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Circulating immune complexes in systemic lupus erythematosus : a search for the antigen

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<u>3/23/84</u>
(Date)

CIRCULATING IMMUNE COMPLEXES IN SYSTEMIC LUPUS ERYTHEMATOSUS:

A SEARCH FOR THE ANTIGEN

A Thesis Submitted to the Yale University School of
Medicine in Partial Fulfillment of the Requirements
for the Degree of Doctor of Medicine

MARK STEIN
1984

ABSTRACT

CIRCULATING IMMUNE COMPLEXES IN SYSTEMIC LUPUS ERYTHEMATOSUS: A SEARCH FOR THE ANTIGEN

Mark Stein
1984

In this study I have examined the composition of cryoprecipitates obtained from the sera of patients with systemic lupus erythematosus and normal donors and sought to determine whether these precipitates contain immune complexes which have small nuclear ribonucleoprotein as antigen component. I found that the cryoprecipitable protein in serum rapidly precipitated when sera were made to contain 3% polyethylene glycol. PEG fractions of normal sera and SLE sera were identical except that the SLE precipitates contained greater amounts of immunoglobulin G and IgM, and proteins with molecular weights between 80K and 60K, and 30K and 20K. Double immunodiffusion demonstrated that both normal and SLE sera contained IgM, C2, C3 and C4, but fibrinogen, ceruloplasmin and C5 were not detected.

Using a DNA probe complementary to the U1 small RNA I searched for snRNP immune complexes in SLE sera. Sera was obtained from four patients with SLE who were known to have anti-RNP or anti-Sm antibodies (both antibodies precipitate the U1 small RNA) while their disease was in its quiescent phase. The U1 small RNA was not detectable in immune complexes isolated from these patients.

These findings further expand our knowledge about the composition of cryoglobulins and suggest that cryoprecipitation is due to the increased concentration of one or more normal serum proteins. The U1 ribonucleoprotein particle which is a major target for the autoimmune response in SLE does not appear to participate in the formation of circulating immune complexes.

ACKNOWLEDGEMENT

DEDICATION

This thesis is dedicated to my parents, and my sister and brother for all their love and guidance.

An honorable mention and a place in my heart also goes to Ken Rosenblum who has been my best friend and constant companion on our journey through medical school.

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I. ABBREVIATIONS USED

ANA	antinuclear antibody
^{o}C	degree centrigade
CIg	cryoimmune globulin
cm	centimeter
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dsRNA	double stranded RNA
ENA	extractible nuclear antigen
^{o}F	degree Fahrenheit
g	gravity
hnRNA	heterogeneous nuclear RNA
hr	hour
IgG,M,A,E	immunoglobulin G,M,A,E
M	molar
MCTD	mixed connective tissue disease
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mRNA	messenger RNA
MW	molecular weight
NBPC	nitrobenzyloxymethylpyridinium chloride
PBS	phosphate buffered saline
PCA	phenol-chloroform-isoamylalcohol
pg	picograms
RBC	red blood cell
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	rotations per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
snRNP	small nuclear RNP
ssDNA	single stranded DNA
ssRNA	single stranded RNA
µg	micrograms
µl	microliters
v	volume
V	volts
WBC	white blood cell
w	weight

II. REVIEW OF LITERATURE

A. Introduction

Systemic lupus erythematosus (SLE) is a disease of unknown etiology that is characterized by inflammatory lesions involving multiple organ systems and multiple immunologic abnormalities (1,2). SLE has a prevalence of 1 per 1,000 population (3) and an incidence of 1 in 15,000 population (2). It occurs three times more frequently in blacks than in whites and is 10 times more common in females than in males (4). The peak age of onset is between 15 and 25 years.

The typical patient with SLE initially presents with fatigue (95%), fever (80%), arthralgia (90%) and a skin rash (75%) (1,4). With the disease in its advanced stages the vital organs most often involved are kidneys (55%), lungs (45%), heart (45%), gastrointestinal tract (40%) and the brain (30%) (1). Much of the tissue injury in SLE results from the deposition of immune complexes.

When immune complexes are deposited, complement becomes activated. Phagocytic cells are attracted and phagocytosis is stimulated. Phagocytosis results in the release of lysosomal enzymes and other cellular products which results in tissue injury. The immune complexes which form in SLE primarily involve autoantibodies. Why the immune system should lose tolerance to self antigens is not fully understood but several explanations have been proposed. Normally, Ts cells downregulate the response of the immune system. This loss of tolerance may be due to the loss of suppressor T lymphocytes (5). Several factors may lead to the loss of Ts cells. There is a strong case for a genetic component. The HLA markers DRW2 and DRW3 are found more frequently among SLE patients than the population at large (6).

There is a 60% concordance rate of SLE in identical twins (7). SLE patients have a higher incidence of other immune system disorders than the population (8). All these examples point to a genetically linked cause.

A second possible cause for the autoimmune phenomenon is a viral infection. Viral nucleocapsids are frequently found in endothelial cells of SLE patients. Although no specific virus has been implicated, antibody titers to various viruses are often elevated in lupus sera. Lastly, several DNA and RNA viruses have been identified which can induce a lupus like syndrome in laboratory animals (9,10).

B. Antigens and Antibodies in SLE

In the sera of lupus patients, autoantibodies have been found against the cellular components listed below (11-13). Clinically, antigen-antibody complexes based on DNA are especially significant as they are the type of immune complexes most often found in deposits around basement membranes of the various organ systems (14). It is therefore thought that such complexes are responsible for much of the pathogenesis of lupus and thus they have been studied extensively.

Antinuclear (including Extractible Nuclear Antigen):	ssDNA
	dsDNA
	ssRNA
	dsRNA
	histones
	Sm
	RNP
	La
Anticytoplasmic:	mitochondria
	lysosomes
	microsomes
	ribosomes
	rRNA
	Ro
Anticell:	RBC
	WBC
	platelet

The first laboratory finding characteristic of SLE was reported by Hargraves and colleagues at the Mayo Clinic (15). They described the appearance of swollen bodies in the cytoplasm of polymorphonuclear leukocytes found in the bone marrow of a patient with SLE and called these unusual polys "LE" cells. The swollen bodies stained with Feulgen reagent and so it was thought that they represented phagocytized nuclei or nucleic acid. By 1957 it was known that LE cells could be experimentally produced by incubating damaged cells with SLE serum containing polys. Holman and Kunkel then showed that by preincubating SLE serum with cell nuclei the serum loses its ability to induce the formation of LE cells (16). Furthermore, when slides containing LE cells were reacted with fluorescent rabbit antiserum to human gammaglobulin the inclusion bodies of LE cells fluoresced brilliantly. From this evidence they correctly reasoned that SLE serum contains antibodies directed against nuclear DNA. When the antibody-coated DNA is phagocytized by polys, LE cells are formed. The LE cell phenomenon thus became the first study of antinuclear antibodies (ANAs). Parallel work on different ANAs was performed in the mid-fifties by Seligman and others (16-20). These attempts involved use of double immunodiffusion or fluorescent antibody techniques to detect reactions between lupus sera and various cellular components. Most commonly, the antibodies reacted with DNA. Unfortunately, these studies were criticized on the grounds that they failed to differentiate between a reaction with naked DNA as opposed to one with DNA associated proteins (21,22). Later work overcame this problem by showing that the reaction no longer took place if the sample was first treated with DNase (22). This showed that DNA

comprised either the entire antigenic site or was a necessary component of the antigenic site. As immunochemical techniques became more sensitive and specific, the true nature of nuclear antigens came to light.

There are essentially three types of anti-DNA antibodies; those which recognize ssDNA only, those which recognize native DNA only and those which recognize both ssDNA and nDNA (19,23-27). Anti-ssDNA antibodies react with determinants hidden in the native molecule. Although the exact nature of the antigenic site is not known, Stollar et al. believe that the antibodies are reacting with purine bases (24,28). They base this conclusion on the observation that purine bases or the denatured end products of DNase digest inhibit the binding of these antibodies to ssDNA. Native DNA does not inhibit this binding.

