-dependent lymphocyte rosette formation (272-274).

c) Cryoprecipitates

Cryoprecipitates occur in a variety of diseases and have been shown to contain IC material (275). Cryoprecipitates when present in the sera of patients with RA appear to be a reliable marker for certain extra-articular manifestations of the disease (276). Cryoprecipitates are also found in SLE and provide a useful source of IC in a concentrated form for study.

IC's have been measured by these assays in a

wide variety of clinical and experimental situations. It is perhaps not surprising that in many instances there has been a lack of correlation between the various assays employed and the disease studied, when one considers the widely differing principles on which the assays are based. It is also likely that few assays will detect the same IC. Studies attempting to relate the IC to disease activity thus have to be viewed with caution unless a number of methods are employed.

Most if not all current IC assays fall short of the ideal. The aim should be to develop an assay which is sensitive, relatively specific but with a wide spectrum of activity for IC's, reproducible, relatively simple to allow for standardisation and it should not require heat inactivation or a particular absorption of the sample before testing.

d) Problem of Specificity

One of the major problems is the inability of many assays to differentiate nonspecifically aggregated Ig's from true IC's. The problem is magnified by any procedure which generates nonspecific aggregates such as repeated freezing and thawing, or heating of samples

at more than 56° before testing. Adequate control experiments should also be performed in the evaluation of any assay to detect the presence of substances which may interfere with IC determination (245).

These measures include:

1 The determination of the size of the material detected since IC's are larger than monometric Ig's.

2 The use of manipulations which alter the size and/or biological properties of IC's such as fractionation in well-defined physicochemical conditions and reduction-alkylation.

3 Confirmation that the test becomes negative after removal of Ig's from the tested sample by immunoabsorption.

4 Treatment with DNase to exclude interference with DNA.

5 Testing in the presence of EDTA to exclude the interference of C-reactive protein.

6 The determination of the various classes and subclasses of Ig's present in the IC. This is relevant to the specificity of those assays which are based on the biological properties of the IC.

Until these aims have been achieved the combined use of a number of assays will be necessary in evaluation of IC disorders.

Bruneau et al (121) compared three assays (C_1q precipitation, anticomplementary activity and DNase treatment) in 38 patients with SLE over periods of up to 4 years. Their observations were correlated with clinical activity (especially proteinuria) and other laboratory parameters (ADA and complement levels). Of the three methods measurement of the anticomplementary activity was found to be the most useful clinically.

More recently (1978) a WHO Collaborative Study (277) was undertaken in which 18 tests were compared in a number of diseases known to be associated with circulating IC's, including idiopathic inflammatory disorders, SLE, RA, nephritis, vasculitis, tropical diseases (leprosy, schistosomiasis, onchocerciasis) and cancer.

Six of the assays fulfilled most of the criteria for the ideal complex assay. These were C_1q binding, C_1q solid phase, conglutinin binding, Raji cell test, monoclonal RF inhibition and platelet aggregation tests.

The sensitivity of the 6 assays was comparable and none require heat inactivation or absorption. The Raji cell and conglutinin binding assays were the most sensitive for aggregated Ig's. Most of the assays were found to be specific for IC's containing IgG antibodies except for C_1q binding assay which also detects IC's containing IgM.

The remaining twelve assays tested were considered suitable only for a particular laboratory investigation.

While different results were obtained in the various disease states investigated by these methods, a certain pattern of reactivity corresponding to each of the groups of diseases was noted, suggesting differences in the biological reactivities of IC's. This point may be illustrated from their data showing that higher levels of IC's were present in RA compared to SLE using the C_1q binding and

monoclonar RF assays, while the inverse was true with Raji cell, platelet aggregation and C_1q solid phase assays.

It therefore seems likely that the apparent discrepancies in IC levels using various methods are due to differences in the constitution and biological properties of the IC, according to the clinical situation.

In summary therefore:

1 A large number of methods are currently available for the detection of IC's.

2 Most of the assays are antigen-nonspecific and are based on the physical or biological properties of the IC.

3 The various assays detect different complex material and it is therefore advisable to employ a number of assays when evaluating IC disorders.

4 Inter-laboratory standardisation allows for more meaningful comparative studies.

5 There is a need to develop more antigen-specific IC assays, for example, DNA-ADA

assays in SLE.

8 THE IMMUNE COMPLEX, COMPLEMENT AND TISSUE INJURY

SLE is regarded as a Type III hypersensitivity (278) autoimmune IC disorder in which circulating IC's deposited in affected organs initiate a sequence of events which leads to tissue damage. It bears a strong resemblance to experimental serum sickness (279-281) where the appearance of arteritis and nephritis coincide with the rapid clearance of antigen as antigen-antibody complexes are formed. Deposition of these IC's in target organs results in tissue damage from activation of the complement system and neutrophils (von Pirquet 1911).

Under normal circumstances IC's are rapidly cleared from the circulation. If, however, there is a constant source of antigen or if the clearance mechanisms are ineffective (pp 49-50) the IC may persist in the circulation and become trapped in vessel walls. In rabbits IC deposition is facilitated by an increase in vascular permeability from two mechanisms (280), namely, the release of platelet aggregating factor from sensitised basophils, and from the release of stored histamine from platelets by their interaction with IC's. Whether these reactions occur in the human situation remain to be established. In addition it is likely that hydrodynamic physical forces also

aid in the localisation of complexes in vessel walls. This stems from a study by Fisher and Bark (282) who showed that IC deposition was more intense in hypertensive animals where the transmural filtration pressure is high.

In the kidney there are additional mechanisms which favour the deposition of IC's. These include C_3b receptors (283) and an affinity for DNA of the glomerular basement membrane (241,242), a filtering action in the kidney for trapping IC's (252) referred to earlier.

The IC itself does not cause tissue injury, but once localised, it initiates the process by activating the complement system. This is achieved by the incorporation of the C_1q subunit of C_1 to a specific binding site on the antibody of the complex, namely the CH_2 area of the Fc fragment (284). The results of complement activation are twofold. Firstly, the IC becomes more soluble due to incorporation in the antigen-antibody lattice of C_3b, derived mainly from the alternate pathway and thought to facilitate the subsequent clearance of IC's by the RES. Secondly, and of far greater importance is the generation and release of various pharmacologically active byproducts. C_3a, C_5a and the intermediate complex $\overline{C567}$ are mediators in the inflammatory response by virtue of their strong chemotactic properties (285). Neutrophils

and other cells (macrophages, mast cells, B lymphocytes and platelets) thus accumulate in and around IC's. These cells have on their surface receptors for both the Fc portion of the IC and for C_3b. The IC's become attached to the cells, a process known as immune adherence (281) and may be regarded as the end result of chemotaxis. Immune adherence is usually followed by phagocytosis of the IC during which time a number of hydrolytic and other active materials are released. These compounds include a number of proteolytic enzymes capable of hydrolysing collagen, elastin and basement membranes and further amplify the inflammatory reaction by an action on the prostaglandin, clotting and fibrinolytic systems. In addition C_3a and C_5a have anaphylatoxic properties which release histamine from mast cells and platelets (286).

The end result is tissue damage which encompasses singly or in combination a number of pathological changes such as acute or chronic inflammation, enzymic degradation of connective tissue and necrosis.

SECTION II : D E O X Y R I B O N U C L E I C A C I D

MATERIALS AND METHODS

1 Chemicals

Acrylamide and N, N-methylene bisacrylamide (British Drug Houses, Poole, U.K.) were recrystalised as described by Loening (287). Ribonucleate 3'-pyrimidino-oligonucleotidohydrolase (RNase), E C No 2.7.7.16, and deoxyribonucleate 5'-oligonucleotidohydrolase (DNase), E C No 3.1.4.5., were chromatographically purified preparations (Miles Laboratories, Indiana). Methyl ^{3}H-thymidine and ^{125}I-iododeoxyuridine were purchased from Radiochemical Centre, Amersham, and ethidium bromide from Sigma Chemical Company.

All other reagents were of Analar or equivalent quality.

2 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, leaving a clear 1 ml (equivalent to about 7 mm) 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 taking care 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) isoamyl-alcohol 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).

3 Preparation of DNA Standards

DNA was prepared essentially as described previously (288). The DAB-1 line of rat hepatoma cells, obtained from Dr C Albrecht, was used for its capacity of growing to high cell densities in monolayer culture, thereby giving a high yield of DNA, and because of its good uptake of labelled pyrimidine nucleosides (289). DNA was also prepared on occasions from the CCL_2 line of HeLa cells and from E. coli K12.

Cells were propogated in 75 cm^2 Falcon Flasks in Eagles minimal essential medium supplemented with 10% foetal calf serum (GIBCO) and in the presence of antibiotics. Cultures were monitored for mycoplasma contamination as described previously (290). Cells were labelled just before reaching confluence with 2 µCi/ml methyl ^{3}H-thymidine or 1 µCi/ml ^{125}I-iododeoxyuridine for 1 hour at 37°C. The medium was then decanted and the monolayer washed once with 5 ml saline.

The monolayer was dissolved by the addition of 3 ml 1% sodium dodecyl sulphate in 1 M sodium perchlorate, pH 7.4, and incubated for 30 minutes at 37°C. The viscous lysate was mixed using a rotomixer with an equal volume of chloroform containing 1% isoamylalcohol and saturated with 1 M sodium perchlorate and the phases separated by centrifugation at 2500 rpm for 10 minutes. The aqueous phase was removed and extracted twice more with chloroform reagent or until clear. DNA was precipitated by the addition of 2 volumes of ethanol. The DNA was then dissolved in 0.1 x saline sodium citrate (SSC), made up to standard strength SSC (0.15 M NaCl and 0.015 M trisodium citrate pH 7.0) and treated with RNase, 1 µg/ml for 30 minutes at 37°C followed by a further incubation with 10 µg/ml Pronase for 30 minutes at 37°C. After two further extractions with the chloroform reagent, DNA was precipitated from the final aqueous phase with ethanol and dissolved in

0.1 M Tris-HCl, pH 7.5 and 10^{-4} M EDTA to give a stock solution of about 200 µg/ml. This was aliquoted and stored at -15°C until use.

To prepare a working preparation of DNA the stock was diluted with 0.01 M Tris-HCl, 0.13 M NaCl pH 7.5 to about twice the final working concentration and filtered through a 0.45 µm millipore filter. The concentration was checked by measuring the radioactivity in an aliquot, the specific activity having been previously determined. Additional Tris buffer was then added to bring the working concentration of DNA to 10 µg/ml. The DNA was sheared to a constant size by rotomixing at full speed for 30 seconds. Typical specific activities for ^{3}H-DNA were 30 000 cpm/µg and 20 000 cpm/µg for ^{125}I-DNA. Three or four 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.

4 Polyacrylamide Gel Electrophoresis

This was performed in Tris-phosphate EDTA buffer essentially as described previously (291). An electrophoresis apparatus was designed to hold 20 cylindrical perspex tubes (4 x 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% crosslinked). 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 100V, 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.

5 Quantitation of Fluorescence and Calculation of DNA Concentration

Fluorescent bands were quantitated with a Vitatron 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 three 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.

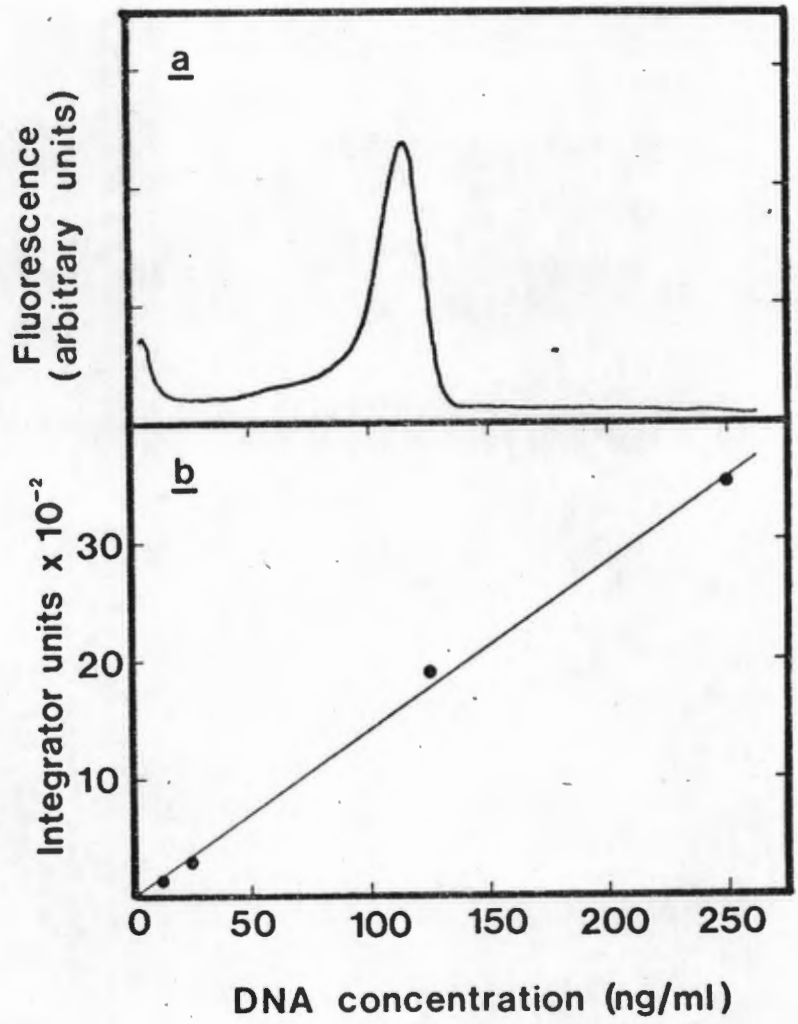


FIG. 1

(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 four 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.

RESULTS

1 Specificity of the Assay for ds-DNA

The staining and mobility characteristics of various preparations of DNA electrophoresed in polyacrylamide gels, are illustrated in the upper panel, Fig. 2. 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 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 only demonstrable as a diffuse smear in the top few mm 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 (291). Plots of this type are illustrated in Fig. 2, lower panel, for the plasma DNA and the standard DNA. Quadratic equations were fitted to the two data sets and standard deviations on the quadratic coefficients were determined.

Statistical evaluation of the two sets using 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.

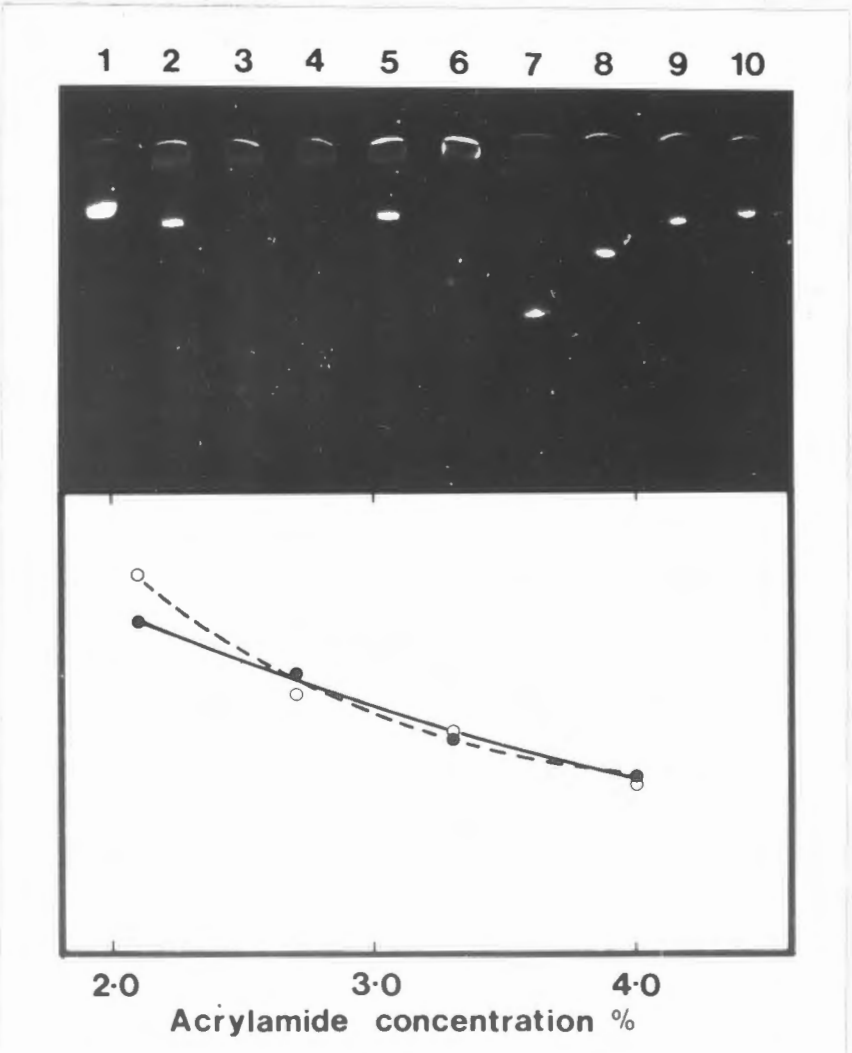


FIG. 2

Upper Panel: PAGE for 2h at 100v of 100 ng standard rat hepatoma DNA (1) or of nucleic acid preparations from either plasma (2,4-10) or serum (3) from one 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 Panel: Gel concentration range analysis of O----O, 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). Quadratic functions were fitted to the two data sets by the method of least squares.

2 Nature and Collection of Blood Samples

The effect on plasma DNA concentrations of using different gauge needles 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 (S.D. $\pm$ 3 ng/ml) which was not significantly different ($p > 0.1$) to the level produced using the heparinised syringe with a 19 gauge needle (63 $\pm$ 4 ng/ml). However, DNA levels were significantly higher in the traumatised sample (126 $\pm$ 33 ng/ml, $p < 0.02$) inferring 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. Cyto-centrifugation (Cytospin, Shandon Elliot), and using May-Grundwald 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 one centri-fugation.

The effect of residual cells on DNA levels, by measuring DNA in the top 2 ml of plasma after a single cycle of centrifugation and compared with DNA levels in the 2 ml below this, leaving 1 ml of plasma undisturbed above the buffy layer is shown in Fig. 3. The top layer of plasma gave a mean value of 114 $\pm$ 75 ng DNA/ml, whereas the lower 2 ml gave a value of 718 $\pm$ 366 ng DNA/ml ($p < 0.001$).

