This is to certify that the

thesis entitled

CANINE ANTINUCLEAR ANTIBODIES WITH
NUCLEOSOME CORE PARTICLE SPECIFICITY

presented by

RANDY LEIMAN ALLEN

has been accepted towards fulfillment
of the requirements for

Masters degree in Pathology

Robert W. Bull

Major professor
Robert W. Bull

Date 7-2-85

CANINE ANTINUCLEAR ANTIBODIES WITH NUCLEOSOME CORE PARTICLE SPECIFICITY

By

Randy Leiman Allen

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1985

ABSTRACT

CANINE ANTINUCLEAR ANTIBODIES WITH
NUCLEOSOME CORE PARTICLE SPECIFICITY

By

Randy Leiman Allen

Antinuclear antibodies (ANA) to deoxyribonucleoprotein (DNP) occur almost exclusively in the autoimmune disease systemic lupus erythematosus. Antibodies to DNP are responsible for the lupus erythematosus cell phenomenon and have been implicated in immune-mediated glomerular nephritis. The DNP previously used in assays to study anti-DNP antibodies was a combination of poorly defined antigens. In this study we developed an enzyme-linked immunosorbent assay (ELISA) to study canine ANA with nucleosome core particle specificity. Nucleosome core particles are a homogeneous population of highly purified DNP. Of nine serum samples positive for ANA by indirect immunofluorescence, four tested positive by the ELISA. Antibodies to nucleosome core particles were demonstrated in sera with homogeneous and speckled immunofluorescent staining patterns. Antibody binding in all sera required intact nucleosome core particles suggesting that antibodies recognized an epitope consisting of DNA and protein.

Dedicated to my

wife, Debbie

to my children, Lisa and Amanda

to my parents, Von and Savannah

to my brother, Dennis

and to my sister-in-law, Wendy

ACKNOWLEDGEMENTS

My thanks to the Animal Health Diagnostic Laboratory and the Department of Pathology at Michigan State University for supporting my research activities. Special thanks to Clay Hodgin for his guidance, advice and friendship both at M.S.U. and at North Carolina State University. Clay has had an immense influence on my career for which I will always grateful.

My deepest thanks to Robert Bull for providing an outstanding environment for scientific work. I am indebted to Dr. Bull for his encouragement, his enthusiasm for science and invaluable help. Special thanks also to Shane Boland for her technical expertise, for her collaboration and for being a close friend.

I wish to express gratitude to my graduate committee consisting of Clay Hodgin, Robert Bull, George Padgett and Ronald Cleveland for their support. Thanks also to Debbie Allen and Wendy Caudill for their help in writing the thesis.

Finally, I want to thank Dennis Allen for his help. His time and expertise made it possible for me to finish my thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

In 1948 the lupus erythematosis (LE) cell phenomenon was described as a diagnostic feature of the autoimmune disease systemic lupus erythematosis (SLE). Subsequent investigations demonstrated that the LE cell phenomenon depended on the presence of serum antibodies to deoxyribonucleoprotein (DNP). A variety of antinuclear antibodies (ANA) have since been described in SLE and in other autoimmune diseases including rheumatoid arthritis, progressive systemic sclerosis (scleroderma), Sjorgren`s syndrome, polymyositis and a clincal syndrome called mixed connective tissue disease (MCTD). Antinuclear antibodies were a spontaneously occurring feature of these diseases.

The identification of the nuclear specificity of ANA is of diagnostic and pathogenic significance. Certain ANA are highly selective in distribution, such as anti-Sm and anti-DNP antibodies which occur almost exclusively in SLE. Other ANA, particularly anti-DNP and anti-DNA antibodies, have been implicated in the pathogenesis of certain autoimmune diseases and are thought to be involved in immune complex-mediated glomerulonephritis.

Among the various ANA, antibodies with specificity for DNP have been of particular interest. Anti-DNP antibodies, however, have been difficult to study in

detail. Immunofluorescence assays, while useful in screening sera suspected of containing ANA, have not proven specific or quantifiable enough for accurate identification of anti-DNP antibodies. The heterogeneity of isolated DNP has also made it difficult to employ more sensitive techniques such as radioimmunoassay or enzyme-immunoassay.

In this study we have developed an enzyme-linked immunosorbent assay (ELISA) for the detection and characterization of canine ANA with nucleosome core particle specificity. Nucleosome core particles are a form of highly purified DNP that retains an in situ configuration upon isolation, unlike the DNP used in previous studies. Nucleosome core particles have also been highly characterized using biochemical and biophysical methods. The study of autoimmune disease in dogs has importance in veterinary medicine and provides an opportunity to study the etiology, pathogenesis and clinical features of autoimmune disease in a large nonhuman species.

In order to better understand the results of this study a brief discussion of ANA is given below. This is followed in Chapter II by a more in-depth review of the literature on ANA, DNP, the structure of nucleosome core particles, and related experimental methods.

Antinuclear Antibodies

The extent of our knowledge of ANA has essentially

paralleled the development of assays used to detect ANA. The indirect immunofluorescence assay described by Coons and Kaplan (1950) has been widely used for detecting antinuclear antibodies on tissue sections or cell smears. Reactivities of the test sera upon dilution are used to determine antibody titers with higher titers usually being more clinically significant (Notman et al., 1975). At least four immunofluorescence patterns have been identified and are of some diagnostic importance. The most common patterns included homogeneous, speckled, peripheral and nucleolar (Table 1).

The immunofluorescence assay is an indispensable diagnostic tool, however, the assay has certain limitations. With sera containing antibodies of multiple specificities, interference can occur between antibodies, and at a given dilution one pattern of nuclear staining may predominate (Tan, 1983). It has also become apparent that a large number of nuclear antigens exist, yet there have been only a limited variety of immunofluorescent staining patterns observed. New techniques and assays are allowing a more in depth and quantitative analysis of ANA, as shown in Table 2. This list is not complete since ANA with as yet undetermined specificity have been observed (Tan, 1983).

Antibodies to DNA

Antibodies to DNA have been divided into three types:

Table 1. Immunofluorescence Patterns with associated antigens and diseases.

Patterns	Antigen	Disease
[1,2] Homogeneous	DNP	SLE
[3] Peripheral	Double-Stranded DNA	SLE (high titre) Other autoimmune disease (low titre)
[4] Speckled	Extractable nuclear antigens: Ribonucleo-protein, Sm, Scl 70, SS-B	MCTD, SLE, Sjorgren's syndrome, scleroderma
[3] Nucleolar	4-5s RNA	Scleroderma Sjorgren`s syndrome

[1] Lachman et al. (1961) [2] Friou (1964) [3] Beck (1963)

[4] Tan and Kunkel (1966)

Table 2. Antibodies to Nuclear Antigens.

Antibodies	Disease	Assay
DNA: Double-stranded	SLE	RIA ID CIE HA CF ELISA
Single-stranded & double-stranded	SLE and other autoimmune diseases	RIA ID CIE HA CF ELISA
Single-stranded	Autoimmune diseases	RIA ID CIE HA CF ELISA
DNP	SLE	RIA ID Latex HA LE cell
Histone	SLE	CF IF
Sm	SLE	ID CIE HA CF
RNP: High titre	MCTD	ID CIE HA CF
Low titre	Autoimmune diseases	
Scl-1	Scleroderma	ID
SS-A	Sjorgren`s syndrome low incidence in other autoimmune diseases	ID CIE
SS-B	Sjorgren`s syndrome low incidence in other autioimmune diseases	ID CIE
Nucleolar	Scleroderma Sjorgren`s syndrome	IF

CF = Complement fixation IF = Immunofluorescence

CIE = Counter immunoelectrophoresis

HA = Passive hemagglutination ID = Double immunodiffusion

RIA = Radioimmunoassay ELISA = Enzyme-linked immunosorbent assay

those reacting with native DNA (double-stranded), those reacting with denatured DNA (single-stranded) and those cross-reacting with both double- and single-stranded DNA (Arana and Seligmann 1967).

Antibodies to native DNA are highly specific for SLE and titers are reported to correlate well with disease activity (Davis and Makinen, 1980; Lightfoot and Hughes, 1976). It has been suggested that the antigenic determinants of double-stranded DNA are related to the double helix configuration, since these antibodies do not react with single-stranded DNA (Tan, 1983). Antibodies that cross-react with both single-stranded and double-stranded DNA have been found in fifty to seventy percent of the sera from patients with SLE, but are also present in other autoimmune diseases (Notman et al., 1975). Studies suggest that the antigenic site is the common deoxyribose backbone, since native and denatured RNA do not inhibit the reactions (Arana and Seligmann, 1967). Antibodies to single-stranded DNA have been found in a number of autoimmune diseases and are thought to recognize the exposed purine and pyrimidine bases (Stollar, 1973).

Antibodies to Histones

Antibodies to histones occur in a majority of patients with drug-induced SLE and in a small proportion of patients with idiopathic SLE (Fritzer and Tan, 1978; Fishbein et al., 1979). Drug-induced SLE can occur in patients who

have taken medication including procainamide, isoniazid, nitrofurantoin and hydralazine for diseases unrelated to SLE. Drug induced SLE, in contrast to idiopathic SLE, is characterized by a less heterogeneous ANA population, with antibodies to histone present in 96% of all patients (Fritzer and Tan, 1978). Fritzer and Tan (1978) have shown that the majority of antibodies in drug-induced SLE are directed against histone fractions H2A and H2B, while Stollar (1973) has shown the presence of antibodies to H1, H2A, H2B, H3 and H4 in idiopathic SLE.

Antibodies to Nonhistone Proteins

Nonhistone proteins are defined as the proteins remaining in the nucleus after the five histones have been removed. Nonhistone proteins are involved in the organization of higher order chromatin, synthesis of nucleic acids and the control of gene expression (Lewin, 1980).

The first immunological description of an ANA to a nonhistone protein was by Tan and Kunkel (1966). The antigen was called Sm and had an extremely high selectivity for SLE. Antibodies to nonhistone proteins designated SS-A and SS-B have been identified in Sjorgren`s syndrome. The presence of anti SS-B antibodies is selective for Sjorgren`s syndrome and high titers correlate with disease activity (Alspaugh and Tan, 1976). Antibodies to RNA complexed with nonhistone proteins (RNP) have been

described in a number of autoimmune diseases; however, high titers have been associated with MCTD (Sharp et al., 1972). Other nonhistone nuclear antigens include Scl-1 recognized by ANA in the sera of patients with scleroderma, and PM-1 recognized by ANA in the sera of patients with polymyositis (Tan and Rodnan, 1975; Wolfe et al., 1977).

