PRODUCTION, CHARACTERIZATION AND SPECIFICITY OF ANTIBODIES AGAINST DEOXYRIBONUCLEIC ACID

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(Asif Ali)
DEDICATION

DEDICATED TO MY PARENTS
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ABSTRACT

The presence of spontaneously occurring autoantibodies to nuclear antigens is a hallmark of diseases collectively known as autoimmune diseases and include systemic lupus erythematosus, progressive systemic sclerosis, rheumatoid arthritis and Sjogren's syndrome. The antigens recognized in these rheumatic diseases include single-stranded DNA, single-stranded RNA, double-stranded DNA, double-stranded RNA, ribonucleoprotein, nucleosomes, histones and nonhistone proteins. Antibodies to DNA are of prime importance in both human and animal diseases. The laboratory detection of circulating anti-DNA antibodies is of great clinical significance not only for the diagnosis of rheumatic diseases but also for the management of patients afflicted with them. A number of techniques have been adopted for the measurement of anti-DNA antibodies but their usefulness is limited by one or more factors.

The incidence of anti-DNA antibodies in various rheumatic diseases was studied by immunoprecipitation and hemagglutination techniques and the sensitivity of these assays was compared with spectrophotometric and fluorometric techniques reported in this thesis. Seventeen percent and 26% of SLE patients were positive for anti-DNA antibodies by immunodiffusion (ID) and counter-immunoelectrophoresis (CIE) respectively as compared to 35% with passive hemagglutination assay (PHA). Antibodies to DNA were positive in 39% and 43% of SLE patients with spectrophotometric
and fluorometric techniques respectively. Anti-DNA antibodies were detected in 14% of SLE patients with overlapping PSS by PHA, spectrophotometric as well as fluorometric techniques. However, none of such sera was positive by immunoprecipitation techniques. These results show that both spectrophotometric and fluorometric techniques are more sensitive than ID and CIE and almost parallel in sensitivity to PHA.

A microplate enzyme-linked immunosorbent assay (ELISA) is described for the measurement of anti-DNA antibodies. The method employs plastic surface for antigen binding and alkaline phosphatase-linked rabbit anti-human IgG for the detection of immune complexes, using colorigenic (PNP-P) and fluorogenic (4MU-P) enzyme substrates. The sensitivity of the two substrates was compared in ELISA under different experimental conditions. Fifty seven percent of SLE patients were positive for anti-DNA antibodies with PNP-P and 71% with fluorogenic substrate. Microplate ELISA as described in this thesis in conjunction with 4MU-P is simple, sensitive and could be of help in the detection of low avidity anti-DNA antibodies.

The sensitivity of ELISA has been further increased by the use of different solid support to increase the surface area for antigen immobilization and by decreasing nonspecificity and background values. We report the successful utilization of nylon as solid phase for antigen binding for use in ELISA in the detection of anti-DNA antibodies. A number of preliminary experiments
were performed to define the experimental conditions for optimum results. The prevalence of anti-DNA antibodies in rheumatic diseases was compared using fluorogenic and colorogenic enzyme substrates. Fifty two percent of SLE sera were positive for anti-DNA antibodies with PNP-P and 56% by 4MU-P as substrates. Fourteen percent of SLE patients with overlapping PSS and 10% of PSS patients showed a positive reaction with both the substrates. ELISA utilizing nylon as solid support is convenient, specific and suitable for use in clinical laboratories where large number of samples are to be screened for the detection of anti-DNA antibodies. Antigen coated beads can be stored for appreciable period without loss in antigenicity. The procedure requires small quantities of antigen or antibody and is therefore highly economical.

Anti-DNA antibodies in the sera of patients with various rheumatic diseases were classified into three types depending upon their avidity, on the basis of their reaction in ID, CIE, PHA and ELISA utilizing nylon as solid phase. Twenty one percent SLE patients, 14% SLE + PSS and 10% PSS patients showed low avidity anti-DNA antibodies. Twenty three percent of SLE patients and 14% SLE + PSS patients demonstrated moderate avidity whereas thirteen percent of SLE patients had high avidity anti-DNA antibodies.

Rabbits immunized with calf thymus ds- and ssDNA-MBSA complex elicited antibodies against ssDNA as detected by immuno-
precipitation and hemagglutination techniques, indicating their specificity for bases and that the antigenic determinants are not related to the backbone of DNA.

Antibodies to native DNA can not be produced by experimental immunization but occur spontaneously in NZB/NZW mice which are genetically predisposed to a disorder closely resembling human SLE. We report the production of antibodies against DNA-psoralen adduct showing crossreactivity with dsDNA. These antibodies were not detectable by immunoprecipitation and hemagglutination techniques. ELISA utilizing nylon as solid support was adapted for the detection of these antibodies. The production of low avidity, conformation oriented antibodies in animals immunized with DNA-psoralen adduct was observed. These antibodies were specific for DNA-psoralen adduct and showed strong crossreactivity with dsDNA. Single-stranded DNA and RNA exhibited low reactivity with immunized sera. SLE anti-DNA antibodies isolated by affinity chromatography using dsDNA-Sepharose 4B columns were used to study the specificity of immune sera. The results showed that affinity purified anti-DNA antibodies have an immunological specificity identical to that of the experimentally immunized sera.

The results presented here suggest that although it is not possible to immunize animals against dsDNA but when DNA is intercalated with psoralen, this apparently nonimmunogenic molecule becomes immunogenic as a result of change in some portion
of DNA molecule induced by intercalation with psoralen. The low reactivity of immunized sera with ssDNA/RNA could probably be due to nonavailability of the antigenic bases to elicit antibody response. It is proposed that similar structural changes might occur in DNA by causative agent(s) making it highly antigenic and thus contributing to the pathogenesis of SLE.
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I. INTRODUCTION

The need for self and nonself discrimination was appreciated long ago and the concept of autoimmunity was considered early in the development of immunology. Autoimmunity can be considered as a two-stage process in which a breakdown of immunological regulation activates immunologic effector mechanism which results in autoantibody formation and tissue destruction.

Immune reactions are mediated by a number of different effector cells. These include B lymphocytes and their plasma cell progeny, T lymphocytes which are active in cellular immunity and delayed hypersensitivity, monocytes and related tissue macrophages. These effector cells are regulated by an intricate network of controlling mechanisms. Autoimmunity may arise not as a primary effector cell abnormality but rather as the consequence of disordered immunologic control. The mechanism of autoimmunity is unknown. It is frequently associated with genetic, immunologic and viral factors that interact with immune system and influence regulation (Talal, 1976, 1978, 1981).

The first attempt to explain how self unresponsiveness might be acquired was put forward by Burnet (1962) as a corollary to his clonal selection hypothesis. This hypothesis postulated that each clone of lymphocytes gives rise to cells making antibody
of only one specificity. He also suggested that antibody diversity is generated by rapid somatic mutation especially in embryonic development. Since some randomly generated clones would necessarily be capable of reacting with self antigens, he surmised that these would be eliminated if they come into contact with the corresponding autoantigen during the critical period of development. Thus all self reactive lymphocytes would be deleted and the rest retained. The above hypothesis for self tolerance is no longer tenable in its simplest form because normal animals show some evidence of autoimmunity including autoantibodies, autoantigen binding B cells and autoantibody secreting plasma cells. These are found even when the autoreactive lymphocytes are known to have been exposed to the corresponding autoantigen from early life (Elson et al., 1979).

Hossal and Pike (1975) revived a hypothesis which proposes that at some stage of their differentiation from stem cells to mature antibody forming precursor cells, B lymphocytes go through a phase during which contact with antigen induces only tolerance and not immunity. This theory is different from Burnet's (1962) in focussing attention on the stage of differentiation of lymphocytes rather than the animal as a whole. The tolerant state induced in differentiating cells is independent of suppressor cells and appears to result from the failure of mature progenitors. This is probably how most self reactive lymphocytes are eliminated.
If most autoreactive B cells are deleted then how do some emerge unscathed? Some may escape because the corresponding antigens are not expressed until after the B cells complete their differentiation or because the concentration of autoantigen is too low. Perhaps the critical factor governing the fate of immature autoreactive B cells is the avidity of antigen binding by their surface immunoglobulin receptors, the higher the avidity then the more complete the irreversible loss of receptors. Support for this contention comes from observations that tolerance in immature B cells is more easily induced with multivalent than with paucivalent hapten-protein conjugates (Metcalf and Klinman, 1976; Elson, 1977) and in high avidity clones than low avidity clones (Nossal et al., 1979).

Of various autoimmune diseases, systemic lupus erythematosus (SLE) is considered to be the prototype. A large varieties of antibodies against nucleic acids, nucleoproteins, cell surface antigens and phospholipids have been studied in this disease. There has been no explanation for the extensive diversity in the serological abnormalities of the disease. Lupus autoantibodies are not as diverse as they seem and are restricted in both their ligand-binding properties and their idiotypic diversity. These findings imply that the process of polyclonal B cell activation, the presumed final common pathway of antibody production in SLE must involve a restricted population of lymphocytes (Schwartz, 1983).
Studies of monoclonal representatives of the major class of lupus autoantibodies—anti-DNA antibodies have disclosed that a single autoantibody can bind to multiple nucleic acid antigens of widely different base composition (Andrzejewski et al., 1981; Koike et al., 1982; Pisetsky and Caster, 1982). Another means of ascertaining the nature and limits of the polyclonally activated population of B cells in SLE is by analysis of the idiotypes of lupus autoantibodies. Several such investigations have been carried out. These investigations strongly suggest that anti-DNA idiotypes originate from a restricted family of germ line genes. Moreover, the study reveals that the population of lymphocytes that can produce anti-DNA antibodies occur not only in lupus prone mice but also in normal mice. The spontaneous production of such antibodies, therefore, must involve the loss of regulatory mechanism that operates either at the level of immunological process or on the expression of immunoglobulin genes (Schwartz, 1983).

The presence of spontaneously occurring autoantibodies to many nuclear antigens occur in disease conditions collectively known as autoimmune diseases which include systemic lupus erythematous (SLE), rheumatoid arthritis (RA), progressive systemic sclerosis (PSS), Sjogren's syndrome (SS), dermatomyositis (DM), and mixed connective tissue disease (MCTD). Nuclear macromolecules recognized as antigens in these autoimmune diseases include double-stranded deoxyribonucleic acid (dsDNA), single-stranded deoxyribonucleic acid (ssDNA), double-stranded ribonucleic acid

Antibodies against dsRNA and DNA–RNA hybrid are found in majority of SLE patients (Schur and Monroe, 1969; Steinberg et al., 1969; Hiroshi, 1980). Autoantibodies against ssRNA and double-stranded synthetic polymer like poly I:C, poly A:U (Eilat et al., 1978), synthetic ssRNA like poly A, poly C (Koffler et al., 1971; Pillarisetty et al., 1975), tRNA (Eilat et al., 1976) and ribosomal RNA (Lamon and Bennet, 1970) have been well established. Antibodies against heterogeneous nuclear RNA have also been demonstrated (Ali and Tan, 1979). Autoantibodies against RNA are also present in the New Zealand mouse, NZB/NZW F₁ hybrid (Eilat et al., 1977). RNA antibodies show marked specificity for RNA and do not show any crossreactivity with native DNA. However crossreactivity was observed with DNA–RNA hybrids (Stollar, 1970).

Antibodies to histones have been reported in 95 percent of patients with procainamide induced lupus erythematosus (Fritzler and Tan, 1978), 30 percent of patients with idiopathic SLE (Kunkel et al., 1960; Tan et al., 1976) and 35 percent of patients with rheumatoid arthritis (Aitcheson et al., 1980).
These antibodies have reactivity against H₁, H₂A, H₂B, H₃ and H₄ histone subpopulations (Rekvig and Hannestad, 1979). It has been reported that the majority of drug induced lupus patients had antibodies directed against the H₂A-H₂B histone complex (Tan and Portanova, 1981). A number of studies have been carried out to determine the precise antigenic determinants on deoxyribonucleoprotein. It has been shown that when the DNA moiety of deoxyribonucleoprotein is destroyed by digestion with DNase, the antigen is no longer reactive with antibody. Similarly when the histone moiety of deoxyribonucleoprotein is digested with trypsin, the remaining DNA is also no longer reactive with antibody. Because of these and a number of other observations it was postulated that the binding of antibodies to deoxyribonucleoprotein is dependent on the presence of both proteins and DNA but is independent of the structural integrity of the entire deoxyribonucleoprotein (Holman and Deicher, 1959; Tan, 1967; Tahourdin and Bustin, 1980).

A variety of other autoantibodies against tissue extracts have been described in the sera of patients with SLE and other connective tissue diseases. Recent studies have explored the significance of antibodies to nuclear nonhistone (acidic) antigen Sm (Tan and Kunkel, 1966), RNP (Sharp et al., 1972), Ro or SS-A (Clark et al., 1969), La or SS-B (Alspaugh and Tan, 1975; Alspaugh et al., 1976; Akizuki et al., 1977), RAP (Alspaugh and Tan, 1976), PM-1 (Reichlin and Mattioli, 1976; Wolfe et al., 1977), Scl-70
(Douvas et al., 1979b) and have demonstrated their diagnostic specificity and association with certain clinical features in these diseases. Microsomal (Deicher et al., 1960), lysosomal (Wiederman and Meischer, 1965), ribosomal (Schur et al., 1967; Lamon and Bennet, 1970) and mitochondrial (Doniach et al., 1966) antibodies have been reported in these diseases. Recently antibodies to neurons (Toh and Mackay, 1981), α-interferon (Panem et al., 1982), acetyl choline receptor (Valesini, 1983), poly ADP-ribose (Morrow et al., 1982) and collagen (Gioud et al., 1982) have been reported in SLE.

Antibodies to DNA is of prime importance in both the human and animal diseases (Koffler et al., 1967; Lambert and Dixon, 1968; Andres et al., 1970; Atkins et al., 1972; Landry and Sams, 1973; Tan, 1976; Swaak et al., 1982). The formation of antibodies to DNA generally occurs in SLE, New Zealand hybrid mice (NZB/NZW), Palmerston North mice, MRL and BXSB mice (Talal and Steinberg, 1974; Walker et al., 1978; Sawada and Talal, 1979; Kolaja and Fast, 1981; Hahn, 1982). Anti-DNA antibodies have been shown to be particularly injurious type of antibodies in terms of production of inflammatory vasculitis in the kidneys of patients with SLE (Tan and Kunkel, 1966). The presence of DNA and antibodies to DNA in circulation was the initial evidence which suggested that these (DNA-anti-DNA) immune complexes might be important in the kidney diseases. Renal involvement is a frequent and serious feature of SLE involving approximately 75
percent of the patients (Tan and Kunkel, 1966; Koffler et al., 1971; Stollar, 1975; Steensgaard and Johansen, 1980; Yamada et al., 1982).

SLE sera contain heterogeneous populations of anti-DNA antibodies (Arana and Seligmann, 1967; Koffler et al., 1971). Although antibodies to native DNA appear to be almost diagnostic of SLE, antibodies to ssDNA are more widespread (Hughes et al., 1971; Koffler et al., 1971). Recently antibodies specific for Z-DNA have been reported in SLE (Lafer et al., 1983). Autoantibodies to DNA occurring in both human and murine SLE sera can be divided into three major groups on the basis of specificity.