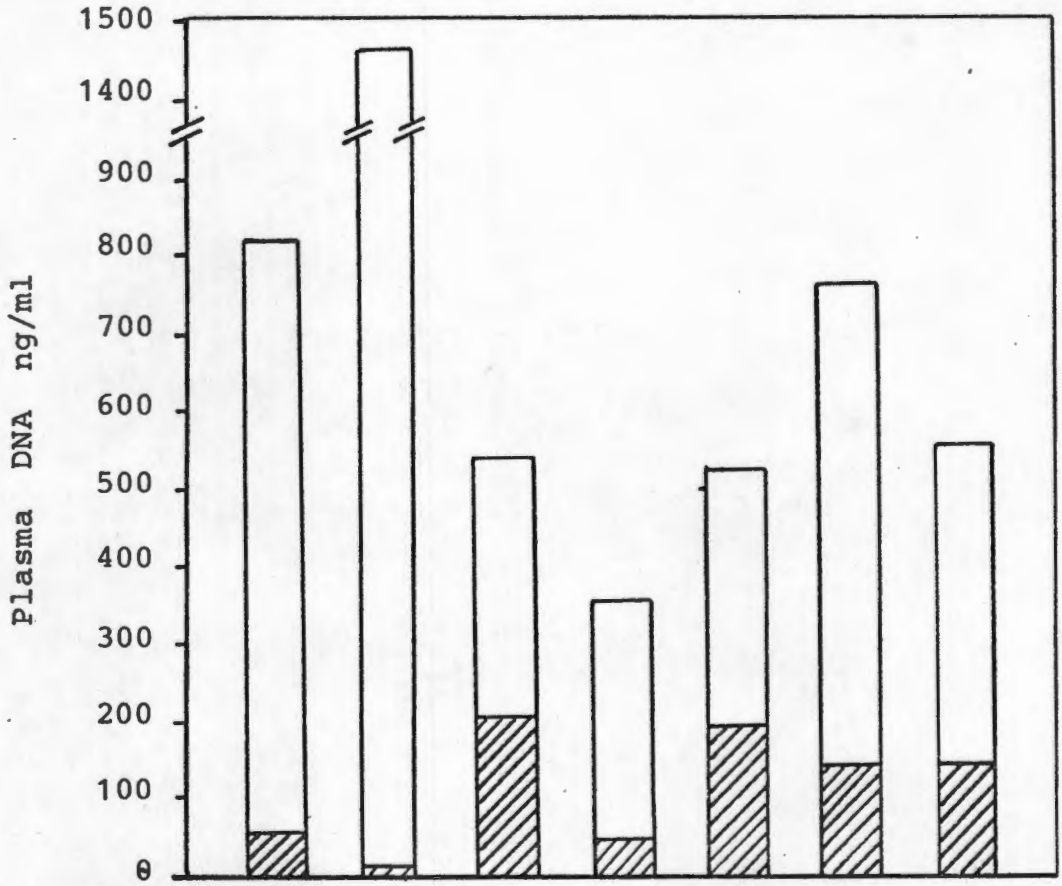


FIG. 3

DNA levels in the lower 2 ml plasma after a single cycle of centrifugation are represented by the open blocks and in the upper 2 ml plasma by the hatched blocks.

3 Stability of DNA in Stored Samples

Selecting a patient with a relatively high concentration of plasma DNA, 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 I). Similarly samples stored at -20°C were analysed at weekly intervals over a period of 4 weeks.

No significant change in DNA concentration was demonstrated under any of the storage conditions described.

TABLE I

Effect on plasma DNA levels of incubation of plasma at various temperatures.

Figures give plasma DNA in ng/ml (mean of duplicate measurements ± 1 S.D.)

	Duration of incubation (h)			
	1	3	6	24
Plasma incubated at 37°C	93±4	96±8	123±21	111±12
Plasma incubated at 20°C	102±2		57±21	96± 8
Plasma incubated at 4°C				90±25
Plasma incubated at -20°C				86± 6

4 Recovery

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 (Table II). The results show that the percentage recovery is in excess of 80% for 50 ng or more but falls appreciably to less than 60% for 25 ng added. Serum treated in similar manner produced no clear bands on the gels.

Only a slight diffuse fluorescence was noted in the top first cm of the gels (compare with Fig. 2, Gel 3).

TABLE II

Recovery of DNA added to plasma before the extraction procedure.

DNA added (ng/ml)	Recovery (%)
25	56
50	89
250	84
500	97

5 Reproducibility

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 µg/ml plasma.

6 Plasma Versus Serum

In 11 subjects DNA levels were measured in both plasma and serum drawn at the same time. In 5 subjects clearly defined DNA bands were visible in the gels of the plasma samples but not in the gels of the equivalent serum samples. Instead, only a diffuse fluorescence in the top 1 cm of the gels similar to that seen for DNA added to serum was visible. The appearance

of 3 of these paired plasma and serum samples is illustrated in Fig. 4. No DNA was detectable in either plasma or serum from the remaining 6 subjects. The diffuse fluorescence precluded accurate quantitation; consequently all DNA determinations were routinely performed on plasma.

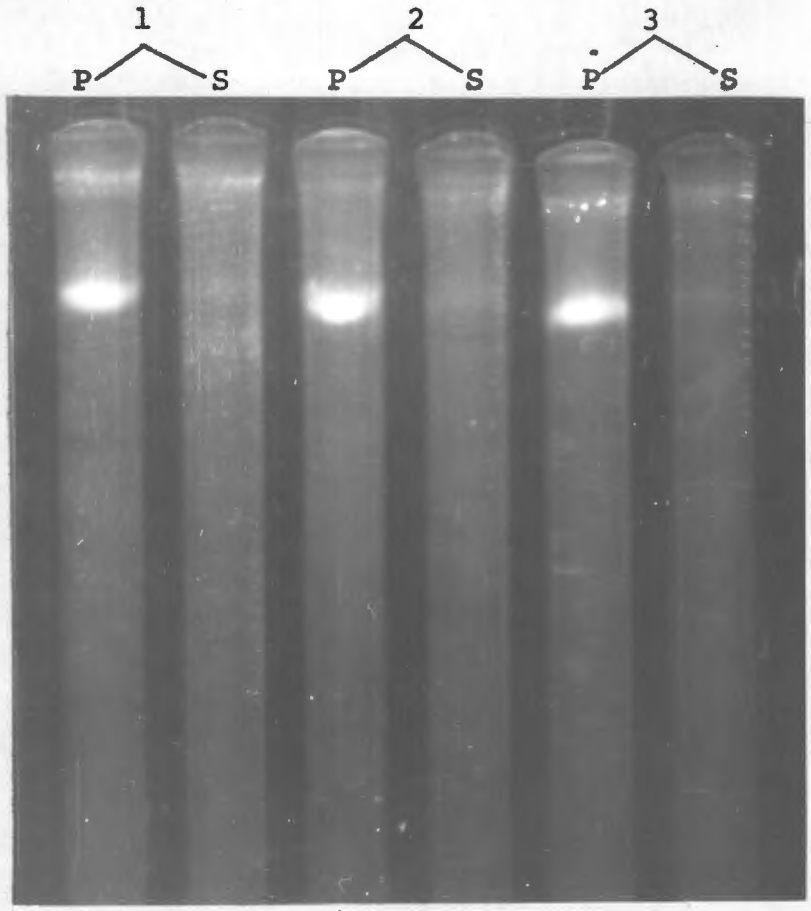


FIG. 4

The staining characteristics of 3 representative paired plasma and serum samples extracted and analysed for DNA. The well-defined bands in the plasma gels (P) and the lack of discernible bands in the serum (S) gels are shown.

Because of the marked difference between DNA levels in heparinised plasma and serum, DNA was assayed in plasma using both heparin and EDTA as anti-coagulants, and compared to levels in serum. The plasma levels were the same irrespective of whether heparin of EDTA was used while the marked discrepancy between plasma and serum levels was again noted. This data excludes the possibility that heparin itself could account for this difference.

7 Normal Range of Plasma DNA

Plasma DNA was measured in 58 normal subjects (Fig. 5) 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 was no significant age or sex difference.

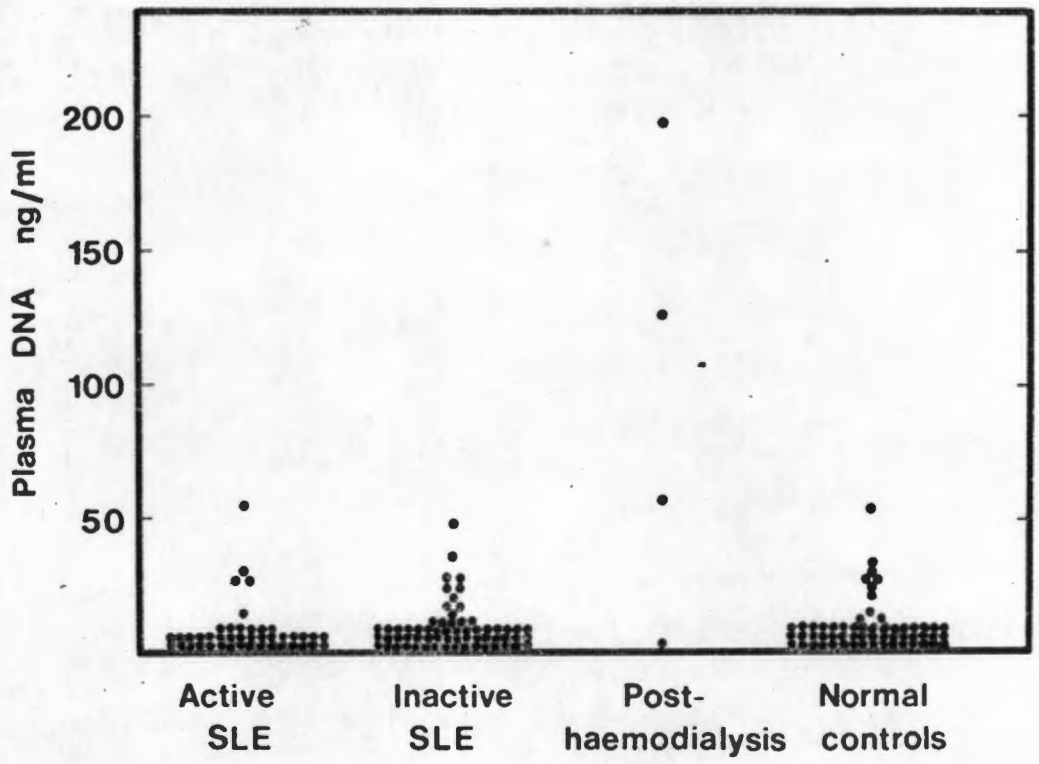


FIG. 5

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.

8 Physiological Variation in DNA Levels

The effect on circulating DNA levels of exposure to the sunburn spectrum of UV light was tested in two normal Caucasian volunteers, a male and a female aged 33 and 34 years respectively, as follows: the minimal erythema dose (MED) was

established for each subject (292) whereupon a whole body exposure of 4 MED was administered to each using 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 for DNA assays and differential white cell estimations. No DNA was detected in any of the samples. While the total leucocyte counts in the subjects remained unchanged, both developed a relative lymphocytosis 48 and 72 hours after exposure. (224).

Five blood samples were drawn from each of 4 male and 4 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 the subjects throughout the 24 hours while all demonstrated fluctuations in the white cell count consistent with the normal diurnal variation. A typical example is illustrated in Fig 6.

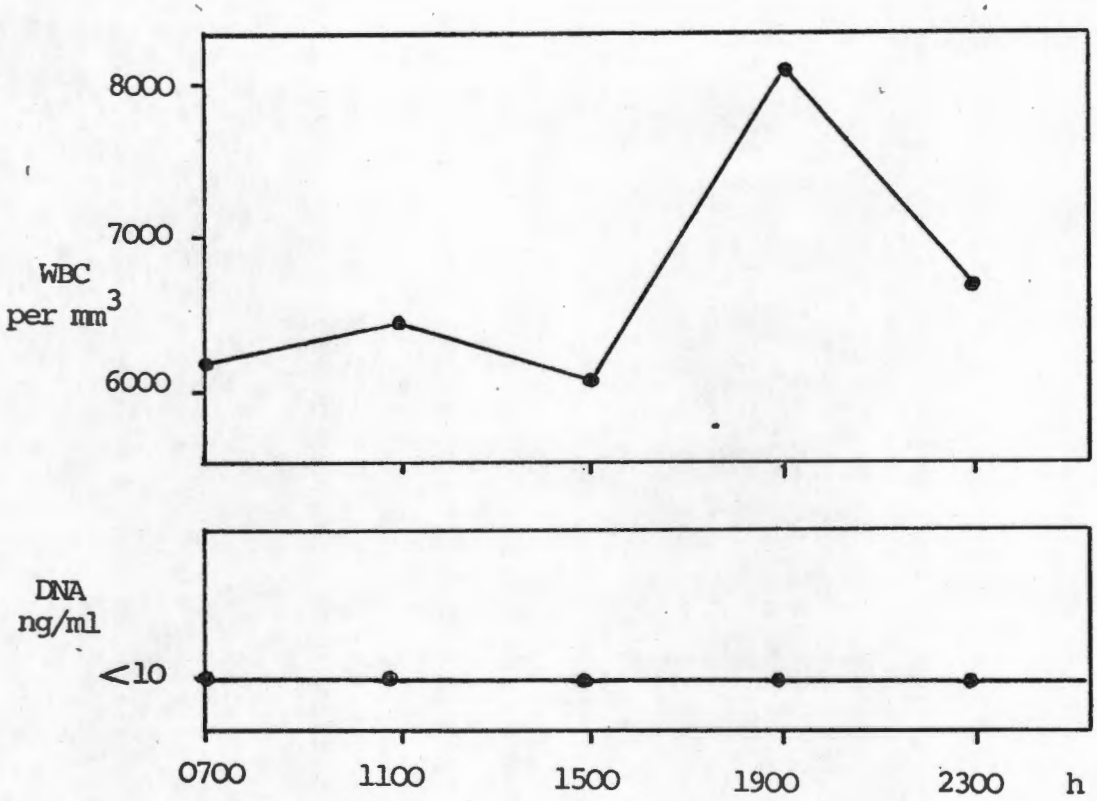


FIG. 6

Leucocyte and DNA levels measured in a subject over 24 hours. DNA remained undetectable throughout while the wide fluctuations in the leucocyte count, consistent with the normal diurnal variation, occurred.

9 Plasma DNA in Pathological Conditions

Plasma DNA levels were measured in 47 patients all of whom fulfilled the preliminary ARA criteria for the classification of SLE (36-41). One hundred and seven assays were performed over a period of 8 months. 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 (Table III) nor were there significant differences in levels between clinically active and quiescent phases of the disease or between SLE as a group and a normal control group (Fig 5). Since high levels of circulating DNA have been reported after haemodialysis (214), plasma DNA levels were also measure 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 5).

TABLE III

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

		No. of samples with levels		
		<10 ng/ml	>10 ng/ml	Total
1	Clinically active (organ or system)			
	Skin	7	1	8
	Musculo-skeletal System	18	2	20
	Kidney	5	0	5
	Nervous System	3	0	3
	Cardiovascular System	0	1	1
	Non-specific (constitutional symptoms)	5	1	6
11	Clinically inactive	53	11	64

DNA DISCUSSION

The specificity of this physico-chemical assay for dsDNA was established by a number of features:

The extraction procedure with phenol and chloroform 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. Their presence, however, does not affect the assay since their mobility characteristics on acrylamide gels are very different to those of polynucleotides. Furthermore, polysaccharides do not fluoresce when stained with ethidium bromide.

Ethidium bromide is an acrydine dye which fluoresces when bound to polynucleotide chains of 4 nucleotides or more (293). The fluorescence produced by ethidium bromide is due to its ability to intercalate into a double helix and as such it is specific for double-stranded polynucleotides although a lesser degree of fluorescence is given by single-stranded species.

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) (291,294). A useful

feature of the gel electrophoresis method is that the electrophoretic mobility of dsDNA is virtually independent of molecular weight above a value of about $5x10^6$ daltons (291). This property is also shown by dsRNA although its electrophoretic mobility at higher molecular weight values is lower than that of DNA (294) and is 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 (291), and can be distinguished from dsDNA by this latter property and by the less well defined bands which they form. The slope of the plot of electrophoretic mobility for the polynucleotides present in the test sample at different gel concentrations was statistically similar to that of a standard DNA preparation (Fig 2). 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 lack of a discernible band after DNase treatment of plasma and its persistence after RNase treatment provide further evidence that the band which fluoresces with ethidium bromide is DNA and not RNA.

Thus 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 confirm unequivocally that we are quantitating native dsDNA.

Since heparin is a highly-charged molecule the possibility that it might in some way account for the discrepancy between plasma and serum DNA levels was considered. This possibility however was excluded on three counts:

1 There was no difference in DNA levels whether heparin or EDTA was used as an anticoagulant.

2 The staining and mobility characteristics of the test DNA are identical to those of the standards, which have never been in contact with heparin.

3 Since heparin does not fluoresce with ethidium bromide the chances of it augmenting the fluorescence attributable to ethidium bromide binding to DNA are remote. This is further supported by the fact that in the recovery experiment the quantity of DNA recovered from the samples did not approach or exceed 100% of that added before the extraction procedure.

It is not known why plasma containing DNA produces a well-defined band on gels whereas serum fails to do so. A hypothesis which would at least partly explain the absence of a discernible dsDNA band in serum is proposed.

It has been shown that while both plasma and serum contain DNase, levels are considerably higher in serum (226). This is thought to be due to the release of DNase during clotting from platelets which contain DNase in a high concentration relative to plasma (295). It has also been shown that serum nucleases are capable of destroying nucleic acids (296,297). Since nuclease activity would create fragments which migrate faster than dsDNA, the DNA would be sufficiently degraded to account for the diffuse fluorescence occasionally seen in the region of, or just below the expected dsDNA migration. This would also explain the absence of a visible band when DNA is added to serum. Since no definitive bands were visible in this region it was not possible to either quantitate or to determine the nature of the polynucleotides present in the fluorescent blush.

The hypothesis would not explain the fluorescence in the top 1 cm of the gel since fluorescence in this region implies that the DNA is in a denatured rather than a degraded form.

Davis and Davis (213) showed that DNA is sporadically released during clotting and therefore considered serum unsuitable for the assay of DNA. It would be interesting to know whether the DNA they refer to is predominantly single-stranded, double-stranded, or partially degraded.

From the appearance on the gel it would appear that in serum some DNA is partially denatured, some is partially degraded and some probably exists as dsDNA. However none is present

in sufficient amounts to be accurately quantitated and the study was confined to dsDNA in plasma. To what extent the serum findings are artifactual due to the clotting process rather than representing the in vivo situation requires further study.