The second type of anti-DNA antibodies recognize determinants common to both ssDNA and nDNA. Again, the exact nature of the antigenic site is not known, but complement fixation studies indicate that it is a small, repeating unit -- possibly deoxyribose (23,26). The third type of anti-DNA antibody binds exclusively to native DNA. The antigenic site in this case is thought to be constituted by the tertiary structural feature of the double helix (26). Not all researchers share this view. They cite evidence which shows that ssDNA, when present in high concentrations, inhibits the binding of these antibodies to nDNA. Others argue that this inhibition is due to the recombination of two ssDNA chains.

Most of the early studies centered around DNA as the most important reactive nuclear antigen. This was partly due to the

simplicity of isolation of DNA. When nuclei are disrupted in isotonic saline, deoxyribonucleohistone is easily recovered as a precipitate (29). In his search for an SLE antigen other than DNA, Holman disrupted nuclei in saline, discarded the precipitated DNA and recovered the supernatant. When this supernatant was saturated with ethanol at room temperature a precipitate formed which, like nucleic acid, bound to antibodies from SLE serum. Since it was extracted from the supernatant, Halman called it an extractible nuclear antigen (ENA). This ENA was found to be composed of acidic, non-histone proteins and RNA and so it is also called ribonucleoprotein (RNP) (29). Unlike DNA and nucleoprotein, RNP is insensitive to digestion by DNase and trypsin. It is nondialyzable and is stable between pH's 5 and 9 and at temperatures less than 56°C. One year after Holman's discovery, Tan and Kunkel described a second ENA (30). The sera used to detect this antigen came from a patient with the last name of Smith and it was thus called Sm. In addition to being resistant to DNase and trypsin, Sm is also resistant to digestion by RNase. Sm and RNP are also referred to as small nuclear ribonucleoproteins (snRNP). Each has a molecular weight less than 150,000 and a sedimentation coefficient of 7S. When bound to antibodies, each can fix complement (29,30).

The molecular identity of the antigen recognized by anti-RNP and anti-Sm antibodies finally became apparent when Lerner and Steitz developed a new assay based on immunoprecipitation of extracts of ^{32}P labeled HeLa cells. In this assay ^{32}P is first incorporated into the cell's RNA. The cells are then disrupted by sonication. This releases the labeled snRNPs and other molecules containing nucleic

acids into the supernatant. The snRNPs are bound to anti-Sm and anti-RNP antibodies from SLE sera and the resulting immune complexes precipitated with staphylococcal protein A. Since snRNPs were thought to be composed of RNA and protein, Lerner and Steitz examined these components separately.

In their protocol, the RNA part is recovered from the immune complexes by extraction with phenol-chloroform. The RNA is separated on a urea-acrylamide gel and the location of these bands compared to known RNA species (31). Anti-RNP precipitated only one band of RNA which is called U1 (see Figure 1). Anti-Sm precipitated 5 bands, labeled U1-U6. These RNAs turned out to be a previously studied snRNA species whose importance and function was not previously appreciated. U1-U6 have since been fully sequenced and range in size from 100 to 200 bases.

To study the protein portion of snRNPs, HeLa cells were first labeled with ^{35}S-methionine. ^{35}S is incorporated in all newly synthesized proteins including the protein portion of snRNPs. Again, snRNPs are precipitated with SLE sera, adsorbed to a staph protein A-sepharose column and then eluted from this column. This procedure separates immune complexes from other proteins. The purified complexes are then fractionated on an SDS-acrylamide gel. It was found that anti-Sm and anti-RNP antibodies can bind to the same seven proteins. The molecular weights of these proteins range from 12,000 to 35,000 Daltons. Each snRNP contains only one snRNA species. The U1 snRNP complex is composed of the U1 snRNA and all seven proteins. snRNPs containing the other U RNAs are composed of the respective RNA and a subset of any five of the seven proteins (25,31,32).

An intact snRNP complex is required for antigenicity. Neither anti-Sm nor anti-RNP can precipitate snRNAs after deproteinization. Likewise, the protein portion of snRNPs cannot be precipitated after the snRNA has been removed (33-35).

C. Role of snRNPs

Although one gene codes for one polypeptide chain, many eukaryotic genes occur in pieces. Within the genome, the informational DNA sequences may be interrupted numerous times by extraneous segments of DNA. These intervening segments are known as introns while the segments bearing information are called exons. The ends of an intron attached to the exon segments are called splice junctions The entire gene, including both introns and exons, is initially transcribed to heterogenous nuclear RNA. The hnRNA is then processed. The introns are cut out and the exons are spliced together to form the final message. Whereas the base sequence of introns vary, the sequence of splice junctions is essentially identical from gene to gene (36).

The mechanism involved in splicing is unknown. Recently, Lerner, Steitz and others proposed that snRNPs are intimately involved in this process (37,38). They reason that several conditions would have to be met for their proposal to be valid. From an evolutionary standpoint, higher eukaryotic species possessing interchangeable transcription systems should also possess the same splicing mechanism. Therefore, the structure of snRNPs should be highly conserved across different species if they are to mediate splicing. snRNPs from man, frogs, mouse, rat, chicken and sea urchins can all be precipitated by SLE serum indicating that they are antigenically similar. When sequenced,

the snRNA from all these species are either highly similar or completely identical to human snRNA (31,37,39,40).

Also, if the theory is correct metabolically active cells should have a higher concentration of snRNPs than inactive cells. In relative terms, liver cells and erythroblasts can be considered metabolically active and mature red blood cells inactive. Studies have shown that liver cells have a 25-fold increase in snRNPs over red blood cells. When the percent of the erythroblast component of blood is increased the above ratio is decreased (37,41).

Further evidence for the theory could be found by showing that removal of snRNPs blocks the splicing process and thus retards the formation of mRNA. Indeed, the addition of anti-Sm or anti-RNP antibodies to a cell free splicing system will block the formation of mRNA (42). This shows that splicing is dependent on the presence of snRNPs.

The most stringent condition that needs to be met is that snRNPs should be able to recognize and bind to splice junctions since this would be necessary to facilitate the splicing mechanism. The sequence of both the 5' and 3' ends of U1 snRNAs are conserved and highly complementary to the base sequence of splice junctions (37,38). Thus, the snRNP complex can both bind to splice junctions and participate in the splicing mechanism. Whether snRNPs serve only to align the splice junctions or also to enzymatically cut out the introns is not known. The function of the other U snRNAs is not yet known but there is growing evidence that they too are involved in the transcription and translation processes (43).

Much less is known about two other ribonucleoproteins, La and Ro

(44-46,79). Early cell fractionation and immunofluorescence studies indicated that Ro and La were cytoplasmic in origin (45). More recent studies have demonstrated the antigenic identity of Ro to SS-A, and La to SS-B. SS-A and SS-B are two nuclear antigens extracted from Wil_2 cells, a lymphoblastoid cell line (44,47,49). La is sensitive to trypsin and both La and Ro are sensitive to ribonuclease. They show many physical similarities to Sm and RNP but do not contain the same snRNA species (Figure 1).

D. Significance of Antinuclear Antibodies

Antinuclear antibodies are often found in the serum of patients with SLE and other rheumatic diseases. While not present in every individual, nor found exclusively in any one disease, certain ANAs are thought to be characteristic of particular illnesses (Table 1). Antibodies to ssDNA and dsDNA are found in 50-60% of all lupus patients. Those against histones and RNP are found in 25-40%. Anti-La is found in 10-15% and anti-Ro in 30-40% of SLE patients (27). While Sm antibodies are present in only one third of SLE patients, they are considered pathognomonic for lupus since they do not exist in the sera of patients with other connective tissue diseases (48). However, the most important point to consider is not the presence of any one specific antibody, but rather the wide spectrum of ANAs present in lupus. For example, although anti-RNP is present in nearly 100% of patients with mixed connective tissue disease (MCTD), they are not diagnostically significant since anti-RNP is also found in patients with lupus and other rheumatic diseases. What is important is that the only other ANA present in MCTD is anti-ssDNA. Therefore, lupus can be easily distinguished from MCTD because of the higher

variability of ANAs in the sera of lupus patients. Diagnostically, therefore, the spectrum of antibodies can be more important than the presence of a specific antibody. One possible exception to this rule is found in patients with neonatal lupus. Nearly all infants and their mothers have antibodies to only the Ro antigen (49-51).