1. **Anti-dsDNA antibodies reactive with only dsDNA.** These antibodies do not react with single-stranded DNA and therefore it has been presumed that the reactive antigenic determinants on DNA should be related to the double helix conformation. This phenomenon has been checked by studies of binding properties of hybridoma anti-DNA antibodies showing striking specificity for dsDNA (Arana and Seligmann, 1967; Koffler et al., 1971; Andrzejewski et al., 1981; Ballard, 1981; Hahn, 1982).

2. **Anti-dsDNA antibodies reactive with both ds- and ssDNA.** This type of autoantibody is present in 50 to 70% of patients with SLE. Low concentrations of this antibody are also present in other rheumatological diseases. Because the antibody appears to recognize similar antigenic determinants present on double-
stranded and single-stranded DNA, most workers postulated that the antigenic determinants should consist at least in part of the deoxyribose phosphate backbone of DNA. The dual reactivity of these antibodies with both ds- and ssDNA may be due to the fact that most dsDNA strands contain single-stranded nicks and most of the ssDNA strands have helical areas. A unique feature of antibodies of this type is the fact that they occur spontaneously in SLE and other diseases but do not appear to be immunogenic in experimental animals (Notman et al., 1975; Samaha and Irvin, 1975; Stollar and Papalian, 1980).

(3) Anti-DNA antibodies reactive with only ssDNA. These antibodies recognize purine and pyrimidine bases exposed in ssDNA as antigenic determinants. These autoantibodies are found in patients with rheumatological diseases as well as nonrheumatological diseases such as chronic infectious processes (Stollar et al., 1962; Levine and Stollar, 1968; Alarcon-Segovia et al., 1970; Lacour et al., 1973, Stollar, 1973). Antibodies to ssDNA cross react extensively with RNA (Levine and Stollar, 1968).

Antibodies in autoimmune diseases appear to belong primarily to the IgG class of immunoglobulins. Antibodies to DNA and antinuclear factors belong to both the IgM and IgG immunoglobulin classes. It is clear from murine and human studies that renal disease and disease activity in general are more closely related to high circulating levels of IgG anti-DNA antibodies
than to IgM antibodies. In mice it is at the time when production of IgM anti-DNA switches to IgG that proteinuria and declining renal function begin. There has been recent report claiming that antibodies to native DNA in patients with SLE are primarily of IgG$_1$ and IgG$_3$ subclasses (Tan and Vaughan, 1979). These subclasses of IgG are known to be associated with high complement fixing activity. IgG antibodies to DNA which are capable of fixing complement are more likely to be associated with clinically significant renal lesions than are noncomplement fixing antibodies (Arana and Seligmann, 1967; Rothfield and Stoller, 1967; Schur et al., 1972; Sontheimer and Gilliam, 1978a; Tan and Vaughan, 1979; Hahn, 1982; Weinstein et al., 1983).

Native DNA is a poor immunogen and its immunogenic form has not yet been identified. Chemical modifications of DNA (with suitable carrier) leads to the immunogenicity, and higher levels of specificity are due to such modifications of structures (Stollar, 1973, 1981). Normal animals have a remarkable tolerance to DNA and are only capable of making antibodies to ssDNA when complexed to methylated bovine serum albumin and presented in Freund's adjuvant. With suitable protein carriers, ssDNA and synthetic polynucleotides can induce antibody formation in normal animals (Plescia et al., 1964, Stollar, 1973). It has been shown that DNA irradiated with UV light is a potent immunogen and antibodies formed react only with UV irradiated DNA showing no cross reaction with native or ssDNA (Tan, 1968; Natali and Tan, 1971).
conflicting results (Steward et al., 1975; Leon et al., 1977; Tron and Bach, 1977; Winfield et al., 1977; Griffiths et al., 1978; Aarden et al., 1979). SLE sera contained at least two distinct populations of anti-dsDNA antibodies. One population was of rather high avidity and dissociated slowly in the presence of excess DNA or high salt. The other population was of considerably lower avidity and dissociated more rapidly under these conditions. The results of a double label dissociation kinetics study provided independent evidence supporting this hypothesis (Riley et al., 1980). Using dsDNA fragments of varying sizes (20 to 1200 bp), the heterogeneity of anti-DNA antibodies in SLE sera and the recognition of the secondary structure of antigen for specific binding was established. Competitive assays with mononucleotides, oligonucleotides, homopolymers and RNA-DNA hybrid indicated that two strands of polydeoxyribonucleotide were required for optimal reaction with these SLE serum antibodies (Ethel and Gunnel, 1980; Papalian et al., 1980; Stollar and Papalian, 1980).

It has been reported that guanosine is the best nucleoside for inducing antibody production in BALB/c mice (Stollar and Borel, 1976). Accumulation of guanine-cytosine enriched low molecular weight DNA fragments have been shown in lymphocytes of patients with SLE (Sano et al., 1983). G-C rich DNA has been known to form unusual conformations such as triple-stranded, left-handed (Z-DNA) and bent-DNA (Podder, 1971; Selsing et al., 1979; Wang et al., 1981). If such specific structures take place in the
antigen fragments, it is possible that antibodies are raised against them. Two classes of DNA fragments have been isolated from DNA anti-DNA immune complexes by electrophoresis. The small fragments averaging molecular weight 25,000 with a length of 30 to 40 base pairs (bp) and the large fragments averaging molecular weight 100,000 with a length of 150 bp. The small fragment contains an average of 55% G-C content and the large fragment contains 45% G-C content. Because average G-C content in total human DNA is 38%, it was concluded that the antigen is rich in G-C content (Sano and Morimoto, 1982).

The heterogeneity of DNA antibody populations in human and murine SLE sera makes it difficult to study physicochemical characteristics and antigenic specificity. Therefore, the development of hybridoma technology by various workers represent a major advance and studies of monoclonal antibodies to DNA and other nuclear and cytoplasmic antigens are entering a new era.

Serological methods are playing an increasingly important role in the diagnosis and epidemiological assessment of various rheumatic diseases. With the proper assay system the diagnostic value of antibodies to dsDNA seems to be beyond doubt. Antibodies to DNA are found in high frequency in serum of patients with active SLE but not in the serum of normal person and patients with a variety of other autoimmune diseases such as SS, RA and MCTD (Pincus et al., 1969; Hasselbacher and Leroy, 1974; Rochmis
et al., 1974; Aarden et al., 1975; Lightfoot and Hughes, 1976; Bustine et al., 1982; Swaak et al., 1982).

A number of techniques have been developed for the measurement of antibodies to DNA which include complement fixation (Robbins et al., 1957), double diffusion in agar (Seligman, 1957), latex particle agglutination (Christian et al., 1958), counter-immunoelectrophoresis (Davis and Winfield, 1974), hemagglutination (Inami et al., 1973), precipitation of radiolabelled DNA-anti-DNA immune complexes with ammonium sulfate (Farr, 1958; Wold et al., Pincus et al., 1969), nitrocellulose membrane technique (Kredich et al., 1973), millipore filter assay (Ginsberg and Keiser, 1973), peroxidase labelled antibody test (Vladutiu, et al., 1979), test utilizing glass fiber filter (Lewis et al., 1973), polyethylene glycol precipitation assay (Riley et al., 1979; Smeenk et al., 1982), solid phase radioimmunoassay (Tan and Epstein, 1973; Smith et al., 1981), immunofluorescence (Sontheimer and Gilliam, 1978b; Somerfield and Wilson, 1980), fluorometric assay (Shepherd et al., 1978) FIAx fluorometric assay (Berne et al., 1982) and enzyme-linked immunosorbent assay (Hillyer and Modeline, 1980; Labrouce et al., 1980; Miller et al., 1981; Kavai et al., 1982; Eaten et al., 1983).

The laboratory detection of circulating DNA antibodies is of great clinical significance not only for the diagnosis of SLE but also for evaluating its prognosis and efficacy of treatment (Pincus et al., 1969; Feltkamp, 1975; Swaak et al., 1982).
Sensitive, rapid and economic procedures are desired for clinical diagnosis and efficient management of these patients. The potential usefulness of most of the techniques presently in use is often limited by one or more factors. These are lack of sensitivity, specificity, complexity, difficulty in quantification, presence of serum inhibitors and length of time necessary to obtain results. There is a great deal of conflicting data concerning the clinical significance of anti-DNA antibody titer. These differences may be attributed in part to variations in the methods employed to detect anti-DNA antibodies. Different laboratories have used different types of DNA as antigen and have used either ammonium sulfate precipitation or cellulose nitrate filtration to separate bound from unbound antigen. It has been shown that both of these variables, antigen and means of detection, can result in a significant difference in the amount of anti-DNA antibody measured in the same serum (Aarden et al., 1975, 1976b).

At present there are three widely accepted assays for the detection of anti-DNA antibodies. They are immunofluorescence, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). Immunofluorescence assay now widely used employs the kinetoplast of Crithidia luciliae as a substrate. It is specific for dsDNA antibody by virtue of the fact that the circular DNA of the organism appears to be free of single-stranded DNA and histone contaminations (Sontheimer and Gilliam, 1978b). In this assay
smears of *Crithidia luciliae* are incubated with sera to be tested followed by fluorescein labelled anti-human immunoglobulin serum. Fluorescence of the kinetoplast indicates the presence of antibodies to dsDNA. Sera of SLE patients almost always have antibodies reacting with cell nuclei i.e. antinuclear antibodies (ANA), therefore a nuclear fluorescence commonly occurs in the *Crithidia luciliae* assay. The nuclear fluorescence is often difficult to differentiate from the kinetoplast fluorescence, especially when sera are diluted for titration purposes (Vladutiu *et al.*, 1979). In practice immunofluorescence is not easy to quantify for antibody assays since it depends on subjective visual assessments of fluorescence and the results are usually expressed as the serial dilutions of serum that gives least fluorescence.

The most extensively used technique for the detection of anti-DNA antibodies in terms of sensitivity and diagnostic specificity is the radioimmunoassay (RIA) of Farr (1958). Although it is a highly sensitive technique, nevertheless expensive and involves the use of sophisticated radioactive measuring instruments which limit its use in laboratories where all these facilities do not exist. Both advantages and disadvantages are derived from radioactive nature of its label (Schall and Tenoso, 1980). Farr assay has recently come under criticism for alleged inability to detect low avidity anti-DNA antibodies. A small percentage of antibody binding was not detected when this assay was performed under the conditions generally used. Conditions of incubation,
buffer, pH, concentration of reactants and molecular weight of antigen are very important for optimal detection of anti-DNA antibodies by ammonium sulfate assay (Rubbin et al., 1978). The high salt concentration of the ammonium sulfate precipitation dissociates all DNA/anti-DNA complexes of low avidity (Griffiths et al., 1978, Aarden et al., 1979).

The introduction of enzyme immunoassays originally described by Engvall and Perlmann (1971, 1972) and van Weemen and Schuurs (1971) has offered an alternative to the RIA, contributing major improvements in reagent stability, waste handling and safety (Schall and Tenoso, 1980). The range of application of ELISA is potentially as wide as that of RIA and they may also reinforce or replace other serological tests such as immunofluorescence. Automation has been applied effectively, although as with RIA, the definitive system has not been reported (Voller et al., 1976, Schall and Tenoso, 1980). Enzyme immunoassays employ antibodies or antigens conjugated to enzymes in such a way that the immunological and enzymatic activity of each moiety is maintained. These assays give objective results and are extremely sensitive. The evaluation of results can either be visual or be made with a rather simple spectrophotometer of the type found in most laboratories (Voller et al., 1976). ELISA has been described for the measurement of anti-DNA antibodies in SLE by various workers (Miller et al., 1981; Kavai et al., 1982; Eaton et al.,
The sensitivity of ELISA can be limited by the factors which include the type of solid phase used for antigen immobilization and method used to monitor enzyme activity. In order to achieve the full potential of such assays, a number of modifications have been made. These include, the use of different materials as solid support to increase the surface area for immobilization of antigen, use of fluorogenic enzyme substrate to monitor enzyme activity and modification in the assay procedure and data expression. Such changes have resulted in the increased sensitivity, decreased nonspecificity and background values and ease in performing the techniques (Hendry and Herrmann, 1980; Schall and Tenoso, 1980; Aitkaci et al., 1981; Labrousse et al., 1982; Malvano et al., 1982; Ishaq and Ali, 1983).

Enzyme immunoassay utilizing nylon for the covalent immobilization of antibodies has been reported (Hendry and Herrmann, 1980). Treatment of nylon with HCl for limited time causes exposure of free amino and carboxyl groups. Such an activated nylon is highly reactive, since a number of macromolecules like DNA, RNA and proteins can be noncovalently adsorbed on its surface.

Ethidium bromide (2, 7 diamino-9-phenyl-10-ethyl phenanthridinium bromide), a cationic dye which intercalates strongly and specifically with double helical DNAs and RNAs, is
widely used in spectrofluorometric studies because of the striking fluorescence enhancement it displays upon binding (Waring, 1965; LePecq and Paoletti, 1967; Gatti et al., 1975). It is generally agreed that strong fluorescence enhancement accompanies intercalation of the dye into the double helix conformation of the nucleic acid but there is also evidence for additional nonintercalative, less fluorescence enhanced sites which are presumed to involve electrostatic binding (Waring, 1965; LePecq and Paoletti, 1967). One dye molecule is bound for each 2-2.5 base pairs of DNA (Mandal et al., 1980). Ethidium bromide (EB) has been used to probe dsRNA sequences within hnRNA from HeLa cells (Paoletti et al., 1980), tRNA structure (Urbanke et al., 1973), ssRNA (Gray and Saunders, 1971; Feunteun et al., 1975), circular DNA (Hudson et al., 1969), chromatin structure (Ide and Baserga, 1976), ribosomal RNA (Lawrence and Daune, 1976), synthetic DNA (Aktipis and Marts, 1974), tRNA protein interactions (Rigler et al., 1971), determination of molecular weight of DNA (Weissman et al., 1976) and in assaying conformational changes of DNA in DNP (Angerer and Moudrianakis, 1972).

When EB intercalates into a double helix, both its solvent environment and equilibrium conformation are modified, and either one or both of these changes might be involved in the enhancement mechanism. It has been suggested that the fluorescence enhancement is due to the immersion of EB in the hydrophobic region of the nucleic acid where it is protected against the aqueous solvent
(LePecq and Paolleti, 1967). Burns (1969) attributed the enhancement to a change in the conformation of EB.

Shepherd et al., (1978) reported EB assay for the quantitation of anti-dsDNA antibodies using IgG fractions of SLE sera. Their technique was based on the inhibition of fluorescence by the dye after displacement from DNA as a consequence of immunoglobulin binding. Since EB intercalates only with double-stranded nucleic acids, only those antibodies which bind dsDNA should prevent intercalation of EB. The amount of dye displaced is proportional to the amount of IgG bound, making direct linear quantitation of anti-dsDNA antibodies.