The marked effect of contaminating leucocytes in plasma on DNA levels must be emphasised. Due to inadequate plasma separation in the preliminary stages of this study a wide range of DNA levels was found in normal subjects and in a variety of disease states. Levels as high as 1000 ng/ml were recorded in normal subjects and it appeared in addition that there was a marked diurnal variation of DNA levels. The reason for these wide fluctuations became clear only after a series of experiments were performed to test the adequacy of sample collection and plasma separation.

DNA levels recorded for blood drawn atraumatically and by the vacutainer method were similar and exclude the possibility that either the vacuum or the needle in the latter traumatise leucocytes to any significant degree. Thus DNA levels measured on blood drawn by the vacutainer method reflect true circulating plasma DNA. This is of importance as we confirmed the finding of Tan et al (207) that sufficient trauma to cells can significantly alter DNA levels.

Of even greater importance are the steps that must be taken to ensure that the plasma is cell-free. Since one 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 greater than 99.9% of leucocytes from the plasma before assay. This is especially relevant when an extraction procedure which disrupts cell membranes prior to assay is used. The high levels of DNA and the presence of cells in the lower 2 ml compared to the significantly lower DNA levels and the absence of cells in the top 2 ml plasma after a single cycle of centrifugation emphasise the care that must be taken to avoid cellular contamination. For this reason 2 centrifugations were performed routinely leaving 1 ml plasma undisturbed above the cellular layer after each centrifugation. From a 10 ml sample of blood sufficient plasma was obtained (about 3 ml) to perform all assays in duplicate. Tan et al (207), recognising the need for serum free from contamination with cellular breakdown products, used the method of Gupta and Herriot (226) for the collection of their samples.

Levels of plasma DNA found in normal subjects were considerably lower than those of some reported series (210, 211, 217), but similar to those reported by others (207, 212, 213, 219). It is most unlikely that this can be explained by differences in the selection of normal subjects and the reasons are presumably methodological since the various techniques are based on widely differing principles. No comment can be made on the effect on DNA levels of contaminating leucocytes in those assays not requiring an extraction procedure, since this aspect was not investigated in the study.

DNA appears to be stable in plasma. Even at 37°C over a 24 hour period, and over a 4 week period at -20°C there is no significant change in DNA concentration. This data together with the fact that there was no difference in DNA levels in samples assayed immediately on plasma freshly separated at 20°C compared to samples stored at -15°C excludes the possibility that DNA is precipitated in the cold (such as in the form of cryoprecipitates) to any significant degree. Furthermore it is likely that the concentration of DNase in carefully collected plasma is too low to materially affect DNA levels, even after prolonged storage. Finally the presence of DNase inhibitors in plasma may interfere with any DNase which might be present. (226,298,299)

The recovery studies demonstrate that DNA in plasma can be measured accurately at levels of 50 ng/ml and above; below 50 ng/ml recovery falls appreciably. The results obtained for 250 ng and for 500 ng added to plasma are similar to those of Cox and Gokcen (211) using a RIA technique. The assay is reproducible even at levels as low as 40 ng/ml and compares favourably with that of Leon et al (218), also using a RIA method.

Since no diurnal fluctuations in DNA were recorded in any of the 8 subjects studied, it must be concluded that no diurnal variation exists and that the release of endogenous DNA into the circulation is not influenced by any of the normal activities of daily living experienced by the subjects, such as eating, sleeping, exposure to moderate physical or emotional

stress, or by the "physiological" variation of leucocytes which was demonstrated in all the subjects. Should variations in DNA indeed occur they are too small i.e. less than about 10 to 20 ng/ml to be detected by this assay.

No effect on circulating DNA levels was recorded in the small series of two subjects exposed to sunburn irradiation. The dose administered was sufficient to cause substantial erythema and lymphocytosis, yet avoided blistering or necrosis which could conceivably affect DNA levels. For ethical reasons patients with SLE were not included in this series. It would be interesting to compare DNA, ADA, CH_{50} and IC levels in those patients who develop an exacerbation of the disease following sun exposure with those who do not.

Raised plasma DNA levels were not observed in the group of patients with SLE. In no instance could a relationship between DNA levels and involvement of a particular organ be demonstrated, nor did plasma DNA levels vary with disease activity. These findings are in conflict with other studies (207-211). In some of these reports it is possible to find an alternative explanation for DNA elevation.

Tan et al (207), for example, detected DNA in 11 of 95 patients with SLE. It is interesting to note that in 2 of the patients who had multiple assays performed the appearance of DNA in the serum coincided with the commencement of high dose corticosteroid therapy, and disappeared from the circulation as therapy was reduced, an association described by Hughes

et al (215). The presence of DNA in the serum of these patients can therefore not necessarily be ascribed to disease activity itself. No reference was made to the therapy administered to the remaining nine patients.

Koffler et al (210) found elevated levels of ssDNA in the serum from 13 of 18 patients with SLE tested randomly. Marked elevations of ssDNA were found in some patients with SLE followed serially when compared to other conditions. In addition antibodies to ssDNA were found throughout most of the clinical course. They suggested that ssDNA has a special significance in the pathogenesis of the disease. Unfortunately they were unable to determine dsDNA directly due to insufficient quantities of antisera with specificity for dsDNA. Their data which emphasises the role of ssDNA in SLE therefore cannot be compared to that of our study where specifically dsDNA is quantitated.

Another study worthy of comment is that of Cox and Gokcen (211). Serum DNA levels (comprising total, ss- and dsDNA) were measured in normal subjects and in a variety of disorders by a RIA technique requiring phenol extraction and using dog serum as a source of DNA-binding protein. Nine patients with SLE were studied. The mean serum DNA concentration in these individuals was twice that of the normal range while marked fluctuations were demonstrated in individual subjects investigated serially, many levels being within the normal range. Reference however was not made to the number of assays performed on these patients or whether the fluctuating levels bore

any relationship to disease activity. dsDNA was undetectable in 2 patients, and in the group as a whole accounted for only 16% (about 116 ng/ml) of the total circulating DNA. They postulate that the low levels of dsDNA relative to ssDNA in patients with SLE may be due to the elimination of dsDNA from the circulation in the form of dsDNA-ADA IC's. This is in accordance with our experience of low dsDNA levels in SLE apart from the fact that the actual levels recorded by them are higher than in our study.

An association between cerebral lupus and systemic vasculitis, and the presence of circulating dsDNA has recently been demonstrated (300). Confirmation of Steinman's findings would be of interest since the evidence for a DNA-IC-mediated pathogenesis in cerebral lupus is scanty. It would be vital in such a study to exclude the coexistence of other organ involvement (such as the kidney) by all available means and to monitor not only dsDNA, but also dsADA and DNA-IC serially in these patients.

A number of aspects pertaining to the assay technique and patient selection must be considered before data from various studies can be compared and conclusions drawn.

These may be summarised as follows:

1 Whether plasma or serum is used.

2 The precautions taken to ensure that the samples tested

are free of cellular material particularly when an extraction procedure with phenol and/or chloroform is used.

3 The specificity of the assay for DNA

4 Whether single-stranded, double-stranded or total DNA is measured.

5 Whether the assay is reproducible and shows a good percentage recovery.

6 The exclusion of other factors which may account for elevated DNA levels, such as corticosteroid therapy.

7 Whether levels are measured during active or inactive phases of the disease.

Evidence has been presented that our assay fulfils all the criteria for the assay of dsDNA and the requirements for the assay have been clearly stated.

From the data presented it is postulated that two alternative explanations may account for the low circulating levels of dsDNA in patients with SLE.

1 The release of dsDNA into the circulation of patients with SLE is no different to that found in normal subjects and perhaps ssDNA or DNA is a slightly altered form not

detectable by our technique are of more importance in the pathogenesis of the disease.

2 Alternatively dsDNA is an important immunogen in SLE and the low circulating levels are due to the fact that any DNA released into the circulation is mopped up by ADA and eliminated in the form of complexes.

SECTION III: THE DNA IMMUNE COMPLEX ASSAY

MATERIALS AND METHODS

1 Chemicals

Deoxyribonucleate 5'-oligonucleotidohydrolase, E C No 3.1.4.5., and the Bgl II, Hind III, and Eco R 1 restriction endonucleases and the wild type lambda DNA were purchased from Miles Laboratories, Indiana. Methyl ^{3}H-thymidine and 2-^{14}C-thymidine were purchased from Radiochemical Centre, Amersham and Dimilume-30 scintillation mixture from Packard.
All other reagents were of Analar or equivalent quality.

2 DNA Immune Complex Assay

To 0.5 ml of serum, heat inactivated for 6o mins at 56^{o}C, is added 4.5 ml of 0.01 M Tris-HCl, 0.13 M NaCl, pH 7.5. The diluted sera are then passed under a pressure difference of 15kPa through 0.45 um pore size 25 mm diameter cellulose nitrate membrane filters (Millipore Corp., Bedford) which are then washed x 3 with 5 ml Tris-HCl buffer to remove any free ADA from the filter. This step reduces nonspecific binding to the filter to a minimum. The filter is then cut accurately into two equal halves. To one half is added 0.5 ml 0.15 M Tris-HCl, pH 7.5 and to the other half 0.5 ml 0.15 M Tris-HCl, 0.003 M $MgCl_2$, pH 7.5, containing 50 µg pancreatic DNase. After incubation at 37^{o}C for 3 hours 0.05 ml 0.5 M EDTA, 0.15 M Tris-HCl,

pH 7.5, 0.03 M $MgCl_2$, containing 50 µg DNase was added to the first half filter, and 0.05 ml 0.5 M EDTA, 0.15 M Tris-HCl, was added to the second half filter to stop the reaction. A blank containing 0.5 ml 0.01 M Tris-HCl, 0.13 M NaCl, pH 7.5, handled in the same way as the serum samples was included with each assay. Triplicate 100 µl aliquots of the DNase digested and undigested mixtures were taken for assay of free ADA based on the Ginsberg and Keiser (67) method as follows:

To each of the triplicated 100 µl aliquots was added 100 µl of a 10 µg/ml lambda DNA solution prepared as described below, and vortex mixed. The samples were incubated for 20 minutes at 37°C and then placed on ice and diluted rapidly by the addition to each of the 4 ml ice cold SSC solution (0.15 M NaCl, 0.015 M sodium citrate). The diluted assay mixture was then passed through 0.45 µm cellulose nitrate membrane filters presoaked in SSC and then washed with 3 x 4 ml volumes of SSC solution. The washed filters were then placed in glass scintillation vials and allowed to dry. After the addition of 6 ml of a commercially prepared scintillation mixture (Packard) the radioactivity was measured in a Beckman's LS 233 Liquid Scintillation Spectrometer.

Calculation

The mean of the counts of the triplicated undigested samples was subtracted from the mean of the digested samples. Using the following formula the DNA-IC levels, in µg DNA bound/ml serum, hereafter referred to as units/ml, were calculated as follows:

$$\text{DNA-IC} = \frac{(\bar{x}^1 \text{ counts/min} - \bar{x}^2 \text{ counts/min}) \times 22 (=\text{Dilution factor}}{\text{Specific Activity } (= \text{counts/min/µg } ^3\text{H-DNA})}$$

where $\bar{x}^1$ = mean of the digested samples

$\bar{x}^2$ = mean of the undigested samples

The result was taken to be significant at a probability of < 0.02 when comparing the triplicated measurements fcr the DNase digested and undigested samples.

3 Lambda DNA Preparation

Lambda DNA was prepared by a modification of the method outlined by Thomas and Davis (301).

Labelled lambda bacteriophage dsDNA was obtained by productive infection of a K 12 strain of E coli obtained from Prof W du T Naude. The organisms were inoculated into oxoid nutrient broth No 2 (ONB) and cultured overnight. 2.5 ml of the culture were added to 250 ml ONB No 2 medium and incubated at 37°C with vigorous aeration for 3 hours to yield a count of approximately 10^8 cells/ml. One drop of silicone

antifoam was added to prevent overflow. At this stage 2.5 ml stock lambda phage were added and the incubation with aeration continued for a further 30 minutes. From predetermined growth curve data (by periodic sampling for phage titration and optical density of .125 measured at 500 nm), either 2-^{14}C-thymidine or methyl ^{3}H-thymidine was then added at a final concentration of 0.2 µCi/ml, and 1 µCi/ml, respectively. Incubation was continued for a further 30 minutes by which time phage-induced lysis was indicated by visible clearing of the incubation mixture. After lysis, $CHCl_3$, was added to 0.5% (v/v) and the culture placed on ice. Bacterial debris was removed by centrifugation at 6000 rpm for 30 minutes using the 3015-BR4 rotor in a Sorvall Superspeed RC-2B centrifuge. The phage was then pelleted by centrifugation at 35000 rpm for 1 hour in the Ti 60 rotor of a Beckman Model L 2-65B ultracentrifuge at 4°C. The phage pellet was resuspended in 1 ml of 10^{-3}M $MgCl_2$, 10^{-2}M Tris-HCl, pH 8. The sample was then layered on a CsCl step gradient consisting of 0.5 ml 4.8 M CsCl (ρ = 1.6 gm/cm^3), 0.5 ml 4.0 M CsCl (ρ = 1.5), 1 ml 3.2 M CsCl (ρ = 1.4) and 1 ml 2.4 M CsCl (ρ = 1.3) prepared in 10^{-3}M $MgSO_4$, 10^{-4}M EDTA and 10^{-2}M Tris-HCl, pH 8. Centrifugation was carried out at 30000 rpm in the SW 50.1 rotor of a Beckman Model L 2-65B ultracentrifuge. The phage layer, identified as a fine band above the 4.0 M CsCl layer, was removed with a 1 ml syringe and a 25 gauge needle or with an LKB

perpex pump at slow speed, mixed with an equal volume of saturated CsCl solution and overlayered with 1 ml each of 4.8, 4.0 and 3.2 M CsCl. After a further centrifugation as above at 15-20^{o}C a single, clear, sharp phage band was visible towards the top of the tube. This reverse step gradient was performed to eliminate bacterial DNA contamination. The band was removed and the DNA extracted by placing the phage in a dialysis bag (pre-washed several times in a 10^{-2}M Na_3 EDTA, 10^{-2}M NaCl, $NaHCO_3$ and absolute ethanol solution) and dialysed for 16 hours against 500 ml 50% v/v formamide, 0.1 M Tris-HCl, pH 8.5 and 10^{-2}M Na_3 EDTA at room temperature. The dialysis bag was then transferred to 0.1 M Tris-HCl, pH 7.5, 10^{-4}M Na_3 EDTA for a further 24 hours at 4^{o}C.
The specific activity was determined using a conversion factor of 58 µg/ml/unit absorbance at 260 nm, and the DNA diluted in Tris-HCl buffer so as to contain approximately 10 µg DNA/ml. The DNA was sheared to a constant size by vigorous vortex mixing and by filtering through a 0.45 µm millipore filter before use.

4 Restriction Endonuclease Digestion

0.5 µg aliquots (7 µl) of ^{3}H lambda DNA were added to 43 µl of the appropriate restriction endonuclease digestion buffers (see Miles Biochemical Catalogue 1979-80) and 3 units of either Bgl II, Hind III or

Eco R1 restriction endonuclease. Incubation was for 1.5 hours at 37°C and 16 hours at 4°C. Unlabelled wild type lambda DNA was digested in the same way. Analysis was on 1.2% agarose gels at 100V for 2½ hours. The restriction endonuclease studies are necessary to confirm that the DNA prepared is lambda in nature. This point is illustrated in Fig. 7 which shows that the bands produced by restriction endonuclease digestion of one of our putative lambda DNA preparations harvested as described, differs markedly from that of the control lambda DNA.

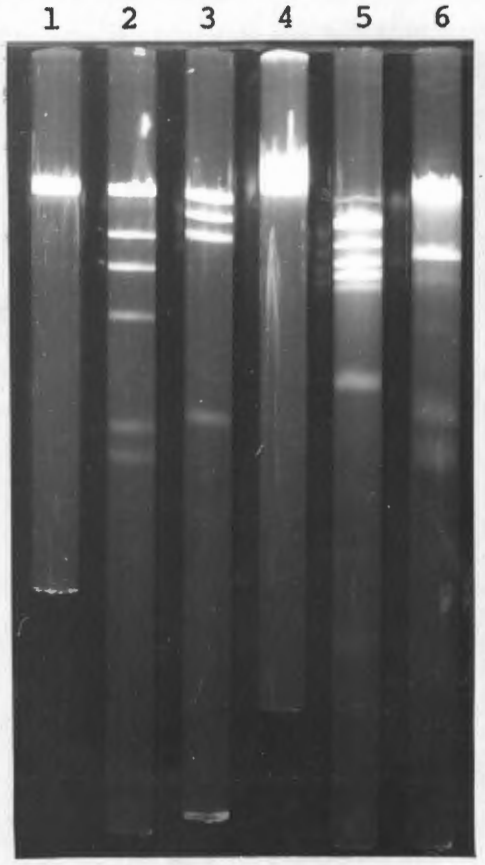


FIG. 7 Hind III and Bgl II restriction endonuclease digestion patterns of a putative lambda DNA preparation, compared with a wild type lambda DNA control, are shown. The band in gel 1 is that of the control lambda DNA, and on gels 2 and 3 the typical Hind III and Bgl II restriction endonuclease patterns respectively. The band on gel 4, produced by the putative lambda DNA preparation is indistinguishable from that of the control but the Hind III and Bgl II restriction endonuclease patterns in gels 5 and 6 show that the nucleic acid harvested was not lambda DNA.

RESULTS

Circulating complexes are quantitated in terms of the quantity of antibody released by DNase digestion of the antigen component of the complex, and the units therefore express the DNA binding capacity of the ADA in a situation of antigenic excess, namely, µg DNA bound/ml of serum. Since the units for IC levels are the same as those for expressing the free ADA in the serum, it was decided to express the quantity of DNA-IC as units/ml in order to avoid confusion.

The accuracy of bisecting the filters into two equal halves was determined by comparing the weights of each pair of 25 cut filters. This gave a standard deviation of 3.1%.

The percentage free ADA removed from the filters by washing with Tris-HCl buffer was evaluated by comparing the counts/min in the saline blank with those in the undigested sample. Typical results in 2 patients with high free ADA levels, and the % free ADA washed through the filters are shown in Table IV. It was found that the degree of sensitivity of the assay was not affected by the levels of free ADA, even in excess of 100 µg DNA bound/ml serum since the washing of the filter was effective in removing virtually all the free ADA.