The serum antigen or antibody concentration often correlates with the severity of the disease. For example, in one patient with SLE, recurrent bouts of very high fever, skin rash and proteinuria coincided with the appearance of DNA in his serum and resolved with the appearance of anti-DNA antibodies (12). High titers of anti-RNP without elevation of anti-Sm is not predictive of the activity of SLE, although it does correlate with the severity of MCTD (52-55). The presence of RNP antibodies in children with lupus is a poor prognostic sign usually indicating renal and cardiac involvement and a high fatality rate. In MCTD, high titers of anti-Ro are associated with polyarthralgia (90%), Raynaud's phenomenon (81%), hypergammaglobulinemia (74%), polyarthritis (74%), swollen hands (73%), esophageal hypomotility (58%), sclerodactyly (50%) and myositis (50%) (56,57).

Research correlating Sm to the severity of SLE shows mixed results. Winn found that patients with Sm antibody and no DNA antibodies have a milder form of lupus than patients who also possess DNA antibodies (58,80). In the latter group of patients, hypertension, central nervous system disease and renal disease occurred less frequently. Specifically, on renal biopsy only 1/23 patients with Sm antibodies alone had diffuse proliferative glomerulonephritis whereas this was true for 6/14 patients who also had DNA antibodies. From this evidence it can be concluded that the

level of antibody or antigen in a patient's serum often correlates to
the severity of a specific disease. Some of the mechanisms for this
phenomenon have been mentioned; others have yet to be discovered.

E. The Property of Cryoprecipitability

In 1946 Lerner first described a group of proteins that
precipitated from cooled serum and named them cryoglobulins (59,60).
The major protein component of this mixture was separated and found to
have many physical properties similar to gamma-globulin. Since that
time, cryoglobulinemia was found to be associated with many disease
processes. Due to their immunochemical diversity, cryoglobulins have
been classified into three groups by Bonet et al. (61). Type I are
composed of isolated monoclonal immunoglobulin: either IgM, IgG, IgA
or Bence-Jones protein. Type II cryoglobulins are mixed cryoglobulins
with a monoclonal component possessing antibody activity against
polyclonal IgG. Type III cryoglobulins are mixed polyclonal
immunoglobulins or nonimmunoglobulin molecules. This last type of
cryoglobulin is predominantly present in inflammatory and autoimmune
diseases such as lupus erythematosus.

Many theories have been put forward to explain
cryoprecipitability. Lerner originally hypothesized that this
property derived from modified gamma-globulins but could not determine
what the modification was (59). His hypothesis represents the
classical theory of cryoprecipitation although it applies more to Type
I and II cryoglobulins than Type III. Many researchers have expanded
on his theory.

Levo proposes that cryoprecipitation is due to immunoglobulins
which differ from normal by lacking sialic acid or other carbohydrate

residues. Removal of these residues leads to decreased solubility of immunoglobulins. Levo based his hypothesis on the results of chemical analysis of immunoglobulin side chains obtained from the sera of patients with cryoglobulinemia. Humans possess three kinds of immunoglobulins: intracellular, membranous and secretory. The secretory kind is found in blood and differs from the intracellular kind in the amount of carbohydrate residues that it possesses. This glycosylation is a post-transcriptional event (66,67). Some secretory immunoglobulin may leak out of B cells before glycosylation has had a chance to take place. This is evidenced by the fact that healthy people contain small amounts of cryoprecipitable immunoglobulins in their blood. Infectious, malignant and autoimmune diseases are often characterized by the intense stimulation of the lymphoreticular system. The glycosylation process may not be able to keep up with this increased protein synthesis rate and so intracellular immunoglobulins will leak out at a faster rate. This, Levo hypothesizes, causes the accompanying increase in cryoglobulin formation.

A different explanation, but one that is also based on the decreased solubility of modified immunoglobulins, was proposed by Erickson et al. (68). They feel that the modification lies in the primary structure of the heavy chain variable regions of immunoglobulins. Point mutations cause different amino acids with different side chains to be inserted in this region in place of the normal amino acids. These novel side chains when directed either inwardly or outwardly, disturb the normal hydrophobic interactions and change the folding pattern of adjacent segments of the protein. The

immunoglobulin then assumes a configuration which is less soluble at low temperatures. They reached this conclusion by sequencing the heavy chain variable regions of monoclonal immunoglobulins obtained from two patients with cryoglobulinemia. Both samples of immunoglobulins contained extra residues in the outer beta sheet structure of the V_H domain which on metric analysis were shown to alter internal and external hydrophobic interactions.

Christian et al. believe that the property of cryoprecipitability is dependent on a heat labile serum factor (69). In their study, SLE sera was first measured for its ability to form cryoprecipitates. The sera was then heated to $56^{\circ}C$ and it was noted that this significantly decreased the amount of precipitate which was later able to form. The researchers concluded that a heat labile serum factor must play a crucial role in initiating cryoprecipitation. By adding back various serum fractions, each enriched in one specific protein, they identified C1 and an 11s component of complement as two factors which could restore cryoprecipitability.

Yet another protein which has been implicated in cryoprecipitation is cold-insoluble globulin (CIg). Although the identity of CIg is not definitively known, its structure is believed to be a two-chain dimer covalently bounded by disulfide bridges and folded into a beta-structure (70-72). CIg causes cryoprecipitation by forming complexes with fibrin-fibrinogen. These complexes are temperature dependent; forming in the cold and separating at body temperature (73)

Some researchers feel that CIg is identical to the protein fibronectin. They base this conclusion on the observation that the

two proteins have similar structures and are antigenically identical
(74). Like CIg, fibronectin also forms insoluble complexes with
fibrin-fibrinogen. In recent studies linking fibronectin to
cryoprecipitation, fibronectin has been found in cryoprecipitates from
synovial fluid of five of five rheumatoid arthritis patients and from
the sera of two of two SLE patients (75,76). In these studies,
removing fibronectin reduced the amount of cryoprecipitable material.

Hardin also suggests a model in which cryoprecipitability is a
property of large normal serum proteins and not of the immune
complexes themselves (77). In one set of experiments, normal non-
immunoglobulin proteins were precipitated from normal sera with
polyethylene glycol. These proteins had the property of precipitating
spontaneously at 4°C and resolubilizing at 37°C. Experimentally
produced, cold soluble antigen-antibody complexes were made and mixed
with the cryoglobulins. When incubated at 4°C, the normal, cold
insoluble serum proteins were able to co-precipitate the immune
complexes. Thus, large serum proteins were shown to act as
cryoprecipitagogues, co-precipitating the immune complexes. Another
line of evidence supporting non-immunoglobulin induced
cryoprecipitation is that sera from some patients can form
cryoprecipitates even though they do not contain any circulating
immune complexes as measured by the ^{125}I-C1q binding assay (78).

Two explanations (which are not mutually exclusive) for
nonimmunoglobulin induced cryoprecipitation have been proposed. The
first, as has been mentioned previously, is that fibronectin binds to
cold-soluble fibrin-fibrinogen complexes to form cold-insoluble
complexes. Fibronectin can be found in both PEG fractions of normal

sera and in cryoglobulin isolated from patient serum (75-77). The
second explanation is that cryoprecipitation is due to a normal
concentration effect. In many disease states the serum concentration
of large proteins normally increases. Intermolecular interactions
become more pronounced and this in turn leads to decreased solubility
in the cold. Thus, this theory is very simple and elegant in that it
does not call on special protein interactions or changes in protein
structure.

The non-immunoglobulin portion of cryoprecipitates has never been
systematically studied. However, in the course of research done to
identify the mechanism of cryoprecipitation, a number of proteins have
been identified. These are: C1 and 11s component of complement (69),
fibronectin (74-77), $beta_1A$ and lipoprotein (61), and fibrin and
fibrinogen (73). It must be cautioned that not all of these proteins
were reported to be present by every investigator.