The presence of antinuclear antibodies in the sera of patients treated with furocoumarins plus 365 nm light (PUV-A photochemotherapy) indicates a disturbed immunological function as exemplified in various rheumatic diseases (Kubba et al., 1981). The finding that PUV-A therapy is effective treatment for several diseases (mycosis fungoides, alopecia areata, vitiligo) has prompted investigators to study the influence of PUV-A on host immune functions (Grekin and Epstein, 1981). It is suggested that furocoumarins plus UVA irradiation does indeed have a profound effect on the immunological system. Lymphocytes taken from patients treated with PUV-A had decreased phytohemagglutination induced DNA synthesis as compared to nontreated control (Friedman and Rogers, 1980). PUV-A may suppress immunological
surveillance in the skin (Bridges and Strauss, 1980). In vitro, the response of T lymphocytes to HLA-D antigen is inhibited if either the stimulator or responder cells are pretreated with PUV-A. PUV-A has a lethal effect in vitro on lymphocytes and monocytes, but not on the majority of the epidermal cells, suggesting that the beneficial effect of PUV-A on patients with psoriasis may be due to an effect on immunocompetent cells rather than on the epidermis (Morhenn et al., 1980).

The furcoumarins, belong to a group of heterocyclic compounds, formed by condensation of coumarin and furan ring. Many natural and synthetic furcoumarins have been synthesised. Of these furcoumarins, psoralen, 8-methoxypsoralen and 4,5,8-trimethylpsoralen are used clinically in the treatment of vitiligo (Fowlks, 1959; Kaufman, 1960; Scott et al., 1976).

It is well known that two types of interactions may occur between a furcoumarin and DNA: (i) formation of a molecular complex, in the dark, consisting of an intercalation of a furcoumarin molecule, in its ground state, between 2 base pairs of DNA, involving very weak forces such as Vander Waal's forces, hydrogen bonding and hydrophilic forces; (ii) photochemical reaction, following irradiation with UVA light, leading to a covalent combination of furcoumarin molecule to pyrimidine bases of DNA (Dall'Acqua et al., 1979; Hearst, 1981; Sherman and Grossweiner, 1981). Only pyrimidine bases thymine, cytosine or
uracil, their nucleosides and nucleotides react with furocoumarins (Musajo et al., 1965; Musago et al., 1966; Krauch et al., 1967; Pathak and Kramer, 1969). No evidence has been obtained from chromatographic or labelled radioactive tracer studies that furocoumarin react with purine bases, adenine or guanine and their nucleosides (Musajo et al., 1965).

It has been suggested that the drugs with the ability to form complexes with DNA and DNP are most likely to induce the ANA formation (Grabar, 1974). Antibodies against histones, ssDNA, nuclear ribonucleoprotein are detectable in patients treated with procainamide and hydralazine (Stollar, 1981). So far no report of the occurrence of anti-dsDNA antibodies in such patients have appeared.

Antinuclear antibodies have been reported in patients undergoing PUVA therapy. However, antibodies to dsDNA have not been detected, although it is known that psoralen forms a covalent photoadduct with DNA in the presence of UVA light and makes it immunogenic (Zarebska et al., 1978). In such studies Farr assay was employed for the detection of antibodies which is under criticism for its inability to detect anti-DNA antibodies of low avidity.

The present work describes the detection and quantitation of anti-DNA antibodies in the sera of patients with various rheumatic diseases and rabbits immunized with ds- and ssDNA by
immunoprecipitation and hemagglutination techniques. The sensitivity of these techniques was compared with spectrophotometric and fluorometric method which were developed in the laboratory for anti-DNA antibodies. An enzyme-linked immunosorbent assay (ELISA) is described for the assay of anti-DNA antibodies employing plastic surface for antigen binding. Experiments were performed to compare the sensitivity of colorigenic and fluorogenic enzyme substrates under different experimental conditions. We report the successful utilization of nylon as solid support for antigen immobilization for use in ELISA in the detection of anti-DNA antibodies. The production of low avidity antibodies against DNA-psoralen adduct showing crossreactivity with dsDNA have also been described. The antigenic specificity of affinity purified anti-DNA antibodies from positive SLE serum was compared with the antibodies elicited by immunization of animals with DNA-psoralen adduct.
II. EXPERIMENTAL

A. MATERIALS

**Chemicals for immunization.**

Bovine serum albumin and calf thymus DNA were purchased from Sigma Chemical Company, U.S.A. Psoralen was a gift from C.D.R.I., Lucknow. Freund’s complete adjuvant was obtained from Difco Chemical Company, U.S.A. Methanol and magnesium turnings were obtained from B.D.H., India and iodine from E. Merck, Germany.

**Chemicals for the determination of anti-DNA antibodies.**

Agarose, 4-methylumbelliferyl phosphate, 2-amino-2 methyl-1-propanol, picrylsulfonic acid, ethidium bromide, alkaline phosphatase (bovine intestine, type VII-S) and goat anti-human IgG and IgM were purchased from Sigma Chemical Company, U.S.A. Calf serum was from Microlab, India. p-nitrophenyl phosphate was obtained from C.S.I.R. Center for Biochemicals, Delhi. Polystyrene flat bottom plates having 96 wells (7 mm diameter) were purchased from Cooke Microtiter Company, U.S.A. Nylon 66 was a product of B.D.H., England. Diphenylamine and diethanolamine were from B.D.H., India. Tween-20 was from Koch-Light Laboratories, England and sodium azide was obtained from Polskie, Poland.
Chemicals for isolation and purification of anti-DNA antibodies.

DEAE-cellulose was obtained from Whatman, England. Sephadex G-200, Blue Dextran-2000 and cyanogen bromide activated Sepharose-4B were purchased from Pharmacia Fine Chemicals, Sweden. Acrylamide, Coomassie Brilliant Blue R and dithiothreitol were from Sigma Chemical Company, U.S.A. Bisacrylamide was from Reanal, Hungary. N,N,N'N'-tetramethylethylenediamine was purchased from B.D.H., England. Glycine was obtained from E. Merck, Germany.

Other chemicals.

RNA (yeast) and orcinol were obtained from Sigma Chemical Company, U.S.A. Hydroxyapatite was obtained from Bio-Rad Laboratories, U.S.A. Glutaraldehyde was purchased from E. Merck, Germany. All other chemicals used were of analytical grade.

Equipment.

Colorimetric reactions were monitored with a Spekol-10 spectrocolorimeter (Veb Carl Zeiss Jena, F.R.G.) and fluorescence measurements were carried out on Aminco-Bowman spectrophotofluorometer. Measurements of absorbance at 260 nm were made by a single beam spectrophotometer (Carl Zeiss Jena VSU 2-P).

Sera.

Normal human sera were obtained from healthy subjects. Sera of patients with various rheumatic diseases were collected.
from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. The samples were transported to the laboratory on ice-sodium chloride mixture. Patients with systemic lupus erythematosus, progressive systemic sclerosis, Sjogren's syndrome and rheumatoid arthritis had features meeting preliminary criteria of the American Rheumatism Association for these diseases (1973).

Blood from rabbits and guinea pigs was collected by heart puncture and sera were separated after 4 hours. All sera were stored in small aliquots at -20°C with 0.1 percent sodium azide as preservative.

B. METHODS

(1) **Determination of protein concentration.**

Protein was estimated by the method of Lowry et al. (1951) using Folin-phenol reagent.

(a) **Preparation of Folin-phenol reagent.** Folin-phenol reagent was prepared by the procedure given by Folin and Ciocalteu (1927).

100 gm of sodium tungstate, 25 gm sodium molybdate, 700 ml of distilled water, 48.2 ml of 85 percent orthophosphoric acid and 100 ml of hydrochloric acid were refluxed in dark for 10 hours. 128 gm of lithium sulfate, 50 ml of distilled water and few drops of bromine were added to the mixture. Excess bromine
was removed by boiling the mixture for 15 minutes. The solution was cooled, diluted to one liter and filtered. The bright yellow reagent was protected from light by storing in amber colored bottle. The above reagent was diluted 4 times with distilled water before use.

(b) **Preparation of alkaline copper reagent.** 50 ml of 2 percent sodium carbonate solution prepared in 0.1 N sodium hydroxide was mixed with 1 ml of 0.5 percent copper sulfate in 1 percent sodium potassium tartrate. The reagent was prepared fresh before use.

(c) **Procedure.** 5 ml of alkaline copper reagent was mixed with 1 ml of the protein sample. The tubes were left for 10 minutes at room temperature and 1 ml of diluted Folin-phenol reagent was added with immediate mixing and allowed to stand at room temperature for 30 minutes. The absorbance was read at 660 nm. Protein content of unknown sample was calculated from a standard plot constructed with bovine serum albumin.

(2) **Determination of DNA concentration.**

DNA concentration was determined by the method of Burton (1956) using diphenylamine reagent.

(a) **Crystallization of diphenylamine.** 2 gm of diphenylamine was dissolved in 100 ml of boiling hexane. Approximately 0.5 gm of activated animal charcoal was added to the boiling mixture. The
solution was filtered while hot and filtrate kept at 4°C till
the crystallization was complete. The crystals were separated
by filtration and air dried at room temperature.

(b) Preparation of diphenylamine reagent. 750 mg of recrystal-
llized diphenylamine was mixed with 50 ml of glacial acetic acid
and 0.75 ml of concentrated sulfuric acid. The reagent was
prepared immediately before use.

(c) Procedure. One ml of DNA sample was mixed with one ml of 1N
perchloric acid. The tubes were incubated at 70°C for 15 minutes
in a water bath and 0.1 ml of 54.3 mM acetaldehyde was added
followed by 2 ml of diphenylamine reagent. The tubes were mixed
and allowed to stand at room temperature for 16-20 hours and
absorbance was recorded at 600 nm. The DNA concentration in
unknown sample was determined from the standard plot.

(3) Determination of RNA concentration.

RNA was estimated by cupric ion catalysed orcinol reaction

(a) Crystallization of orcinol. 5 gm of commercial orcinol was
dissolved in 100 ml of boiling benzene and decolorized with 1 gm
of activated animal charcoal. The suspension was filtered while
hot and kept at room temperature for 1 hour and then at 4°C till
the crystallization was complete. The crystals were separated
by filtration and dried at room temperature.
(b) **Cupric ion reagent.** 150 mg CuCl$_2$·2H$_2$O was dissolved in 100 ml of concentrated HCl.

(c) **Stock orcinol solution.** 12.5 gm of recrystallized orcinol was dissolved in 95 percent ethyl alcohol and the volume made to 25 ml. The solution was kept in dark.

(d) **Orcinol reagent.** 2 ml of orcinol stock solution was mixed with 100 ml of cupric ion reagent. The reagent was prepared fresh before use.

(e) **Procedure.** 2 ml of RNA solution and 2 ml of orcinol reagent were mixed and kept in boiling water bath for 35 minutes. Tubes were cooled and absorbance recorded at 660 nm. RNA concentration of unknown sample was calculated from a standard plot drawn by using yeast RNA.

(4) **Separation of dsDNA and ssDNA by hydroxyapatite column chromatography.**

Separation was done as described by Bernardi (1971).

(a) **Chromatography of dsDNA.** Native DNA at a concentration of 1 mg/ml in 0.01 M phosphate buffer, pH 7.2 was passed through a 5.5 cm x 1.5 cm column of hydroxyapatite. Stepwise elution was carried out using 0.01M - 0.3M phosphate buffer, pH 7.2. Fractions of 3 ml were collected at a flow rate of 40 ml/hour. DNA in each fraction was estimated by the method of Burton (1956).
Double-stranded DNA was found to be eluted at 0.25 M phosphate buffer, pH 7.2 (Fig.1). 0.25 M fractions were pooled and used as dsDNA antigen.

(b) Chromatography of ssDNA. Native DNA in 0.01 M phosphate buffer, pH 7.2 was denatured by heating for 15 minutes in boiling water bath. Samples were fast cooled and then passed through hydroxyapatite column. Denatured DNA showed chromatographic behavior quite different from that of native DNA as it was eluted at 0.2 M phosphate buffer, pH 7.2 (Fig.2). Fractions eluting at 0.2 M phosphate buffer were used as ssDNA antigen.

(5) Preparation of antigens for eliciting antibodies with dsDNA and ssDNA.

(a) Preparation of methylated bovine serum albumin. Methylated bovine serum albumin (MBSA) was prepared by the method of Sueoka and Cheng (1962). One gm of bovine serum albumin was dissolved in 100 ml of absolute methanol and 0.84 ml of 12 N HCl was added. The protein was readily soluble at first but precipitates as it becomes methylated. The reaction mixture was allowed to stand with occasional mixing at room temperature in the dark for at least a day. Thereafter, the mixture was centrifuged and the precipitate washed four times with methanol followed by two washings with anhydrous ether. Residual ether was removed from MBSA by evaporation in air and residual acid by keeping it over KOH in a vacuum desiccator. The dried MBSA was stored in a vacuum desiccator over KOH.
Fig. 1: Chromatography of native calf thymus DNA on hydroxyapatite column.

1.5 mg of DNA in 1.5 ml of 0.01 M phosphate buffer, pH 7.2 was loaded on a column of hydroxyapatite (5.5 cm x 1.5 cm). The column was washed with 0.01 M, 0.1 M and 0.2 M phosphate buffer and dsDNA eluted at 0.25 M phosphate buffer, pH 7.2.
Fig. 2: Chromatography of denatured DNA on hydroxyapatite column.

Native DNA (1 mg/ml in 0.01M phosphate buffer, pH 7.2) was denatured by heating and applied onto a column of hydroxyapatite (5.5 cm x 1.5 cm). Stepwise elution was made from 0.01M-0.3M phosphate buffer, pH 7.2. Fractions of 3 ml were collected and monitored for DNA. ssDNA was eluted at 0.2M phosphate buffer, pH 7.2.
(b) **Formation of DNA-MBSA complex.** Suitably immunogenic protein conjugates were prepared by mixing equal volumes of DNA (1 mg/ml) and MBSA (0.5 mg/ml). The reaction was carried out at room temperature with gentle mixing.

(c) **Immunization schedule.** Rabbits (nine to twelve months old, weighing 1 to 1.5 kg) were injected intramuscularly for 3 weeks with freshly prepared antigen-MBSA complex containing 350 μg of hapten in a total volume of 1.4 ml. A single rabbit received a total of 1.05 mg of hapten in the course of three injections. Antisera were separated from blood obtained by cardiac puncture 7 to 10 days following the last injection.

(6) **Isolation of IgG.**

Serum IgG was isolated by DEAE-cellulose adsorption method of Stanworth (1960). IgG was also isolated by DEAE-cellulose chromatography of the crude immunoglobulin precipitate obtained by 40% ammonium sulfate saturation of serum.

(a) **Isolation of IgG by DEAE-cellulose adsorption method.** Approximately 17 gm of moist DEAE-cellulose previously washed and equilibrated with 0.01 M sodium phosphate buffer containing 0.015 M NaCl, pH 7.5 was mixed with 2.5 ml of cold phosphate buffer, 2.5 ml of serum (previously dialyzed for 16 hours at 4°C against phosphate buffer) was added dropwise to the exchanger with continuous mixing. The mixture was kept for 5 hours at 4°C.
Thereafter 5 ml of cold buffer was added with stirring. The resulting slurry was centrifuged in cold for 15-20 minutes. The supernatant was removed and cleared of cellulose, if any, by recentrifugation. The purity of the isolated IgG was checked on Sephadex G-200 column (54.6 cm x 1.8 cm) using 0.1 M phosphate buffer, pH 6.5 as eluent and by polyacrylamide gel electrophoresis using 10 percent gel. The protein was found to be essentially homogeneous.