Antibodies to Nucleolar Antigens

The sera of some patients with scleroderma and Sjorgren`s syndrome contain antibodies specific for nucleoli (Beck, 1962). Few studies however have been performed to characterize the nucleolar reactive sera. Pinnas et al. (1973) has shown that sera staining nucleoli by immunofluorescence reacts with 4-5s RNA and that the reaction is sensitive to spleen phosphodiesterase. Miyawaki and Ritchie (1973) suggest that anti 4-5s RNA is probably only one of several ANA reactive with nucleolar antigens.

CHAPTER II

ANTIBODIES TO DEOXYRIBONUCLEOPROTEIN (DNP)

Antibodies to DNP were first described by Holman and Kunkel (1957) in association with the lupus erythematosus cell phenomenon (LE cell). Lupus erythematosus cells were found to occur in patients with systemic lupus erythematosus (SLE), resulting from the phagocytosis of altered nuclei by polymorphonuclear (PMN) leukocytes (Hargraves et al., 1948). Haserick et al. (1950) demonstrated that nuclei incubated with sera that induced LE cell formation were readily phagocytized by PMN cells due to adsorption of a serum factor displaying the properties of an antibody. The serum factor eluted from the nuclei possessed a sedimentation coefficient equal to the gammaglobulins (7S) and could be quantitatively precipitated by antisera to normal gammaglobulin. Holman and Kunkel (1957) demonstrated that inclusion bodies of LE cells were fluorescent when reacted with fluorescein-conjugated antisera to normal gammaglobulin, whereas the nucleus of the phagocytic cell was not fluorescent. Particulate nucleoprotein, extracted from calf thymus nuclei in 1M NaCl and reprecipitated, was able to adsorb out the ability of sera to induce LE cell formation. Following adsorption, the nucleoprotein was readily phagocytized. The ability of particulate nucleoprotein to extract LE cell factor was

largely removed by the digestion of the nucleoprotein with deoxyribonucleases or by the extraction of histones. The data suggested that the LE cell serum factor had an affinity for nucleoprotein and that DNA and histones were involved in the reaction (Holman and Diecher, 1959).

Friou (1957), using a fluorescent antibody technique, demonstrated that synthetic nucleoprotein complexes would also react with the LE cell factor. The synthetic nucleoprotein was a combination of purified DNA and histone resulting in a complex free of much of the residual nonhistone proteins invariably present in particulate nucleoprotein preparations.

Tan (1967) isolated DNP in soluble form (soluble nucleoprotein, sNP) and demonstrated by enzyme degradation, ultracentrifugation and immunologic analysis that sNP was a complex of DNA and a moiety susceptible to proteolytic digestion. The soluble antigen reacted with SLE sera by double immunodiffusion precipitation, and immunologic identity was obtained between sNP and synthetic DNA-histone complexes. The sNP precipitation reaction was lost by prior digestion of the sNP with DNase I or trypsin. Soluble nucleoprotein preparations were not composed entirely of DNA and histone, but were shown to contain material antigenically related to DNA and at least one other unidentified antigen when reacted with SLE sera containing antibodies with multiple specificities. The serum factor precipitated by sNP migrated in the gammaglobulin region by

immunoelectrophoretic analysis and by sedimentation studies. The serum factor was also shown to be identical to IgG by double immunodiffusion reactions.

Tan (1967) demonstrated antibodies to sNP in 51% of 65 SLE sera tested by the double immunodiffusion technique. Antibodies to sNP were shown in these studies to be more common than antibodies to DNA in SLE sera. Antibodies to sNP were not found in 92% of sera from patients with rheumatoid arthritis, dermatomyositis, polymyositis, scleroderma, autoimmune hemolytic anemia and Sjorgren`s syndome, thereby demonstrating the selectivity of anti-sNP antibodies for SLE.

Stollar (1967) examined an SLE serum which reacted with synthetic DNA-histone complexes and with sNP. The relative contribution of DNA and histone in the formation of the antigenic determinants was studied using a complement fixation assay. Complement fixation was carried out with reactive DNA-histone complexes in the presence of excess DNA or excess histone. With excess histone, the complement fixation curve was displaced to higher antigen concentrations, but with excess DNA complement fixation was completely inhibited. Based upon these experiments, DNA appeared to be the major contributor to the antigenic determinants.

A radioimmunoassay for the detection of anti-sNP antibodies was developed by Robitaille and Tan (1973) using I^{125} labeled sNP. Antibody-sNP complexes were precipitated

by ammonium sulfate (Farr assay) and radioactivity counted. Unlabeled DNA was included in the assay to inhibit the reaction of anti-DNA antibodies to free DNA contaminating the sNP preparations. Significant binding values were observed almost exclusively with sera from patients with SLE (21/36 or 58%). Nearly all the positive sera also contained antibodies to double stranded DNA (18/21 or 86%). In previous studies there was a good correlation between the presence of anti-double-stranded DNA antibodies and disease activity, suggesting to Robitaille and Tan that such a correlation might exist for antibodies to sNP. Soluble nucleoprotein has also been eluted from the glomeruli of several patients with SLE nephritis (Koffler et al., 1967; Krishman and Kaplan, 1967).

Rekig and Hannestad (1979) described antibodies that were able to cross-react with polynucleosome preparations and with the plasma membrane of human leukocytes. The cross-reacting antibodies were partially purified by adsorption to and elution from leukocytes in a glycine/HCl buffer. A radioimmunoassay to detect anti-nucleosome antibodies was developed using preparations of polynucleosomes as the solid phase. Immunofluorescence and radioimmunoassay studies revealed that the ANA activity of the leukocyte eluates was inhibitied by mononucleosomes, but not by high molecular weight double-stranded DNA or histones. Using the radioimmunoassay previously described, Rekig and Hannestad (1980) established that cross-reacting

antibodies recognized an epitope formed when the core histones associated with the nucleosomes interacted in the absence of double-stranded DNA. This reactivity was lost in 0.15M salt unless the histones were allowed to interact with double-stranded DNA to generate the nucleosome structure. Activity of the antibody was also lost when nucleosomes were subjected to trypsin digestion, which removed the NH_2-terminal end of each core histone, suggesting that the location was confined to the N-terminal region. Rekig and Hannestad (1980) showed that nucleosomes adsorbed LE cell factor, but that LE cell factor did not bind to leukocyte membranes. This suggested that the cross-reacting antibodies described by Rekig and Hannestad and the LE cell factor differed in immunologic specificity.

NUCLEOSOMES

Chromatin Subunits

Chromatin was once thought to be duplex DNA surrounded extensively by proteins and generally not accessible to macromolecules. Hewish and Burgoyne (1973) were the first to propose that chromatin was organized into a simple basic repeating substructure. When chromatin was digested with an endogenous endonuclease it was possible to isolate a series of subunits of a basic size. This phenomenon could not be repeated by the digestion of double-stranded DNA. Noll (1974) presented evidence that chromatin could also be

converted to subunits by digestion with micrococcal nuclease (an endonuclease endogenous to Staphyococcus). The degradation of chromatin into multimeric subunits by micrococcal nuclease and endogenous endonuclease implied that this was a feature of chromatin structure and not a characteristic of a particular enzyme. Noll showed that when double-stranded DNA isolated from the digested chromatin was electrophoresed through acrylamide gel a series of bands appeared. These bands were composed of double-stranded DNA from the subunit monomers, dimers, trimers, etc. Digestion was monitored by the conversion of DNA to acid-soluble nucleotides consisting of the DNA degradation products. When 13% of the DNA was converted to acid-soluble nucleotides upon digestion, all the DNA applied to the acrylamide gels entered in the form of monomers or multiples of monomers. This implied that 87% of the DNA was in a subunit form and not accessible to nuclease digestion. The basic subunit or monomer contained about 205 base pairs of DNA and had a sedimentation coefficient of 11.2s. In an electron microscopic study, chromatin fibers were visualized by Olins and Olins (1974) as "particles on a string". The chromatin fibers were composed of spherical particles (termed v-bodies) ranging from 60 to 80 angstroms in diameter connected by a thin filament 15 angstroms in diameter. Oudet et al. (1975) described particles identical to the v-bodies for which they coined the term, nucleosome.

Histone Octomer

Wilkens and co-workers revealed a structure repeating at intervals of about 100 angstroms along the chromatin fibers by X-ray diffraction (Wilkens et al., 1959). A repeating structure similar to the X-ray patterns were observed when tetramers of histones H3 and H4 and oligomers of histones H2A and H2B were added to DNA. Histone H1 was not required for formation of the repeating unit (Kornberg, 1974). From the preceding work, Kornberg (1974) accurately proposed a structural model of chromatin with repeating units of an octomer of histones (two each of the four histones H3, H4, H2A and H2B) containing about 200 base pairs of DNA. The model proposed by Kornberg suggested that chromatin subunits contained equal amounts of the histones H3, H4, H2A and H2B. Thomas and Kornberg (1975) described experiments in which the amino groups of adjacent chromosomal histones were cross-linked by dimethyl suberimidate. Isolation of the histones and electrophoretic separation of cross-linked products produced protein bands with a regular progression of spacing and intensity. Since all four histones differed considerably in molecular weight, a random mixture of all possible cross-linked products would have given a complex pattern of bands or a smear. Analytical data presented by Olins et al. (1976) indicated that nucleosomes consisted of equimolar amounts of two each of the four histones H3, H4, H2A and H2B. The total molecular weight of the subunit proteins was calculated

to be 116,000 - 122,000 daltons, about twice the sum of the combined molecular weights of H3, H4, H2A and H2B (55,400 daltons). Compelling evidence for the histone octomer was presented by Sollner-Webb et al. (1976). Particles reconstructed from DNA and a large number of histone combinations were subjected to five enzymatic probes (DNase I, DNase II, micrococcal nuclease, trypsin and chymotrypsin). Only the combination of histones H3, H4, H2A and H2B resulted in digestion products identical to those of native chromatin.

Core Particles

Nucleosome monomers released from nuclei by brief digestion with micrococcal nuclease contained about 200 base pairs of DNA; however, only about 140 base pairs of DNA are actually associated with the histone core. The nucleosome monomers are thought to be derived from chromatin by a single enzymatic clip somewhere in the remaining 60 base pairs of DNA. As digestion proceeds, the DNA ends of the nucleosome monomers are trimmed to 140 base pairs of DNA and are known as "core particles". The sixty base pairs of DNA between the nucleosome core particles are known as the linker region (Lewin, 1980).