TABLE IV: The percentage removal of free ADA from the filters by washing with Tris-HCl buffer in 2 representative patients with SLE

FREE ADA (µg DNA bound/ml)	SALINE BLANK ($\bar{x}$ CPM)	UNDIGESTED SAMPLE ($\bar{x}$ CPM)	DIFFERENCE (CPM)	% ADA REMOVED
76	217	301	84	99.9
114	266	266	0	100.0

Negative values, significant at a probability of <0.02, were not found more frequently than expected.

For low levels at the limit of sensitivity for the assay the significance was determined by the student t test on the triplicated undigested and digested data values. Circulating DNA-IC levels were taken to be significant at a P value of less than 0.02 for levels of 0.03 units/ml or greater.

The relationship between DNA-IC and ADA levels during active SLE was investigated in a series of 40 patients, 37 of whom fulfilled the preliminary ARA classification criteria and the remaining 3 with unequivocal SLE.

Five distinct categories of patients emerged:

I Elevated free ADA levels with no complexes detectable.

II Elevated free ADA levels and low but significant levels of complexes.

III Elevated free ADA with relatively high levels of complexes.

IV Normal or only slightly elevated ADA and high relative levels of complexes.

V Normal free ADA and undetectable complexes.

A representative patient from each of the categories is shown in Table V.

TABLE V: Serological data of 5 representative patients with SLE from each of the categories based on free ADA and DNA-IC levels during active disease.

CATEGORY	PATIENT	FREE ADA (µg DNA bound/ml)	DNA-IC (units/ml)
I	JS	85	0.0
II	TN	129	0.41
III	MA	80	2.93
IV	NP	6	3.17
V	PR	0	0.0

In a study of individual patients during active disease, it was found that the majority remained "true" to category irrespective of the system involved with each attack or whether they were tested early or late in the acute episode. An analysis of these parameters and of CH_{50}, singly or in combination, in relation to disease activity is presented in Section IV.

DNA-IC levels were assayed in 20 normal subjects and in 20 patients with active rheumatoid synovitis. The levels obtained are shown in Fig. 8.

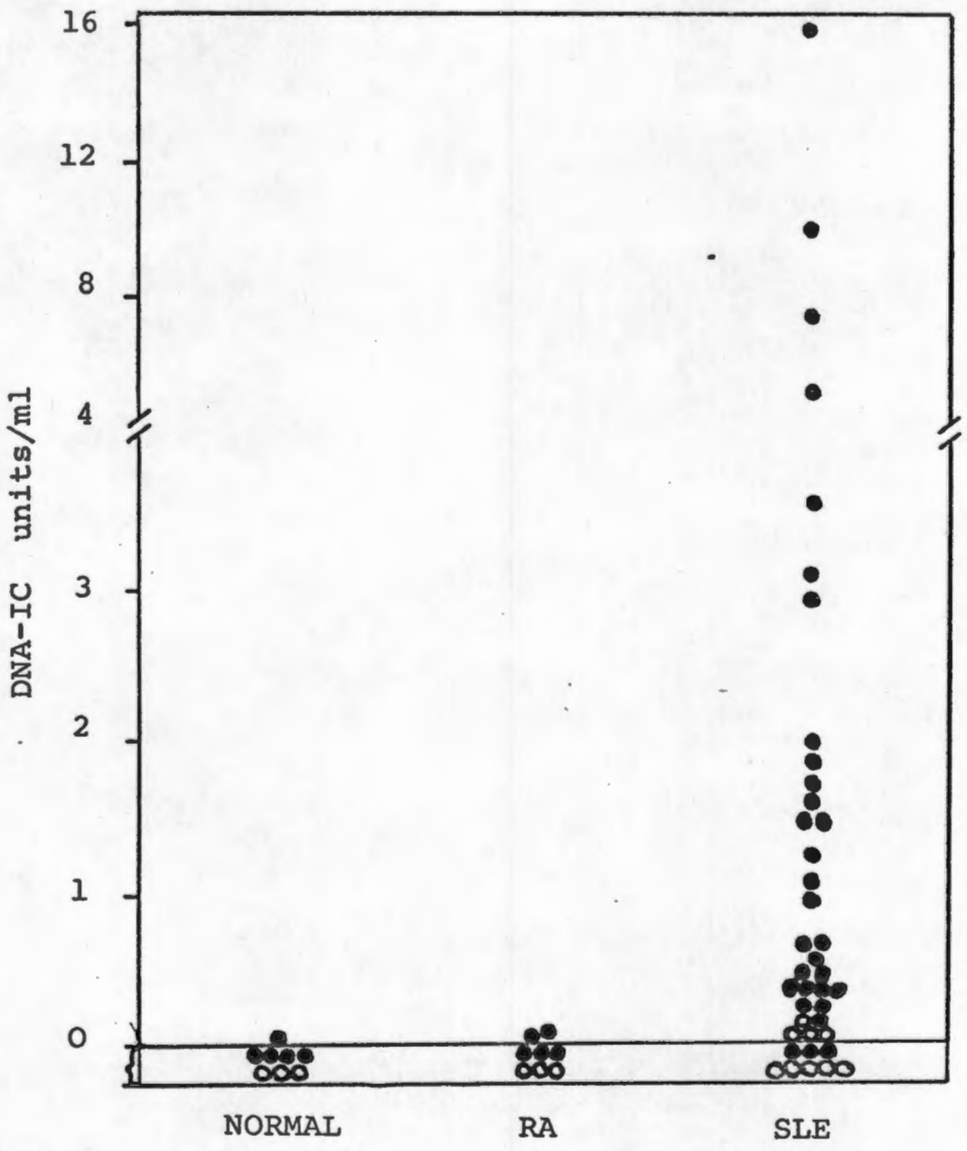


FIG. 8 DNA-IC levels in 20 normal subjects and in 20 patients with active RA compared with levels found during 76 acute episodes in 40 patients with SLE.

Closed circles = 1 assay
Open circles = 5 assays

In normal subjects complexes were undetectable in 19 (95%), while in the remaining subject (5%) a level of 0.03 units/ml,

significant at the 0.02 level, was recorded. In the RA group complexes were undetectable in 18 (90%) patients, with levels of 0.10 and 0.13 units/ml in 2 (10%), significant at the 0.02 level. By contrast there was a good correlation between DNA-IC levels and active SLE, both quantitatively and qualitatively, elevated levels being present in 47 of 76 (62%) active episodes in the 40 patients studied, reaching levels as high as 16 units/ml.

The possibility that complexes small enough to pass through the 45 μm millipore filter and thus escaping detection was investigated by refiltering through 0.22 μm filters, the effluent from the 45 μm pore size filters used in the standard assay. In none of the samples tested was complex material detected on the 0.22 μm filters.

The further possibility considered was that a proportion of the material detected by this assay is due to nonspecific aggregates rather than true complexes. DNA-IC's were therefore measured in freshly collected lupus sera and compared to levels in aliquots of serum either heat inactivated at 56°C for one hour, or repeatedly frozen and thawed or heat inactivated plus freezing and thawing, conditions known to promote the formation of aggregates. In none of the samples tested in this way was there any difference between complex levels in the control sera when compared to either heat inactivation or repeated freezing and thawing. In only one of the samples which was both

heat inactivated and repeatedly frozen and thawed was there a significant increase ($P < 0.02$) of 0.18 units/ml over the control.

To ensure that there was quantitative release by DNase digestion of antigen from the complexes bound to filters, artificial complexes were prepared by the addition of a small quantity of ^{14}C-DNA to the serum of patients with known high levels of free ADA and no circulating complexes. The proportion of ^{14}C-DNA released by DNase treatment from the artificial complexes bound to the filters and of the control samples without DNase showed that binding of more than 95% to the filter is obtained in the former, and release is virtually quantitative. The minor quantity of counts released into the supernatant from the half filter not exposed to DNase presumably reflects dissociation of ADA from the complex since virtually all the free ADA is removed from the filters by the washes as referred to earlier.

The kinetics of DNA binding to ADA was investigated by adding an excess of labelled DNA to serum containing a high level of free antibody and incubating at 37^{o}C. DNA binding was measured before incubation and in duplicate samples removed at 2½, 5, 10, 20, 30, 60, 120 and 240 minute intervals. The results are shown in Fig. 9.

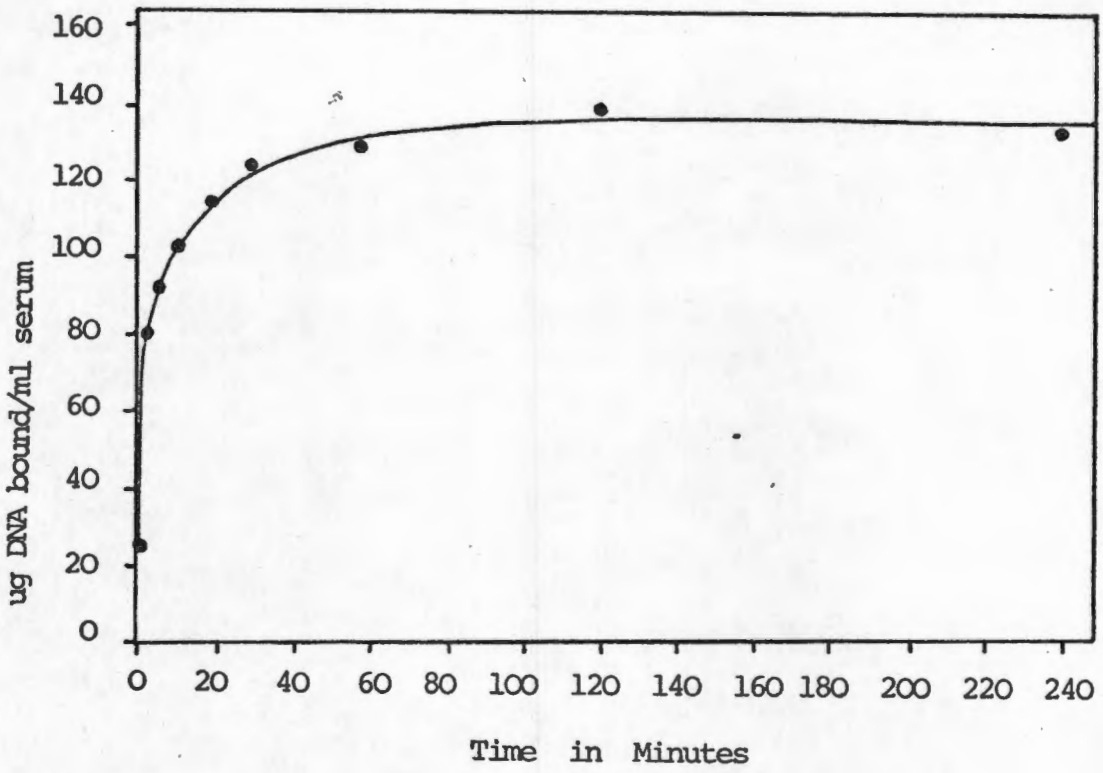


FIG. 9 The kinetics of DNA binding.
DNA binding to ADA is rapid, 94% occurring by 60 mins. Thereafter the curve flattens out and the reaction is virtually complete by 240 mins.

DNA-IC levels were measured in 16 patients with SLE and compared with the % binding by the C_1q assay (302), a technique which has only recently become available to us as a routine assay. The preliminary results are shown in Table VI. Levels in excess of 3% binding by the C_1q assay, and more than 0.03 units/ml for the DNA-IC assay are taken as significant.

TABLE VI: DNA-IC and C_1q binding assays in 16 patients with active and inactive SLE.

PATIENT	C_1q binding (%)	DNA-IC (units/ml)	ACTIVITY
1	6.8	0.41	+
2	1.4	0.00	-
3	12.9	0.92	+
4	13.1	2.35	+
5	2.0	0.14	+
6	1.6	0.00	+
7	5.3	0.00	+
8	1.4	0.30	-
9	1.5	0.03	-
10	1.7	0.13	+
11	7.9	0.00	-
12	1.0	0.40	+
13	2.1	0.04	+
14	45.0	1.05	+
15	6.0	0.28	+
16	8.5	0.00	-

The results show that in the 11 patients with active SLE, levels by both techniques were abnormal in 5, elevated DNA-IC levels with normal C_1q binding were present in one, and in one patient only the C_1q showed significant binding. In one patient with active disease both DNA-IC and C_1q binding were within normal limits. In inactive SLE, levels were normal by both assays in one patient while in the remaining 4 patients, 2 had normal DNA-IC levels with significant C_1q binding, and 2 had elevated DNA-IC levels with normal C_1q binding. While of interest, the numbers

are too small for an analysis of the data.

DISCUSSION

The need for the development of new assays for the measurement of IC's in SLE was emphasised in two recent editorials (303,304) as there is considerable evidence that IC's deposited in tissues can induce pathological lesions such as vasculitis and glomerulonephritis. As far as SLE is concerned our understanding of the pathogenetic mechanisms involved has been greatly increased since IC's have been studied more carefully. While of undoubted value, most of the assays currently available lack specificity and the antigens involved are unknown (245,253,254). While it is likely that IC systems other than DNA may be of importance in the pathogenesis of the disease, it has been established that antibodies to dsDNA in serum are highly specific for SLE (68) and there is furthermore strong evidence that DNA-IC's play an important role in the pathogenesis of the disease (188,189).

Harbeck et al (117) described a method for determining specific concentrations of DNA-IC's in sera and showed that they are present during active disease, and also found that their presence correlates with nephritis (117,119,120), one of the major causes of mortality in the disease. Adequate accuracy by their method was only obtained by analysis of the samples in quintuplicate. It also lacks sensitivity.

The method developed in our laboratory for the detection of specific DNA-IC's is an extension of the principle used by Ginsberg and Keiser for the measurement of ADA by millipore filtration (67). In their method sufficient labelled DNA (antigenic excess) is added to serum to form artificial complexes with all the free ADA present. These complexes are then trapped on a cellulose nitrate millipore filter while any unbound DNA passes through (305), and the ADA is expressed in terms of the DNA bound in the form of these artificially created complexes. In our method any naturally occurring complexes are trapped on the filter by prefiltering the serum diluted in Tris-HCl buffer to ensure that the filter is uniformly covered while virtually all the free ADA passes through, assisted by the 3 washes with Tris-HCl. All the ADA released from the natural complexes by DNase digestion is then bound to the added radioactively labelled DNA to form artificial complexes and the DNA-IC levels are calculated in terms of DNA bound/ml serum. In order to avoid confusion with the units used for the standard free ADA assay, DNA-IC levels are expressed as units/ml.

A crucial step in the assay is the ability of DNase to release the DNA bound to ADA in the form of complexes trapped on millipore filters. DNase was shown to effectively release more than 95% of the bound DNA.

Mg^{++} facilitates the action of DNase while EDTA, which inhibits the action of DNase, is added at the end of the incubation period to stop the reaction.

While DNA prepared from rat hepatoma cells is used successfully in the assay, we prefer to use lambda DNA since the counts in the undigested samples and in the saline blanks are lower than with rat hepatoma DNA. This is presumably due to the more uniform size and lower molecular weight (30 million) of lambda DNA. These factors would reduce the tendency of the DNA to form clumps which may be trapped on the filter.

It was important to determine the accuracy with which the filters were bisected since the quantitation of complexes in the serum is a measure of the difference between the counts registered for the DNase digested and undigested half-filters. The results obtained were taken into account in the evaluation of the assay.

Ideally a control serum containing a known quantity of DNA-IC should be included with each assay. Apart from the lack of available control samples in sufficient quantities (0.5 ml per assay) which precludes their inclusion as a routine procedure, it should be established whether DNA-IC's remain stable with prolonged storage. A solution to the problem of a control would be the preparation of standard quantities of artificial DNA-IC's.

The object of refiltering the effluent from the 0.45 μm filter through filters of smaller pore size was to establish whether small complexes escape detection. Our data suggests that this is not the case. However, it is not possible to

equate the molecular weight of complexes with their ability physically to pass through millipore filters. A more direct study of the complexes themselves is necessary to determine the characteristics of the DNA-IC's detected by this assay.

An important issue and one which plagues many current assays is whether nonspecific aggregates rather than true complex material is being detected. Since nonspecific binding of DNA to, or entrapment by aggregates may occur, and since aggregates of sufficient size may be trapped on millipore filters, it is possible that some of the material detected by this assay is not true DNA-IC in nature. Some of the factors known to promote the formation of aggregates were therefore investigated. Our studies have shown that neither heat inactivation nor freezing and thawing affected the DNA-IC levels. In only one instance did these factors in combination give rise to a small, but significant increase over the levels in the control sample. In order to avoid such increases as far as possible, heat inactivation is no longer performed. Because it is not always possible to perform the assay immediately, samples which have been frozen only once are used, and assayed within a few days of collection. It is likely that most of the counts on the filters of the undigested samples are due to DNA binding to nonspecific aggregates. By subtracting these counts from those in the digested samples and by adhering strictly to the measures already mentioned, we believe that the values obtained reflect almost exclusively true circulating

DNA-IC levels.

Why patients with SLE should show such a wide variation in free ADA and DNA-IC levels during active disease is not clear, especially since no correlation was demonstrable between the pattern of the serological parameters and the nature of organ involvement or of the timing of sample collection during the acute episode. Factors pertaining to the size and properties of the free antibody and of the complex itself, and to the site of complex formation almost certainly play an important role in this respect.

From the data presented, the DNA-IC assay appears to be specific for SLE especially for levels in excess of 0.13 units/ml since in none of the 10% of the control and RA group where complexes were detectable were these levels exceeded. Because of this specificity the assay is of diagnostic value. On the other hand, the diagnosis of SLE cannot be excluded where complexes are not detectable since this occurred in 38% of patients with active, and in 68% with inactive disease (Section IV). In this respect the situation is analogous to the ADA assay. Data will also be presented showing that actual complex levels correlate with the severity of the acute attack.

SECTION IV: CLINICAL APPLICATION OF THE DNA IMMUNE COMPLEX ASSAY IN SLE

MATERIALS AND METHODS

1 Patients

Forty patients were studied prospectively.

They were selected from ward admissions and from patients attending the lupus clinic at Groote Schuur Hospital which serves all the racial groups namely Negro-Caucasoid, Caucasian and Black living in the metropolitan area of Cape Town and the Western Cape. The selection of patients was based on the clinical picture so as to include as wide a spectrum of the disease as possible during both active and inactive phases. Serial studies were conducted on 8 patients and during plasma exchange on one patient.