(b) Preparation of crude immunoglobulins. 4 ml of 100 percent saturated ammonium sulfate solution was slowly added to 6 ml of serum in cold and the mixture was left at 4°C for 1 hour for complete precipitation of the immunoglobulins. The precipitate was recovered by centrifugation and washed thrice with 40 percent saturated ammonium sulfate solution. The washed precipitate was dissolved in and dialyzed against 10 mM phosphate buffer, pH 8.0.

(c) DEAE-cellulose chromatography. Dialyzed crude immunoglobulins were loaded onto a column of DEAE-cellulose (20 cm x 2 cm) previously equilibrated with the starting buffer (10 mM phosphate buffer, pH 8.0). The column was eluted at room temperature with linear ionic strength gradient of 10mM - 300mM phosphate buffer, pH 8.0. 3 ml fractions were collected and monitored for protein (Fig.3). First peak of the chromatogram was pooled and used as purified IgG. The material was homogeneous and gave a single band in polyacrylamide gel electrophoresis.
Fig. 3: Fractionation of serum immunoglobulins by DEAE-cellulose chromatography.

40% saturated ammonium sulphate precipitated serum fraction was dissolved and dialyzed against 10 mM phosphate buffer, pH 8.0 and loaded on a column of DEAE-cellulose (20 cm x 2 cm). The elution was performed by applying linear ionic strength gradient of 10 mM - 300 mM phosphate buffer, pH 8.0. Fractions of 3 ml were collected and monitored for proteins.
(7) Detection and quantitation of anti-DNA antibodies by immunoprecipitation and hemagglutination techniques.

Sheared DNA was used as antigen in immunodiffusion, counterimmunoelectrophoresis and passive hemagglutination techniques for the measurement of anti-DNA antibodies. It was prepared by passing 1 mg/ml dsDNA in PBS (0.01 M phosphate buffer in 0.15M NaCl, pH 7.2) or McIlvaine buffer (0.05 M citric acid, 0.1M Na₂HPO₄, pH 4.9) three times through a sterilized 21-gauge needle. Single-stranded DNA was prepared by heat denaturation.

(a) Immunodiffusion. The precipitin reactions were carried out by Ouchterlony double diffusion using glass petri dishes as described by Seligmann (1957) and Tan et al. (1966). 6 ml of molten, filtered, 0.4 percent agarose in PBS containing 0.1 percent sodium azide was poured in 5 cm x 1.5 cm glass petri dishes and allowed to harden at room temperature. The petri dishes were stored at 4°C. All the sera tested were decomplemented by heating at 56°C for 30 minutes. 50 μl of each serum and antigen was placed in the wells and the reaction was allowed to proceed for 2-4 days in a moist chamber at room temperature. Petri dishes were washed with 5 percent sodium citrate for 4 hours to remove nonspecific precipitin lines. The precipitin lines were analysed visually and the results recorded.

(b) Counterimmunoelectrophoresis. Counterimmunoelectrophoresis (CIE) was performed as described by Davis and Winfield (1974).
3 ml of 0.6 percent molten agarose prepared in 25 mM barbital buffer, pH 8.4 containing 0.1 percent sodium azide, was pipetted onto alcohol cleaned microscopic slides (2.5 cm x 7.5 cm) and allowed to solidify at room temperature. The slides were stored in a moist chamber at 4°C. 4 mm wells were cut on the slide at a distance of 5 mm between the two opposite wells. Antigen was placed in the cathodal well and decomplemented serum in the anodal well (25 µl in each well). Electrophoresis was performed in 50 mM barbital buffer, pH 8.4 with a current of 3-4 mA per slide. Electrophoresis was performed for 30-50 minutes. Nonspecific precipitin lines were abolished by washing the slides with 5 percent sodium citrate for 2-4 hours. Finally the slides were examined for precipitin lines and the results recorded.

(c) Passive hemagglutination technique. Microhemagglutination test of Inami et al. (1973) with slight modifications was used for the detection of anti-DNA antibodies in the sera of patients with various rheumatic diseases and animals immunized with dsDNA, ssDNA and DNA-psoralen adduct. Sheared DNA was used as the source of antigen and formalinized sheep erythrocytes were employed as their passive carriers. Sheep erythrocytes were formalinized according to the procedure of Inami and associates (1973).

To 0.3 ml of packed cells, 4.2 ml of 0.005 percent tannic acid in PBS was added. The resulting suspension was incubated
at 37°C for 45 minutes with occasional shaking. The cells so treated were washed 4 times with 20 volumes of PBS. The fifth and final wash was carried out with McIlvaine buffer (citric acid 0.05M, Na₂HPO₄ 0.1M, pH 4.9). Formalized tanned cells were coated with dsDNA, ssDNA and DNA-psoralen adduct. DNA (50 µg/ml) in McIlvaine buffer was added to the packed cells to give 4 percent suspension. Mixture was incubated at 37°C for 1 hour with frequent shaking. The cells were packed and washed 4 times with PBS and resuspended in PBS containing 0.07 percent BSA to a final concentration of 2.5%. The antigen coated cells were stored at 4°C and then used within a week.

All decomplemented sera tested were adsorbed for 30 minutes at room temperature with equal volumes of fresh packed sheep erythrocytes that had been washed 4 times with PBS. This was done to get rid of heterophilic antibodies that would otherwise nonspecifically agglutinate the antigen coated erythrocytes.

(i) Check of specificity by inhibition studies. The specificity of hemagglutination reaction was checked by inhibition experiments. In a parallel row of wells, 50 µl of ds- or ssDNA (100 µg/ml in PBS containing 0.2 percent bovine serum albumin) was placed in all but the first well. To the first well were added 50 µl of ds- or ssDNA at a concentration of 200 µg/ml in PBS and 50 µl of test serum, the solutions were mixed and serially diluted in subsequent wells containing constant level of antigen. The plates
were shaken and incubated at 37°C for one hour and at 4°C for 16 hours. 25 µl of ds- or ssDNA coated cells were added to each well, mixed and read after 2 hours. Decrease in the hemagglutination titer compared to test without added DNA confirmed the presence of anti-DNA antibodies in the test serum.

(8) Spectrophotometric assay for the detection of anti-DNA antibodies.

A spectrophotometric procedure utilizing purified calf thymus DNA as antigen was developed and carried out as follows.

(a) Procedure. 50 µg of ds- or ssDNA (purified by hydroxyapatite column) in 0.9 ml of 0.15 M borate sodium chloride buffer, pH 8.0 was mixed with 0.1 ml of decomplemented serum. The mixture was incubated at 37°C for 1 hour followed by 16-24 hours incubation at 4°C. One ml of saturated ammonium sulfate solution was added and the contents mixed thoroughly. The mixture was kept for 1 hour at 4°C for the complete precipitation of immune complexes. The contents were centrifuged and supernatant separated. Absorbance of the supernatant was recorded at 260 nm. Each test serum was run along with a control which contained all reagents except the antigen. The results were expressed in terms of percent DNA bound to anti-DNA antibodies, calculated according to the formula:

\[
\text{Percent DNA bound} = 100 - \left[ \frac{A_{\text{test}} - A_{\text{control}}}{A_{50 \, \mu g \, \text{of DNA}}} \right] \times 100
\]
(b) **Specificity of antigen-antibody reaction in spectrophotometric assay.** The specificity of anti-DNA antibody measurement by spectrophotometric assay was studied by an inhibition experiment. Varying concentrations of DNA were mixed with 0.1 ml of anti-DNA antibody positive SLE serum to a final concentration of 2 to 50 µg DNA/ml and incubated at 37°C for one hour and at 4°C for 16 hours. Incubated contents were used in the assay instead of serum.

(9) **Fluorometric assay for anti-DNA antibodies using ethidium bromide as intercalating dye.**

(a) **Fluorescence measurement.** The excitation and emission spectra of ethidium bromide (EB), DNA and EB-DNA complexes were obtained by plotting fluorescence intensity (FI) versus wavelength. The excitation and emission wavelengths were 300 nm and 590 nm respectively.

(b) **Principle of the fluorometric assay.** EB forms a fluorescent complex with double-stranded DNA which shows strikingly enhanced fluorescence intensity as compared to free DNA and EB. The increase in fluorescence is a function of DNA concentration (Fig.4). The method described measures the amount of DNA bound in the immune complexes by the enhanced fluorescence due to dye intercalation. The fluorescence intensity is proportional to the amount of DNA which in turn is dependent on the concentration of the anti-DNA antibodies.
Fig. 4: Emission spectra of EB, DNA and EB-DNA complex.

Inset: Fluorescence intensity of EB-DNA complex as a function of DNA concentrations.

Emission spectra were recorded with excitation wavelength of 300 nm. Concentration of DNA in EB-DNA complex: 2 µg (●●), 4 µg (●●●), 6 µg (●●●●), 8 µg (●●●●), 10 µg (●●●●●●), free EB (●●●●●●), 10 µg of free DNA (●●●●●●●). The concentration of EB was kept constant (5 µg/ml).
(c) **Procedure of the fluorometric assay.** 10 μg of hydroxyapatite purified dsDNA in 0.5 ml of borate buffer (0.15 M borate-sodium chloride buffer, pH 8.0) was mixed with 0.5 ml of decomplemented serum in siliconized plastic tubes. All the sera were tested at a dilution of 1:5. The mixture was incubated at 37°C for one hour and at 4°C for 20 hours. One ml of saturated ammonium sulfate solution was added and the resulting fifty percent saturated mixture was held at 4°C for one hour for complete precipitation of immune complexes. The precipitated immune complexes were separated by centrifugation (12,000 rpm for 15 minutes at 4°C) and dissolved in 4.5 ml of borate buffer at 4°C. 25 μg of EB in 0.5 ml of borate buffer was added and fluorescence intensity recorded. Each test serum was run along with a control which contained all reagents except antigen. The fluorescence reading of the control was subtracted from the test and the results were expressed in terms of percent DNA bound in the immune complexes. The amount of DNA bound to the immune complexes was calculated from a standard plot of fluorescence intensity of EB-DNA complexes as a function of DNA concentration.

(d) **Inhibition experiment.** The specificity of the assay was checked by performing an inhibition experiment as described previously in the spectrophotometric assay.
(10) **Enzyme-linked immunosorbent assay (ELISA) for anti-DNA antibodies on polystyrene plates.**

(a) **Buffers and other reagents.**

(i) **Bicarbonate buffer.** 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH adjusted to 9.6. Sodium azide (0.02%) was added as preservative.

(ii) **Phosphate buffered saline-Tween (PBS-T).** 136 mM sodium chloride, 2 mM potassium dihydrogen orthophosphate, 8 mM disodium hydrogen phosphate, 0.5 ml Tween-20/lit, pH adjusted to 7.4.

(iii) **Diethanolamine buffer.** 1 M diethanolamine containing 100 mg/lit MgCl$_2$$\cdot$6H$_2$O, pH adjusted to 9.8 with hydrochloric acid. 0.02 percent sodium azide was added as preservative.

(iv) **2-amino-2-methyl-1-propanol (AMP) buffer.** 150mM AMP, 3mM MgCl$_2$, 0.1 ml Triton x-100/lit, pH adjusted to 10.3. The solution was filtered before use.

(v) **Substrates.** 5 x 10$^{-3}$M p-nitrophenyl phosphate in diethanolamine buffer and 1 x 10$^{-3}$M 4-methylumbelliferyl phosphate (4MU-P) in AMP buffer.

(b) **Preparation of rabbit anti-human-IgG alkaline phosphatase conjugate.** Rabbits were immunized with four weekly intramuscular injections of purified normal human IgG in PBS with equal volume of Freund's complete adjuvant. Each rabbit received 4 mg
IgG per injection. The animals were bled a week after 4th injection and IgG was isolated by DEAE-cellulose chromatography from the pooled sera.

2 mg of isolated rabbit anti-human IgG in 0.1 ml of PBS was mixed with 5 mg of alkaline phosphatase. The mixture was dialyzed for 20 hours against PBS at 4°C with 3 changes of buffer. 25% glutaraldehyde was added to a final concentration of 0.2%. The resulting mixture was kept at room temperature for 2 hours and then dialysed at 4°C for 16 hours against PBS with 3 changes of buffer. Dialysis sack containing the conjugate was transferred to 0.05M Tris buffer pH 8.0 and dialyzed for 16 hours at 4°C. Finally the dialysis sack contents (conjugate) were diluted to 4 ml with Tris buffer containing 1% BSA and 0.02% sodium azide.

The conjugate was partially purified by gel filtration on 58 cm x 1.9 cm column of Sephadex G-200 in 0.1M phosphate buffer, pH 7.2 and the active fractions were pooled and stored at 4°C.

(c) ELISA procedure. Technique of Voller et al. (1976) with slight modification was used for the detection and quantitation of anti-DNA antibodies. DNA in coating buffer (12.5 μg/0.25 ml in 0.05 M bicarbonate buffer, pH 9.6) was added to the wells of the microtiter plate which was then incubated at 4°C for 16 hours. The wells were washed three times with PBS-T and unoccupied sites
were saturated with fetal calf serum (1% v/v in PBS-T) by incubation at 37°C for 1 hour. After washing the plate with PBS-T, 0.25 ml of serum (1:100) or IgG (1:100 at 12 mg/ml) was added and the plate was incubated at 37°C for one hour. 0.25 ml of rabbit antihuman IgG alkaline phosphatase conjugate was added, after washing the excess antibody. The plate was once again incubated at 37°C for 2 hours. At the end of incubation period, the plate was emptied and washed. 0.20 ml of substrate (PNP-P, 5x10^{-3} M in diethanolamine buffer, pH 9.8 or 4MU-P, 1x10^{-3} M in AMP buffer, containing 1.5x10^{-3} M Mg^{2+} pH 10.3) was pipetted into each well. The plate was incubated at 37°C for one hour and the reaction was stopped by the addition of 0.05 ml of 3N NaOH. The contents of each well were either read at 400 nm or fluorometric measurements were made, depending upon the substrate. Excitation and emission wavelengths were 365 nm and 455 nm respectively.

(11) **ELISA with nylon as solid phase.**

A highly sensitive enzyme-linked immunosorbent assay was developed for the detection and quantitation of anti-DNA antibodies in the sera of patients with various rheumatic diseases and animals immunized with DNA-psoralen adduct, using nylon as solid support for antigen immobilization.

(a) **Activation of nylon.** Nylon beads (3mm x 2mm) were treated with 3.5N HCl for 36 hours at 37°C with occasional shaking and
thereafter washed thoroughly with distilled water till free of acid. The exposed amino groups were detected by the treatment of activated beads with picrylsulfonic acid (Synder and Soborinski, 1975).

(b) Procedure. Activated nylon beads were treated with DNA (100 μg/ml) in McIlvaine buffer (citric acid 0.05M, Na₂HPO₄ 0.1M, pH 5) for 24 hours at 4°C with occasional shaking. The antigen coated beads were washed 4 times with ST-20 (0.15M NaCl, 0.05% Tween-20) to remove the unreacted DNA. Unoccupied sites were saturated with 1% BSA in ST-20 by keeping the beads immersed in the BSA solution for 12 hours at 4°C. The beads were washed 4 times with ST-20, dried on Whatman filter paper and stored at -20°C until use. Activated beads treated with BSA, without prior antigen treatment, were used as control. Six beads were used for ELISA. All the sera were tested at a dilution of 1:100.