The digestion of monomers to core particles has been shown to proceed in a stepwise manner. Micrococcal nuclease initially cleaves the linker DNA between nucleosome core particles and then rapidly removes the newly generated

DNA ends before being substantially slowed by the histone octomer (Sollner-Webb and Felsenfield, 1975). Unlike nucleosome monomers, in which the base pairs of DNA have ranged from 154 in Aspergillus (Morris, 1976) to 241 in sea urchin sperm (Spadafora et al., 1976), nucleosome core particles consistantly contained 140 base pairs of DNA. Nucleosome core particles with 140 base pairs of DNA have been isolated from calf thymus (VanHolde et al., 1977), duck reticulocytes (Sollner-Webb and Felsenfield, 1975), chicken erythrocytes (VanHolde, 1977; Shaw et al., 1976), pea seedlings (McGhee and Engle, 1975) and yeast (Lohr and VanHolde, 1975). The physical properties of core particles have been determined by Olins et al. (1976): $E^{1\%}_{260nm}$ = 93.12 $\pm$ 0.52, $S^{o}_{20,w}$ = 11.41 $\pm$ 0.31, M_w (S,D) = 209,547 $\pm$ 9,634 and the protein/DNA ratio (wt/wt) = 1.24-1.30.

Organization of Nucleosomal DNA

Noll (1974) first presented evidence for the organization of nucleosomal DNA by digesting rat liver nuclei with DNase I. When the isolated double-stranded DNA was electrophoretically separated on gels, poorly resolved bands occurred representing multiples of 200 base pairs of DNA. By analyzing the DNA under denaturing conditions and separating the single-stranded DNA by gel electrophoresis, a series of distinct bands with chain lengths corresponding to multiples of ten base pairs were identified. Sollner-Webb et al. (1976) demonstrated that isolated nucleosome core

particles, monomers and dimers digested with DNase I produced bands by single stranded DNA gel electrophoresis that were indistinquishable from those produced by DNase I digestion of nuclei and chromatin. The same periodicity was shown to be generated by DNase II (Alterburger et al., 1976) and by extensive micrococcal nuclease digestion (Sollner-Webb et al., 1976).

Two basic models have been presented to explain the ten base periodicity. Noll (1974) proposed that the sites of cleavage were reflected in the pitch of the DNA helix wound smoothly around the histone octomer. Theoretical calculations have shown this to be possible (Levitt, 1978; Sussman and Trifonor, 1978). In the second model Crick and Klug (1975) suggested that the DNA was not wound smoothly around the histone octomer, but consisted of relatively straight stretches joined by large kinks. Crick and Klug had difficulty in estimating how much energy was required to wind DNA smoothly around such a small unit as the histone octomer; however, they formed the impression that the energy needed would be high as opposed to their kinked structure.

EXPERIMENTAL TECHNIQUES

The majority of experimental techniques described in the materials and methods are well established procedures and require little introduction. The enzyme-linked

immunosorbent assay (ELISA) is a relatively new technique and an essential component of this work; therefore, a brief review of ELISA procedures is presented below.

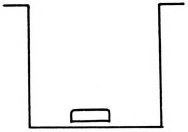
ELISA

A schematic of the enzyme-linked immunosorbent assay (ELISA) used in this study is shown in Figure 1. Nucleosome core particles were attached to wells of a microtiter plate (the solid phase) which was then washed. The diluted test serum was added, incubated and the wells washed. Alkaline phosphatase conjugated anti-globulin was added and allowed to react, then washing was repeated and the enzyme substrate added. The amount and rate of degradation of the substrate was related to the amount of anti-nucleosome core particle antibody present in the serum. In some of the ELISA experiments, reagents were added to the serum to inhibit antibody binding to the nucleosome core particles. Degradation of the substrate was then inversely proportional to the amount of inhibition.

Many of the principles for the ELISA were first developed for the radioimmunoassay. Catt and Tregear (1967) demonstrated that polystyrene tubes had the property of irreversibly binding proteins including antibodies and that adsorbed antibodies retained activity for antigen. Binding of various antigens to polystyrene was also demonstrated by Catt and Tregear (1967) and allowed measurement of antibody binding to antigen. The ELISA was

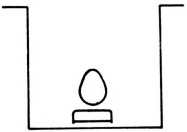
Figure 1. Schematic diagram of the enzyme-linked immunosorbent assay (ELISA) for the detection of serum antibodies with nucleosome core particle specificity.

Antigen adsorbed to plate



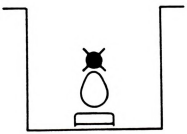
Wash

Add serum (specific antibodies attach)



Wash

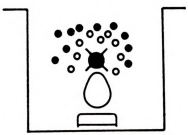
Add enzyme - labelled antiglobulin



Wash

Add substrate

Amount hydrolized = amount of antibody



made possible by the ability to couple enzymes to other proteins. Avraneas (1969) demonstrated that peroxidase or alkaline phosphatase could be coupled to sheep immunoglobulin G, producing conjugates that retained a substantial part of their immunological and enzymatic activity. This led to the development of the ELISA by Engvall and Perlmann (1971) to measure rabbit IgG. Alkaline phosphatase-conjugated sheep anti-rabbit IgG was used to measure rabbit IgG bound to polystyrene tubes. The sensitivity and precision was fully comparable to that of the radioimmunoassay (Johansson et al., 1968). Rabbit IgG could be detected at concentrations of lng/ml using the ELISA. The stability of the enzyme-conjugated immunoglobulins was shown to exceed one year.

The microplate method was introduced by Voller et al. (1974) in an ELISA for the measurement of antibodies to malaria. The wells of the microtiter plate were coated with malarial antigen. Test sera were incubated in the wells and bound antibody detected by alkaline phosphatase-conjugated anti-human IgG. This method was found to be simple, inexpensive and easy to carry out on a large scale or in the field.

The ELISA has not been extensively adapted to the study of autoimmune diseases. The major application of the ELISA in the study of ANA has been in the detection of antibodies to DNA. An ELISA measuring the amount of antibody to double-stranded DNA in patients with SLE was developed by

Pesce et al. (1974). The assay consisted of adsorbing excess double-stranded DNA to polystyrene tubes and incubating with test sera, followed by anti-human IgG conjugated to horseradish peroxidase. The conversion of product was measured by a spectrophotometer. The method had considerable variability, but offered a new approach to the measurement of antibody in autoimmune disease. Engvall later (1976) demonstrated that polystyrene was a poor binder of double stranded DNA. Klotz et al. (1979) precoated polystyrene tubes with protamine sulfate followed by the adsorption of double stranded DNA. The ELISA demonstrated better reproducibility and correlated well with the glass microfiber filter assay using tritiated DNA as described by Lewis et al. (1973).

The full potential of the ELISA for ANA or other autoantibodies has not been fully exploited (Voller et al., 1979); however, many autoimmune-related assays have been developed. Leinonen et al. (1978) developed an ELISA to measure antibodies to histones. An assay was developed by Voller et al. (1979) to detect antibodies to thyroglobulin and obtained good correlation with passive agglutination tests. The measurement of immune complexes by ELISA was described by Ahlstedt et al. (1976) and the measurement of rheumatoid factor has been described by Willems and Klaassen ed Kort (1978) and Maiolini (1979).

CHAPTER III

MATERIALS AND METHODS

Isolation of Nucleosome Core Particles

The isolation and characterization of nucleosome core particles was performed by methods described by Rill et al. (1977) with only slight modification.

Preparation of Nuclei

Fifteen to thirty grams of frozen calf thymus (Pel-Freeze) was finely minced into 40ml of ice cold TKMC buffer (pH 7.5) consisting of 0.05M tris-HCl, 25mM KCl, 5mM $CaCl_2$ and 1mM phenylmethylsulfonyl fluoride (PMSF). Phenylmethylsulfonyl fluoride was added immediately before use from a 100mM stock solution dissolved in isopropanol (PMSF is an inhibitor of serine proteases and is slowly hydrolyzed by water). The minced calf thymus was homogenized at low speed in a Sorvall "Omini Mixer" for 7 min; pausing to remove trapped tissue from the rotor after 2 min and 5 min. Homogenization and all subsequent steps were carried out at 4°C. Tissue homogenates were then filtered through 4 layers of rinsed gauze followed by filtration through 2 layers of rinsed Miracloth (Chicopee Mills Inc.).

Nuclei were isolated from cellular debris by sedimentation through sucrose. Two volumes of 2.3M sucrose in TKMC buffer also containing 0.75% triton X-100 and 0.1mM

PMSF were added to 1 volume of homogenate. This mixture was layered over 1 volume of 2.3M sucrose in TMKC buffer containing 0.1mM PMSF and centrifuged at 18,000rpm for 20 min in an SW-27 rotor at 4°C. The nuclear pellet was then removed and resuspended in micrococcal nuclease digestion buffer consisting of 0.25M sucrose, 15mM NaCl, 10mM $MgCl_2$, 0.1mM PMSF (adjusted to pH 6.5 with solid cacodylic acid) or dispersed in 0.25M sucrose in TKMC buffer containing 25% glycerol and stored at -80°C. Nuclear fractions were examined for purity by staining with 0.2% crystal violet in 0.25M sucrose. Cells resistant to the immediate penetration of the dye were assumed to be intact whole cells. The concentration of nuclei was determined by disrupting a 0.1ml aliquot of nuclei in 9.9ml of 2.2M NaCl, 5.5M urea using a Potter homogenizer and measuring the absorbance of the homogenate at 260nm (1 absorbance unit at A_{260nm} equals approximately 50ug of DNA).

Micrococcal Nuclease Digestion

Nuclei that had been resuspended directly in micrococcal nuclease digestion buffer or stored at -80°C were washed twice in digestion buffer and incubated with 2 to 10 units of micrococcal nuclease (Worthington) for each A_{260nm} absorbance unit of nuclei. Routinely, 220 A_{260nm} absorbance units of nuclei were used per digestion. Digestions were terminated after various lengths of time by the addition of 1/10 volume of 0.15M EDTA in distilled water

and then dialyzed for 2 to 3 hr at 4°C (with vigorous stirring) against 2 changes of 10mM tris, 1mM EDTA and 0.1mM PMSF (pH 7.5). Particulate matter was removed from the solution by centrifugation at 12,000rpm for 10 min at 4°C in a Ti-57 rotor. Digestions were monitored by the production of acid soluble nucleotides and by double-stranded DNA gel electrophoresis.

Sucrose Gradient Centrifugation

The isolation of nucleosome core particles from the micrococcal nuclease digestions was accomplished by centrifugation through 5-20% linear sucrose gradients. Two milliliters of nuclease digest with a maximum of 220 A_{260nm} absorbance units were layered on 40ml gradients and centrifuged at 25,000rpm for 16-24 hr at 4°C in an SW-27 rotor. Nucleosome core particle sedimentation distance was determined by concurrently running catalase in phosphate buffered saline (catalase sediments at a distance equal to nucleosome core particles). The fraction containing the nucleosome core particles was collected and the nucleosome core particles were precipitated by the addition of 10mM $MgCl_2$ followed by the addition of 2 volumes of 95% ethanol. Histone H1 was removed by this procedure. The precipitate was resuspended in storage buffer consisting of 10mM tris-HCl and 0.1mM EDTA (pH 7.5) and dialyzed for 2 days against 2 liters of storage buffer containing 3mM NaN_3 with 2 changes of buffer. The nucleosome core particle

preparation was then further purified by a second identical sedimentation process. The quantity of nucleosome core particles recovered was determined from the extinction coefficient ($E^{1\%}_{260nm}$ = 93.12) and the purity was determined by double-stranded DNA gel electrophoresis.