Of the 40 patients, 37 were women and 3 were men, 27 were Negro-Caucasoid, 8 were Caucasian and 5 were Black. Their ages ranged from 15 years to 54 years (mean 30). Eight were studied during the initial presentation of SLE while in the remaining 32 patients the duration of the disease ranged from 1 to 26 years (mean 5.9).

Thirty seven patients (92.5%) satisfied the preliminary criteria of the ARA for the classification of SLE (36-41). The three patients (7.5%) not fulfilling the criteria were considered to have unequivocal

evidence of SLE on the following grounds: One patient presented with acute polyarthritis, an erythematous butterfly rash, pericarditis, fever and severe depression with hypocomplementaemia, ANA positive to a titre of 1000 and significant elevation of ADA levels. The second patient had a long-standing history of non-deforming polyarthritis, neuropsychiatric manifestations and a butterfly rash with ANA positivity, hypocomplementaemia, significant ADA elevation and granular casts on a number of occasions during the course of the disease. The third patient presented with generalised cutaneous vasculitis, polyarthritis and alopecia with ANA positivity and ADA elevation.

Flow sheets were designed to record:

a) The presence or absence of clinical activity, and in the case of active disease the nature of the manifestations,

b) the therapy administered, and

c) the laboratory results.

The patients were assessed by the author at each visit for evidence of clinical activity and graded accordingly. This was done before the results of the laboratory investigations were known. The

manifestations considered to be indicative of active SLE after other causes had been excluded are listed in Table VII.

TABLE VII: Parameters used in the assessment of disease activity in SLE.

Organ Involvement	Criteria
Musculoskeletal	Polyarthralgia Polyarthritis Tenosynovitis Synovial effusion Significant morning stiffness (>30 min)
Cutaneous	Alopecia Oral ulceration Vasculitis Maculopapular eruption Erythematous butterfly rash Exclusions: Raynaud's phenomenon, CDLE, Photosensitivity, Pigmentory changes, leg ulcers, ? digital gangrene
Renal	Red cell or granular casts Active involvement on histology Significant or increasing proteinuria Azotaemia Increasing impairment of GFR
Neurological	Psychosis Coma Recent neuropsychiatric manifestations Recent focal neurological signs
Serositis	Pericarditis Pleurisy Peritonitis
Haematological	Haemolytic anaemia Thrombocytopenia ($<100\ 000/mm^3$) Leucopenia
Constitutional	Fever Malaise Fatiguability Weight loss

Flares were classified as minor, major or life-threatening depending on the severity and nature of the involvement, and on the therapy required to control

the flare. A flare was judged to be minor if non-steroidal anti-inflammatory agents were sufficient to control musculoskeletal symptoms or if skin lesions could be adequately controlled with local corticosteroid applications. Major flares usually required admission to hospital and almost invariably corticosteroid and/or immunosuppressive therapy to achieve control of the flare. Life-threatening episodes consisted of that small group of patients presenting with severe fulminating cerebral, renal or multisystem disease which are often unresponsive to therapy.

2 Laboratory Tests

At each visit urine was examined chemically and microscopically, and blood was drawn for the following investigations:

a) Serum complement

CH_{50} was assayed on freshly collected serum based on the method of Kent et al (306). The lower limit of the normal range was 160 units/ml.

b) Anti-DNA antibody

ADA was quantitated on serum stored at -20^{o}C by a modification of the millipore filtration technique of Ginsberg and Keiser (67) using rat hepatoma cell (pp 67-69) or λ DNA prepared from the K12 strain of E coli labelled with methyl

^{3}H-thymidine or ^{14}C-thymidine as described on pp 100-103. The normal range for our laboratory is between 0 and 5 µg DNA bound/ml serum. Levels of between 5 and 15 are regarded as suggestive, while levels above 15 µg DNA/ml are virtually diagnostic of SLE.

c) DNA immune complex
Specific DNA-IC's were assayed by the millipore filtration and DNase digestion technique as outlined on pp 98-104.

d) Where indicated, other investigations including full blood count, serum chemistry, tests of renal function, renal or other biopsies, radiological and neurological tests were performed.

The following correlations between the clinical evaluation and the serologic parameters were determined:

i) The clinical status and the coincident CH_{50}, ADA and DNA-IC levels, where for statistical analysis the clinical status was designated active or inactive, and each of the parameters as normal or abnormal.

ii) The relationship between different grades of disease activity and actual coincident DNA-IC levels, and

iii) The relationship between elevated levels of DNA-IC in patients with clinically inactive disease and the subsequent development of flares in these patients. In this instance actual DNA-IC levels and the grade of activity which ensued were evaluated.

3 Statistical Evaluation

The relative risk of the disease being active when any one of the three parameters, CH_{50}, ADA and DNA-IC was abnormal, was determined from Haldane's modified formula described elsewhere (307-309).

This formula allows for the inclusion of both normal and abnormal levels of each parameter present in both active and inactive disease and also corrects for those instances where the numbers are relatively small.

The entries of the appropriate 2x2 table were a, b, c and d where:

a = number of abnormal levels in active disease
b = number of normal levels in active disease
c = number of abnormal levels in inactive disease
d = number of normal levels in inactive disease

Thus, the relative risk, $X = \frac{(2a+1)\ (2d+1)}{(2b+1)\ (2c+1)}$

y is the natural logarithm of x (In x), with a variance given by

$$V = \frac{1}{a+1} + \frac{1}{b+1} + \frac{1}{c+1} + \frac{1}{d+1}$$

and weight, $w = 1/V$.

The significance in each case was estimated by calculating $\chi^2 = wy^2$ and Fisher's exact probability (P) values.

By selecting a, b, c and d for each parameter and using the same formula, the relative risk of the disease being active when two or all three parameters in combination were either normal or abnormal, was also determined. Even though the relative risk may appear to be higher in certain combinations, Fisher's exact P value, which takes into account differences in sampling size, is used to assess the true significance.

In order to establish whether there was a statistically significant difference in DNA-IC levels in the different grades of disease activity, the following hypotheses were tested:

a) Null hypotheses (H_o) ie. that the means of DNA-IC levels in the different grades were equal (H_{o1}: $\mu_1 = \mu_2$, H_{o2}: $\mu_2 = \mu_3$ and H_{o3}: $\mu_3 = \mu_4$ for inactive/minor, minor/major and major/life-threatening

disease respectively) where μ = actual population mean.

b) Alternative hypotheses (H_1) ie. that the means were progressively higher for each grade of activity (H_{11}: $\mu_1 < \mu_2$, H_{12}: $\mu_2 < \mu_3$ and H_{13}: $\mu_3 < \mu_4$).

The test statistic, T, for the difference of means was chosen under the assumption of normality and equal but unknown population variances.

$$T = \frac{\bar{x}_1 - \bar{x}_2}{S\sqrt{\frac{1}{m} + \frac{1}{n}}}$$

where $\bar{x}_i$ = means, $i = 1,2$

S = the pooled standard deviations

m = number in sample one

n = number in sample two

and $$S^2 = \frac{(m-1)S_1^2 + (n-1)S_2^2}{m + n - 2}$$

T has t distribution with m+n-2 degrees of freedom ($t_{\alpha;m+n-2}$).

The significant levels, α, was set at 0.05.

RESULTS

Of the 40 patients, 15 remained clinically inactive during the 30 month period of study while in the remaining 25 patients 44 acute flares of the disease were recorded. In the latter group 13 were on corticosteroids and/or immunosuppressive therapy at the time of the flare. The dosage was increased in 9 patients in order to control symptoms, while therapy was instituted in 9 who were not on therapy at the time of the flare.

The clinical manifestations of the 25 patients who developed active disease and their relationship to the presence or absence of circulating DNA-IC are shown in Table Arthritis and skin involvement accounted for the majority of active episodes both when they were involved alone (12 and 11 episodes respectively) or when involved as part of a more general flare with other organs (21 and 20 episodes respectively).

Histological evidence of renal disease was present in 12 patients. Of these 50% were focal or mesangial, 8% were membranous and 28% were membranoproliferative. In all these patients the urinary sediment was abnormal on numerous occasions, the nephrotic syndrome was present in three, and of the two patients with uraemia and low GFR one has died. The presence on one or two occasions of a few granular casts without deterioration in renal function in four patients during the study was not considered an indication for renal

biopsy. These patients were not included.

TABLE VIII:Organ involvement in 25 patients who had 44 acute episodes of SLE in relation to the presence or absence of DNA-IC

ORGAN INVOLVEMENT	NO. OF ACTIVE EPISODES	DNA-IC PRESENT*	NOT PRESENT*
Musculoskeletal	12	16	7
Skin	11	10	8
Renal	5	2	5
Musculoskeletal/ Skin	3	3	2
Skin/CNS	2	4	1
Musculoskeletal/ CNS/Renal	2	2	2
Skin/Renal	2	2	1
CNS	1	1	-
Musculoskeletal/ Skin/Renal	1	2	-
Thrombocytopenia	1	1	-
Pericarditis	1	-	1
Musculoskeletal/ Renal	1	1	-
Musculoskeletal/ CNS/Pleurisy	1	2	1
Musculoskeletal/ Skin/Renal/Peri-carditis/Lung	1	1	1
TOTAL	44	47	29

* Number of assays

A total of 136 IC assays were performed, 76 during active and 53 during quiescent phases of the disease (total:129). The remaining 7 were additional assays performed over a 24 hour period on two patients during active disease. The mean

of these levels for each patient was taken as representing one IC level in terms of the particular episode. ADA levels were measured in 188, and CH_{50} in 176 samples from the patients during the same period of study. The results obtained, the relative risk of disease activity for each parameter separately and in combination, the chisquare and the P values are shown in Table IX.

Abnormalities of the three parameters either singly or in combination, were all significant as monitors for the presence of active disease. Of the parameters assessed singly, CH_{50} was better than either ADA or DNA-IC. When two parameters were assessed in combination the values obtained for ADA + DNA-IC and for CH_{50} + DNA-IC were significantly better than for any of the parameters singly or for ADA + CH_{50}. The combination of all three parameters while superior to ADA + CH_{50} did not reach the significance of either CH_{50} + DNA-IC or ADA + DNA-IC as tests of active disease. Furthermore it is noteworthy that CH_{50} as a single monitor is better than ADA + CH_{50}. Thus, the most useful combination of monitors are firstly, CH_{50} + DNA-IC, followed by ADA + DNA-IC and ADA + CH_{50} + DNA-IC.

TABLE IX Relationship between clinically active and inactive SLE and the serologic parameters ADA, CH_{50} and DNA-IC, singly and in combination together with the chisquare and P values.

Serologic Parameter	Active Disease Number of assays		Inactive Disease Number of Assays		Relative Risk of Active Disease (X)	χ^2	P Value
	Abnormal	Normal	Abnormal	Normal			
ADA	69 (69%)	31 (31%)	45 (51%)	43 (49%)	2.11	6.19	0.009
CH_{50}	66 (76%)	21 (24%)	42 (47%)	47 (53%)	3.46	14.73	7.77×10^{-5}
DNA-IC	47 (62%)	29 (38%)	17 (32%)	36 (68%)	3.36	10.73	0.0008
ADA+CH_{50}	50 (81%)	12 (19%)	26 (46%)	30 (54%)	4.65	14.24	0.0001
DNA-IC + CH_{50}	37 (86%)	6 (14%)	9 (32%)	19 (68%)	11.84	19.14	4.54×10^{-6}
ADA + DNA-IC	37 (74%)	13 (26%)	5 (19%)	21 (81%)	10.86	18.36	5.56×10^{-6}
ADA + CH_{50} + DNA-IC	28 (85%)	5 (15%)	4 (22%)	14 (78%)	16.70	16.94	1.57×10^{-5}

DNA-IC levels in active and inactive disease are shown in Fig. 10. The highest levels for inactive disease and for minor flares were 1.77 and 1.63 units/ml respectively. Except for one level of 7.40 units/ml recorded in a patient with a major flare, levels above 3.50 units/ml were confined to life-threatening disease.

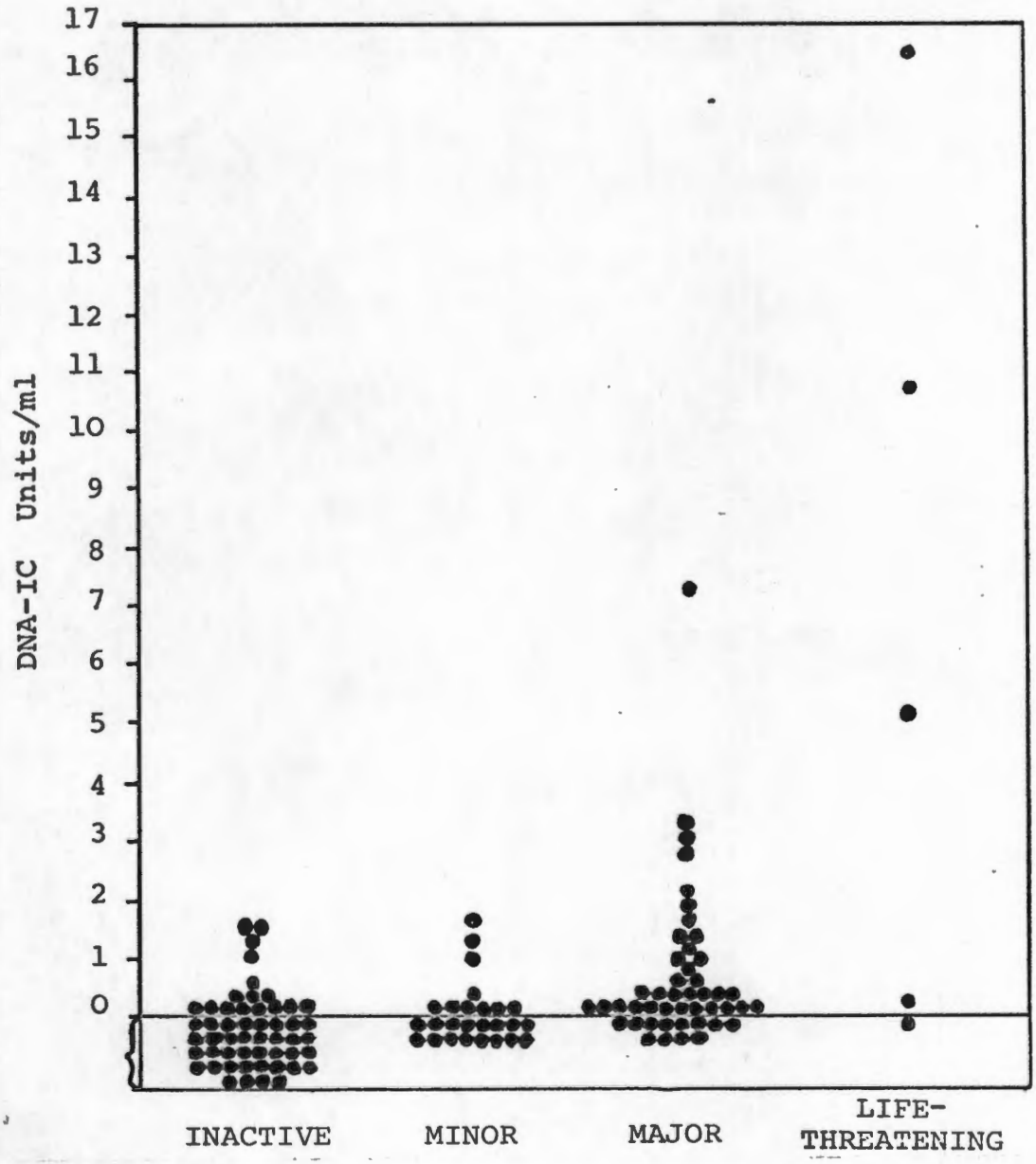


FIG. 10 DNA-IC levels in active and inactive SLE are shown. Complexes were undetectable in 36/52 (69%) patients with inactive disease and in 16/26 (61%) with minor, 12/46 (26%) with major and in 1/5 (20%) with life-threatening disease. Full statistical analysis of levels in the various grades is shown in the following table.

In Table X, the data obtained from an analysis of DNA-IC levels in the different grades of disease activity shows that while the difference between DNA-IC levels in inactive disease compared to minor activity is not significant, the mean levels in the three groups, inactive/minor, major and life-threatening activity, are significantly different at the 0.02 significance level. It should however be noted that in the life-threatening category there were only five samples.

TABLE X: Statistical analysis of DNA-IC levels in patients with active and inactive SLE.

Statistic	Inactive n=52	Minor n=26	Major n=46	Life-Threatening n=5
$\bar{x}$ *	0.16	0.19	0.74	6.47
S_x **	0.43	0.44	1.33	6.72
$S_{\bar{x}}$ ***	0.06	0.09	0.20	3.10
S^2		0.19	1.21	5.33
S		0.43	1.10	2.35
T		-0.29	-2.04	-5.18
$t_{0.05,m+n-2}$		1.67	1.67	1.68
		$T > -t$ Accept H_o ($\mu_1 = \mu_2$)	$T < -t$ Accept H_1 ($\mu_2 < \mu_3$)	$T < -t$ Accept H_1 ($\mu_3 < \mu_4$)

* = estimated sample mean
** = standard deviation
*** = standard error of the mean

Five of the patients (12%) died, one from uraemia and cerebral involvement, two from cerebral lupus, one from suspected cerebral lupus (who died at home), and one from myocardial infarction, polyserositis, pulmonary oedema and a midbrain haemorrhage. Of the deaths only one occurred during the study. In the remaining four patients none had previously had life-threatening disease, three had had major, and one had had minor episodes in the past. Of the two patients who survived life-threatening episodes one has remained in remission and the other has been lost to follow-up. In the other two groups two patients with major and one with minor disease have been lost to follow-up. The clinical course of two of these patients, the one who died during the study and one of those who died from cerebral lupus subsequent to the study, are described later in this Section.

Those patients in whom DNA-IC's were present without evidence of clinical activity were observed for the subsequent development of active disease. The outcome of this group of 11 patients in whom 17 elevated DNA-IC levels were recorded is shown in Table XI. Three patients did not develop any evidence of active disease over the 30 month period of observation, 6 (55%) had minor and 2 (18%) had major flares. The onset of the active episodes ranged from 1 to 9 months. As shown on the Table, where more than one IC level in any particular patient was measured during inactive disease, each has been related individually to the onset of the active episode. One patient who developed a minor flare died 11

months later from suspected cerebral lupus. The levels of DNA-IC recorded bore no relationship to the severity of the acute episodes which developed.