Beads (test and control) in siliconized glass tubes were incubated with 0.5 ml of serum for 1 hour at 37°C with occasional shaking. The beads were washed 4 times with ST-20 and incubated with 0.5 ml of conjugate (1:1000 dilution or 11 μg/ml protein) for 2 hours at 37°C. Unreacted conjugate was washed four times with ST-20. The final washing was done by distilled water. The beads were dried on Whatman filter paper and transferred to a clean tube containing 1 ml of substrate (PNP-P or 4MU-P). The tubes were incubated at 37°C, for 30 minutes with occasional shaking. The
reaction was stopped with 1 ml of 3N NaOH for PNP-P and 1 ml of 1M \( \text{K}_2\text{HPO}_4 \)-KOH buffer, pH 10.4 for 4MU-P. The absorbance (A) was monitored at 420 nm. Excitation and emission wavelengths were 365 nm and 455 nm respectively. Results were expressed as \( \frac{A_{\text{test}} - A_{\text{control}}}{F_{\text{I}}} \).

(c) Inhibition ELISA. The specificity of antigen-antibody reaction in ELISA was checked by inhibition experiments. Varying concentrations of DNA were mixed with an anti-DNA positive SLE serum to a final concentration of 2 to 20 \( \mu \text{g} \) DNA/ml. The mixture was incubated at 37°C for 1 hour and then at 4°C for 20 hours and used in ELISA instead of serum.

(12) Production of anti-DNA antibodies by immunization with DNA-psoralen adduct.

(a) Preparation of DNA-psoralen adduct. The formation of inter-strand cross-linkages by photoreaction of psoralen with dsDNA was studied by column chromatography on hydroxyapatite. The renaturation capacity shown by drug intercalated DNA after heat denaturation was the basis of adduct formation (Lawley and Brookes, 1967).

1 mg of hydroxyapatite purified dsDNA in TNE (0.001M Tris, 0.015M NaCl, 2x10^{-5}M EDTA, pH 7.5) buffer was thoroughly mixed with 100 \( \mu \text{g} \) of psoralen in the same buffer at 25°C for one hour.
The mixture was irradiated at a dose rate of $2\times10^6$ ergs cm$^{-2}$ min$^{-1}$ for 30 minutes using illuminating wavelength of 365 nm. Samples of DNA and psoralen without irradiation were the experimental control. Both samples were dialyzed against PBS to remove unbound psoralen. Dialyzed contents were heated for 10 minutes in a boiling water bath and then immersed in ice-sodium chloride mixture for 15 minutes. Each sample was chromatographed on a column (5.5 cm x 1.5 cm) of hydroxyapatite. Stepwise elution was carried out using 0.01M - 0.3M phosphate buffer, pH 7.4. Fractions of 3 ml were collected at a flow rate of 40 ml/hour. DNA in each fraction was estimated with diphenylamine reagent. Fractions eluting at 0.25M phosphate buffer (cross-linked) were pooled, dialyzed against PBS and used as DNA-psoralen antigen for eliciting antibodies in guinea pigs.

(b) Equilibrium dialysis. The amount of psoralen intercalated with DNA was evaluated by equilibrium dialysis measurements as described by Romeu et al. (1976).

Dialysis bags with 4 ml of DNA-psoralen adduct were immersed in 60 ml of TNE buffer in stoppered round bottom flask. Equilibrium was allowed to attain by shaking the flask for 30 hours at 4°C. The fluorescence intensity of the dialysate was recorded at 450 nm (320 nm excitation). The concentration of psoralen dialyzed out was quantitated with the help of a standard plot obtained by drawing fluorescence intensity of irradiated
psoralen as a function of its concentration. The amount of psoralen thus intercalated with DNA was determined.

(c) Immunization. DNA-psoralen-MBSA complex was prepared as described previously for DNA-MBSA. Guinea pigs (6 to 8 months old, weighing 400 to 500 gm) were injected intramuscularly in the hind limbs with the complex (2 ml) weekly for seven weeks. A single animal received a total of about 1.5 mg intercalated DNA in the course of seven injections. Serum was separated from blood obtained by cardiac puncture 7 days after the last injection.

(d) Reaction of DNA-psoralen immunized sera with various nucleic acids by ELISA with nylon as solid phase. Preimmunized and DNA-psoralen immunized guinea pig sera at different dilutions were tested by ELISA for the reaction against adduct, dsDNA, ssDNA and RNA using goat anti-guinea pig IgG alkaline phosphatase conjugate (prepared as for rabbit anti-human IgG alkaline phosphatase conjugate).

Activated nylon beads were treated with DNA-psoralen adduct, dsDNA, ssDNA and RNA (100 µg/ml) in McIlvaine buffer, pH 5 for 24 hours at 4°C with occasional shaking. Remaining steps were as described earlier. Sera were tested at a dilution of 1:100 in siliconized glass tubes with antigen sensitized and control beads. Immune complex attached to nylon beads was
detected by incubating the beads in 0.5 ml of goat anti-guinea pig IgG alkaline phosphatase conjugate (1:500 dilution or 28 μg/ml protein).

(13) Isolation and purification of anti-DNA antibodies.

(a) Preparation of DNA-Sepharose column. DNA was coupled with CNBr activated Sepharose-4B according to the method for Arndt-Jovin et al. (1975).

1 gm of CNBr activated Sepharose-4B was swollen in 20 ml of 1 mM HCl for 15 minutes. The gel was transferred onto sintered glass filter and washed with 1 mM HCl, 10 mM potassium phosphate buffer, pH 8.0. The contents were transferred to a stoppered cylinder containing hydroxyapatite purified double-stranded DNA (500 μg/ml) in 50 ml of 10 mM potassium phosphate buffer, pH 8.0 (coupling buffer). The mixture was rotated end-over-end for 2 hours at room temperature and then kept at 4°C for 12 hours. The DNA-Sepharose product was washed on a sintered funnel with 10 mM potassium phosphate, pH 8.0 followed by 1 M potassium phosphate, pH 8.0 and 1 M KCl. The gel was then washed with 0.2 M glycine, pH 8.0 and transferred to a stoppered cylinder containing 20 ml of 0.2 M glycine. The contents were mixed end-over-end for 2 hours at room temperature and filtered. The gel was then washed with coupling buffer and finally equilibrated with TEDG buffer.
(50mM Tris, 0.1mM EDTA, 0.1mM dithiothreitol and 5% glycerol, pH 7.9). The gel was stored at 4°C.

(b) Purification of anti-DNA antibodies by affinity chromatography.

IgG was isolated from an anti-DNA positive SLE serum by DEAE-cellulose adsorption method and dialyzed against equilibrating buffer at 4°C for 24 hours with 3 changes of buffer. The dialyzed contents were applied to affinity column (7 cm x 2 cm). The column was washed with equilibrating buffer followed by 50mM Tris, 0.1mM EDTA, 0.1mM dithiothreitol, 5% glycerol and 0.1M NaCl buffer, pH 7.9. Bound protein was eluted with 1M NaCl in TEDG buffer. The eluate was dialyzed at room temperature against 0.01M phosphate buffer containing 0.015M NaCl, pH 7.5 and concentrated.

(c) Reaction of affinity purified material with different nucleic acids by CIE and ELISA. The specificity and crossreactivity of the affinity purified material was checked by CIE and ELISA.

ELISA was carried out at various antibody dilutions (120 μg/ml to 0.06 μg/ml) employing DNA-psoralen adduct, dsDNA, ssDNA and RNA sensitized nylon beads. Rest of the steps were as described previously.

(d) Demonstration of IgG/IgM response. Crude immunoglobulins were isolated from an anti-DNA positive SLE serum by 40 percent satu-
rated ammonium sulfate solution. The precipitate was dissolved in and dialyzed against equilibrating buffer.

Dialyzed contents were applied on affinity column and bound protein was eluted as described previously. IgG/IgM response in purified material was checked by ELISA using goat anti-human IgG alkaline phosphatase (1:1000 dilution) and goat anti-human IgM alkaline phosphatase (1:1000 dilution) conjugate employing dsDNA coated beads.
III. RESULTS

(1) Determination of precipitating and hemagglutinating anti-DNA antibodies in rheumatic diseases and rabbits immunized with DNA.

Sera of patients with SLE, PSS, SLE with overlapping PSS, SS, RA and rabbits immunized with ds- and ssDNA-MBSA complex were monitored for the presence of precipitating and hemagglutinating anti-DNA antibodies by immunodiffusion, counterimmunoelectrophoresis and passive hemagglutination techniques.

(a) Immunodiffusion and counterimmunoelectrophoresis.

Circulating antibodies against dsDNA were found in 14 and 23 percent of patients with SLE by immunodiffusion (ID) and counterimmunoelectrophoresis (CIE) respectively. Antibodies to ssDNA were positive in 19 percent of SLE patients when detected by ID and 30 percent with CIE. Normal human sera (NHS) and sera of patients with SLE overlapping with PSS, PSS, SS and RA were negative for precipitating anti-DNA antibodies by ID and CIE (Table 1).

Rabbits immunized with calf thymus ds- and ssDNA complexed to MBSA responded by eliciting antibodies against ssDNA. 14 and 56 percent of dsDNA immunized rabbits elicited antibodies against ssDNA as checked by ID and CIE respectively. Thirteen and 50
<table>
<thead>
<tr>
<th>Type of serum</th>
<th>Number tested</th>
<th>Percent positive</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dsDNA</td>
<td>ssDNA</td>
<td>dsDNA</td>
<td>ssDNA</td>
</tr>
<tr>
<td>SLE</td>
<td>43</td>
<td>14</td>
<td>19</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>SLE + PSS</td>
<td>7</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>PSS</td>
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<tr>
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<td>7</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>56</td>
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<tr>
<td>ssDNA immunized rabbit sera</td>
<td>8</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Preimmunized rabbit sera</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ID was performed in 0.4% agarose in PBS. CIE was carried out using 0.6% agarose. Electrophoresis was performed in 0.05M barbital buffer, pH 8.4 with a current of 3 mA/slide. The DNA concentration was 100 µg/ml in PBS.
percent of ssDNA immunized sera were positive for anti-ssDNA antibodies. No reactivity was observed with dsDNA in either of the immunized animals. Anti-dsDNA and anti-ssDNA antibodies were absent in the sera of preimmunized animals (Table 1).

(b) **Passive hemagglutination technique.**

The incidence and titer of anti-DNA antibodies in rheumatic diseases and rabbits immunized with ds- and ssDNA were also studied by passive hemagglutination (PHA) technique using ds- and ssDNA coated sheep erythrocytes. Thirty five and 51 percent of SLE sera were found positive with ds- and ssDNA respectively. Sera positive against dsDNA also reacted with ssDNA with varying degree of crossreactivity. The titer of agglutination reaction of antibody positive SLE sera ranged from 1:8-1:512 with dsDNA and 1:16 to 1:1024 with ssDNA. One out of seven SLE patients with overlapping PSS was positive with a titer of 1:16 and 1:32 with ds- and ssDNA respectively. Sera of patients with PSS, SS, RA were negative for anti-DNA antibodies. Normal human sera had a titer of 0-1:8 with either of the antigen (Table 2).

Immunization of rabbits with ssDNA-MBSA complex along with Freund's complete adjuvant produced antibodies reacting only with ssDNA having a titer of 1:128-1:512. Rabbits immunized with dsDNA-MBSA complex elicited antibodies against ssDNA. The titer of agglutination reaction was 1:64-1:256 with ssDNA coated...
<table>
<thead>
<tr>
<th>Type of serum</th>
<th>Number tested</th>
<th>Number positive</th>
<th>Titer range</th>
<th>dsDNA</th>
<th>ssDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE + PSS</td>
<td>43</td>
<td>15</td>
<td>1:8 - 1:128</td>
<td>1:16</td>
<td>1:1024</td>
</tr>
<tr>
<td>SLE</td>
<td>7</td>
<td>1</td>
<td>1:8 - 1:16</td>
<td>1:16</td>
<td></td>
</tr>
<tr>
<td>SLE + PSS</td>
<td>10</td>
<td>0</td>
<td>0 - 1:2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PSS</td>
<td>6</td>
<td>0</td>
<td>0 - 1:4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>12</td>
<td>0</td>
<td>0 - 1:4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>40</td>
<td>0</td>
<td>0 - 1:4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>10</td>
<td>0</td>
<td>0 - 1:4</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Formalinized tanned sheep erythrocytes were coated with ds- or ssDNA at a concentration of 50 µg/ml. The coating was performed at 37°C for 1 hour. 2.5% cell suspension in 0.07% bovine serum albumin was used in aggregation reaction.
cells. Very low reactivity was observed with dsDNA (1:4-1:16). Preimmunized rabbit sera showed a titer not exceeding 1:8 and 1:4 with ss- and dsDNA respectively (Table 2).

The specificity of agglutination reactions was checked by hemagglutination inhibition studies. Inhibition experiments were carried out with three anti-DNA positive SLE sera, two dsDNA immunized rabbit sera and one ssDNA immunized rabbit sera, employing ds- and ssDNA coated sheep erythrocytes. A significant decrease in the titer of the hemagglutination reaction was observed in the presence of 5 µg of DNA (Table 3).

(2) Spectrophotometric assay for the detection and quantitation of anti-DNA antibodies.

In order to have an alternative method to ID, CIE and PHA, we developed a spectrophotometric technique for the detection and quantitation of anti-DNA antibodies. In spectrophotometric method DNA in the supernatant after precipitation of immune complexes with ammonium sulfate, was conveniently estimated by recording the absorbance (A) at 260 nm. Results were expressed in terms of percent DNA bound to immune complexes calculated according to the formula:

$$\text{Percent DNA bound} = 100 - \left( \frac{A_{\text{test}} - A_{\text{control}}}{A_{50 \mu g \text{ DNA}}} \right) \times 100$$

NHS and sera of patients with rheumatic diseases were
TABLE 3

Inhibition of agglutination of SLE sera and sera of rabbits immunized with ds- and ssDNA.

<table>
<thead>
<tr>
<th>Sera (patients/ immunized animals)</th>
<th>Uninhibited titer</th>
<th>Titer after inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dsDNA</td>
<td>ssDNA</td>
</tr>
<tr>
<td>MK</td>
<td>1:512</td>
<td>1:512</td>
</tr>
<tr>
<td>JS</td>
<td>1:256</td>
<td>1:1024</td>
</tr>
<tr>
<td>NG</td>
<td>1:256</td>
<td>1:512</td>
</tr>
<tr>
<td>IR&lt;sub&gt;1&lt;/sub&gt;-dsDNA</td>
<td>1:2</td>
<td>1:256</td>
</tr>
<tr>
<td>IR&lt;sub&gt;2&lt;/sub&gt;-dsDNA</td>
<td>1:8</td>
<td>1:128</td>
</tr>
<tr>
<td>IR&lt;sub&gt;3&lt;/sub&gt;-ssDNA</td>
<td>1:2</td>
<td>1:128</td>
</tr>
</tbody>
</table>

Agglutination inhibition was performed in the presence of 5 µg of DNA. MK, JS and NG represent three different positive SLE sera. IR<sub>1</sub> and IR<sub>2</sub> are two rabbits immunized with dsDNA whereas IR<sub>3</sub> is the ssDNA immunized rabbit.
tested with dsDNA (hydroxyapatite purified) as antigen for the presence of anti-DNA antibodies (Fig. 5). Sera showing DNA binding activity of more than 30 percent were considered as positive. With this criteria 37 percent of SLE patients were positive for anti-DNA antibodies. The range of percent DNA binding activity of these sera was 35 to 93, depending upon the severity of the disease. Three patients with inactive SLE had DNA binding activity of 35 to 43 percent. NHS and sera of patients with RA, SS, PSS were all negative. One out of seven SLE patients with overlapping PSS showed a binding of 38 percent.