DNase I Digestion

Nucleosome core particles were washed twice in DNase I digestion buffer consisting of 0.01M tris-HCl, 0.01M NaCl and 0.003M $MgCl_2$ (pH 7.9) and incubated with 10 units of DNase I (Type 1, Sigma Chemical Co.) for each A_{260nm} absorbance unit of nuclei. Digestions were terminated after various periods of time by the addition of 1/10 volume of 0.15M EDTA in distilled water and dialyzed for 2 to 3 hr (with vigorous stirring) against 2 changes of 2 liters of 10mM tris-HCl (pH 7.5), 1mM EDTA and 0.1mM PMSF. Particulate matter was removed from the solution by centrifugation at 12,000rpm for 10 min at 4°C in a Ti-57 rotor. Digestions were monitored by the production of acid soluble nucleotides and by single-stranded DNA gel electrophoresis.

Determination of Acid Soluble Oligonucleotides

Samples from micrococcal nuclease and DNase I digestions were added to an equal volume of 10mM EDTA (in distilled water) in an ice bath. After all samples had been prepared, 8 sample volumes of ice-cold 0.5M perchloric acid

containing 0.5M NaCl were added to each tube. The tubes were shaken for 1 hr at 5°C and then centrifuged at 4,500rpm for 15 min at 4°C in an IEC HN-SII rotor. Supernatants were heated for 30 min at 70°C, cooled to room temperature and absorbance measured at 260nm. The pellets were resuspended in 2 to 10ml of 2% sodium dodecyl sulfate (SDS) in 50mM tris (pH 7.5) and heated for 1 hr at 70°C. An equal volume of 20% $HClO_4$ containing 2M NaCl was then added and the samples were heated for an additional 30 min at 70°C. All solutions were processed simultaneously since sucrose and perchloric acid react at high temperatures to give reaction products that absorb at 260nm. The percentage of acid-soluble material was calculated by the following equation.

$$\frac{\text{Supernatant } (A_{260nm})}{\text{Supernatant } (A_{260nm}) + \text{Pellet } (A_{260nm})} \times 100$$

DNA Isolation

Samples were dispersed in a buffer with a final concentration of 0.15mM tris base (pH 8.0), 0.2M NaCl, 10mM EDTA and 2% SDS. Protease K (Sigma Chemical Co.) was added to a concentration of 10ug/100ug of DNA and samples were incubated for 2 hr at 37°C. Protease digestion was stopped by mixing the sample for 10 min with an equal volume of

distilled phenol that had been saturated with the above buffer minus SDS. This was followed by the addition of 1 sample volume of chloroform for an additional 5 min with mixing. After brief centrifugation to facilitate phase separation, the aqueous upper layer was removed and the extraction repeated. DNA was precipitated from the aqueous layer by the addition of 2 volumes of ice-cold ethanol with overnight incubation at 4°C. DNA was collected by centrifuging the sample at 4000 x g for 20 min at 4°C, washing twice in 70% ethanol and drying under vacuum.

Double-Stranded DNA Gel Electrophoresis

Electrophoresis of double stranded DNA was performed as described by Maniatis et al. (1975) on a gel apparatus similar to one developed by Dewachter and Fiers (1971). The DNA was examined on vertical slab gels (40cm x 20cm x 0.4cm) containing 2.5% acrylamide supplemented with 0.5% agarose. Gels were prepared by the addition of 17.5ml of acrylamide solution (14.29gm acrylamide, 0.714gm N,N^1-methylene-bis-acrylamide) to 33ml of 10X TBM buffer (0.09 tris-borate, pH 8.3, 5mM $MgCl_2$, containing 1.6ml N,N,N^1,N^1-tetramethylethylenediamine per liter). The solution was heated to 50°C and 49.5ml of 1% agarose and 0.5ml of freshly prepared 10% ammonium sulfate (polymerizing agent) were added, mixed and the gel was poured. Isolated DNA was resuspended in 10X TBM buffer containing 5% glycerol, 0.025% xylene cyanol FF (Bio Rad Laboratory) and

0.025% bromphenol blue (Sigma Chemical Co.). Twenty-five microliter aliquots of DNA were applied and electrophoresed at room temperature at a constant voltage of 200V (10V/cm). Gels were stained for 30 min with 10ug/ml of ethidium bromide. Double-stranded DNA from nucleosome core particles co-migrated with bromphenol blue.

Single-Stranded DNA Gel Electrophoresis

Electrophoresis of single-stranded DNA was performed as described above except for the substitution of 12% acrylamide TBM gels containing 7M urea. The gels were prepared by mixing 31.5g of urea, 7.5ml of 10X TBM buffer, 30ml of 30% acrylamide, 3ml of 1.6% ammonium persulfate and enough water to bring the final volume to 75ml. The solution was degassed and polymerized by the addition of 25ul of N,N,N^1,N^1-tetramethylethylenediamine. The smallest DNA fragments from DNase-I digested nucleosome core particles co-migrated with bromphenol blue.

Serum Samples

The study consisted of 9 canine serum samples with ANA that had been submitted to the Clinical Immunology section of the Animal Health Diagnostic Laboratory, Michigan State University. The presence of ANA was assayed by indirect immunofluorscence (described below). Negative control samples consisted of sera from healthy laboratory dogs determined to be negative by the ANA immunofluorescence

assay. The positive-negative threshold for the ELISA was determined by 75 serum samples that had been submitted to the Clinical Immunology section and determined to negative by the ANA immunofluorscence assay.

Immunofluorescence

Indirect immunofluorescence used in ANA assays, pattern determinations and end point titrations were performed on frozen rat liver sections commercially available from Zeus Products Inc. Slides were allowed to equilibrate to room temperature, placed in a moist chamber and incubated for 30 min with sera. All assays included a reference positive and negative serum sample. Serum samples were routinely screened for ANA at a dilution of 1/20 in phosphate buffered saline (PBS), pH 7.2. After incubation, samples were washed twice in PBS for 15 min each time and incubated for 30 min in a moist chamber with fluorescein-labeled rabbit anti-dog IgG (Miles Laboratories). Slides were washed twice in PBS for 15 min each time and stained for 1 min with a solution of Evans blue (0.1%). Slides were then rinsed in PBS and mounted in a solution containing 10% PBS and 90% glycerol. Immunofluorescent staining was examined with a transmitted light microscope (Zeiss Photomicroscope III) using a 12 volt Halogen light source, FITC interference filter (maximum transmission 485 - 495nm), BG - 38 red suppressor filter (maximum transmission 650nm) and a 530nm LP barrier filter.

Immunoglobulin - Enzyme Conjugation

Immunoglobulin - enzyme conjugates were prepared by the method of Voller et al., (1976). The IgG fraction of rabbit anti-dog IgG (Cappel Laboratories) was conjugated to bovine intestinal alkaline phosphatase Type VII-S (Sigma Chemical Co.). Alkaline phosphatase (5mg) was dissolved in 1ml of 0.05M PBS (pH 7.5) containing 2mg of immunoglobulin and dialyzed overnight at 4°C against 2 liters of PBS with 2 changes of buffer. Glutaraldehyde (25%) was then added to the solution to give a final glutaraldehyde concentration of 0.2% (v/v) and incubated for 2 hr at room temperature. The solution was dialyzed overnight at 4°C against 2 liters of PBS with 2 changes of buffer. The dialysis bag was transferred to 2 liters of 0.05M Tris base (pH 8.0) containing 1mM $MgCl_2$ and dialyzed overnight at 4°C with 2 changes of buffer. The mixture was then chromatographed on a column of sephadex G-200 (Pharmacia Fine Chemicals) in 0.05M Tris base (pH 8.0) containing 1mM $MgCl_2$. The first 280nm peak was collected as conjugate. Bovine serum albumin (1% w/v) and sodium azide (0.02% w/v) were added for stabilization and the conjugate was stored in the dark at 4°C.

ELISA

Disposable 96-well microtiter plates (Dynatech Laboratory Inc.) were coated with isolated nucleosome core particles prepared as described above. The nucleosome core

particles were diluted in carbonate-bicarbonate buffer and 200ul aliquots were incubated in each well overnight at 4°C. The carbonate-bicarbonate buffer (9.6) consisted of 1.59gm of Na_2CO_3, 2.93gm of $NaHCO_3$, 0.2gm of NaN_3 in 1 liter of distilled water and was stored at room temperature for not more than 2 weeks. The wells were washed 3 times in PBS-Tween consisting of 8gm of NaCl, 0.2gm of KCl, 0.5ml of Tween 20 and 0.2gm of NaN_3 in 1 liter of distilled water (pH 7.4). The wells were incubated with 200ul of sera (in duplicate) diluted in PBS-Tween for 2 hr at room temperature and washed 3 times in PBS-Tween. The wells were then incubated with 200ul of conjugate (diluted in PBS-Tween) for 3 hr at room temperature and washed 3 times with PBS-Tween. The amount of enzyme bound to the wells was determined by adding 200ul of 1mg/ml solution of p-nitrophenyl phosphate (substrate for alkaline phosphate) in substrate buffer consisting of 97ml of diethanolamine, 800ml of distilled water, 0.2gm of NaN_3, 100mg of $MgCl_2 \cdot 6H_2O$. The pH was adjusted to 9.8 with 1N HCl and distilled water was added to bring the volume to 1 liter. The plates were incubated for 30 min at room temperature and the reaction was stopped by the addition of 50ul of 3M NaOH in distilled water.
The absorbance of each well was read at 405nm on a Gilford spectrophotometer. Substrate solution containing 50ul of 3M NaOH was used as the blank.

Inhibition Assays

Inhibition studies using the ELISA were performed as described above, but either RNA (Type IV, Sigma Chemical Co.), DNA (Type I, Sigma Chemical Co.) treated as described under DNA isolation, or DNase I-treated nucleosome core particles was mixed with the sera immediately before incubation with the nucleosome core particle-coated wells. The difference in absorbance between treated and untreated sera indicated the amount of inhibition.

Statistical Methods

The within-run reproducibility of the ELISA was estimated by making 2 determinations on 14 different serum samples. The coefficient of variation was calculated using the following equation:

$$\sqrt{(\Sigma d^2)/2n}$$

The term d represented the percentage difference between duplicates and n the number of terminations. The between-run reproducibility of the ELISA was estimated using a reference serum sample run on different days. The coefficient of variation was calculated by the following equation:

$$\sqrt{[\Sigma (x-\bar{x})2]/(n-1)}$$

The term x represented the individual values, $\bar{x}$ the mean value and n the number of determinations.