TABLE XI: Outcome in 11 patients with SLE in whom DNA-IC was present without evidence of clinical activity.

Patient	DNA-IC levels	Outcome (Months)	Severity of Acute Episode	
			Minor	Major
FJ	1.73	4	Polyarthralgia	
IM	0.03	2		Oral ulceration
	0.59	1		Headache Butterfly rash Alopecia
MM	1.17	9		
	0.04	7	Polyarthralgia	
	0.19	6		
MN*	0.28	3	Polyarthralgia	
NP	0.26	8		
	0.02	7	Malaise	
	1.68	4		
JR	0.06	2		Skin vasculitis Alopecia Malaise
TS	0.60	9	Polyarthritis	
UT	1.77	2	Polyarthralgia	
	0.10	1		
SE	0.18	30	No Activity	
FG	0.22	30	No Activity	
AM	0.14	30	No Activity	

* Died 11 months later from suspected cerebral lupus.

Evidence of previous renal involvement was sought in the same group of patients, namely those with increased DNA-IC levels with no evidence of clinical activity. Definite histological evidence of renal lupus was present in 6 of the patients, accounting for 11 of the 17 elevated DNA-IC levels in the group of patients with inactive disease. Two further patients in this group (who were not biopsied) had had granular casts on a few occasions while the remaining three had never shown any evidence of renal involvement.

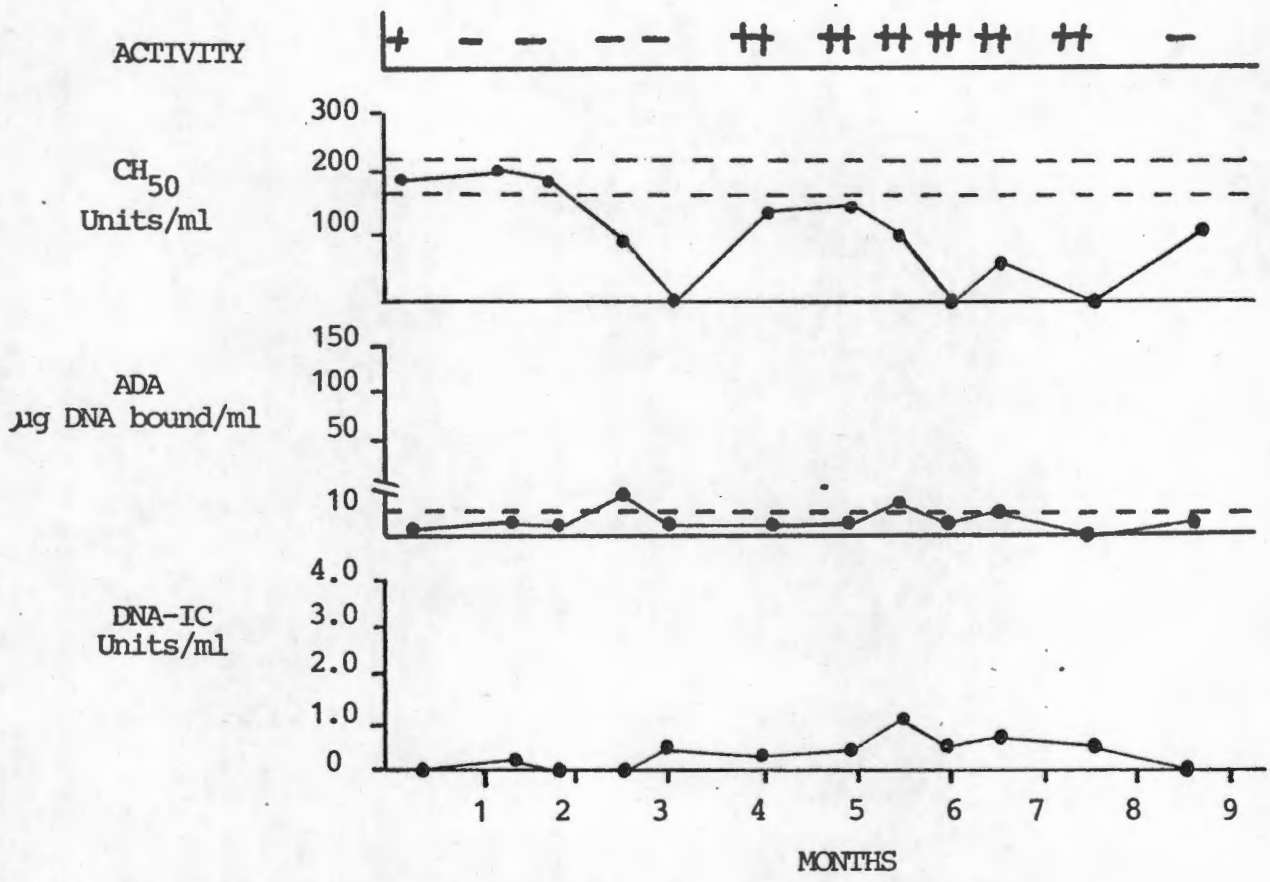
The clinical course and management in 2 of the 3 patients with life-threatening disease are described on pp 141 and 149. There was no uniformity in the pattern of the three laboratory parameters in any of these patients. In Patient 9 ADA levels in excess of 1000 µg DNA bound/ml serum were present with high IC levels and unrecordable CH_{50}. In patient 4 who subsequently died there was never more than a modest increase in IC and ADA levels. In the third patient the presentation was that of polyarthritis, alopecia, butterfly rash, pericardial effusion, and vasculitis. Laboratory investigation revealed positive LE cells on numerous occasions, a biological false positive test for syphilis, unrecordable CH_{50}, ANA 2500, ADA elevation, leucopenia, Coombs positive haemolytic anaemia and granular casts. During the time that high circulating complexes were present in this patient, 6 assays were performed over 24 hours. The levels were the highest yet recorded by this assay ranging from 10.6 to 16 units/ml.

CASE REPORTS

PATIENT 1

In 1971 this patient, an Asian male (IM) aged 14 years was diagnosed as viral encephalitis. Four months later he was readmitted with the nephrotic syndrome, microscopic haematuria and granular casts in his urine. LE cells and ANA were positive and complement levels were unrecordable. He was treated with prednisone and azothiaprine. In 1977 he developed a butterfly rash, alopecia, oral ulceration and a Coombs positive haemolytic anaemia. LE cells and ANA were again positive and the ADA was 86 µg DNA bound/ml serum. Proteinuria of 8g/24 hours and numerous granular casts were present. The only available information of the renal histology was of focal hypercellularity with capsular adhesions. His subsequent course has been one of frequent polyarthralgia, cutaneous vasculitis, oral ulceration, memory impairment, headaches, unexplained chest pain and malaise. In July 1979 there was an exacerbation of the nephrotic syndrome. A renal biopsy revealed diffuse membranous changes with hypercellularity, segmental hyalinisation, pericapsular adhesions, fibrosis and areas of calcification. Treatment consisted of high-dose corticosteroids and cyclophosphamide.

Serial studies of CH_{50}, ADA and IC levels over 9 months during periods of activity and inactivity are shown in Fig 11.



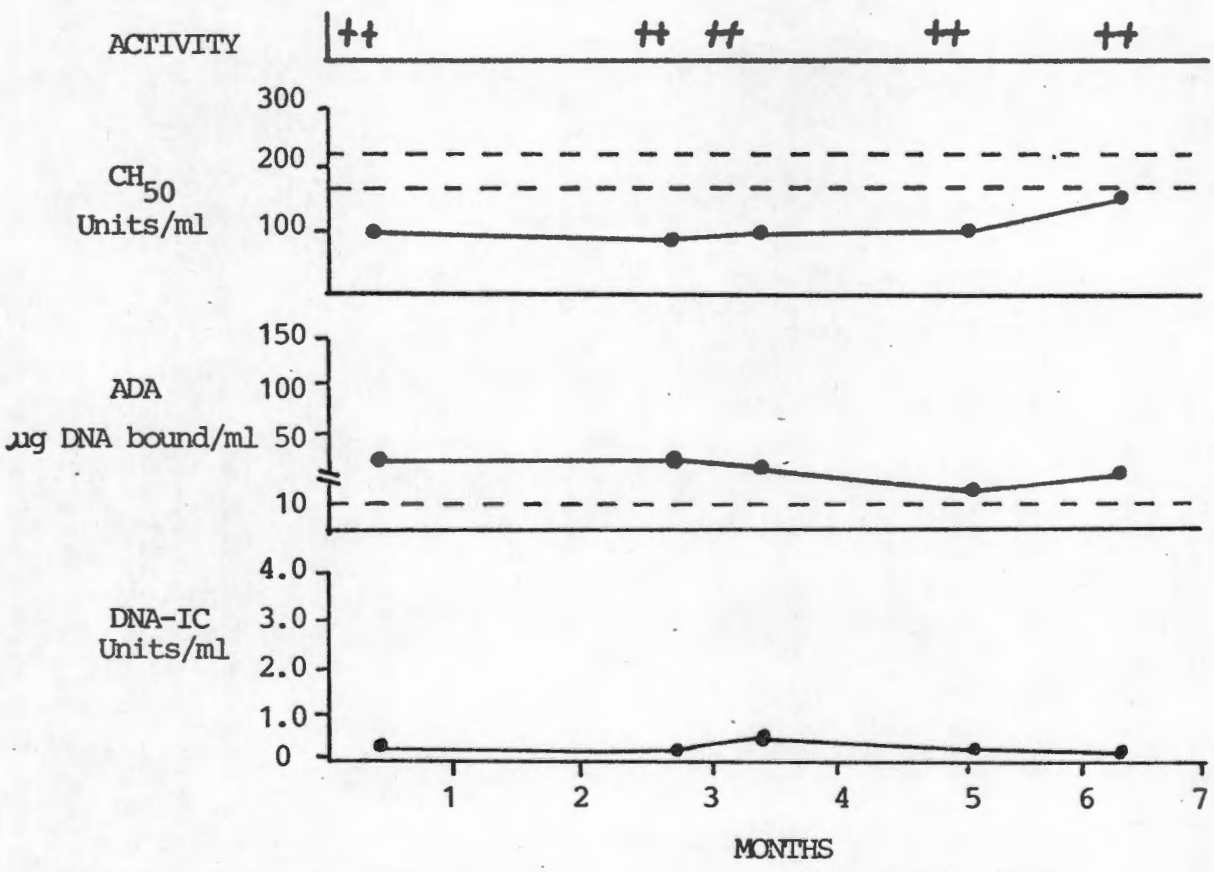
A good correlation between CH_{50} and DNA-IC levels in active and inactive disease is demonstrated. Of interest is the appearance of circulating complexes one month before the major flare, and the fact that ADA levels throughout the course remained within normal limits apart from two minor elevations.

PATIENT 2

At the age of 30 years this White female patient (CP) developed seronegative polyarthritis. SLE was established some years later on the grounds of alopecia, LE cells, a biological false positive test for syphilis, positive ANA

and ADA elevation. Her course has been one of intermittent episodes of polyarthritis, urticaria, blotchy erythematous skin lesions, malaise and depression.

The serological data over a 6 month period of active disease is illustrated in Fig.12.

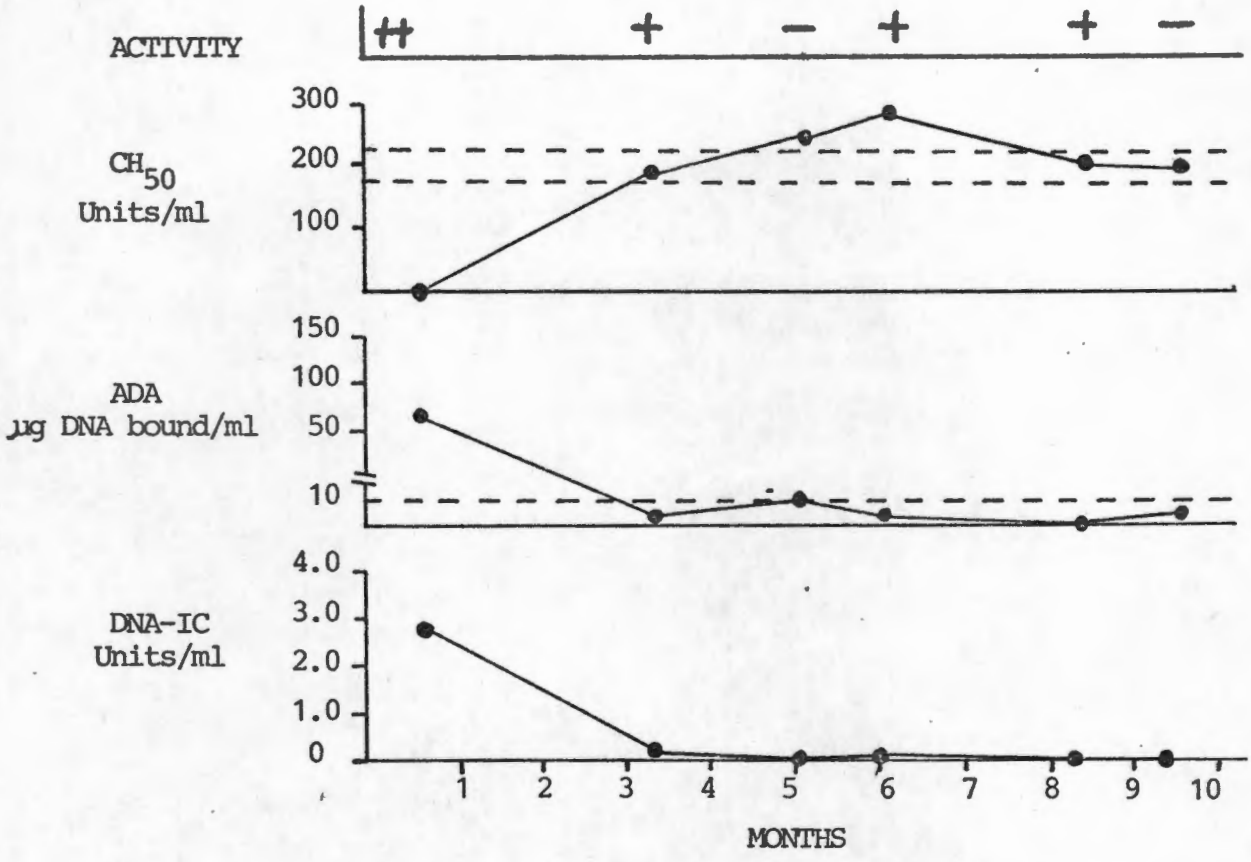


In this patient there was persistent elevation of ADA and DNA-IC levels in addition to hypocomplementaemia throughout the monitoring period.

PATIENT 3

This 53 year old Coloured female (MA) presented in May 1977 with polyarthritis, vasculitic skin lesions, thrombocytopenia, and Coombs positive haemolytic anaemia. LE cells were present, ANA was positive to a titre of 2500 and an ADA level of 80 µg DNA bound/ml serum was present on admission. Urinalysis revealed normal chemistry but on microscopy numerous granular casts were noted. A renal biopsy showed the features of diffuse membranoproliferative nephritis and subendothelial and subepithelial deposits were demonstrated on electronmicroscopy. Therapy with high-dose corticosteroid and cyclophosphamide was instituted. An initial creatinine clearance was 87 ml/min. This fell to 38 and gradually reverted to normal over the next two months. The patient became asymptomatic after three months of therapy and apart from intermittent episodes of microscopic haematuria and granular casts there was no evidence of active disease. At no stage had there been any evidence of cerebral involvement but in January 1980 she developed acute cerebral lupus and died 3 days after admission despite intensive therapy.

ADA, CH_{50}, and IC levels at presentation and over the next 9 months of her course are illustrated in Fig. 13.



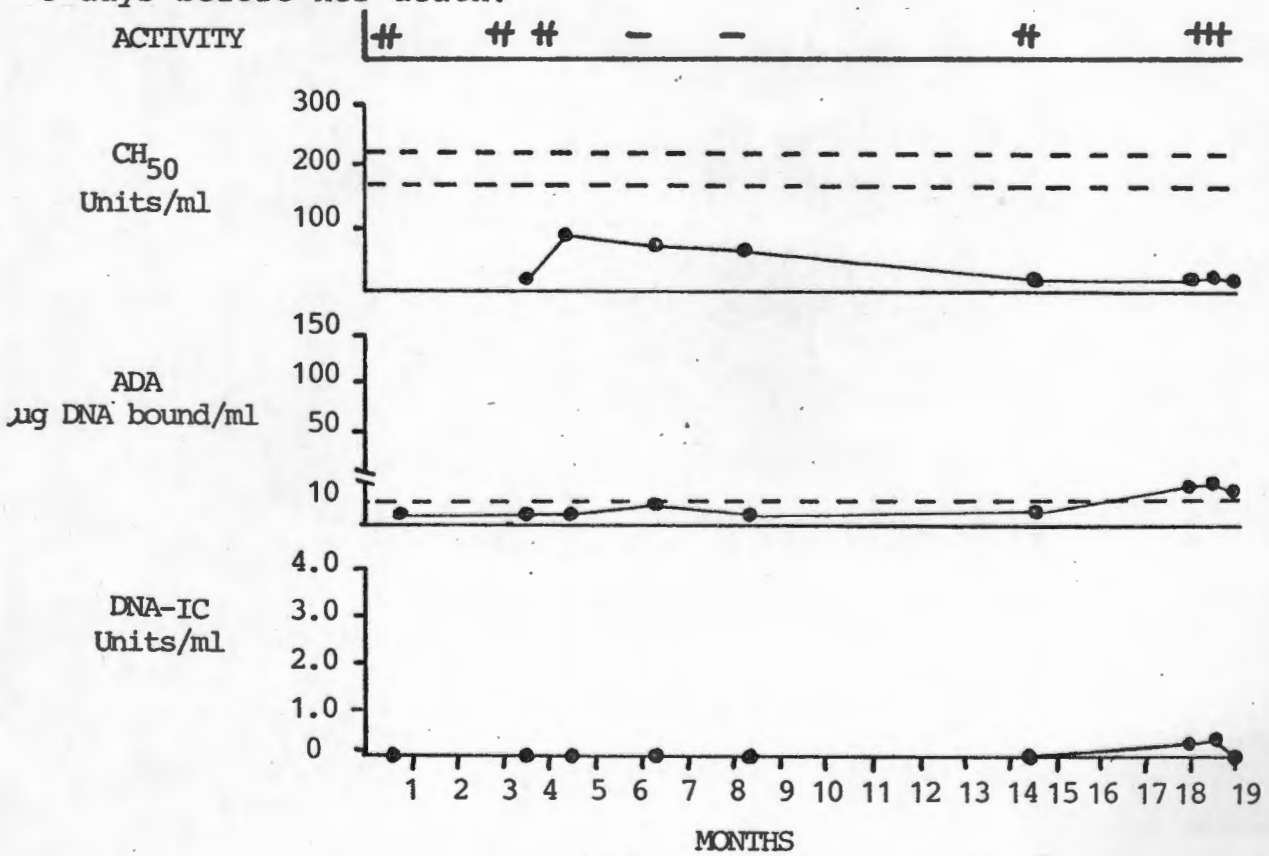
Undetectable CH_{50} and high ADA and DNA-IC levels accompanied the initial presentation in this patient. Thereafter the levels of the three parameters remained essentially normal despite subsequent episodes of active disease.