F. Purpose of this Thesis

Two broad goals guided the work of this thesis. My first
objective was to determine whether the sera of patients with SLE
contain immune complexes which have small nuclear ribonucleoprotein as
the antigen component. As has been discussed, patients with lupus
produce antibodies which bind to specific snRNPs in vitro. The level
of these antibodies often correlates to the activity of the disease
and for this reason it is often clinically useful to monitor anti-
snRNP levels. Likewise, the presence of snRNP immune complexes may
also have prognostic and therapeutic implications. As Tan and
colleagues pointed out, the onset of an acute flare in an SLE patient
was heralded by an increase in the DNA antigen whereas a remission was

preceded by an increase in DNA immune complexes (12). Since much of the pathogenesis of SLE is due to the deposition of immune complexes it may prove clinically important to ascertain the presence or absence of snRNP immune complexes in various end organs. Thus far the search for these complexes has been hampered by the lack of a sensitive technique with which to detect them. Recently, with the cloning of the U1 gene and the development of hybridization techniques, a powerful method for identifying the presence of snRNP immune complexes has become available and has made the study of this problem possible.

My second goal was to examine the composition of cryoprecipitates from normal and SLE sera. Although cryoglobulins have been studied extensively over the past 25 years, many fundamental questions remain. We still do not fully know the composition of cryoprecipitates or understand the mechanism for cryoprecipitation. Determining the former may provide an answer to the latter. For example, if it were found that the only difference between cryoglobulins from lupus sera and those from normal sera was an increase in the concentration of immune complexes then this would support the theory that cryoprecipitation is due to increased concentration of modified immunoglobulins. Likewise, if it was found that the sole difference was an increase in the concentration of normal serum proteins, it would support that mechanism of cryoprecipitation. For these reasons I identified some of the components of cryoglobulins by immunological techniques and compared normal and lupus cryoprecipitates electrophoretically.

III. MATERIALS AND METHODS

A. Solutions

PBS
    0.01M PO_4 (1.42gm/L)
    0.15M NaCl (8.76gm/L)
    pH 7.4

PEG (6% or 3%)
    6gm of Polyethylene Glycol M.W. 6,000 in 100ml Borate Buffer
    3gm of Polyethylene Glycol M.W. 6,000 in 100ml Borate Buffer

Borate Buffer
    0.1M H_3BO_3 (6.18gm/L)

    0.025M $Na_2B_2O_7 \cdot 10H_2O$ (9.53gm/L)

    0.075M NaCl (4.38gm/L)
    pH 8.3

Sucrose Solutions (10%, 40%, 60%)
    10gm, 40gm or 60gm of sucrose in 100ml of PBS

NET Buffer
    50mM Tris (6.05gm/L)
    150mM NaCl (8.7gm/L)
    pH 7.5

NET-1
    0.gm NP-40 in 100ml NET Buffer

NET-2
    0.05gm NP-40 in 100ml NET Buffer

PCA
    Phenol (redistilled) 50ml
    Chloroform 50ml
    Isoamylalcohol 1ml
    8 Hydroxyquinoline 0.1gm

TBS
    0.05M Tris-HCl (7.9gm/L)
    0.150M NaCl (8.7gm/L)
    pH 7.5

TBE
    0.1M Tris (12.11gm/L)
    0.1M Borate (6.18gm/L)
    0.002M EDTA (0.74gm/L)
    pH 8.3

Sample Buffer for RNA Electrophoresis
    7M Urea (420g/L)
    0.1% Bromophenol Blue
    0.1% Xylene Cyanol-FF
    dissolved in TBE

Acetate Buffer
    0.2M NaOAc (16.4gm/L)
    pH with 0.2M Acetic acid (12ml/L Glacial Acetic Acid)
    pH 4.0

Solution for Stacker Part of Protein Gels
    1.6ml of 2M Tris-HCl pH 6.8
    0.5ml of 0.2M EDTA
    0.5ml of 10% SDS
    34ml of H_2O

    9ml of 30% Acrylamide
    0.05ml of TEMED
    5ml of Ammonium Persulfate (0.38gm/25ml H_2O)

Solution for Lower Part of Protein Gels
    11.88ml of 2M Tris-HCl pH 8.8
    0.63ml of 0.2M EDTA
    0.63ml of 10% SDS
    24.38ml of H_2O

    18.75ml of 30% Acrylamide
    0.06ml of TEMED
    6.25ml of Ammonium Persulfate (0.38gm/25ml H_2O)

Running Buffer for Protein Gels (Per 4L Solution)
    Glycine 147gm
    Tris 24.2gm
    0.2M EDTA 40ml
    10% SDS 40ml
    pH 8.6

2X Sample Buffer for Protein Gels
    61.5 µl of 2M Tris
    400 µl of 10% SDS
    100 µl of B-Mercaptoethanol
    400 µl of Glycerol
    10 µl of 0.5% B-cartoene dye
    28.5 µl of H_2O
    pH 6.8

10X NT
    0.5M Tris-HCl (79gm/L)
    0.5M MgCl
    0.1M B-Mercaptoethanol
    500 µg/ml BSA
    pH 7.5

Hybridization Buffer
    50% Formamide (500ml/L)
    0.9M MaCl (52.6gm/L)
    0.05M EDTA (1.86gm/L)
    0.05M Na_2HPO_4 (7.1gm/L)
    0.1% SDS (1gm/L)
    1X Denhardt's Solution:
    0.02% BSA
    0.02% Ficoll
    0.02% Polyvinylpyrrolidone
    Salmon Sperm DNA (250 μg/ml), sonicated and denatured
    pH 7.0

B. Enzymes, Radionucleotides and Reagents

2'deoxyguanosine 5'triphosphate	New England Nuclear (NEN)
2'deoxythymidine 5'triphosphate	NEN
^{32}P-2'deoxyadenosine 5'triphosphate	NEN
^{32}P-2'deoxycytidine 5'triphosphate	NEN
DNA Polymerase I	Boehringer Mannheim
DNAse	Worthington
G25 Sephadex	Pharmacia
Pansorbin	Cal Biochem
Minimal essential medium	GIBCO
Antisera against whole serum	Meloy
$\quad$ IgG	Meloy
$\quad$ C_3	Meloy
$\quad$ C_4	Meloy
$\quad$ C_5	Meloy
$\quad$ fibrinogen	Meloy
$\quad$ ceruloplasmin	Meloy

C. Human Subjects

5-50ml of blood was obtained from 6 patients and 12 controls after obtaining informed consent. The consent consisted of a full explanation of the purpose for which the blood specimens will be used and the potential risks of venipuncture. All patients were followed at the Yale Rheumatology Clinics and had systemic lupus erythematosus as defined by the American Rheumatism Association (82).

D. Composition of Cryoprecipitates

1. Preparation of Serum. Approximately 100ml of blood was obtained by venipuncture from patients and controls and allowed to clot at 37°C for 30 min., followed by 30 min. at 4°C. The clots were

centrifuged at 1000g for 10 min. at 4°C in an ICC clinical centrifuge. 50ml of serum was recovered from each sample and clarified further by re-centrifuging for 10 min. Each was then divided into two equal aliquots of 25ml (A_1 and A_2).

2. Isolation of Cryoprecipitates. A_1 aliquot serum was incubated for 48 hr. at 4°C. The precipitate which formed was sedimented at 12,000g for 10 min. at 4°C in a Sorvall RC-5B centrifuge. The pellet was washed 3 times with 10ml of PBS at 4°C. The washing steps were carried out at 12,000g for 10 min. each. The thrice washed cryoprecipitate was re-solubilized in 1ml of PBS by incubating for 30 min. at 37°C. Any remaining insoluble material was removed by centrifugation. The cryoglobulins were allowed to re-precipitate at 4°C and were stored in this form (77).

3. Isolation of Putative Cryoprecipitogogues. All steps were carried out at 4°C unless otherwise noted. A_2 serum was added to an equal amount of 6% PEG in Borate buffer and incubated for 1 hr. The resulting suspension was sedimented at 12,000g for 10 min. and the pellet washed with 3% PEG in Borate buffer. The washed pellet was solubilized in 1ml PBS by incubating for 30 min. at 37°C. Any insoluble material was removed by centrifugation. The solution (containing the putative cryoprecipitogogue) was dialyzed in 1cm dialysis tubing for 24 hr. against 6 liters of PBS. The resulting precipitate was collected and stored in 1ml of PBS at 4°C (77,83).