The specificity of antigen-antibody reaction in the assay was studied by inhibition experiment. Percent inhibition obtained at different DNA concentrations, using an anti-DNA positive SLE serum is shown in Fig. 6. High degree of specificity is evident since 70 percent inhibition was achieved with 20 µg of DNA. Besides being specific, the assay described is highly reproducible. Table 4 shows the intra- and inter-assay coefficient of variations for these measurements.

(3) **Fluorometric assay for anti-DNA antibodies using ethidium bromide as intercalating dye.**

We studied the detection and quantitation of anti-DNA antibodies in the sera of patients with various autoimmune diseases by exploiting the fact that ethidium bromide (EB) and dsDNA form fluorescent complex which has strikingly enhanced
Fig. 5: Levels of DNA binding activity in patients with rheumatic diseases.

The mean DNA binding values of positive SLE sera, NHS and sera of patients with other rheumatic diseases with standard deviations are indicated at the bottom of the figure.
Fig. 6: Demonstration of specificity of spectrophotometric assay by inhibition experiment.

Varying concentrations of DNA (2 to 50 µg/ml) were mixed with 0.1 ml of an anti-DNA positive SLE serum. The mixture was incubated at 37°C for 1 hour and at 4°C for 16 hours. Incubated contents were used in the assay instead of serum and percent inhibition obtained at different DNA concentrations was plotted.
TABLE 4

Reproducibility data for anti-DNA antibody assay of positive SLE sera.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Variation ± S.D. a</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td>84.4 ± 6.6</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>52.1 ± 3.2</td>
<td>6.07</td>
</tr>
<tr>
<td></td>
<td>35.0 ± 1.50</td>
<td>4.23</td>
</tr>
<tr>
<td></td>
<td>81.4 ± 10.1</td>
<td>12.41</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>55.0 ± 5.2</td>
<td>9.45</td>
</tr>
<tr>
<td></td>
<td>35.9 ± 2.8</td>
<td>7.80</td>
</tr>
</tbody>
</table>

Number of samples analyzed in each case is 10.

a Standard deviation.
fluorescence intensity (F.I.) as compared to free DNA and EB. The method involves the precipitation of DNA-anti-DNA complexes and the measurement of DNA bound to immune complexes by the enhanced fluorescence as a result of EB intercalation with the antigen.

Apparent excitation and emission characteristics of EB-DNA complexes were studied at different DNA concentrations keeping EB concentration constant (5 μg/ml). With DNA concentration of 2 to 4 μg, a prominent excitation at 300 nm was observed. On increasing DNA concentration there was a shift in the excitation maxima from 300 to 310 nm (Fig.7). The emission spectra of EB-DNA complex showed a prominent maxima at 590 nm (excitation 300 nm; Fig.4). The inset of Fig.4 shows linear increase in fluorescence on complex formation with increase of DNA concentration at 590 nm. This linear relationship was utilized for the quantitation of DNA in the immune complexes.

Effect of serum proteins and ammonium sulfate on F.I. of EB-DNA complex was studied to decide the dilution of serum and to choose the fraction (supernatant or pellet) suitable for the fluorescence measurements. Effect of serum proteins on F.I. of EB-DNA complex were studied using different dilutions of NHS. The quenching of fluorescence of EB-DNA complex was observed in the presence of serum proteins. The quenching was dependent on serum dilution. On increasing serum dilution, fluorescence quenching was released. At a serum dilution of 1:5, 13 percent
Fig. 7: Excitation spectra of EB, dsDNA and EB-DNA complex.

Excitation spectra were recorded keeping emission wavelength at 590 nm. Concentration of DNA in EB-DNA complex: 2 μg (○), 4 μg (■), 6 μg (□), 8 μg (△), 10 μg (□□), free EB (○○), 10 μg of free DNA (○△). EB concentration was kept constant (5 μg/ml).
quenching was observed. Similarly the effect of ammonium sulfate on F.I. of EB-DNA complex was studied. At 2.9 M ammonium sulfate (almost 50 percent saturation) the percent quenching was 52. On decreasing its concentration, fluorescence quenching was released, but even at 0.59 M ammonium sulfate the value was 24 percent (Fig.8). Since both the serum proteins and ammonium sulfate were found to be fluorescent quenchers of EB-DNA complex, the pellet was chosen for the assay as the concentrations of both these interfering agents was quite low in the pellet as compared to the supernatant and the quenching effect could largely be minimized.

NHS and sera of patients with various rheumatic diseases were tested for the presence of anti-DNA antibodies (Fig.9). Sera showing DNA binding activity of more than 20 percent were considered positive. Using this criteria, all NHS, sera of patients with SS and RA were negative for anti-DNA antibodies. Forty three percent of SLE sera were positive for anti-DNA antibodies. One out of 7 SLE patients with overlapping PSS and one out of ten PSS patients were found to be positive for antibodies against DNA.

The specificity of the assay was checked by inhibition experiment. Percent inhibition obtained with different DNA concentrations using an anti-DNA antibody positive SLE serum is shown in Fig.10. The antigen-antibody reaction is specific because of 75 percent inhibition obtained with 8 µg of DNA.
Fig. 8: Fluorescence quenching of EB-DNA complex in the presence of different dilutions of NHS and varying concentrations of ammonium sulfate.

EB-DNA complex was prepared in varying concentrations of ammonium sulfate (0.58 M to 2.9 M) and different dilutions of NHS (neat to 1:64) as described. Fluorescence intensity was recorded at 590 nm (excitation wavelength of 300 nm) and percent fluorescence quenching was calculated.
The graph shows the relationship between serum dilution and percent fluorescence quenching. The x-axis represents the log of serum dilution, while the y-axis represents the percent fluorescence quenching. The graph includes two curves: one represented by circles (-o-) and the other by dots (---). The x-axis values range from 20 to 0, and the y-axis values range from 50 to 0.

The data points correspond to different concentrations of ammonium sulfate (M). The concentration values are 0.4, 0.8, 1.2, and 1.6 M, and these are marked on the x-axis. The corresponding fluorescence quenching percentages are indicated on the y-axis.

The graph suggests that as the serum dilution increases, the percent fluorescence quenching also increases, indicating a positive correlation between the two variables.
Fig. 9: Prevalence of anti-DNA antibodies in various rheumatic diseases as measured by fluorometric assay.

The mean DNA binding values of positive SLE sera, NHS and other sera with standard deviations are indicated at the bottom of the figure.
Fig. 10: Demonstration of specificity of fluorometric assay by inhibition experiment.

Inhibition of antigen-antibody reaction was performed in the presence of varying concentrations of DNA (1 to 10 µg/ml). Other experimental details were as described in legend to Fig. 6.
In order to compare the sensitivity of spectrophotometric and fluorometric techniques with those of commonly used techniques (immunoprecipitation and hemagglutination), various sera were tested for the presence of anti-DNA antibodies. These results (Table 5) show that both spectrophotometric and fluorometric methods are more sensitive than ID and CIE and almost parallel in sensitivity to PHA.

(4) A microplate enzyme-linked immunosorbent assay (ELISA) for anti-DNA antibodies using fluorogenic and colorigenic substrates.

Recently a lot of interest has been focussed in the use of ELISA because of simplicity and high sensitivity. ELISA was developed for the detection and quantitation of anti-DNA antibodies in the sera of patients with SLE. The technique involves the adsorption of calf thymus DNA to the surface of microtiter plate and saturation of excess sites with fetal calf serum. After the antigen-antibody reaction, the immune complexes were detected by an enzymatic reaction involving alkaline phosphatase conjugated rabbit anti-human IgG. Measurement of enzyme activity was facilitated by the use of p-nitrophenyl phosphate (PNP-P) and 4-methylumbelliferyl phosphate (4MU-P) as substrates. Experiments were intended to compare the sensitivity and reliability of the two substrates in ELISA under different experimental conditions.
TABLE 5

Comparison of incidence of anti-DNA antibodies in rheumatic diseases by various techniques.

<table>
<thead>
<tr>
<th>Sera</th>
<th>Number tested</th>
<th>ID</th>
<th>CIE</th>
<th>PHA</th>
<th>Spectrophotometric assay</th>
<th>Fluorometric assay</th>
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</thead>
<tbody>
<tr>
<td>SLE</td>
<td>23</td>
<td>17</td>
<td>26</td>
<td>35</td>
<td>39</td>
<td>43</td>
</tr>
<tr>
<td>SLE + PSS</td>
<td>7</td>
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<td>0</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>PSS</td>
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<tr>
<td>RA</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NHS</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Rabbit anti-human IgG-alkaline phosphatase conjugate purified by gel filtration on Sephadex G-200 gave an IgG-enzyme ratio of 4. The activity of the enzyme in conjugate was measured at increasing dilutions with a constant concentration of the substrates. The reaction end point was found to be 1:2560 in case of 4MU-P and 1:80 for PNP-P providing 32 fold higher sensitivity in the detection of enzyme activity with the fluorogenic substrate (Fig.11). Similarly when enzyme activity of the conjugate was measured at different substrate concentrations, very little of 4MU-P (1 µg) gave a detectable reaction. The detectable concentration for PNP-P was 100 µg indicating almost 100 fold greater sensitivity with fluorogenic substrate in the detection of enzyme activity. The amount of antigen bound to the plastic surface was estimated by measuring optical density at 260 nm after overnight incubation at 4°C and found to be about 12% of the total antigen placed in the well.

Detailed study of the sensitivity of the two substrates in ELISA was performed using serum and IgG of an SLE patient (DK) who had higher titer anti-DNA antibody activity. The end point of the reaction with PNP-P was 1:6400 for serum and 1:1600 for IgG. With 4MU-P as substrate the reaction titer was found to be 1:51200 for serum and 1:12800 for IgG. Fluorescence intensity (F.I.) and optical density (O.D.) of less than 10 and 0.1 respectively were taken as cut off points. An 8 fold greater sensitivity of the reaction with fluorogenic substrate is evident.
Fig. 11: Plot showing the enzyme activity as a function of different conjugate dilutions using PNP-P and 4MU-P as substrates.

0.3 ml of serially diluted conjugate (1:10 to 1:20480) was mixed with 1 ml of substrate solution (PNP-P, $5 \times 10^{-3}$ M in diethanolamine buffer or 4MU-P, $1 \times 10^{-3}$ M in AMP buffer) and incubated at 37°C for 30 minutes. Reaction was stopped by adding 5 ml of 0.2 N NaOH. Absorbance and fluorescence intensity were recorded.
The sensitivity of the two substrates was further compared at different dilutions of the conjugate using native DNA as antigen and at a constant concentration of purified IgG from DK (1:100 at 12 mg/ml). The titration end point was found to be 1:40960 for 4MU-P and 1:2560 for PNP-P. Therefore, the fluorogenic substrate increased the sensitivity of the assay almost 16 times (Fig. 12). The optimum concentration of the conjugate was almost same for both the substrates. This concentration was found to be 43 μg conjugate protein/ml in our experiments.

Fig. 13 shows the results obtained with IgG in ELISA using different concentrations of DNA. With PNP-P the minimum concentration of antigen for a detectable reaction was 18 nM bp whereas it was 2 nM bp for 4MU-P. Thus the fluorogenic substrate increased the sensitivity of the assay 9 times. The antigen concentration used, for both the substrates, was 19 uM bp. On varying the period of incubation, a linear relationship with the amount of PNP formed upto 90 minutes was observed (Fig. 14). The reaction followed linearity upto 150 minutes with fluorogenic substrate. An incubation time of 60 minutes was used in all our experiments.

These characteristics of fluorogenic substrate were exploited in the detection and quantitation of anti-DNA antibodies in the sera of patients with SLE. Results were compared with the assay using PNP-P as substrate. Tables 6 and 7 show the characteristics of various sera tested by ELISA using PNP-P and 4MU-P as substrates. Each serum was tested at various dilutions
Fig. 12: Comparison of the sensitivity of the two substrates in ELISA at different conjugate dilutions.

Serial dilutions of the conjugate (neat to 1:81920) and a fixed concentration of DNA (19 μM bp) and antibody (1:100 of serum or 12 mg/ml IgG) were used. The reaction product were monitored as described in Methods.
Fig. 13: Effect of antigen concentration on the sensitivity of ELISA with the two substrates.

The experiment was performed by coating different amount of DNA (1 nM bp to 23 μM bp) in the wells of microtiter plate. ELISA was carried out with PNP-P and 4MU-P. A 1:5 dilution of the conjugate and 1:100 dilution of IgG (12 mg/ml) were used in the assay.
Fig. 14: A plot of alkaline phosphatase activity in conjugate against incubation time in ELISA.

The assay was carried out at an antigen concentration of 19 \( \mu M \) bp. The concentration of the conjugate and antibody were as in legend to Fig. 13. Immune complex-conjugate adduct was incubated with PNP-P and 4MU-P for different time intervals and the product formed was monitored by recording absorbance and fluorescence intensity.
<table>
<thead>
<tr>
<th>log serum dilution</th>
<th>Normal human sera (40)</th>
<th>Positive SLE sera (8)</th>
<th>Negative SLE sera (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean O.D. ± S.D. x 10^3</td>
<td>Coefficient of variation (%)</td>
<td>Mean O.D. ± S.D. x 10^3</td>
</tr>
<tr>
<td>2</td>
<td>78 ± 12</td>
<td>16</td>
<td>212 ± 66</td>
</tr>
<tr>
<td>2.3</td>
<td>71 ± 14</td>
<td>20</td>
<td>181 ± 60</td>
</tr>
<tr>
<td>2.6</td>
<td>60 ± 13</td>
<td>22</td>
<td>147 ± 63</td>
</tr>
<tr>
<td>2.9</td>
<td>56 ± 14</td>
<td>24</td>
<td>122 ± 61</td>
</tr>
<tr>
<td>3.2</td>
<td>54 ± 14</td>
<td>26</td>
<td>101 ± 42</td>
</tr>
<tr>
<td>3.5</td>
<td>46 ± 15</td>
<td>33</td>
<td>77 ± 32</td>
</tr>
</tbody>
</table>

Figures in parentheses represent numbers of sera tested. SLE serum at 1:100 dilution having O.D. less than 0.10 has been arbitrarily classified as negative serum.
TABLE 7

ELISA for anti-DNA antibodies in various sera with 4MU-P as substrate.