CHAPTER IV

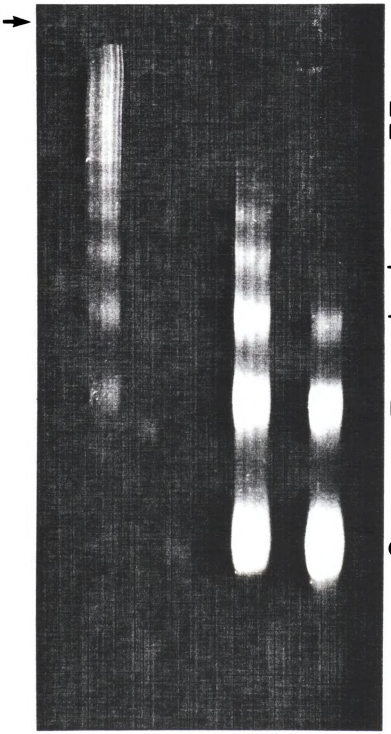
RESULTS

Isolation of Nucleosome Core Particles

Nuclei isolated from calf thymus were used in the preparation of nucleosome core particles. The isolation procedure based on the method described by Rill et al. (1977) yeilded approximately 30 $A_{260}{}^{nm}$ absorbance units of nuclear material for each gram of calf thymus. Only preparations of nuclei containing less than 10% whole cells were used.

Nucleosome core particles were released from nuclei by digestion with micrococcal nuclease. The time course of nuclease digestion was monitored by the release of acid-soluble nucleotides and by electrophoresis of DNA on polyacrylamide gels. The lengths of DNA products of micrococcal nuclease digestions corresponded to nucleosome core particles, dimers, trimers, etc., as illustrated in figure 2 a, c and d. The percentage of acid-soluble nucleotides measured for different digestion times shown in figure 2 a, c and d were as follows: lane a, 8 percent; lane c, 18 percent; lane d, 30 percent. Analysis by gel electrophoresis (figure 2) revealed that a substantial portion of the chromatin had been enzymatically released as nucleosome core particles in the 18% acid-soluble digest and was not contaminated with minor subnucleosomal DNA bands

Figure 2. Gel electrophoresis of double-stranded DNA fragments produced by micrococcal nuclease digestion of calf thymus nuclei. a, Nuclei digested for 15 min. b, Purified nucleosome core particles. c, Nuclei digested for 30 min. d, Nuclei digested for 60 min. Arrow indicates origin of gel.


Nucleosome
Repeat Lengths
Tetramer
Trimer
Dimer
Core
Particle
a
b
c
d

which were present in the 30% acid-soluble digest. Digestions corresponding to the release of 18% of the DNA as acid-soluble nucleotides were used in the preparation of nucleosome core particles for the ELISA.

Nucleosome core particles were isolated from the dimers, trimers, etc. using linear sucrose gradients. The elution patterns of nucleosome core particles and catalase after centrifugation are shown in Figure 3. Catalase marks the distance where nucleosome core particles should sediment under these conditions (catalase and nucleosomes core particles have identical sedimentation coefficients of 11.2S). As shown in figure 3, catalase and nucleosome core particles sedimented to the same position during centrifugation. Nucleosome core particles were further purified by centrifugation through a second linear sucrose gradient (figure 4). The second purification step resulted in a nearly homogeneous preparation as demonstrated by DNA gel electrophoresis (figure 2, line b).

DNase I Digestion

The time course of digestion of the nucleosome core particles with DNase I is shown in Figure 5. DNase I digestion products were used in inhibition studies described below and also provided further data on the integrity of the nucleosome core particles used in this study. The results corresponded favorably with data published by Noll (1974) and Sollner-Webb (1976) and clearly demonstrated the

Figure 3. Sucrose gradient fractionation of nucleosome core particles from nuclei digested with micrococcal nuclease. C Catalase, NCP Nucleosome Core Particles.

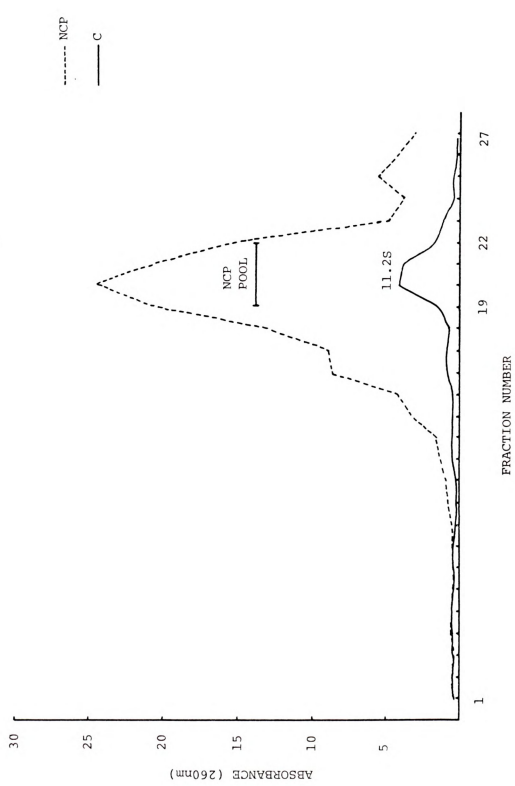

NCP
C
NCP POOL
11.2S
ABSORBANCE (260nm)
30
25
20
15
10
5
1
19
22
27
FRACTION NUMBER

Figure 4. Resedimentation of nucleosome core particles by centrifugation on sucrose gradients. NCP Nucleosome Core Particles.

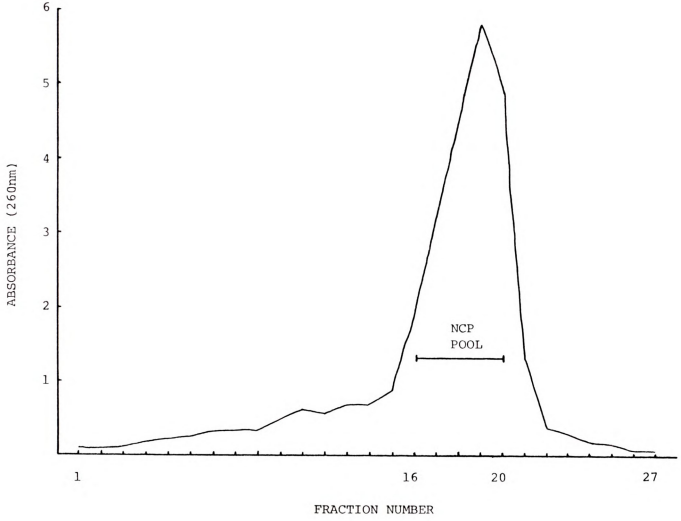
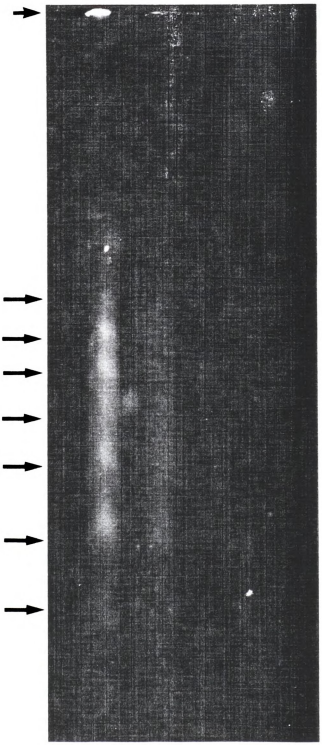

6
5
4
3
2
1
ABSORBANCE (260nm)
NCP
POOL
1
16
20
27
FRACTION NUMBER

Figure 5. Gel electrophoresis of single-stranded DNA fragments produced by DNase I digestion of nucleosome core particles. Digestion times: a, 5 min; b, 15 min; c, 30 min; d, 60 min. Large arrows indicate ten base pair spacing of digestion products. Small arrow indicates origin of gel.


a
b
c
d

The time course of digestion was monitored by the release of acid-soluble nucleotides and by DNA gel electrophoresis. The precentage of acid-soluble nucleotides measured for different digestion times shown in figure 5 a, b, c and d were as follows: lane a, 28 percent; lane b, 56 percent; lane c, 78 percent; lane d, 82 percent. The ten base periodicity was easily observed in lane b, corresponding to the release of 56 percent of the DNA as acid-soluble nucleotides, without the residual DNA observed in the loading well of lane a. Nucleosome core particles digested to approximately 56 percent acid-soluble nucleotides were used in this study.

ELISA

The ELISA was developed using sera A and B as strong and moderate ANA, respectively. It was assumed that sera A and B would react with nucleosome core particles based on reports associating homogeneous immunofluorescence staining patterns with anti-DNP antibodies. This assumption was substantiated by the
ELISA.

Checkerboard titrations were performed to determine optimum reagent concentrations for the detection of antibodies to nucleosome core particles by the ELISA. A 1:50 dilution of conjugate was used in the titrations which corresponded to a 1:500 dilution of prechromatographed conjugate. Based on the results shown in Table 3, a

Table 3. Results of checkerboard titrations using nucleosome core particles and reference sera (O.D. 405nm).

Nucleosome Core Particles	Serum Dilutions $(A)^{-1}$				Serum Dilutions $(B)^{-1}$				Serum Dilutions $(C)^{-1}$			
ug/ml	100	200	400	800	100	200	400	800	100	200	400	800
20	2.79	2.085	1.740	0.997	1.485	1.380	1.278	1.184	0.342	0.310	0.385	0.981
10	1.57	1.467	1.459	0.970	1.813	1.670	1.443	0.993	0.256	0.114	0.274	0.322
1	1.067	0.740	0.648	0.231	1.419	1.320	1.220	1.115	0.156	0.250	0.856	1.540

nucleosome core particle concentration of 10ug/ml and a test serum dilution of 1/200 was selected for the ELISA. This combination of reagents a) resulted in ELISA values that gave a good distinction between the positive and negative reference sera (without exceeding the limit of resolution of the spectrophotometer of 2.0 absorbance units), b) was economical with antigen and c) produce a good substrate reaction within a reasonable amount of time. The ratio of absorbance readings of sera A and B to the negative reference sera was approximately 13:1 and 15:1, respectively. Ratios of 5:1 to 10:1 are considered excellent in this type of assay when comparing a highly positive sample to that of a negative (Voller et al., 1979). The absorbance value of the negative reference serum was the lowest at the 1:200 dilution.