PATIENT 4

A diagnosis of SLE was made on this White female patient (PR) aged 33 years at the time on the basis of nephritis, polyarthritis, alopecia, butterfly skin rash, severe Raynaud's phenomenon, neurological involvement, positive LE cells and high titre ANA. Her course over the years was stormy, punctuated by episodes of digital gangrene,

lethargy and persistent hypoalbuminaemia which, despite intensive investigation for evidence of malabsorption, protein-losing enteropathy or as a result of renal or hepatic involvement remained unexplained. She developed hypertension and terminally her renal function deteriorated. She was admitted to hospital with a myocardial infarct in August 1977 and died 14 days later. Autopsy revealed coronary atherosclerosis, subendocardial anteroseptal myocardial infarction, polyserositis, Libman-Sacks endocarditis, pneumonitis, a solitary hepatic angioma, multiple renal and hepatic infarcts, pulmonary oedema and a midbrain haemorrhage. Unfortunately due to autolysis the kidneys and bowel were unsuitable for histological examination.

Fig 14 illustrates CH_{50}, ADA and IC levels over an 18 month period. The last series of figures reflect the situation 3 days before her death.

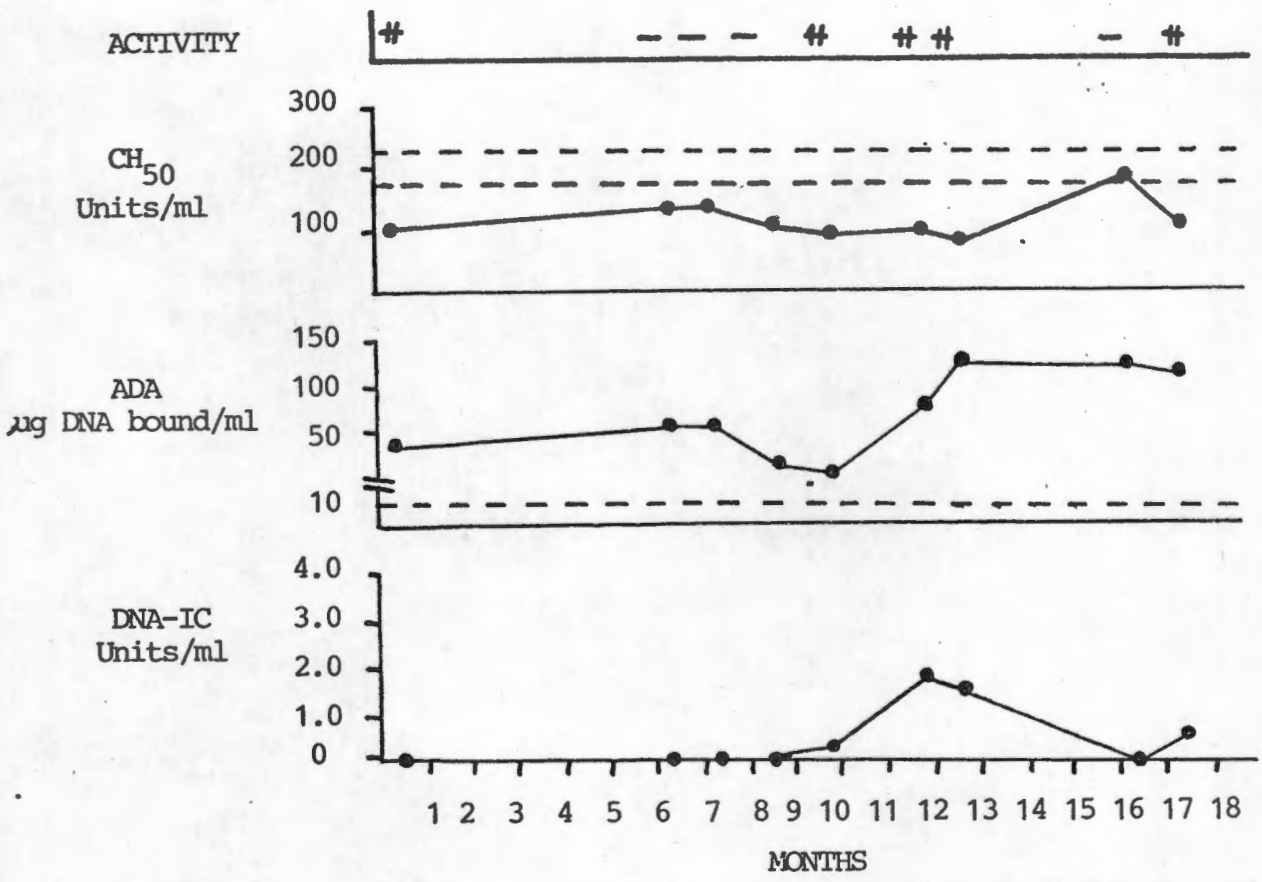


Persistent hypocomplementaemia characterised the clinical course both during active and inactive disease. ADA and DNA-IC levels remained normal until the terminal episode.

PATIENT 5

The patient, a White male (TN) developed seronegative polyarthritis in 1970 at the age of 20 years. Two years later SLE was diagnosed on the basis of LE cells, leucopenia, positive ANA, hypocomplementaemia, proteinuria and granular casts. A renal biopsy was not performed at the time due to a persistently low prothrombin index and a prolonged KCT which did not respond to Vitamin K. He was treated with indomethacin and chloroquin initially but due to inadequate control was given small doses of prednisone. A renal biopsy performed 4 years later showed mild mesangial proliferation, basement membrane thickening with complete fibrosis of some glomeruli. The major emphasis of his disease over the years has been on the musculoskeletal system. He has had recurrent episodes of tenosynovitis and has required surgery for spontaneous rupture of the right tendo Achilles, both patellar ligaments and the right fifth extensor tendon of the right hand. He has also developed marked Boutonniere deformities of his hands.

His laboratory parameters during two musculoskeletal flares are shown in Fig. 15.



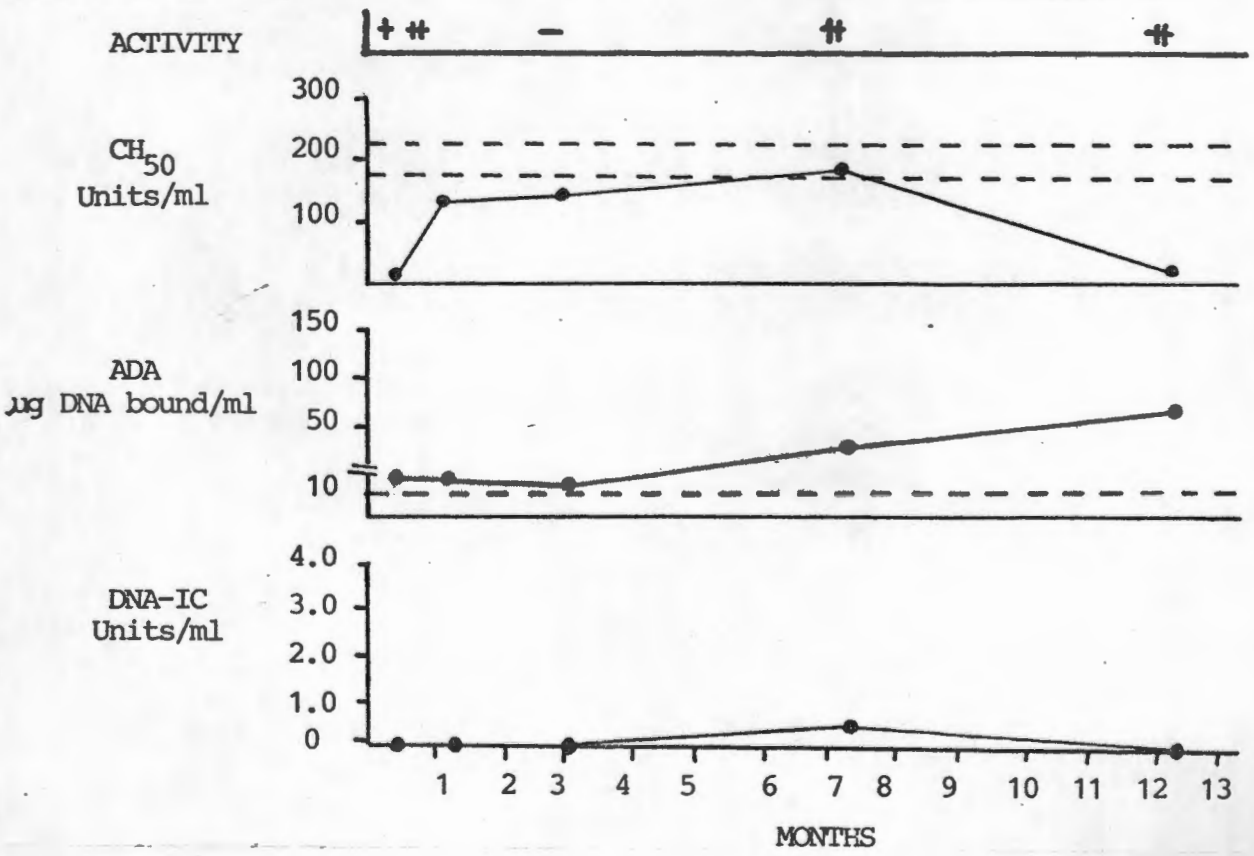
In general a good correlation is present between the three parameters and acute exacerbations. During inactive disease complexes were undetectable but hypocomplementaemia and ADA elevation persisted.

PATIENT 6

This White female patient (JS) presented in 1974 at the age of 19 years with polyarthritis, a butterfly rash, photosensitivity, alopecia and pyrexia. LE cells were positive on numerous occasions, ANA was positive to a titre of 1000, and an ADA level of 70 µg DNA bound/ml serum, hypocomplementaemia and leucopenia were recorded. She

responded well to corticosteroid therapy but over the next three years had persistent polyarthritis, alopecia and active skin lesions which were not adequately controlled despite continued treatment with prednisone and non-steroidal anti-inflammatory agents. In August 1978 there was a flare of arthritis and she developed pericarditis. This episode was controlled with an increase of steroid dosage. Her subsequent course has been uneventful apart from an attack of herpes zoster and the development of granular casts in the urine. Renal biopsy revealed patchy increase in the mesangial matrix and focal hypercellularity. The basement membrane was normal.

The serological findings during a phase of arthritis and cutaneous vasculitis, and also during the episode of pericarditis are shown in Fig. 16.

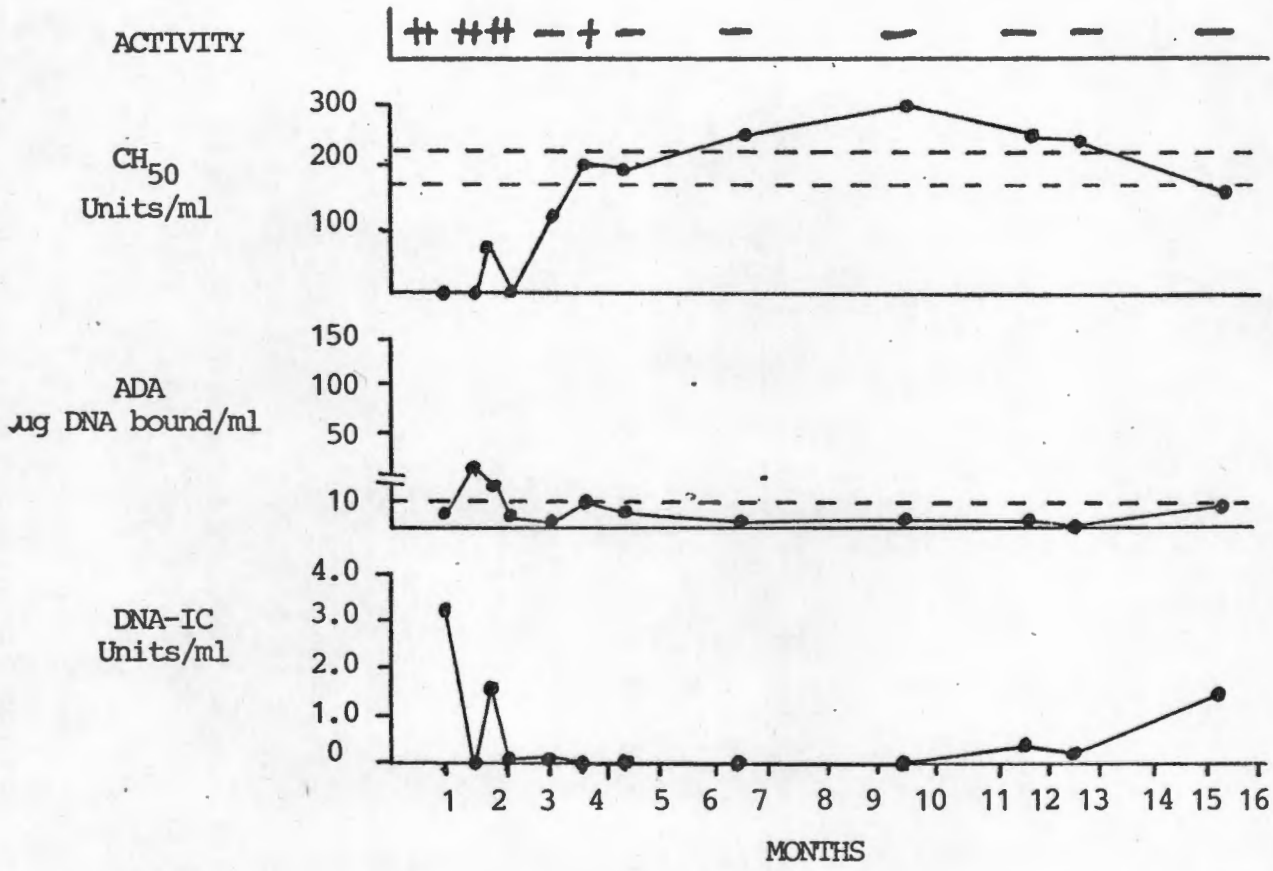


ADA levels followed the clinical course most closely. During the active episodes two of the three parameters (i.e. elevated ADA with either hypocomplementaemia or detectable complexes) were abnormal.

PATIENT 7

SLE was diagnosed in this White female patient (NP) in 1970 at the age of 28 years. The presenting features were a butterfly rash of the face, maculopapular eruption of the palms, Raynaud's phenomenon, polyarthritis, photosensitivity, a biological false positive test for syphilis, LE cells, thrombocytopenia and a positive Coombs test (without anaemia). In 1976 she developed hypertension, heavy proteinuria and granular casts in the urine. Renal histology was that of membranoproliferative glomerulonephritis. She was treated with high-dose corticosteroids and cyclophosphamide. Apart from occasional episodes of cutaneous vasculitis and alopecia (the latter possibly due to cyclophosphamide) she remained well clinically. A repeat renal biopsy in February 1978 showed active focal proliferative changes.

Fig 17 illustrates CH_{50}, ADA and IC levels measured during both active and inactive phases of this patient's illness over a 15 month period.



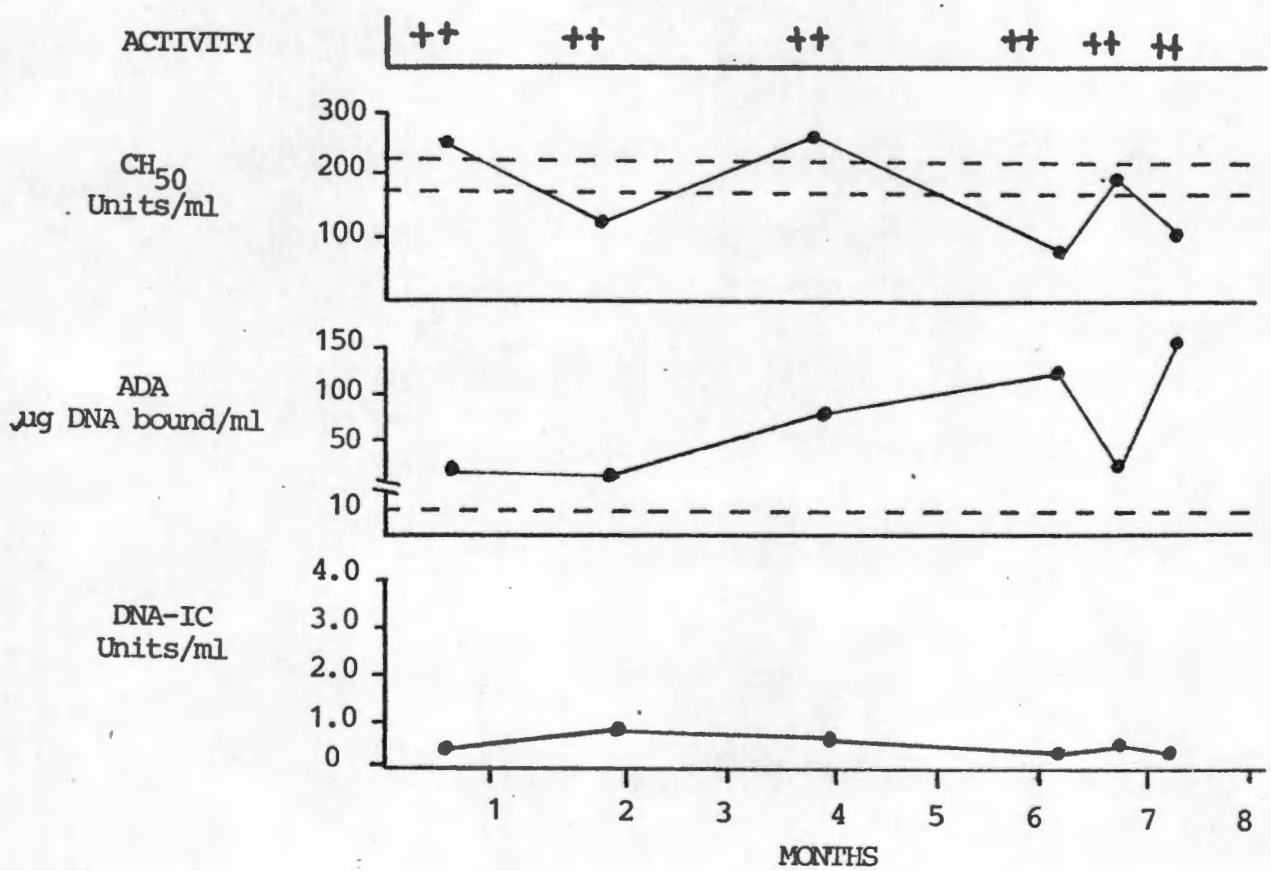
But for the appearance of complexes during the later stages of clinically inactive disease, a good correlation of the three parameters with the clinical status is demonstrated.