<table>
<thead>
<tr>
<th>-log serum dilution</th>
<th>Normal human sera (20)</th>
<th>Positive SLE sera (10)</th>
<th>Negative SLE sera (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean F.I. ± S.D.</td>
<td>Coefficient of variation (%)</td>
<td>Mean F.I. ± S.D.</td>
</tr>
<tr>
<td>2</td>
<td>6.3 ± 1.3</td>
<td>16</td>
<td>26 ± 12.3</td>
</tr>
<tr>
<td>2.3</td>
<td>5.4 ± 1.3</td>
<td>14</td>
<td>23 ± 11.6</td>
</tr>
<tr>
<td>2.6</td>
<td>5.0 ± 1.4</td>
<td>28</td>
<td>20 ± 10.5</td>
</tr>
<tr>
<td>2.9</td>
<td>4.6 ± 1.4</td>
<td>30</td>
<td>19 ± 8.9</td>
</tr>
<tr>
<td>3.2</td>
<td>4.2 ± 0.9</td>
<td>21</td>
<td>16 ± 8.8</td>
</tr>
<tr>
<td>3.5</td>
<td>3.4 ± 0.8</td>
<td>24</td>
<td>13 ± 8.0</td>
</tr>
<tr>
<td>3.8</td>
<td>2.8 ± 0.7</td>
<td>24</td>
<td>10 ± 7.2</td>
</tr>
<tr>
<td>4.1</td>
<td>2.3 ± 0.7</td>
<td>32</td>
<td>8 ± 6.1</td>
</tr>
</tbody>
</table>

Figures in parentheses represent numbers of sera tested. SLE serum at 1:100 dilution having F.I. of less than 10 has been arbitrarily classified as negative serum.
starting from 1:100. Average O.D. and F.I. were calculated at each serum dilution with separate groups of normal and disease patients. The reaction was considered negative when O.D. and F.I. of the reaction product were less than 0.1 and 10 respectively at a serum dilution of 1:100. With this criteria of assay reaction, all normal human sera studied were negative for anti-DNA antibodies. Fifty seven percent of SLE sera were positive with colorigenic substrate and 71 percent were positive with fluorogenic substrate.

In order to compare the sensitivity of ELISA with that of PHA, SLE sera were checked for hemagglutinating anti-DNA antibodies. Five out of 14 sera (36 percent) were positive by PHA. All sera positive by PHA were also positive with ELISA. In addition, some of the serum samples negative by PHA were positive by ELISA (Table 8). These results indicate approximately 1.5 to 2 fold increase in sensitivity, depending on the substrate, of ELISA over that of PHA.

(5) A high sensitive ELISA for anti-DNA antibodies using nylon as solid phase.

The sensitivity of ELISA can be limited by factors which include the type of solid phase and method used to monitor enzyme activity. As mentioned earlier, an increase in the sensitivity of ELISA was observed by the use of fluorogenic substrate for the measurement of enzyme activity. The sensitivity of ELISA has
TABLE 8

Comparative study of anti-DNA antibody titer of SLE sera by ELISA and passive hemagglutination technique.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Titer with ELISA</th>
<th>Hemagglutination titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PNP-P</td>
<td>4MU-P</td>
</tr>
<tr>
<td>DK-serum</td>
<td>1:6400</td>
<td>1:51200</td>
</tr>
<tr>
<td>DK-IgG</td>
<td>1:1600</td>
<td>1:12800</td>
</tr>
<tr>
<td>PM-serum</td>
<td>1:200</td>
<td>1:800</td>
</tr>
<tr>
<td>BW-serum</td>
<td>1:200</td>
<td>1:800</td>
</tr>
<tr>
<td>ST-serum</td>
<td>1:400</td>
<td>1:3200</td>
</tr>
<tr>
<td>AS-serum</td>
<td>&lt; 1:100</td>
<td>1:100</td>
</tr>
<tr>
<td>SH-serum</td>
<td>&lt; 1:100</td>
<td>1:100</td>
</tr>
</tbody>
</table>

The concentration of purified DK-IgG was adjusted to 12 mg/ml. Hemagglutination titer of up to 1:4, fluorescence intensity units of less than 10 and O.D. of less than 0.10 of serum samples at 1:100 dilution are treated as negative. Sera PM, BW, ST, AS and SH were negative for hemagglutinating antibody whereas, AS and SH were negative by ELISA with PNP-P as substrate. All sera were positive when 4MU-P was used as substrate.
been further increased by the use of different solid supports to increase the surface area for the immobilization of antigen. We developed a solid phase ELISA for the detection of antibodies to DNA in various rheumatic diseases employing nylon as solid support for antigen immobilization. The anti-DNA antibody incidence in various sera was compared using fluorogenic and colorigenic enzyme substrates.

Nylon beads were chosen as solid support because it provided greater surface area for antigen binding, ease in washing and low background values. The reaction conditions were established by a series of preliminary experiments using a positive anti-DNA antibody SLE serum. All the sera were tested in triplicate and the A/F.I. values were expressed as the arithmetic mean.

Optimum time for the activation of nylon beads was studied by their incubation with 3.5 N HCl for different time periods. After each time interval the beads were washed, coated with antigen and used in ELISA. As shown in Fig. 15 the extinction values using a positive SLE serum increased up to 72 hours. However, 36 hours activation was routinely done as the beads tend to crack after longer incubation.

Activated beads were treated with different DNA concentrations (5 µg to 400 µg) at 4°C for 24 hours. Amount of antigen coated on the beads was estimated by recording the absorbance of
Fig. 15: Activation of nylon beads as a function of time.

Beads were activated for different time intervals (6-72 hours) by incubating in 3.5 N HCl at 37°C and ELISA was performed with a positive anti-DNA antibody SLE serum.
the residual solution at 260 nm. It appears that at 100 μg of DNA, maximum antigen gets coated on the beads (Table 9). When antigen coating was compared at 37°C and at 4°C for different time periods, maximum coating was obtained at 4°C for 24 hours.

The effect of pH variation on antigen coating was studied by performing the coating with antigen prepared in McIlvaine (pH 3 to 8) and carbonate (pH 9 to 10) buffers. The coated beads were processed for ELISA as described using an antibody positive SLE serum. The coating was pH dependent (Fig.16) with a maxima at pH 5.

ELISA was performed with varying number of antigen coated beads (1-10) to optimize the number of beads to be used in the experiment. Six beads were chosen since maximum absorbance values were obtained using this number (Fig.17). Coefficient of variation of 20 different sets of six beads with an anti-DNA antibody positive SLE serum was around 10 percent.

The specificity of antigen antibody reaction in ELISA was checked by inhibition experiments. Varying concentrations of DNA were mixed with an anti-DNA antibody positive SLE serum to a final concentration of 2 to 20 μg DNA/ml. The mixture was incubated at 37°C for 1 hour and then at 4°C for 20 hours and used in ELISA instead of serum. Percent inhibition obtained at different DNA concentrations is shown in Fig. 18. It is evident that the reaction is specific because of high inhibition (53%) with only 4 μg of DNA.
TABLE 9
Uptake of DNA by nylon beads.

<table>
<thead>
<tr>
<th>DNA concentration (µg/ml)</th>
<th>Uptake of DNA per bead (µg) x 10^{-2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>25.0</td>
<td>12.5</td>
</tr>
<tr>
<td>50.0</td>
<td>23.5</td>
</tr>
<tr>
<td>100.0</td>
<td>35.5</td>
</tr>
<tr>
<td>200.0</td>
<td>35.0</td>
</tr>
<tr>
<td>400.0</td>
<td>35.0</td>
</tr>
</tbody>
</table>

The concentration of antigen was varied between 5 µg to 400 µg/ml and antigen uptake was estimated by checking the residual DNA in the supernatant at the end of incubation by recording the absorbance at 260 nm.
Fig. 16: Effect of pH on antigen coating.

Activated beads were coated with DNA (100 μg/ml) in McIlvaine (pH 3 to 8) and carbonate (pH 9 to 10) buffers at 4°C for 24 hours. Antigen coated beads were processed for ELISA using a positive SLE serum.
Fig. 17: ELISA with varying number of antigen coated beads.

Increasing number of DNA coated beads (1 to 10) were used in ELISA. Other experimental details are as described in Methods.
Fig. 18: Inhibition ELISA for anti-DNA antibodies.

Varying concentrations of DNA (2 to 20 μg/ml) were mixed with a positive anti-DNA antibody SLE serum (1:100) and incubated at 37°C for 1 hour and at 4°C for 20 hours. Incubated contents were used in ELISA instead of serum.
Effect of storage of antigen coated beads on the antigenicity was studied. ELISA was performed with antigen coated beads, stored dry at -20°C for different weeks. The results obtained show that ELISA can conveniently be performed with beads stored up to 4 weeks without any appreciable loss in the antigenicity. Decrease in the absorbance values is observed, if the beads are not stored dry.

Normal human sera and sera of patients with rheumatic diseases (SLE, SLE + PSS, PSS, SS, RA) were tested for the presence of anti-DNA antibodies by ELISA using colorigenic and fluorogenic enzyme substrates. Sera showing FI/A of more than 12/0.12 were considered positive. Using this criteria all normal human sera and sera of patients with RA, SS were negative for anti-DNA antibodies. Fifty two and 56 percent of SLE sera were positive for antibodies to DNA by PNP-P and 4MU-P respectively. Fourteen percent of SLE patients with overlapping PSS and 10 percent of PSS patients showed a positive antibody profile with both the substrates (Figs. 19 and 20).

The assay described can easily be miniaturized in conditions where small amounts of reagents (antigen or antibody) are available. We have successfully decreased the volume of reagents (0.1 ml instead of 0.5 ml) and used 2 beads for the measurement of anti-DNA antibodies in those conditions where very low serum samples were available for screening.
Fig. 19: Incidence of anti-DNA antibodies in rheumatic diseases by ELISA with nylon as solid phase, using PNP-P as enzyme substrate.

NHS and sera of patients with SLE, SLE + PSS, PSS, SS and RA were tested for the presence of anti-DNA antibodies using PNP-P as substrate. The mean absorbance values with standard deviations are indicated at the bottom of the figure.
Fig. 20: Incidence of anti-DNA antibodies in rheumatic diseases by ELISA with nylon as solid phase, using 4MU-P as enzyme substrate.

ELISA was performed with 4MU-P as substrate. Other details are given in legend to Fig. 19.
(6) **Classification of anti-DNA antibodies on the basis of avidity.**

SLE sera and sera of patients with PSS, SLE + PSS, SS and RA were tested by ID, CIE, PHA and ELISA with nylon beads using 4MU-P as enzyme substrate, for the presence of anti-DNA antibodies. On the basis of the antibody level, these sera were arbitrarily classified into three major groups.

I. Sera with high avidity anti-DNA antibodies (F.I. > 25) — detectable by ID, CIE, PHA and ELISA.

II. Sera with moderate avidity anti-DNA antibodies (F.I. between 15 and 25) — detectable by PHA and ELISA.

III. Sera with low avidity anti-DNA antibodies (F.I. between 12 and 15) — detectable only by ELISA.

On the basis of above classification 13 percent of SLE patients belonged to group I, 23 percent of SLE patients and 14 percent SLE + PSS patients to group II and 21 percent SLE patients, 14 percent SLE + PSS and 10 percent PSS patients to group III (Table 10).

(7) **Experimentally induced anti-DNA antibodies by immunization**

with DNA-psoralen adduct.

Before eliciting anti-DNA-psoralen antibodies, the extent of cross-linking between DNA and psoralen was evaluated by
### TABLE 10

Determination of low, moderate and high avidity anti-DNA antibodies by various techniques.

<table>
<thead>
<tr>
<th>Sera</th>
<th>Percent sera detectable by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ID, PHA and ELISA</td>
</tr>
<tr>
<td></td>
<td>F.I. &gt; 25 (High avidity)</td>
</tr>
<tr>
<td></td>
<td>PHA and ELISA F.I. between 15 and 25 (Moderate avidity)</td>
</tr>
<tr>
<td></td>
<td>ELISA F.I. between 12 and 15 (Low avidity)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>SLE + PSS</th>
<th>SS</th>
<th>PSS</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SLE + PSS</td>
<td>23.0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SS</td>
<td>21.0</td>
<td>14</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>PSS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Anti-DNA antibodies in rheumatic diseases were arbitrarily classified into 3 broad groups on the basis of results obtained by ID, CIE, PHA and ELISA using nylon as solid support.
studying the spontaneous renaturation capacity of cross-linked DNA by means of column chromatography. The separation of renatured fractions from irreversibly denatured DNA was performed by column chromatography on hydroxyapatite. DNA-psoralen photoadduct after denaturation was eluted at 0.25 M phosphate buffer, pH 7.2 almost similar to that of native DNA indicating cross-linking between the two. Sample of DNA and psoralen without irradiation was the experimental control which was eluted at 0.2 M phosphate buffer, pH 7.2 similar to that of denatured DNA (Fig.21).

The amount of psoralen intercalated with DNA was determined by equilibrium dialysis measurements. The amount of psoralen dialyzed out was quantitated with the help of standard plot obtained by drawing F.I. of irradiated psoralen as a function of its concentration. The binding ratio of 14M psoralen per 56 μM bp of DNA has been found by equilibrium dialysis.

Antibodies to DNA-psoralen adduct, dsDNA, ssDNA and RNA in the sera of guinea pigs immunized with DNA-psoralen adduct were studied by ELISA, immunoprecipitation and hemagglutination techniques. No reactivity was observed with any of the antigen by ID, CIE, PHA. Immunized sera showed a positive reaction in ELISA on nylon with DNA-psoralen adduct and dsDNA whereas ssDNA and RNA exhibited low reactivity with the immunized sera (Table 11).
Fig. 21: Hydroxyapatite chromatography of DNA-psoralen adduct after denaturation.

The covalent photoadduct was heat denatured, fast cooled and then passed through a hydroxyapatite column (5.5 cm x 1.5 cm). The contents were eluted with 0.20 and 0.25 M phosphate buffer, pH 7.2. Sample of DNA and psoralen without irradiation was the experimental control and was eluted at 0.2 M phosphate buffer, pH 7.2 (---). The fraction eluted at 0.25 M phosphate buffer, pH 7.2 (•••) was taken as DNA-psoralen adduct and used as antigen.
### TABLE 11

Reactivity of DNA-psoralen immunized guinea pig sera with various nucleic acids by ELISA.

<table>
<thead>
<tr>
<th>Sera</th>
<th>-log serum dilution</th>
<th>Antigens</th>
<th>F.I. x 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>o</td>
<td>DNA-psoralen</td>
<td>dsDNA</td>
</tr>
<tr>
<td>DNA-psoralen</td>
<td>2</td>
<td>150</td>
<td>135</td>
</tr>
<tr>
<td>immunized guinea pig</td>
<td>2.3</td>
<td>145</td>
<td>105</td>
</tr>
<tr>
<td>serum</td>
<td>2.6</td>
<td>125</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>105.0</td>
<td>30.0</td>
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<tr>
<td></td>
<td>3.2</td>
<td>75.0</td>
<td>25.0</td>
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<td></td>
<td>3.5</td>
<td>60.0</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>45.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>30.0</td>
<td>0</td>
</tr>
<tr>
<td>Preimmunized</td>
<td>2</td>
<td>30.0</td>
<td>15.0</td>
</tr>
<tr>
<td>serum</td>
<td>2.3</td>
<td>20.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
<td>5.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Preimmunized and DNA-psoralen immunized guinea pig sera at different dilutions were tested by ELISA with nylon as solid phase, using various nucleic acids as antigens.
(8) **Specificity of affinity purified antibodies.**

DK was an SLE patient with a serum titer of 1:51200 by ELISA using 4MU-P as enzyme substrate. The serum was positive for precipitating and hemagglutinating anti-DNA antibodies by ID, CIE and PHA. CIE of the serum revealed precipitin lines with dsDNA, ssDNA, buffalo thymus RNA, E. coli RNA and DNA-psoralen adduct as antigens. Faint precipitin reactions were observed with yeast RNA and purified Sm/RNP antigens. IgG isolated from this serum by DEAE-cellulose chromatography gave identical lines with these antigens showing complete reaction of identity with serum lines. When affinity purified anti-DNA antibodies were used in CIE, a strong line was seen with dsDNA but no other lines were observed with any of the antigen indicating the specificity of anti-dsDNA antibody. Similar results were obtained by ID.