4. Electrophoresis of Cryoprecipitates. Protein electrophoresis was carried out using a modified O'Farrell technique (86). A 9% acrylamide pH 8.8 gel was used with a 5% acrylamide pH 6.8 stacker. Gel dimension was 0.8 x 12 x 18cm. Gels ran at 100V for

4-6 hr. or at 50V overnight. Sample buffer was prepared at 2X concentration and mixed 1:1 (v/v) with the sample solution. This mixture was heated to 100°C for 1 min. before loading onto the gel. After the run was finished, gels were stained with either Coomassie Blue or "Silver Stain III" (87).

5. Double Immunodiffusion. Double immunodiffusion was performed using a modified Ouchterlony and Tan and Kunkel method (30,88) (Figure 2). 1% agarose in PBS, with 0.1% sodium azide was layered 1mm thick on glass microscope slides. The agarose was allowed to harden and 6 wells were punched out. 10 μl aliquots of solubilized cryoglobulins were placed into wells in the outside ring. The center well contained specific antisera. Diffusion was allowed to proceed for 24 hr. before reading.

E. Identification of snRNPs in Circulating Immune Complexes

1. Detection of Immune Complexes. Immune complexes in patient sera were quantified by Dr. John Hardin using the ^{125}I-C1q binding assay (89).

2. Isolation of Immune Complexes. Immune complexes were isolated by modification of the method previously described. These modifications were instituted to decrease the temperature and amount of time required for isolation with the goal of decreasing the degradation of RNA contained in the snRNP complexes by contaminating RNase. Briefly, blood was clotted for 30 min at 0°C. The clot was removed by centrifuging once at 1,000g for 10 min. at 0°C. The serum was added to an equal volume of 6% PEG in Borate buffer and slowly stirred for 1/2 hr. at 0°C. The precipitate was washed once with 3% PEG and resuspended in PBS (v/10 starting volume) (83).

3. <u>Sucrose Density Gradients</u>. The suspension (1-2ml) was layered on a 10%-40% linear sucrose density gradient with a 60% sucrose base. Nitrocellulose centrifuge tubes were used and the sample spun at 27,000 rpm for 28 hr. at $4^{\circ}C$ in a Beckman SW27 rotor. At the end of each run, gradient tubes were punctured from the bottom and fractions collected dropwise (approximately 1ml per fraction). The OD_{280} of each fraction was automatically recorded with a Pharmacia model UV-1 spectrophotometer which was connected in series with the fraction collector. The peak containing immune complexes was pooled and used immediately in the next step (77).

4. <u>Extraction of RNA</u>. The pooled immune complexes were adsorbed to Pansorbin, a commercial preparation of staphylococci protein A, according to the method of Kessler (90). All steps were carried out at $0^{\circ}C$ unless otherwise stated. Briefly, 2ml of Pansorbin was pelleted by centrifuging in a 10ml Corex tube at 7,000 rpm for 5 min. in a SS34 rotor. The pellet was washed once with NET-1 buffer and twice with NET-2 buffer. It was then resuspended in 2ml of NET-2 and saved up to four days. 100 μl of washed Pansorbin was incubated with the immune complex peak for 30 min. This step allows protein A to adsorb the immune complexes. The suspension was washed five times with NET-2 and resuspended in 400 μl of NET-2. This was then mixed with 400 μl of PCA in an Eppendorph tube and allowed to stand at room temperature for 25 min. The two-phase mixture was spun for 1 min. in a Beckman B microfuge and the aqueous (top) phase removed and saved in a new Eppendorph tube. 2 μl of carrier RNA (10mg/ml) was added along with 1.5ml of absolute ethanol. The tube was let stand at $-20^{\circ}C$ for 1-2 hr., until all RNA precipitated. The tube was then centrifuged

for 5 min., the supernatant poured off and any remaining ethanol lyophilized away.

5. Preparation of known snRNP Immune Complexes. snRNPs were isolated from HeLa cells and incubated with anti-Ro, anti-La and anti-Sm antisera (31). All steps were performed at $0^{\circ}C$ unless otherwise noted. HeLa cells were grown in minimal essential medium to a concentration of 4×10^5 cells/ml. 100ml of cells were sedimented at 2,000 rpm for 5 min. in a SS34 rotor and the supernatant discarded. The pellet was washed with 10ml of TBS and then resuspended in 2ml of TBS. The cells were broken by sonication for 3 x 30 sec. at setting 3 of a Branson sonifier. The suspension was clarified by centrifugation at 8,000 rpm for 10 min. and the supernatant retained as the source of antigen. 100 μl of antigen was incubated in an Eppendorph tube for 15 min. with 20 μl of known antisera kindly provided by Dr. John Hardin. On completion of the incubation step, 100 μl of washed Pansorbin was added and the RNA isolated as described in part 4.

6. Electrophoresis of RNA. RNA from immune complexes and control RNA were dissolved in 30 μl of sample buffer. The samples were heated at $60^{\circ}C$ for 5 min. to denature the RNA and then loaded onto the gel (31). In addition, ^{32}P labeled RNA standards, kindly provided by Dr. Joan Steitz, were run as controls to ensure proper transfer to Northern paper. The gel was 0.8 x 10 x 13cm and 10% acrylamide-4M urea. It was run at 300V for approximately 4 hr. (until the second dye was about to run off). TBE was used as the running buffer. Following completion of electrophoresis the gel was washed 3 x 20 min. with acetate buffer (31).

7. Northern Transfer. The Northern procedure allows

electrophoretically separated nucleic acids to be covalently bound to cellulose paper (91). The paper-RNA is used as a solid support for hybridization. All steps were carried out in a hood. Whatman 540 filter paper was cut to 10 x 12cm size and treated with a solution of NBPC and sodium acetate (2.3mg nitrobenzyloxymethyl pyridinium chloride and 0.7mg sodium acetate in 28.5 μl of water per square centimeter of paper) at 60°C for 3-5 min. or until the paper was nominally dry. The paper was then more fully dried in a 65°C oven for 20 min. and then at 135°C for 40 min. It was then washed four times with distilled water and three times with acetone. These steps result in the covalent linkage of n-nitrobenzyl groups to the cellulose. The paper was then incubated at 60°C for 30 min. in 150ml of 20% (w/v) sodium dithionite. It is then washed with water and 30% acetic acid. These steps result in the reduction of the nitro group to an analine group. The paper is incubated for 1/2 hr. in ice cold 1.2M HCl (0.3ml/cm^2) and sodium nitrate solution (2.7ml of 10mg/ml $NaNO_2$ solution per 100ml of HCl used). This converts the analine group into a diazo group that can covalently bind RNA. The transfer itself is similar to Southern's. Transfer is carried out overnight at room temperature (Figure 3) (91).

8. Preparation of Radioactive Probe. Plasmid DNA containing the U1-RNA gene was kindly provided by Dr. Alan Weiner. 1 μl of DNA (0.4 μg/ μl) is added to an Eppendorph tube containing 2.5 μl of 10X NT buffer and 2.5 μl of two nucleotides (10 μM), usually 2'deoxyguanosine 5'triphosphate and 2'deoxythymidine 5'triphosphate. 1 μl DNAse (100pg freshly diluted from 1mg/ml) is added and the mixture incubated at 25°C for 10 min. (47,92). The DNAse creates

numerous nicks in the plasmid. 7 µl of ^{32}P-2'deoxycytidine 5'-triphosphate and 2.5 µl (10 units) of DNA polymerase I are added and the mixture incubated at 15°C for 1 hr. In this step, the polymerase incorporates the hot and cold nucleotides in the spaces where nicks were previously created. The reaction is stopped by passing the mixture over a 3ml G25 Sephadex column equilibrated with 10mM Tris, pH 7.0. The first peak contains the radioactive probe which is then used for hybridization. 0.5ml fractions were collected from which 5 µl aliquots were withdrawn and tested for radioactivity in a Packard 3385 scintillation counter.