A positive-negative threshold of 0.400 was selected for the ELISA. The threshold was determined by testing the sera of 75 dogs judged negative by the ANA immunofluorescence assay and by setting the value at the upper limit of these negative samples (Figure 6).

The sera positive by the indirect immunofluorescence for ANA assay was categorized by their staining patterns and end point titrations (Table 4). Nuclear staining patterns are shown in figure 7. Sera A and B stained with a homogeneous pattern, sera C, D and I produced speckled patterns, serum H stained with an unusual large-grained speckled pattern, sera F and G gave peripheral staining

Figure 6. Distribution of ELISA values for a control population of 75 dogs screened negative by the ANA immunofluorescence assay.

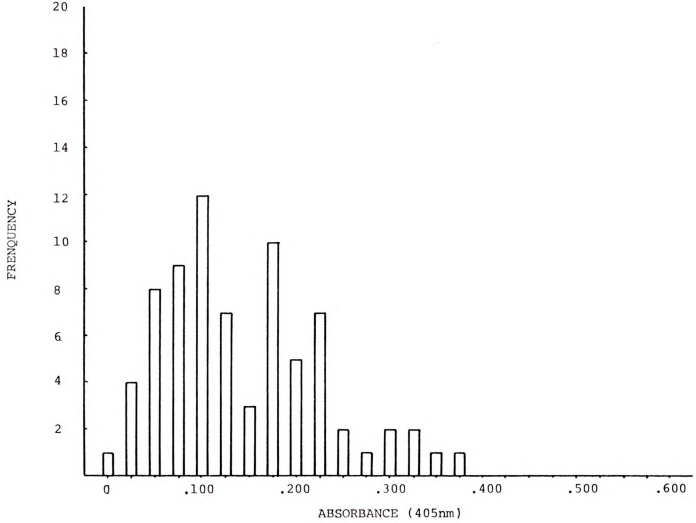
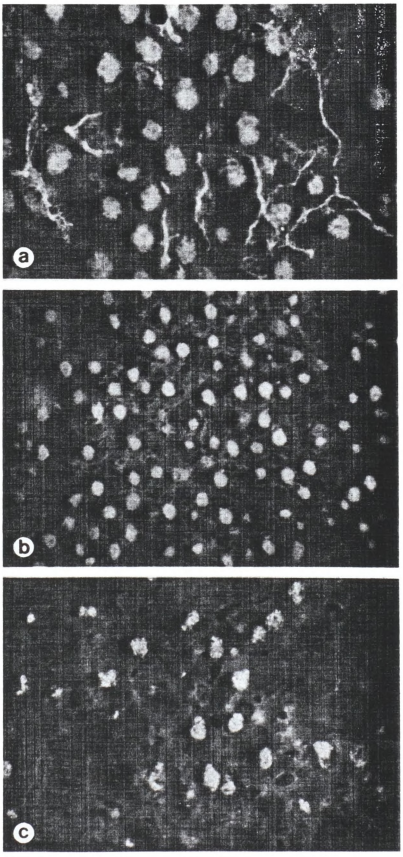

20
18
16
14
12
10
8
6
4
2
FRENQUENCY
0
.100
.200
.300
.400
.500
.600
ABSORBANCE (405nm)

Table 4. The Results of Indirect Immunofluorescence and ELISA assays.

	Immunofluorescence		ELISA	
Serum	Pattern	Titration	Absorbance	Titration
A	Homogeneous	640	1.690	1280
B	Homogeneous	160	1.960	1280
C	Speckled	40	0.513	320
D	Speckled	80	0.409	640
E	Peripheral	40	0.160	ND
F	Peripheral	10,240	0.221	ND
G	Speckled (Large Grain)	2560	0.083	ND
H	Nucleolar	5120	0.164	ND
I	Speckled	20	0.143	ND

Figure 7. Indirect immunofluorescence demonstrating patterns of nuclear staining by sera A through I.


a
b
c

d

e

f

g

h

i

patterns while serum G stained the nucleoli.

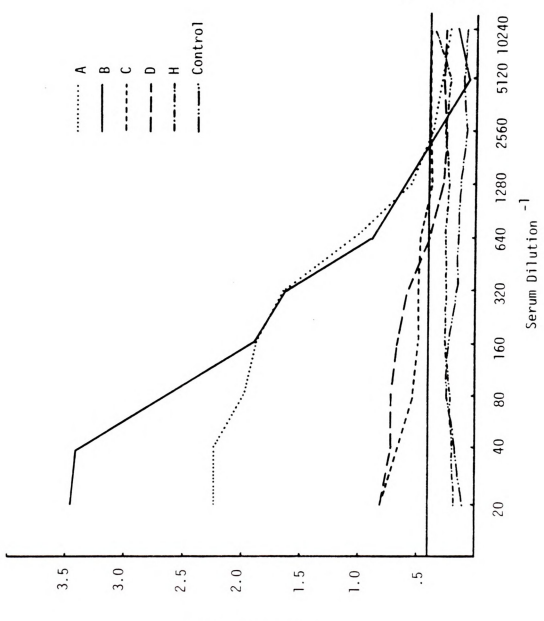
Sera A through I were then tested by the ELISA for antibodies with nucleosome core particle specificity. Sera A through D registered absorbance values greater than 0.400 by ELISA and were considered positive, while sera E through I registered negative values (Table 4). As shown in Table 4, the ELISA proved to be more sensitive upon titration than the immunofluorescent assay. The titration curves for sera containing antibodies to nucleosome core particles (A through D) are shown in Figure 8.

Inhibition studies were performed to determine the antibody specificity of sera A through D. Nucleosome core particles, when added to the sera immediately before performing the ELISA, significantly inhibited antibody binding to the nucleosome core particles bound to the wells of the microtiter plate (Figure 9). Interestingly, nucleosome core particles digested with DNase I (introducing nicks in the nucleosomal DNA every 10 base pairs) were not inhibitory as shown in Figure 10.

Double-stranded DNA was added to the sera before performing the ELISA to determine if antibodies specific for double-stranded DNA were cross-reacting with nucleosomal DNA. The addition of double-stranded DNA inhibited the ELISA values for all four sera, although not as effectively as free nucleosome core particles (Figure 11).

The inhibition by double-stranded DNA was unexpected, especially in view of the results obtained using DNase I-

Figure 8. Titration of sera by ELISA. Sera with absorbance values above 0.400nm were positive for ANA with nucleosome core particle specificity.


A
B
C
D
H
Control
+
-
3.5
3.0
2.5
2.0
1.5
1.0
.5
20
40
80
160
320
640
1280
2560
5120
10240
Serum Dilution -1
ABSORBANCE (405nm)

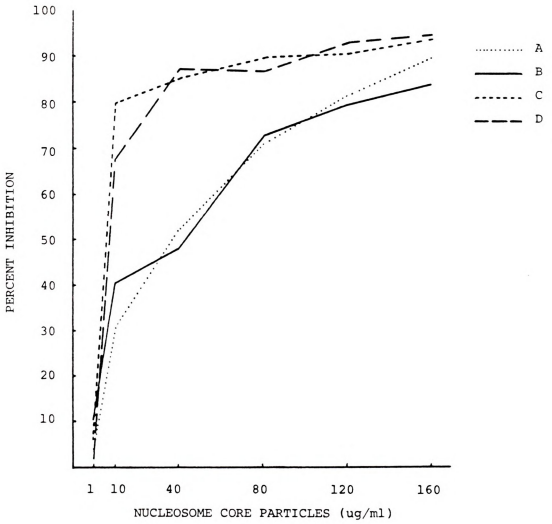


Figure 9. Inhibition of ELISA values of sera A through D by the addition of increasing amounts of nucleosome core particles immediately before testing.

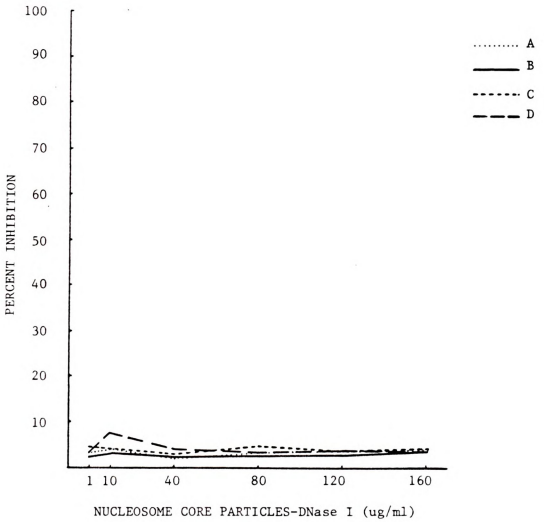


Figure 10. Inhibition of ELISA values of Sera A through D by the addition of increasing amounts of DNase I digested nucleosome core particles to the sera immediately before testing.

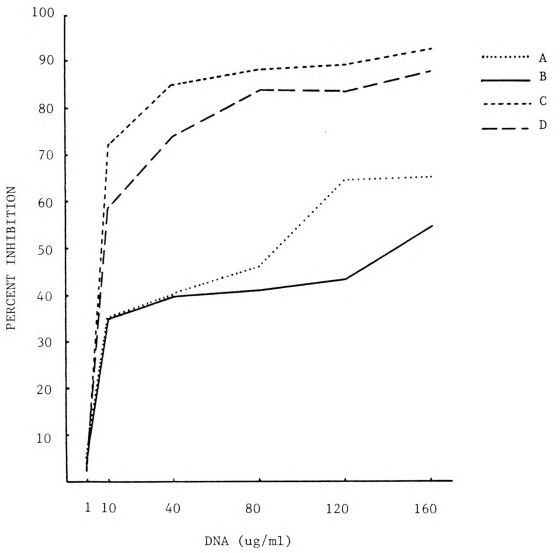


Figure 11. Inhibition of ELISA values of sera A through D by the addition of increasing amounts of double-stranded DNA to the sera immediately before testing.

digested nucleosome core particles which contain DNA but did not inhibit the ELISA. When RNA, double-stranded DNA and nucleosome core particles were incubated with serum B immediately before performing the ELISA, RNA produced more inhibition than either double-stranded DNA or nucleosome core particles (Figure 12). This suggested that another mechanism besides competition for the serum antibodies to nucleosome core particles might be responsible for the inhibition. RNA and double-stranded DNA were incubated with bound nucleosome core particles to determine if the nucleic acids were reacting directly with the antigen. The RNA and DNA were washed out of the wells prior to the addition of serum B. As illustrated in Figure 13, marked inhibition occurred.

Statistics

The between-run reproducibility of the ELISA was estimated from serum B repeated in assays which were run on ten different days (Table 5). The coefficient of variation was 6.2%. The within-run reproducibility was estimated from the titration of serum B (Table 6). The coefficient of variation between duplicates samples over a wide absorbance range was 7.9%.