PATIENT 8

This Coloured female patient (LJ) presented in 1972 at the age of 30 years with alopecia, Coombs positive haemolytic anaemia, positive LE cells on numerous occasions, positive ANA, ADA of 77 µg DNA bound/ml serum and the nephrotic syndrome with heavy proteinuria and granular casts. In 1975 she developed pleurisy. Renal biopsy revealed focal

proliferative glomerulonephritis. She could not be adequately controlled on relatively high doses of prednisone and despite the addition of cyclophosphamide to the regime, control of the disease was not achieved. Polyarthralgia with significant morning stiffness has since developed and she still has persistent malaise, fatigue and the nephrotic syndrome.

IC's were monitored with the usual indices of disease activity over a 7 month period, shown in Fig. 18.

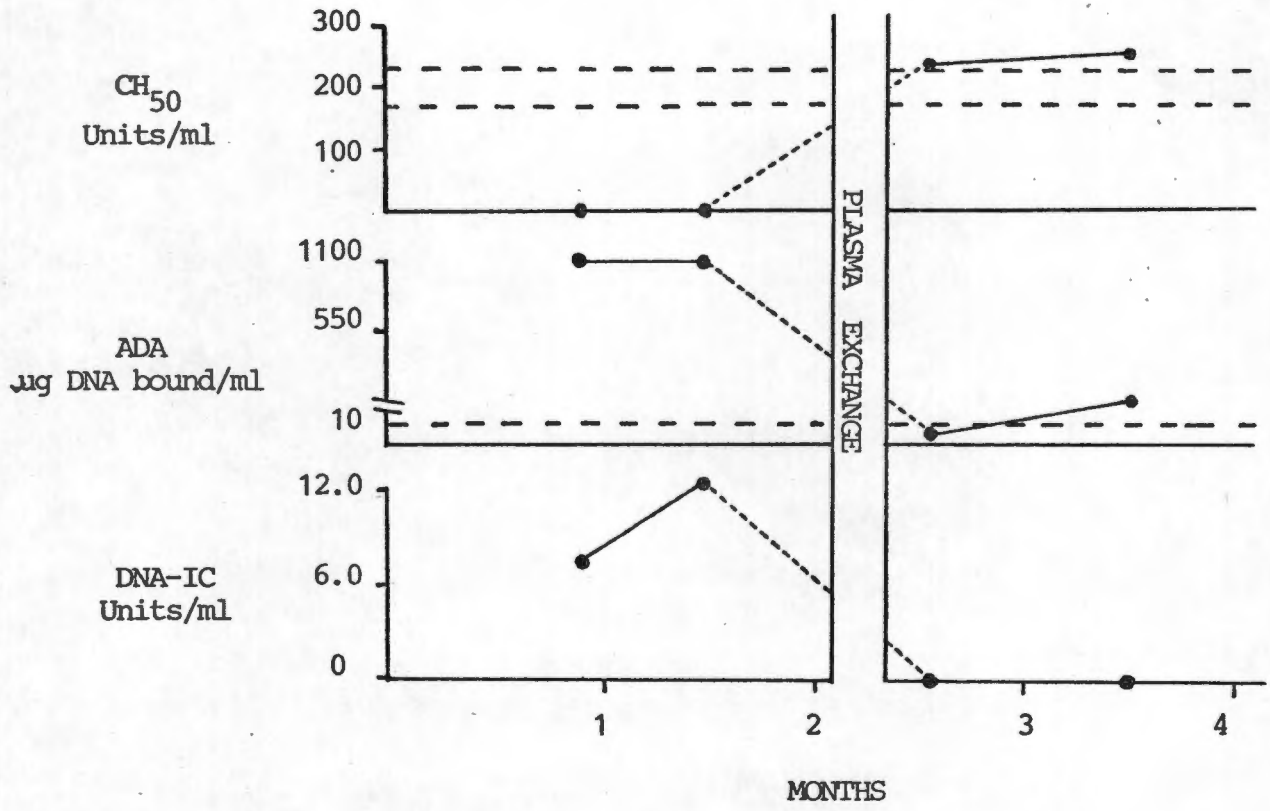


In this patient who has never been in remission clinically, high levels of ADA and significant levels of complexes were a persistent feature. By contrast hypocomplementaemia was only present intermittently.

PATIENT 9

At the age of 35 years this Asian lady (AM) developed severe polyarthritis, Raynaud's phenomenon, malaise, pleuritic chest pain, fever, headache, skin vasculitis and positive LE cells. Blood urea was normal with a creatinine clearance of 22 ml/min, and an ADA level of 1063 µg DNA bound/ml serum, hypocomplementaemia, ANA 2500 and leucopenia were recorded. Renal biopsy revealed focal mesangial hypercellularity and wire-looping. IgA 2+ was demonstrated on immunofluorescence. She was treated with high-dose corticosteroids. Despite this she developed psychotic manifestations and convulsions. Cyclophosphamide was commenced and because of the severity of the presentation five plasma exchanges were performed over a 7 day period. There was a dramatic improvement in the clinical state apart from the psychosis. The latter resolved over several weeks and she was discharged on prednisone and cyclophosphamide. Her subsequent course has been uneventful and she has remained in virtual remission over a 2 year period of follow-up.

CH_{50}, ADA and IC levels before and after plasma exchange are shown in Fig. 19.



The dramatic return of the three parameters to normal levels following plasma exchange is evident.

DISCUSSION

While the usefulness of the ARA criteria for the classification of SLE is not disputed, a significant number of patients who are excluded from clinical or laboratory studies because of failure to fulfil the criteria undoubtedly do have SLE.

In common with the experience of others (26-28) we have a number of patients with unequivocal SLE with a deforming arthropathy indistinguishable from RA. Still at a clinical level a number of patients attending our clinic who are not overtly psychotic, and who do not have any organic neurological disturbance or a history of epilepsy, at one time or another exhibit "odd" behavioural tendencies which are difficult to categorise and which we believe to be part of the lupus syndrome. With the introduction of the antineuronal antibody, LCA and 15 O_2 scanning techniques, we hope to investigate further this aspect of minor neuropsychiatric manifestations in SLE.

While the LE cell test, one of the ARA criteria, is still performed as a routine diagnostic test in SLE we find the ANA, while also nonspecific, and the ADA, which is more specific, of considerably more value in the diagnosis of SLE. The ADA is particularly useful as a diagnostic aid, since we found significant elevation of 15 µg DNA bound/ml serum using our laboratory standards in 49% of 186 patients with SLE (which includes both active and inactive disease)

and in only 2% of 176 normal subjects. No levels in excess of 15 µg DNA bound/ml serum were recorded in 21 patients with RA.

The three patients included in the present series who do not fulfil the criteria for SLE should therefore not strictly be classified as such , but for the reasons mentioned, there was little doubt of the diagnosis and they were included because they illustrate important aspects pertinent to the study. A revision of the criteria, which should at the very least include ADA, is awaited.

The clinical evaluation of patients for evidence of disease activity poses a number of problems. While the assessment is relatively straightforward in most cases, particularly when there is objective evidence of arthritis, pleurisy or pericarditis or when typical skin changes are present, it may be extremely difficult in the case of renal or cerebral involvement. A number of schemes have been devised including the use of scoring indices, grading according to the degree of activity, criteria for major or minor flares or schemes based on the need for increased steroid therapy in order to control symptoms (80,100,310, 311). The system used in this study is based on these but with a few modifications. The need to institute steroid therapy or to increase the dose in those patients already on treatment permits a reasonably clear differentiation between minor and major flares. In our experience most minor flares can be controlled adequately without steroid

or immunosuppressive therapy. The third category of patients, those presenting with life-threatening disease was included in order to study the role of the DNA-IC in this small but important group separately.

No system is entirely satisfactory since the evaluation has to depend on a number of subjective parameters such as malaise, fatigue, polyarthralgia or the duration of morning stiffness. Even some of the more objective parameters may be difficult to evaluate. As far as cutaneous manifestations are concerned Raynaud's phenomenon is not generally regarded as an index of disease activity and although difficult to treat is usually associated with milder disease (312). Whether leg ulcers, urticaria or digital gangrene are indicative of activity is also open to question. In order to avoid as far as possible false positive assessments of activity these cutaneous manifestations were not included in the scheme.

Furthermore the question may be asked: Do cellular casts in the urine indicate active renal lupus? Red cell casts and "significant" numbers of granular casts are suggestive (310) but should not be used in isolation. Certainly their absence does not exclude active renal involvement as casts may be shed intermittently. If it were practical the total number of casts excreted in 24 hours would be of more value. The degree of, or an increase in proteinuria is frequently used as a parameter of disease activity (78).

The absence of significant proteinuria however does not exclude active renal disease. It must be emphasised that before ascribing any of these manifestations to SLE itself, other causes must be considered and carefully excluded.

The main object of this study was to evaluate a new specific DNA-IC assay in patients with SLE in the following terms: firstly, to establish whether its inclusion as a parameter of disease activity is justified; secondly, whether their presence in the circulation in the absence of clinical activity may be used to predict the onset of active disease; thirdly, whether their presence in the circulatien reinforces the concept that complexes with DNA are important in the pathogenesis of the disease.

Using a DNA-specific IC assay Harbeck et al demonstrated a correlation between IC levels and active disease (117,119, 120). In their series as a whole DNA-IC's were present in 58% of cases during acute episodes. This is in agreement with our data where circulating complexes were detected in 62% of acute flares. They also claimed that increased levels were only present during active cerebral and/or renal lupus. Their latter claim was based on a subdivision of their series of 50 patients into 4 groups according to the predominant organ involved. Unless the criteria for the exclusion of certain organs are clearly defined, a system such as this must be viewed with caution. This is particularly relevant to renal involvement where, as has been pointed out, normal renal function and urinalysis alone

are not sufficient to exclude renal activity. It was not stated whether renal biopsies were performed in all their patients.

Biopsies were not performed routinely in our series during acute episodes unless there was clinical or laboratory evidence of renal involvement. Therefore a clinical subdivision such as Harbeck's was not attempted.

The interesting correlations between the three serological parameters require comment. In the first instance, why is CH_{50} a better monitor of SLE than either ADA or DNA-IC? The most likely explanation would seem to be that CH_{50} consumption occurs during the formation of any IC which is complement-fixing, whether the complex is DNA in nature or not. Therefore if it is true that both DNA and non-DNA IC systems share in the pathogenesis of SLE it is logical to assume that CH_{50} would be a good monitor of disease activity providing that the complexes formed are complement-fixing.

Why the DNA-IC should be a better monitor of disease activity than ADA is also not clear but at least two possibilities may considered to account for this discrepancy.

1 ADA in itself is not capable of producing tissue damage so that its presence in the circulation does not necessarily imply that IC's are being formed and deposited. Circulating complexes on the other hand, because of

their potential to initiate tissue injury if deposited, are more likely to be associated with tissue damage and therefore with evidence of disease activity.

2 Even if IC formation is an inevitable consequence when circulating ADA is present, the IC formed, because of its size (for example, large in a situation of antibody excess) or for some other reason, may be rapidly cleared by the RES so that tissue deposition does not occur. Because of the rapid clearance of large complexes from the circulation they are more likely to escape detection.

Whatever the reasons, it has been shown conclusively that the DNA-IC is a valuable addition to the existing serological monitors of disease activity in SLE.

With regard to the predictive value of the IC, 8 (73%) of the eleven patients with raised DNA-IC levels in the presence of inactive disease subsequently developed evidence of activity. While the numbers are too small to draw any firm conclusions, patients with detectable complexes should be observed more closely. As shown, however, it is not possible to predict the nature or the severity of the attack from the levels of circulating complexes although none of the levels was markedly elevated. A longterm study on a larger group of patients is at present in progress but if this small study is representative it would not seem logical to treat patients on the basis of raised IC levels alone.

The possibility that subclinical renal activity might have been present in some of the patients with elevated DNA-IC levels in clinically inactive disease cannot be excluded. The important question of whether elevated DNA-IC levels are a better monitor than CH_{50} or ADA for active renal disease can only be resolved by performing renal biopsies in all patients with increased IC levels whether or not evidence of renal activity is present at the time.

A close relationship has been shown between DNA-IC levels and the severity of clinical manifestations in SLE. The difference in levels between inactive/minor, major and life-threatening, allowing for the fact that there were only 5 samples in the last group, was striking. This data suggests that DNA-IC may not only be of qualitative significance in SLE, but that quantitatively it may be of considerable importance in the pathogenesis of the tissue lesion.

Tan et al (207) suggested that acute exacerbations in SLE are preceded by a rise in ADA levels and that, with the onset of symptoms, ADA disappears from the circulation and coincides with the appearance of free circulating DNA, implying that DNA-IC formation is responsible for the clinical manifestations.

In our experience, firstly, DNA levels bear no relationship whatsoever to disease activity in SLE. Secondly, while ADA levels preceeding acute attacks have been recorded in some of our patients this is by no means the rule. As

outlined in Section III (p106) we recognise five categories of patients with respect to ADA and DNA-IC levels during acute exacerbations.

The serial studies of these parameters in the patients described, while demonstrating a good correlation between the three monitors and the presence of active disease (Patients 1, 2, 3, 4 and in Patient 5 during her initial presentation) and inactive disease (Patients 2 and 5), this was by no means the rule, not only in the group as a whole but also in individual patients during different acute episodes.

Of interest in Patient 1 was the finding that, while there was a good correlation between hypocomplementaemia and raised IC levels during disease activity, the free ADA was generally within the normal range or showed only mild elevation. DNase digestion showed that virtually all the ADA was present in the form of complexes and thus escaped detection. Furthermore hypocomplementaemia and IC elevations in this patient antedated the appearance of clinical manifestations by 6 weeks. He was receiving 30 mg of prednisone at the time and since it is not our policy to treat on the basis of laboratory parameters alone, additional therapy was not instituted. However it subsequently became necessary to institute immunosuppressive therapy which was continued for 8 months before control was achieved of the major flare which ensued.

This raises the question of whether earlier recourse to more aggressive therapy might have aborted or at least have modified the exacerbation with easier control of the disease. While earlier therapy might have benefitted this patient, the arguments against this approach have already been discussed.

The clinical course of Patient 4 prior to her death was one of chronic low-grade activity characterised by persistent hypocomplementaemia in the face of normal ADA levels and absent IC's, and it was not until her terminal illness that these parameters became abnormal. But for mild elevation of ADA levels in Patient 6, a similar trend was noted during her first acute attack. A possible explanation for this phenomenon of normal ADA and absent IC's in the face of active disease is that other non-DNA-containing IC's which are complement-consuming played a role in the pathogenesis of the disease. An alternative explanation is that local formation of DNA complexes occurs as suggested by Izui et al (241).

But for the cerebral manifestations, there was a striking improvement in the clinical condition after plasma exchange of Patient 9 who presented with life-threatening disease and high circulating IC's. This is in agreement with the experience of Verrier Jones et al (313) who found that patients with detectable complexes showed greater improvement than those without. The clinical improvement in our

patient was commensurate with the disappearance of IC's from the circulation and one presumes that they were of importance pathogenetically. The possibility cannot of course be excluded that non-DNA-containing IC's were also removed by plasmaphoresis.

Also of interest in this patient is the fact that despite a life-threatening presentation there has been no major recurrence of the disease after over two years of observation. It is possible that her future course will be one of minor symptomatology or of complete remission. This phenomenon of "once-only" disease has been noted in several (at least 12) of our patients observed over periods of between 2 and 20 years. This phenomenon in SLE is being increasingly recognised even in patients who present with major or life-threatening manifestations. Whether earlier or more aggressive therapy in these patients bears any relationship to the long-term outcome is at present unknown.

In conclusion two important questions must be asked: Firstly, has this new specific DNA-IC assay made a contribution to our understanding of the pathogenesis of SLE as an IC disorder? Secondly, is its inclusion as an additional parameter in the investigation and management of SLE justified?

In answer to the first question, numerous studies have

demonstrated the presence of DNA-containing IC material in organs such as the kidney and skin of patients with SLE suggesting that they are of importance pathogenetically. Furthermore, clinical studies have shown a good correlation between active disease and circulating IC's using various antigen nonspecific techniques for their detection. By contrast there are very few reports investigating the relationship between specific DNA-IC levels and disease activity. One of the reasons for this lack is the paucity of techniques available for their detection.

Recognising the need for an additional assay which is both practical and easy to perform the principle of millipore filtration and DNase digestion was utilised. The successful application of the assay is evidenced by the data obtained in the study of active and inactive SLE and may be summarised as follows:

1 A good correlation was shown between active disease and the presence of DNA-IC, both as a single monitor (where it is superior to ADA), or in combination with CH_{50} and/or ADA.

2 A good correlation also exists between the levels of circulating complexes and the severity of the acute attack. While of prognostic value for the immediate attack, the long-term effect of high levels of circulating complexes on prognosis requires further study.

3 Evidence has also been presented that DNA-IC is of predictive value in that its appearance in the circulation in a substantial number of cases antedates the onset of active disease. Until more data is available the appearance of DNA-IC alone is not considered to be an indication for instituting corticosteroid or immunosuppressive therapy.

4 The specificity of the assay for SLE has been discussed in Section III.

This data reinforces the belief that DNA is an important immunogen in SLE. The participation of other IC systems is not challenged. The nature and molecular weight of DNA involved in complex formation remains to be determined. Indirect evidence supporting the suggestion of Bruneau et al (123) that the DNA involved in complex formation is of low molecular weight was the failure to demonstrate free high molecular weight DNA in the majority of our patients with SLE. However in order to resolve the question, the size and nature of the DNA actually present in the IC's must be determined directly.

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