9. Hybridization. The Northern paper which is now covalently bound to the electrophoretically separated RNA is incubated with 15ml of hybridization buffer plus 1% glycine at 42°C for 48 hr. During this time, the hybridization vessel is constantly, gently agitated. After hybridization, the paper is washed 2 x 15 min. with 250ml of 2X SSC buffer plus 0.1% SDS, and then 2 x 15 min. with 250ml of 0.2X SSC plus 0.1% SDS at 45°C (91).

The paper is blotted dry, covered with Saran Wrap and placed on top of Kodak XRP-1 X-ray film. The film is exposed for 48-96 hr. at -70°C.

IV. RESULTS

A. Composition of Cryoprecipitates

1. Comparison of Cryoglobulins to PEG Precipitates.

Cryoglobulins are serum proteins that precipitate at low temperature.

Often, only very little precipitate is obtainable from normal serum.

PEG is known to precipitate immune complexes as well as large amounts

of other reversibly cryoprcipitable proteins (77,83). Since a large

supply of cryoglobulins is not easily available, it would be helpful

to know if PEG cuts could be used as a substitute. For this reason

the protein composition of PEG cuts was compared to cryoglobulins

obtained by precipitating in the cold.

Normal volunteers donated 100ml of blood each, from which 50ml of

serum was obtained. The sera were divided into two fractions; one

treated with PEG and the other left at 4°C to allow cryoglobulin to

precipitate. Three sets of cryoglobulins and three PEG cuts were

loaded into separate wells in an acrylamie gel (84,85). A lane

containing five standard proteins of MW 94K, 67K, 43K, 30K and 20K and

a lane containing IgG and IgM were also included. After completion of

the run, the gels were stained and photographed. A total of 20 bands

were visualized in each of the cryoglobulin and PEG lanes. Two of

these bands clearly corresponded to IgG and IgM. Each band from the

cryoglobulin samples matched up with a corresponding band from the PEG

samples. This experiment demonstrates that cryoglobulins and PEG cuts

of sera are identical in their protein composition (Figure 4).

The above experiment was repeated, only this time using serum

from patients with SLE. Again, 20 bands were visualized and there

were no differences noted between the PEG fraction and the cold

precipitated fractions.

2. Differences between Cryoprecipitates from Normal Sera and SLE
Sera. The differences in cryoprecipitates between normal and SLE sera
is of great importance. This information will increase our knowledge
of the mechanism of cryoprecipitation and aid our understanding of the
pathophysiology of lupus. In the first set of experiments,
cryoglobulins from SLE and normal sera were compared
electrophoretically (84,85). 1ml of serum was obtained from four SLE
patients and four healthy controls. Cryoglobulins were precipitated
with PEG and each sample loaded onto an acrylamide gel along with a
mixture of five standard proteins. After running for the appropriate
time, the gel was stained with Coomassie Blue or Silver Stain III
(86). As in the previous experiment, 20 bands were visualized in each
lane. The SLE lanes were identical to the normal cryoglobulin lanes
in their protein composition. Each protein band from the patient
samples had a matching counterpart in the control lanes. The protein
concentration of these samples were, however, markedly different.
Essentially, there was a generalized increase in concentration of
proteins from the SLE samples. Specifically, proteins with MW between
80K and 67K, and 30K and 20K were most markedly increased in
concentration (Figure 5). This evidence gives further support to the
theory that cryoprecipitation may be due solely to a concentration
effect.

Type III cryoglobulins contain polyclonal immunoglobulins as well
as numerous serum proteins. Some of these proteins have been
identified although the majority remain unknown. In order to
establish the identity of new proteins and confirm previous findings,

double immunodiffusion on Ouchterlony plates was performed (30,87).

1% agarose was used and the diffusion allowed to proceed for 24

hours. Cryoglobulins were obtained from the sera of six patients and

eight controls and tested against antisera to the following: C_3 , C_4 ,

C_5 , fibrinogen, ceruloplasmin, IgG and whole serum. As expected,

antisera against IgG and whole serum showed very strong reactions. C_3

and C_4 antisera each precipitated a line of identity with

cryoglobulins from four of six patients and five of seven controls.

C_5 showed only a weak reaction with one patient and no controls.

Fibrinogen and ceruloplasmin did not precipitate lines of identity

with any sera (Table 2).

B. Absence of Circulating snRNP Immune Complexes from Sera of Four
   Patients with SLE.

Lupus patients often possess antibodies against two extractible

nuclear antigens, Sm and RNP (31). The molecular identity of their

RNA has been established as the nucleoplasmic snRNA species U1 (RNP)

and U1, U2, U4-6 (Sm) (31). Although antibodies to these snRNPs have

been found in SLE sera, intact immune complexes have never been

identified. Recently, with the cloning of the U1 gene a powerful

technique to identify the presence of snRNP complexes has become

available.

Four patients with SLE donated 50ml of blood from which 25ml of

serum was recovered. All patients were previously found to possess

antibodies against Sm or RNP or both. Cryoglobulins were precipitated

with 6% PEG and loaded onto a 10-40% sucrose density gradient. After

completion of the run, the gradient was fractionated and each fraction

tested spectrophotometrically for its protein content. Three peaks

were identified (Figures 6, 7). The first peak (bottom of gradient)
represents sedimented debris. The second is found at the 19S level
and represents free serum proteins (77). Fractions representing the
second peak were pooled and adsorbed to Pansorbin. Any RNA that was
present was extracted with PCA and loaded onto a urea-acrylamide gel.
Control snRNP immune complexes were prepared and the RNA extracted as
described with the experimental samples. Likewise, ^{32}P labeled RNA
standards were also loaded. After completion of the gel run and
transfer procedure, the Northern paper was hybridized with the U1
probe.

An autoradiograph visualizes lanes 5, 6 and 7 (Figure 8). Lanes
5 and 6 represent control immune complexes and signify three things.
The first is that RNA could not have been lost or somehow degraded
during the extraction steps. The second is that the transfer
procedure was performed properly. The fact that the radiolabeled RNA
standards located in lane 7 are also visualized serves as a second
control for this step. Lastly, visualizing a band in lanes 5 and 6
indicates that hybridization must have taken place. Three reasons can
explain these findings. The first is that the method used is not
sensitive enough to visualize naturally occurring immune complexes.
This explanation is unlikely since the Northern procedure can
visualize as little as 10pg of RNA per band. The second reason is
that the RNA in the complexes was degraded by ribonucleases (29,30).
This is also unlikely since Sm is resistant to RNases and the
isolation procedure was carried out rapidly and at low temperature.
The third possibility is that circulating snRNP immune complexes are
not present in vivo.

V. DISCUSSION

A. Composition of Cryoprecipitates

In 1973, Creighton et al. reported that immune complexes can be precipitated by polyethylene glycol (83). Recently, Hardin described a group of reversibly cryoprecipitable non-immunoglobulin proteins which precipitate from normal sera treated with 3% PEG (77). Considered together, these immune complexes and serum proteins resemble the cryoglobulins first described by Lerner (59,60).

This work found that a PEG cut of normal serum is electrophoretically identical in its protein composition to cryoglobulins precipitated in the cold. For the proteins tested, the two samples are also immunologically identical. Studies of cryoglobulins can thus be carried out using PEG cuts rather than proteins precipitated in the cold. One immediate benefit is the reduction in time needed to perform the isolation steps from three days to just a few hours. Using PEG cuts will allow experiments to be run which were previously difficult and costly to perform. Large quantities of serum are required to obtain usable amounts of protein, even from SLE patients who have relatively large quantities of cryoglobulins. PEG causes nearly complete precipitation so even a small amount of serum provides sufficient amounts of cryoprecipitates.