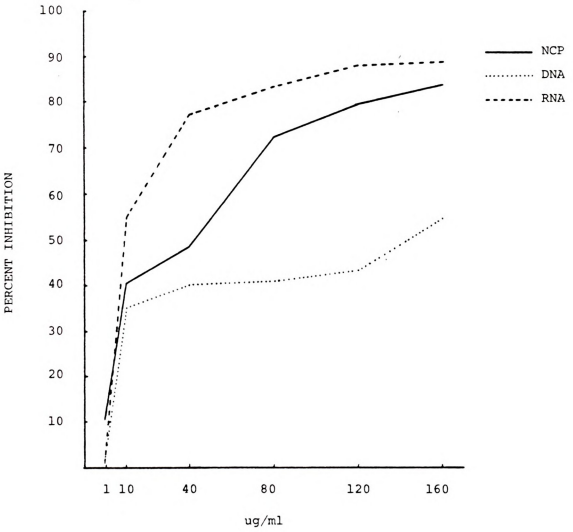


Figure 12. Inhibition of ELISA values of serum B by the addition of increasing amounts of nucleosome core particles, double stranded-DNA or RNA to the serum immediately before testing.

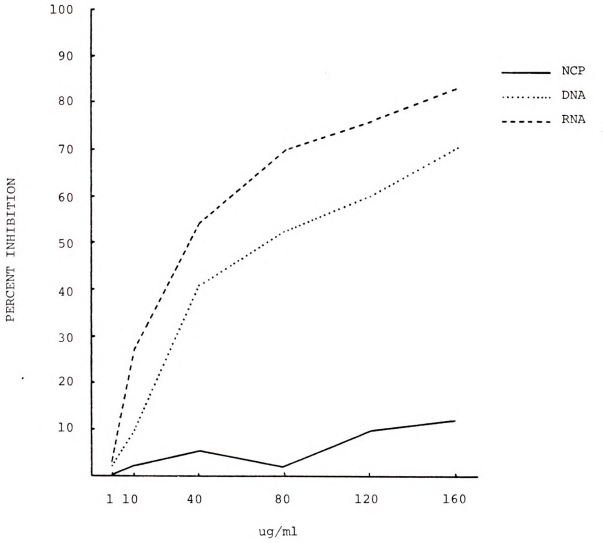


Figure 13. Inhibition of ELISA values of serum B by preincubation of microtiter wells with nucleosome core particles, double-stranded DNA or RNA prior to testing.

Table 5. Day to Day Reproducibility of the ELISA.

Reference Serum	$(x - \bar{x})$
1.892	0.076
1.839	0.023
1.864	0.048
1.805	0.011
1.857	0.041
1.963	0.147
1.646	0.170
1.864	0.048
1.837	0.021
1.589	0.227
Mean = 1.816	
SD = 0.113	
CV = 6.2%	

SD, Standard Diviation

CV, Coefficient of Variation

Table 6. Within-Run Repeatability.

Sera (Duplicate)		$\bar{x}$	d
3.129	3.060	3.095	0.035
3.102	2.964	3.011	0.069
2.358	2.352	2.355	0.003
1.680	1.695	1.688	0.008
1.456	1.464	1.460	0.004
0.835	0.732	0.784	0.056
0.572	0.467	0.520	0.053
0.282	0.266	0.274	0.008
0.045	0.059	0.052	0.007
0.129	0.133	0.131	0.002
0.139	0.143	0.141	0.002
0.163	0.183	0.173	0.010
0.099	0.108	0.104	0.004
0.213	0.202	0.208	0.006

Mean = 1.0

SD = 0.077

CV = 7.69%

SD, Standard Diviation

CV, Coefficient of Variation

CHAPTER V

DISCUSSION

Antibodies to deoxyribonucleoprotein (DNP) have been described in the sera of patients with SLE since 1957 (Holman and Kunkel) and numerous immunological studies performed. However, the nature of anti-DNP antibodies and their role in autoimmune diesease has remained obscure. Sensitive assays to detect and characterize anti-DNP antibodies have been difficult to develop because of the heterogeneity of traditionally prepared DNP which was contaminated by non-nucleosomal DNA and non-histone proteins. The DNP was also extracted from sheared chromatin and the shear forces that pulled apart covalent bonds connecting the DNA disrupted the noncovalent bonds connecting the DNA and histone octomer (Noll et al., 1975). Nuclease and protease digestion studies suggested that recombination of DNA and histones after this type of dissociation resulted in a variety of altered conformations (Noll et al., 1975; Sollner-Webb and Felsenfield, 1975). Endogenous protease activity was also disregarded in earlier studies, but it was shown that even mild digestion altered DNP conformation (Rill et al., 1977).

In this study we have developed an enzyme-linked immunosorbent assay (ELISA) with nucleosome core particles as the solid phase. Nucleosome core particles are DNP that

have been isolated in such a way as to retain properties found in vivo. The biochemical and biophysical properties of nucleosomes have been stringently established because of their importance in the study of chromatin structure, replication, gene expression and transcription. The assay was used to study canine antinuclear antibodies (ANA) with nucleosome core particle specificity.

The nucleosome core particles isolated in this study and used in the ELISA were shown by sedimentation anaylsis and gel electrophoreis to be comparable to nucleosome core particles isolated in published reports (Olins et al., 1976; Rill et al., 1977). The homogeneity of the nucleosome preparations was also reflected in the ELISA readings. The between-run reproducibility and the within-run reproducibility were equal to or better than results reported for radioimmunoassays used with a variety of antigens (Belanger et al., 1976; Rosenthal et al., 1976; Moiolini et al., 1975; Engvall and Perlmann, 1972 and 1971; Engvall et al., 1971). These results demonstrated the suitability of nucleosome core particles in formation of the solid phase, since variablility of antigen binding to the solid phase has been the major factor in determining the precision of all solid-phase immunoassays (Engvall and Pesce, 1978).

Nine sera, designated A through I were tested for antibodies with nucleosome core particle specificity by the ELISA. Sera A through D were positive having ELISA

values greater than 0.400nm. Sera A and B stained by indirect immunofluorescence with a diffuse homogeneous pattern which has been typical of sera with antibodies to DNP. Sera C and D, also positive for anti-nucleosome core particle antibodies by the ELISA, produced speckled patterns by indirect immunofluorescence. Speckled patterns have normally been associated with the presence of Sm, Scl-1, SS-B or RNP antibodies. It should be pointed out, however, that a limitation of the immunofluoresence assay for ANA has been the inability to distinguish between antibodies with different specificities in the same serum sample. The detection of anti-nucleosome core particle antibodies in sera C and D may have been a result of the ELISA`s increased sensitivity and specificity.

Sera E through I regeistered ELISA values below 0.400nm and were considered negative for anti-nucleosome core particle antibodies. However, it was interesting to note the immunofluoresence staining patterns of these antibodies. Sera E and F gave peripheral staining patterns which have been associated with anti-DNA antibodies. Serum F contained a very high titer antibody by immunofluoresence, yet registered negative by the ELISA. This was particularly significant since nucleosome core particles contain DNA, and suggested that either epitopes on the DNA reacting with anti-DNA antibodies were hidden or that nucleosomal DNA was immunologically altered from the configuration of free double-stranded DNA by interacting with the histone

octomer. Sera G gave a high titer nucleolar staining pattern correlating with antibodies to RNA. The lack of reactivity of sera F and G by the ELISA provided indirect evidence that antibodies to DNA or RNA were not cross-reacting with the DNA associated with nucleosome core particles. Sera I gave a typical speckled pattern; however, sera H had a large grained speckled that has only recently been described in the literature in 3 dogs with SLE (Monier, 1980).

Nucleosome core particles have three possible antibody binding moieties: DNA, histones and DNA-histone complex. The specificity of antibodies in sera A through D were further characterized by adding DNA, RNA and nucleosome core particles to the sera immediately before performing the ELISA. Nucleosome core particles were effective competitors with the bound counterparts for antibodies as expected; however, DNA and RNA also significantly inhibited the ELISA. In studies where DNA, RNA and nucleosome core particles were incubated in the wells with bound antigen and rinsed prior to the addition of the sera, the ELISA was inhibited by both DNA and RNA but not nucleosome core particles. Inhibition of the ELISA values could have occurred by DNA and RNA binding to the nucleosome core particles and inhibiting antibody binding by steric hinderence or possibly displacing the nucleosome core particles bound to the polystyrene by neutralizing the charges responsible for their adherence. Data also

suggesting that serum antibodies to DNA or RNA were not binding to nucleosome core particles included the negative ELISA values registered with sera F and H, presumed to have high titers of antibodies to DNA and RNA, respectively, based on immunofluorescent staining patterns.

Nucleosome core particles digested with DNase I did not inhibit ELISA values. DNase I cleaves nucleosomal DNA every ten base pairs which suggests that these sites may contain the antibody binding site. Alternatively, the cleavage sites could be essential for maintaining the immunological integrity of the nucleosome core particle. However, this study demonstrates that DNA and histones are required for antibody binding to nucleosome core particles.

Future experiments should be directed at defining the antibody binding sites on nucleosome core particles and better defining antibody specificity. The ELISA developed in this study proved to be a sensitive, reliable and economical technique for detecting anti-DNP antinuclear antibodies. With the development of an assay incorporating a well-defined DNP particle, the importance of DNP antibodies in the diagnosis and pathology of autoimmune disease should become increasingly clear. Antibodies to DNP (nucleosome core particles) may also provide probes into nucleosome structure and function including the regulatory function of nucleosomes in transcription and the binding sites of regulatory proteins.

LIST OF REFERENCES

References

Ahlstedt S, Hanson LA, Wadsworth C (1976) A Clq Immunosorbent assay compared with thin-layer gel filtration for measuring IgG aggregates. Scand J Immunol 5:293-304.

Alspaugh MA, Tan EM (1976) Serum antibody in rheumatoid arthritis reactive with a cell-associated antigen. Demonstration by precipitation and immunofluorescence. Arthritis Rheum 19:711-719.

Altenburger W, Horz W, Zachau HG (1976) Nuclease cleavage of chromatin at 100 nucleotide pair intervals. Nature 264:517-522.

Arana R, Seligmann M (1967) Antibodies to native and denatured deoxyribonucleic acid in systemic lupus erythematosus. J Clin Invest 46:1867-1882.

Avarameas S (1969) Coupling of enzymes to proteins with glutaraldehyde: Use of the conjugate for detection of antigens and antibodies. Immunochemistry 6:43.

Beck JS (1962) Partial Identification of the "Speckled" nuclear antigen. Lancet i:241-242.

Beck JS (1963) Autoantibodies to cell nuclei. Scott Med J 8:373-388.