The specificity and reactivity of affinity purified material at different concentrations varying from 0.06 μg/ml to 120 μg/ml was also checked by ELISA utilizing dsDNA, ssDNA, DNA-psoralen adduct and RNA coated beads. The results obtained are tabulated in Table 12. These antibodies were found to be highly specific for double-stranded DNA and exhibited strong reactivity with DNA-psoralen adduct. Very poor reactivity was observed with ssDNA and buffalo thymus RNA.

(a) **IgG/IgM response.**

In order to find out specific antibody response in SLE
### TABLE 12

**Reactivity of affinity purified antibodies with various nucleic acids by ELISA.**

<table>
<thead>
<tr>
<th>Antibody dilution (µg/ml)</th>
<th>DNA coated beads</th>
<th>DNA-psoralen coated beads</th>
<th>ssDNA coated beads</th>
<th>RNA coated beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>120.0</td>
<td>23.0</td>
<td>17.8</td>
<td>6.9</td>
<td>7.2</td>
</tr>
<tr>
<td>60.0</td>
<td>21.0</td>
<td>16.0</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>30.0</td>
<td>19.5</td>
<td>14.5</td>
<td>6.4</td>
<td>6.5</td>
</tr>
<tr>
<td>15.0</td>
<td>16.1</td>
<td>13.8</td>
<td>6.4</td>
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<td>7.5</td>
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<td>3.75</td>
<td>15.9</td>
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<td>5.3</td>
<td>3.6</td>
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<td>0.23</td>
<td>3.2</td>
<td>3.9</td>
<td>3.6</td>
<td>2.2</td>
</tr>
<tr>
<td>0.12</td>
<td>1.9</td>
<td>2.9</td>
<td>2.6</td>
<td>1.6</td>
</tr>
<tr>
<td>0.06</td>
<td>1.3</td>
<td>1.6</td>
<td>0.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Normal human IgG**

<table>
<thead>
<tr>
<th>Antibody dilution (µg/ml)</th>
<th>DNA coated beads</th>
<th>DNA-psoralen coated beads</th>
<th>ssDNA coated beads</th>
<th>RNA coated beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>120.0</td>
<td>4.0</td>
<td>3.8</td>
<td>4.2</td>
<td>3.2</td>
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<td>60.0</td>
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<td>30.0</td>
<td>1.8</td>
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<td>1.2</td>
<td>1.0</td>
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</tbody>
</table>

Anti-dsDNA antibodies were isolated by affinity chromatography using dsDNA-linked Sepharose 4B column. The purified antibodies were used in ELISA using dsDNA, ssDNA, RNA and DNA-psoralen coated beads.
serum (DK), anti-DNA antibodies were isolated by affinity chromatography using dsDNA-linked Sepharose 4B column. ELISA was carried out with different antibody concentrations varying from 0.06 μg/ml to 120 μg/ml, using dsDNA coated beads. Immune complex attached to nylon beads was detected by enzymatic reaction involving alkaline phosphatase conjugated goat anti-human IgG and IgM. The results obtained are shown in Fig. 22. IgG and IgM ratio in the purified antibody preparation was 5:1.
Fig. 22: Determination of IgG/IgM ratio in the affinity purified antibodies by ELISA.

ELISA was performed with varying antibody concentrations (0.06 μg/ml - 120 μg/ml) employing dsDNA coated beads. Goat anti-human IgG (—○—) and IgM (—●—) alkaline phosphatase conjugates at dilutions of 1:1000 were used in the assay.
IV. DISCUSSION

The pathogenic significance of anti-DNA antibodies has been well documented in SLE and the antibody levels are claimed to correlate well with disease activity (Swaak et al., 1979). However, the qualitative and quantitative evaluation of anti-DNA antibodies is still being investigated for the perfection as reflected by the number of different methods that have been developed for their measurements. Radioimmunoassay (RIA) has been extensively used for the detection of anti-DNA antibodies (Talal and Pillarisetty, 1976). Sophisticated radioactive measuring instruments limit its use in laboratories where all these facilities are not available. These problems emphasize the need for a new simplified approach to measure anti-DNA antibodies.

The results obtained by spectrophotometric and fluorometric techniques are clearly indicative of their greater sensitivity than ID and CIE and comparability with PHA. Both the assays could therefore be employed for the detection of low affinity antibodies produced by immunized animals and SLE patients having low circulating concentrations of anti-DNA antibodies. Such antibodies practically act as univalent antibody and are not able to form stable precipitating complexes with antigen (Marghi et al., 1980). The reason for low incidence of anti-DNA antibodies by immunoprecipitation has not been established. Avidity, restricted
heterogeneity and the formation of soluble complexes are known factors which may effect the precipitin reaction.

High binding values were observed in normal human sera with dsDNA by spectrophotometric assay. The DNA binding of almost the same extent has been reported by other methods of measurements. This could be due to either low level of anti-DNA antibodies in normal human subjects or the nonspecific association of proteins and other materials found in serum with polyanionic DNA. Moreover, antibodies to nucleic acids have been found increased in aged healthy individuals (Schuller et al., 1981) which can further contribute to increased binding observed in apparently normal individuals. Both spectrophotometric and fluorometric methods described here are simple, specific, reproducible and suitable for use in clinical laboratories where facilities for radioimmunoassay do not exist.

SLE serum contain heterogeneous population of anti-DNA antibodies with varying degree of avidity. Some authors have reported that in humans circulating antibodies with high avidity for DNA are more likely to be associated with severe active disease and nephritis (Leon et al., 1977). Others have correlated low affinity circulating antibody with severe nephritis (Asano and Nakamoto, 1978). In mice, low avidity antibodies in the circulation appear at the time nephritis begins (Steward et al., 1975). Differences between avidity studies may reflect (i) different methods employed for the detection of anti-DNA antibodies.
difficulty in calculating avidity with a bivalent antibody and a large relatively undefined DNA substrate and (iii) antibody heterogeneity (Hahn, 1982). In recent years, Farr assay and Millipore filter assays are under criticism for alleged inability to detect low avidity anti-DNA antibodies (Rubbin et al., 1978). Smeenk et al. (1982) reported the detection of low avidity antibodies in SLE serum by high sensitivity polyethylene glycol precipitation assay, not detected by conventional techniques.

Recently much interest has been directed in the use of ELISA in view of its operational simplicity and high sensitivity (Hendry and Herrmann, 1980; Aitkaci et al., 1981; Goodburn et al., 1981). Antibodies against native DNA have been evaluated by ELISA using glucose oxidase labelled antibodies (Labrouce et al., 1980). Their results were similar with those of Farr assay. ELISA has also been developed and evaluated for the detection of antibodies to DNA in experimental animals (Hillyer and Modeline, 1980). Recently ELISA for the measurement of anti-DNA levels has been reported (Kawai et al., 1982) using horse radish peroxidase-linked IgG, IgM and IgA conjugates. The substrate for monitoring the enzyme activity has generally been PNP-P (Voller et al., 1976; Harris et al., 1979).

In order to increase the sensitivity of ELISA, the conventional PNP-P has been replaced by a fluorescent substrate 4MU-P (Shalev et al., 1980). We adopted ELISA for the detection
and quantitation of anti-DNA antibodies using both PNP-P and 4MU-P as enzyme substrates. In the present study a 100 times lower concentration of the product (4MU) was detected by fluorometry in relation to PNP. This is not in full agreement with that of 1000 fold increased sensitivity of the fluorogenic substrate reported earlier (Shalev et al., 1980). This difference could possibly be accounted on the basis of different source of alkaline phosphatase used for preparing the conjugate resulting in lower specific activity. At different dilutions of antigen or antibody 9 times greater sensitivity was observed with fluorogenic substrate. However, at different conjugate dilutions the sensitivity of the assay was 16 fold higher with 4MU-P and are in full agreement to those reported by Shalev et al. (1980). We found an incidence of 57% of circulating DNA antibodies in SLE patients by ELISA with colorigenic and 71% positive with fluorogenic substrate. These results are clearly indicative of the greater sensitivity of fluorogenic substrate and could therefore be employed for the detection of low avidity antibodies. The same serum samples when tested by passive hemagglutination technique have an incidence of 36% for DNA antibodies. Some of the sera negative by PHA showed a strong positive reaction with ELISA. An agglutination inhibiting factor has been reported in thyrotoxic serum (Wilkin et al., 1979). It is possible that a similar factor is also present in some SLE sera inhibiting agglutination. The absence of hemagglutinating
antibodies in these sera could possibly be another contributory factor for low incidence.

We report the successful utilization of nylon as solid support for binding of DNA for use in ELISA in the detection of anti-DNA antibodies. High background noise poses major limitations in the success of enzyme immunoassays. Our experiments indicate that background activity can considerably be reduced by introducing nylon as solid support instead of conventional polystyrene surface. High background values obtained due to nonspecific binding of the conjugate onto the walls of the test tube, have been reduced substantially in the present method by introducing last washing with distilled water and carrying out the enzymatic reaction with dried beads in separate tubes. ELISA in conjunction with nylon as solid support combines the advantages of (i) the high sensitivity obtained by the use of fluorogenic enzyme substrate (ii) low background values and (iii) facility for prolonged storage of antigen coated solid phase, making the assay highly sensitive, specific and economical. The ability of DNA coated nylon beads to retain antigenicity for a considerable period of time is of special importance since the cumbersome daily antigen coating procedure is avoided. The method is simple and could be conveniently employed for the detection of low avidity anti-DNA antibodies which were not detectable by commonly used techniques. It may allow an earlier diagnosis of the disease which might be helpful in the management of patients. The
possibility of distinguishing between different disease patterns is also feasible. Moreover, in the miniaturized form the method has scope in conditions where very small amounts of patients sera are available for assay.

The high incidence of anti-dsDNA antibodies detectable in patients with inactive SLE suggests that ELISA is potentially useful for diagnostic screening of asymptomatic individuals and patients with syndromes suggestive of SLE. The sensitivity of ELISA is comparable with that of radioimmunoassay and avoids the known dangers of isotope work. The ELISA test circumvents several problems inherent to radioimmunoassay procedure. Molecular weight of antigen does not influence the reaction of antibodies with DNA immobilized on solid phase. On contrary, in RIA only DNA molecules of molecular weight of $1 \times 10^7$ daltons or less are precipitated by a single antibody molecule, and therefore high molecular weight DNA requires a molar antibody-antigen ratio of greater than one for precipitation by ammonium sulfate (Aarden et al., 1976b). Antibody binding avidity is another factor which may account for differences between ELISA and RIA. ELISA directly measures gamma globulins thereby excluding interference by nonimmunoglobulin interactions with DNA such as C1q globulin and DNA binding proteins. In addition, the test system may be used to assay a variety of properties of the antibody like immunoglobulin class, complement fixing property and relative avidity. The ability to objectively quantitate antibody as well as the
potential for automation indicates that the solid phase immunoassay is potentially a useful procedure for the study of the variety of antibodies occurring in the sera of patients with SLE.

Intramuscular injections of dsDNA and ssDNA complexed with MBSA elicited antibodies against ssDNA in immunized rabbits as detected by immunoprecipitation and hemagglutination techniques. The comparison of immunological behaviour of DNA antibodies of SLE sera and sera of immunized animals demonstrated the difference in specificities of the two types of antibodies. Experimentally induced antibodies reacted with ssDNA but not with dsDNA indicating their specificity for bases and that the antigenic determinants are not related to the backbone of nucleic acid. Earlier studies in rabbits suggested that nucleic acids were haptens (Plescia and Braun, 1967). Later work clearly demonstrated that antibodies to double-stranded DNA could not be induced whereas antibodies to double-stranded RNA, synthetic double-stranded DNA, single-stranded DNA, oligonucleotides, nucleotides, nucleosides and bases could easily be elicited (Stollar and Ward, 1970). Anti-ss DNA appears frequently in diseases other than lupus (Bell et al., 1975). These antibodies are frequently induced inadvertently in humans, without appearance of antinative DNA antibodies, during treatment with procainamide and certain other drugs which are known to induce antinuclear antibodies. Many drugs have been implicated in the pathogenesis of SLE-related syndromes which include procainamide, hydralazine,
sulfonamides, isoniazid, trimethadione, phenylbutazone, streptomycin, tetracycline and griseofulvin (Blomgren et al., 1972). It has been suggested that these drugs with the ability to form complexes with DNA and DNA-protein are most likely to induce antinuclear antibody formation (Grabar, 1974). Recently antinuclear antibodies have been reported in psoriatic patients undergoing photochemotherapy. Antibodies to dsDNA have not been detected although, it is known that psoralen forms a covalent photoadduct with DNA in the presence of UV light and makes its immunogenic (Zarebska et al., 1978). In these studies Farr assay was employed for the detection of such antibodies which has been shown to be unable to detect low avidity anti-DNA antibodies.

ELISA on nylon as solid support has been successfully utilized for the detection of antibodies against DNA, RNA, ssDNA and DNA-psoralen adduct in animals immunized with DNA-psoralen adduct. We report that on intercalation with psoralen in the presence of UV-A light, DNA becomes immunogenic and antibodies formed have reactivity against DNA-psoralen adduct and dsDNA. These antibodies showed poor crossreactivity with ssDNA and RNA indicating that the antibodies probably are conformational directed. Our results show that although it is not possible to immunize animals against dsDNA but when DNA is intercalated with psoralen, this apparently nonimmunogenic molecule become immunogenic. This might be as a result of some structural changes in the molecule induced by intercalation with psoralen. The low
reactivity of immunized sera with ssDNA and RNA could probably be due to non availability of the antigenic bases to elicit antibody response resulting in the failure of the appearance of anti-ssDNA specific antibodies.

SLE anti-DNA antibodies isolated by affinity chromatography using dsDNA-Sepharose 4B columns were used to study the specificity of immune sera. SLE antibodies reacted specifically with dsDNA and showed strong reactivity with DNA-psoralen adduct while single-stranded DNA and RNA exhibited low reactivity. The antigenic specificity of these antibodies was indistinguishable from antibodies raised by immunization of animals with DNA-psoralen adduct as is clear from the results obtained in ELISA.

The specific etiological factors that cause SLE have eluded identification to the present time with the exception of patients who have a lupus like syndrome in relation to exposure to certain drugs (Hess, 1982). Genetic, viral, hormonal and environmental factors are all probably involved in the pathogenesis of this disease providing evidence for a truely multifactorial etiology. In drug induced lupus it has been suggested that these agents interact with DNA-protein complex in vivo and the resulting interaction renders it immunogenic and result in the production of autoantibodies. The formation of covalent steroid-protein adducts on incubation of albumin with cortisol or 16 α-hydroxyestrone and its possible involvement in the patho-
genesis of SLE has recently been attempted (Bucala et al., 1982). On the basis of our observation that covalent DNA-psoralen adduct elicit anti-DNA antibodies in experimental animals, it could be assumed that similar structural changes in DNA by interaction with above mentioned agent(s), might render it highly antigenic in susceptible individuals initiating and contributing to the pathogenesis of SLE.
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