When cryoglobulins from normal and lupus patients were electrophoretically compared, the band pattern showed that the protein composition of the samples is identical. The only noted difference was an increased concentration of proteins in the lupus samples. This result sheds some light on the mechanism of cryoprecipitation in SLE. First, it essentially rules out the possibility of disease specific

proteins as a cause of cryoglobulinemia. Since no new bands were
visualized in the SLE samples, cryoprecipitation must be due to the
increased concentration of one or more proteins normally present in
the serum. Although immunoglobulin was one of the components found
increased in concentration, it is unlikely that it is the predominant
cause of increased precipitation. As Hardin and others have pointed
out cryoprecipitation can occur in serum deficient in immune complexes
(77,78). Also, removing immune complexes from PEG cuts does not
eliminate the reversible cryoprecipitability of the remaining
proteins. Thus, Levo's, Erickson's and Christian's theories on
cryoprecipitability do not apply to SLE (62-64,68,69). There remain
two likely causes of cryoprecipitation. The most likely candidate for
a normal protein causing cryoprecipitation is fibronectin.
Fibronectin reversibly binds fibrinogen in the cold to form complexes
around which other proteins precipitate. Its removal from sera
significantly decreases the amount of precipitate that will form (75-
77). The other explanation is that cryoprecipitation is due to normal
molecular interactions of proteins in a saturated solution resulting
in precipitation in the cold. These two explanations are not mutually
exclusive and most likely act in concert. Hopefully, future
experiments will show the relative contribution of each mechanism.

Although cryoprecipitation can occur without immune complexes
being present, that cryoglobulinemia is intimately linked to an
aberrant immune response is undeniable. Consequently, it is not
surprising that this study found C_3, C_4 and possibly C_5 to be present
in cryoprecipitates. This evidence further extends our knowledge
about the protein identity of cryoprecipitates. Future experiments may

want to determine the location of the various proteins in an acrylamide gel by using radiolabeled antisera. This would unambiguously identify which proteins are increased in concentration in lupus sera and thus further elucidate the mechanism of cryoprecipitation.

B. Absence of Circulating snRNP Immune Complexes from Sera of Four Patients with SLE.

As mentioned previously, lupus patients have antibodies which bind to snRNPs in vitro and so are also thought to have the complexes in vivo. In the last part of this work, we set out to identify circulating snRNP immune complexes in the blood of lupus patients. The hybridization technique used is very sensitive and can detect less than 10pg of RNA per band (91). Controls were used at each step and were positive throughout.

snRNP complexes were not visualized. One explanation is that they do not exist in vivo. SLE patients produce a broad spectrum of antibodies. Although each is targeted against one particular antigen, they may cross react with an unrelated though antigenically similar compound. Thus, snRNPs may not be the in vivo target of these antibodies but rather represent a cross reaction in vitro. However, this can only be proven by exclusion (by ruling out all other explanations) or by finding another antigen in lupus sera which also reacts with the same antibodies.

A second explanation is that the techniques used were not sensitive enough to detect the immune complexes. In light of the sensitivity and specificity of the techniques used, this seems unlikely. However, it may be that while antibodies to snRNPs are

always present in the sera of some patients with SLE, the immune complexes themselves are only present during an acute phase of the disease. Once a patient gets over the acute phase, the immune complexes may be quickly cleared from the blood. Since blood samples were obtained during a quiescent phase, this may explain why no complexes were found.

A third explanation is that RNA portion of the snRNPs was degraded by ribonucleases during the isolation steps. This is also unlikely since all steps were carried out rapidly and at either $0^{\circ}C$ or $4^{\circ}C$. Furthermore, snRNPs are relatively insensitive to attack by ribonucleases (29,30,47). The last explanation is that the immune complexes were not found due to a technical error. This can only be proven by further repeating the experiments.
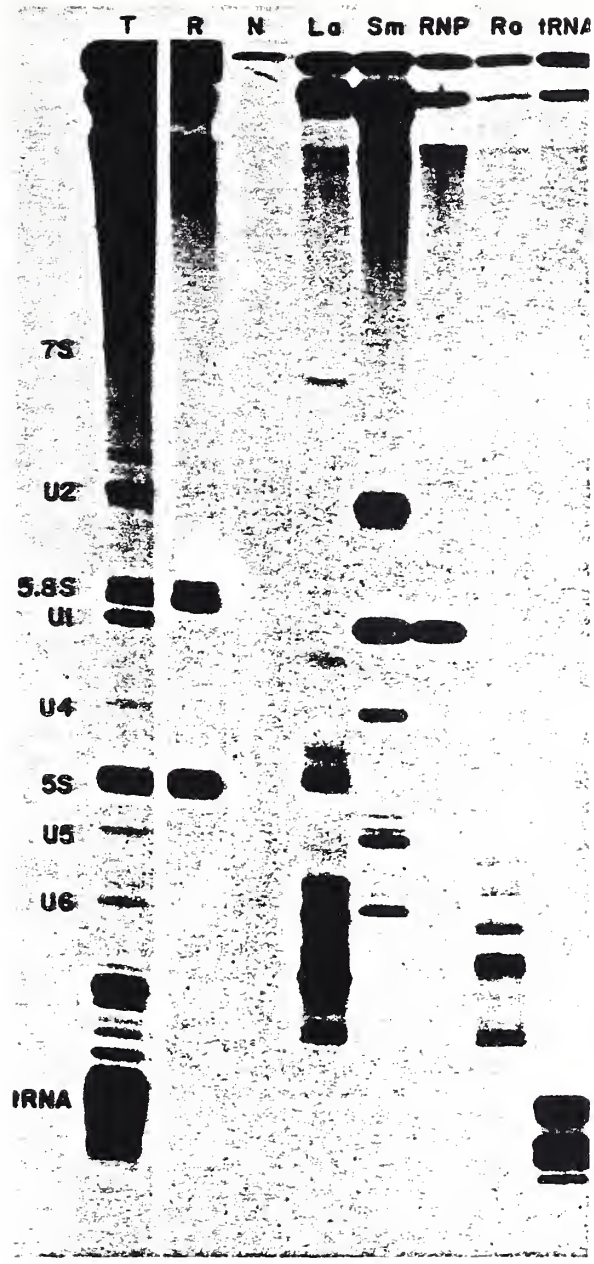
Figure 1. Polyacrylamide gel electrophoresis of ^{32}P-labeled nucleic acids from HeLa cells. Total cell sonicate (lane T). Bands in other lanes were obtained by immunoprecipitation of cell extracts with patient anti-rRNA (lane R), normal human serum (lane N), patient anti-La (lane La), patient anti-Sm (lane Sm), patient anti-RNP (lane RNP), patient anti-Ro (lane Ro), and patient anti-tRNA (lane tRNA). The bands labeled 5.8S and 5S are characteristic of rRNA. See text for the description of other bands.

Figure 1. From Lerner, E.A., Lerner, M.R., Hardin, J.A., Janeway, C.A. Jr., and Steitz, J.A. Deciphering the mysteries of RNA-containing lupus antigens. **Arthritis Rheum.** 25:761-766, 1982.

| Antibody | % Frequency | |
Specificity	SLE	MCTD
dsDNA	50-60	5
ssDNA	50-60	20-25
Histones	25-30	5
Sm	25-30	5
RNP	30-40	95-100
La	10-15	5
Ro	30-40	5
PcNA	10	5