Belanger L, Hamel D, Dufour D, Pouliot M (1976) Double antibody enzyme immunoassay applied to human alpha-fetoprotein. Clin Chem 22:198.

Catt K, Treager GW (1967) Solid-phase radioimmunoassay in antibody-coated tubes. Science 158:1570.

Coons AH, Kaplan MH (1950) Localization of antigen in tissue cells. J Exp Med 91:1-13.

Crick FHC, Klug A (1975) Kinky helix. Nature 255:530-533.

Davis P, Makinen (1980) Antibody to synthetic poly dAT: correlation with antibody to native DNA and specificity for SLE. J Clin Pathol 33:648-652.

De Wachter R, Fiers W (1971) Fractionation of RNA by electrophoresis on polyacrylamide gel slabs. In Method in Enzymology Vol XXI Nucleic Acids Part D (ed Grossman L, Moldave K) pp 167-178, Academic Press, New York.

Engvall E (1976) Determination of antibodies to DNA by ELISA. Lancet ii:1410-1410.

Engvall E, Jonsson K, Perlmann P (1971) Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim biophys Acta 251:427.

Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871-874.

Engvall E, Pesce AJ (1978) Quantitative enzyme immunoassay. Scand J Immunol Suppl No 7, 8:1-62.

Fishbein E, Alarcon-Segovia D, Vega JM (1979) Antibodies to histone in systemic lupus erythematosus. Clin exp Immunol 36:145-150.

Friou GJ (1957) Identification of the nuclear component of the interaction of lupus erythematosus globulin and nuclei. J Immunol 80:467-481.

Fritzler MJ, Tan EM (1978) Antibodies to histones in drug induced and idiopathic lupus erythematosus. J Clin Invest 62:560.

Hargraves MM (1949) Production in vitro of L.E. cell phenomenon. Proc Staff Meet Mayo Clin 27:419-423.

Haserick JR, Lewis LA, Bortz DW (1950) Blood factor in acute disseminated lupus erythematosus. I. Determination of gamma globulin as specific plasma fraction. Am J Med Sci 219:660.

Hewish DR, Burgoyne LA (1973) Chromatin substructure. The digestion of chromatin DNA at regularly spaced sites by a nuclear DNase. Biochem Biophys Res Commun 52:504-510.

Holman H, Deicher HR (1959) The reaction of the lupus erythematosus (L.E.) cell factor with deoxyribonucleoprotein of the cell nucleus. J Clin Invest 38:2059-2072.

Holman HR, Kunkel KG (1957) Affinity between the lupus erythemtosus serum factor and cell nuclei and nucleoprotein. Science 126:162-162.

Johansson SGO, Bennick H, Wide L (1968) A new class of immunoglobulin in human sera. Immunol 14:265-272.

Koffler D, Schur P, Kunkel HG (1967) Immunological studies concerning the nephritis of systemic lupus erythematosus. J Exp Med 126:607-623.

Klotz Jl, Minami RM, Teplitz RL (1979) An enzyme-linked immunosorbent assay for antibodies to native and denatured DNA. J Immunol Methods 29:155-165.

Kornberg RD (1974) Chromatin Structure: A repeating unit of eight histone molecules and about 200 DNA base pairs. Science 184:868-871.

Krishman C, Kaplan MH (1967) Immunopathologic studies of systemic lupus erythematosus. II. Antinuclear reaction of gammaglobulin eluted from homogenates and isolated glomeruli of kidneys from patients with lupus nephritis. J Clin Invest 46:569-579.

Lachmann PJ, Kunkel HG (1961) Correlation of antinuclear antibodies and nuclear staining patterns. Lancet 2:436.

Leinonen M, Jarvinen T, Valkonen K (1978) Enzyme-linked immunosorbent assay (ELISA) for anti-histone antibodies. Scand J Immunol 8:164-164.

Levitt M (1978) How many base pairs per turn does DNA have in solution and in chromatin? Some theoretical calculations. Proc Nat Acad Sci USA 75:640-644.

Lewin B (1980) The nucleosome: Structure of the particle. In Eucaryotic Chromosomes. Vol 2 (ed Lewin B), pp 332-427, John Wiley and Sons, New York.

Lewis RM, Stollar BD, Goldberg E (1973) A rapid, sensitive test for the detection of antibodies to DNA. J Immunol Methods 3:365-374.

Lightfoot RW, Hughes GRV (1976) Significance of persisting serologic abnormalities in SLE. Arthritis Rheum 19:837-843.

Lohr D, Van Holde KE (1975) Yeast chromatin subunit structure. Science 188:165-166.

Maiolini R, Ferrua B, Masseyeff (1975) Enzymoimmunoassay of Human alpha-fetoprotein. J Immunol Methods 6:355-362.

Maniatis T, Jeffrey A, Van deSande H (1975) Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. Biochemistry 14:3787-3794.

McGhee JD, Engel JD (1975) The subunit structure of chromatin is the same as plants and animals. Nature 254:449.

Miyawaki S, Ritchie RF (1973) Nucleolar antigen specific for antinucleolar antibody in the sera of patients with systemic rheumatic disease. Arthritis Rheumat. 16:726-736.

Monier JC, Dardenne M, Rigal D, Costa O, Fournel C, Lapras M (1980) Clinical and laboratory features of canine lupus syndromes. Arthritis and Rheum 23:294-301.

Morris NR (1976) Nucleosome structure in Aspergillus nidulans. Cell 8:357-364.

Noll M (1974) Subunit structure of chromatin. Nature 251:249-251.

Noll M, Thomas JO, Kornberg RD (1975) Preparation of native chromatin and damage caused by shearing. Science 187:1203-1206.

Notman DD, Kurata N, Tan EM (1975) Profiles of antinuclear antibodies in systemic rheumatic diseases. Ann Intern Med 83:464-469.

Olins AL, Carlson RD, Wright EB, Olins DE (1976) Chromatin nu bodies: isolation, subfractionation and physical characterization. Nucl Acids Res 3:3271-3291.

Olins AL, Olins DE (1974) Spheroid chromatin units (V bodies). Science 183:330-332.

Oudet P, Gross-Bellard M, Chambon P (1975) Electron microscopic and biochemical evidence that chromatin is a repeating unit. Cell 4:281-300.

Pesce AJ, Mendoza N, Boreisha I, Gaizutis MA, Pollak VE (1974) Use of enzyme-linked antibodies to measure serum anti-DNA antibody in systemic lupus erythematosus. Clinical Chem 20:353-359.

Pinnas J, Northway JD, Tan EM (1973) Antinucleolar antibodies in human sera. J Immunol 111:996-1003.

Rekvig OP, Hannestad K (1979) Properties of antinuclear antibodies that cross-react with plasma membranes. Scand J Immunol 9:325-332.

Rekvig OP, Hannestad K (1980) Human autoantibodies that react with both cell nuclei and plasma membranes display specificity for the octamer of histones H2A, H2B, H3, and H4 in high salt. J Exp Med 1720-1733.

Rill RL, Shaw BR, Van Holde (1977) Isolation and Characterization of Chromatin Subunits. In Methods in Cell Biology Vol XVIII (ed Stein G, Stein J), pp 69-102.

Robitaille P, Tan EM (1973) Relationships between deoxyribonucleoprotein and deoxyribonucleic acid antibodies in systemic lupus erythematosus. J Clin Invest 52:316.

Rosenthal AF, Vargas MG, Klass CS (1976) Evaluation of enzyme-multiplied immunoassay technique (EMIT) for determination serum digoxin. Clin Chem 22:1899.

Sharp GC, Irwin W, Holman HR (1972) Mixed connective tissue disease-an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen (ENA). Am J Med 52:148-159.

Shaw BR, Herman TM, Kovacic RT, Beaudreau GS, Van Holde KE (1976) Analysis of subunit organization in chicken erythrocyte chromatin. Proc Nat Acad Sci USA 73:505-509.

Sollner-Webb B, Camerini-Otero RD, Felsenfeld G (1976) Chromatin structure as probed by nucleases and proteases: evidence for the central role of histones H3 and H4. Cell 9:179-194.

Sollner-Webb B, Felsenfeld G (1975) A comparison of the digestion of nuclei and chromatin by staphyloccocal nuclease. Biochemistry 14:2915-2920.

Spadafora C, Bellard M, Compton JL, Chambon P (1976) The DNA repeat lengths in chromatins form sea urchin sperm and gastrula cells are markedly different. Febs Lett 69:281-285.

Stollar BD (1967) Studies on nucleoprotein determinants for systemic lupus erythematosus serum. J Immunol 99:959-965.

Stollar BD (1973) Nucleic acid antigens. In The Antigens Vol 1 (ed M Sela), pp 1-85, Academic Press, New York.

Sussman JL, Trifonov EN (1978) Possibility of nonkinked packing of DNA in chromatin. Proc Nat Acad Sci USA 75:103-107.

Tan EM, Kunkel HG (1966) Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. J Immunol 96:464-471.

Tan EM (1967) An immunologic precipitin system between soluble nucleoprotein and serum antibody in systemic lupus erythematosus. J Clin Invest 46:735-745.

Tan EM, Rodnan GP (1975) Profile of antinuclear antibodies in progressive systemic sclerosis. Arthritis Rheum 18:430.

Tan EM (1983) Antinuclear antibodies in diagnosis and management. In The Biology of Immunologic Disease (ed Dixon FJ, Fisher DW), pp 314-321, Sinauer Associates, Inc., Massachusetts.

Thomas JO, Kornberg RD (1975) An cotamer of histones in chromatin and free in solution. Proc Nat Acad Sci USA 72:2626-2630.

Van Holde KE, Shaw BR, Tatchell K, Pardon J, Worcester D, Wooley J, Richards B (1977) The structural subunit of chromatin. In The Molecular Biology of the Mammalian Genetic Apparatus (ed P Ts˜o), pp 239-254 Elisevier.

Voller A, Bidwell DE, Bartlett A (1976) Enzyme immunoassays in diagnostic medicine. Theory and practice. Bull World Health Organization 53:55-65.

Voller A, Bidwell DE, Bartlett A (1979) In The Enzyme Linked Immunosorbent Assay (ELISA), pp 1-125, Dynatech Laboratories Inc., Virginia.

Voller A, Bidwell DE, Huldt G, Engvall E (1974) A microplate method of ELISA. Bull Wld Hlth Org 51:200.

Wilkens MFH, Zubay G, Wilson HR (1959) X ray diffraction studies of the molecular structure of nucleoshistone and chromosomes. J Mol Biol 1:179-185.

Willems F, Klaassen De Kort CCM (1978) ELISA for rheumatoid. Lancet i:994-994.

Wolfe JF, Adelstein E, Sharp GC (1977) Antinuclear antibody with distinct specificity for polymyositis. J Clin Invest 8:176-178.