Table 1. Specificities of ANAs found in SLE and in mixed connective tissue disease (MCTD). From Tan, E.M. Special antibodies for the study of systemic lupus erythematosus: An analysis. **Arthritis Rheum.** 25:753-756, 1982.
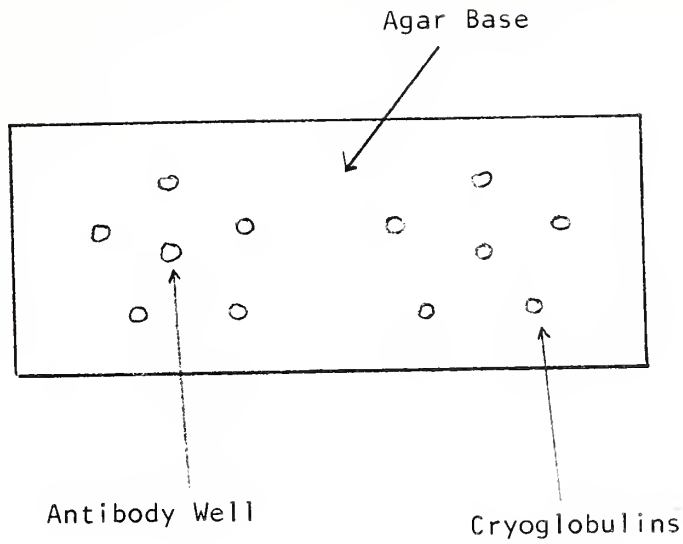
Figure 2. Diagram of a double immunodiffusion slide. 1% agarose in PBS was layered 1mm thick on a glass microscope slide. Six wells were punched out on both halves of the slide. The center well contained the antisera and the five surrounding wells contained cryoglobulins from various patients and controls.
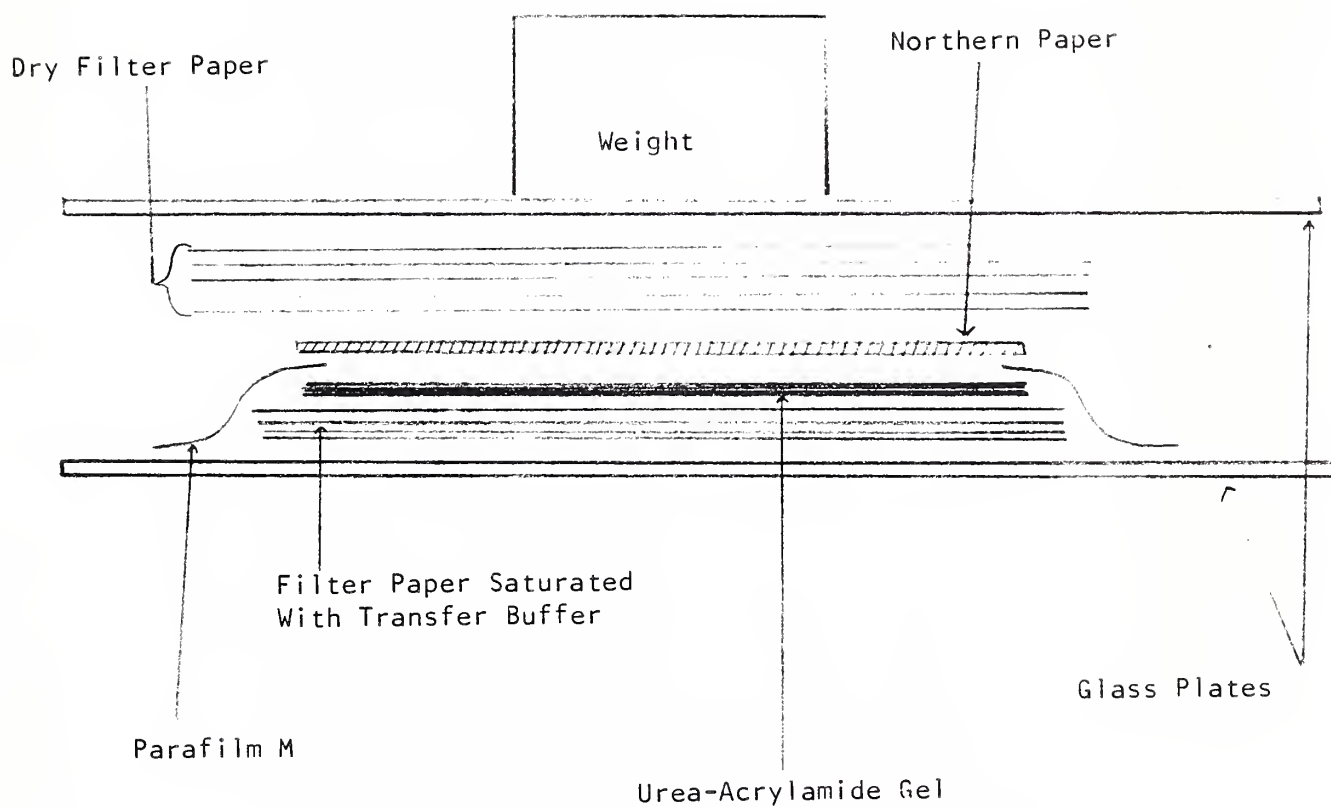
Figure 3. Apparatus used to transfer RNA from a urea-acrylamide gel to Northern paper. Transfer is carried out overnight at room temperature.
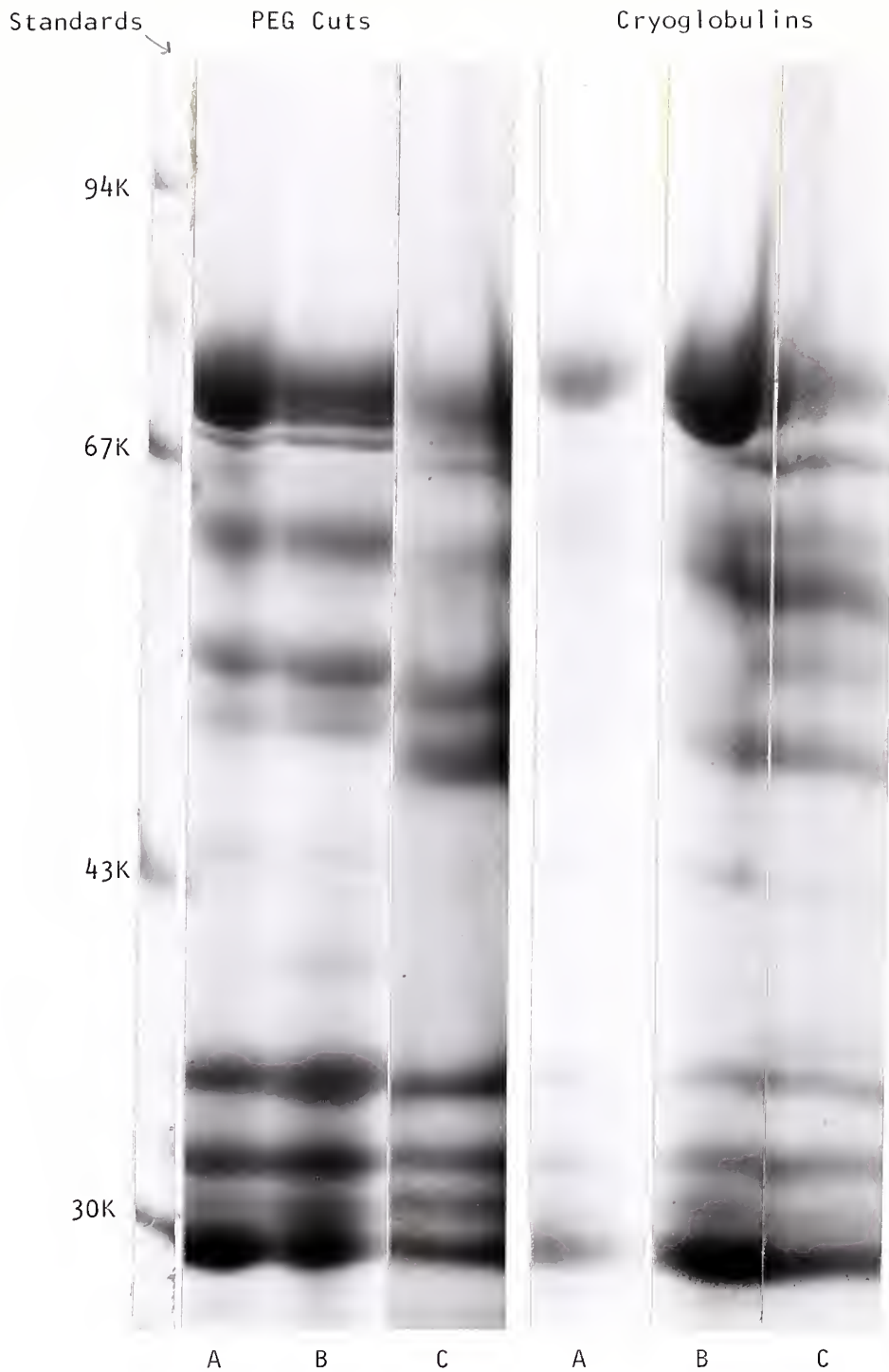
Figure 4. Comparison of PEG cuts to cryoglobulins precipitated in the cold. Sera was obtained from three patients: A, B, C. Control lane had proteins of MW 94K, 67K, 43K, 30K, and 20K.

94K

67K

43K

30K

20K

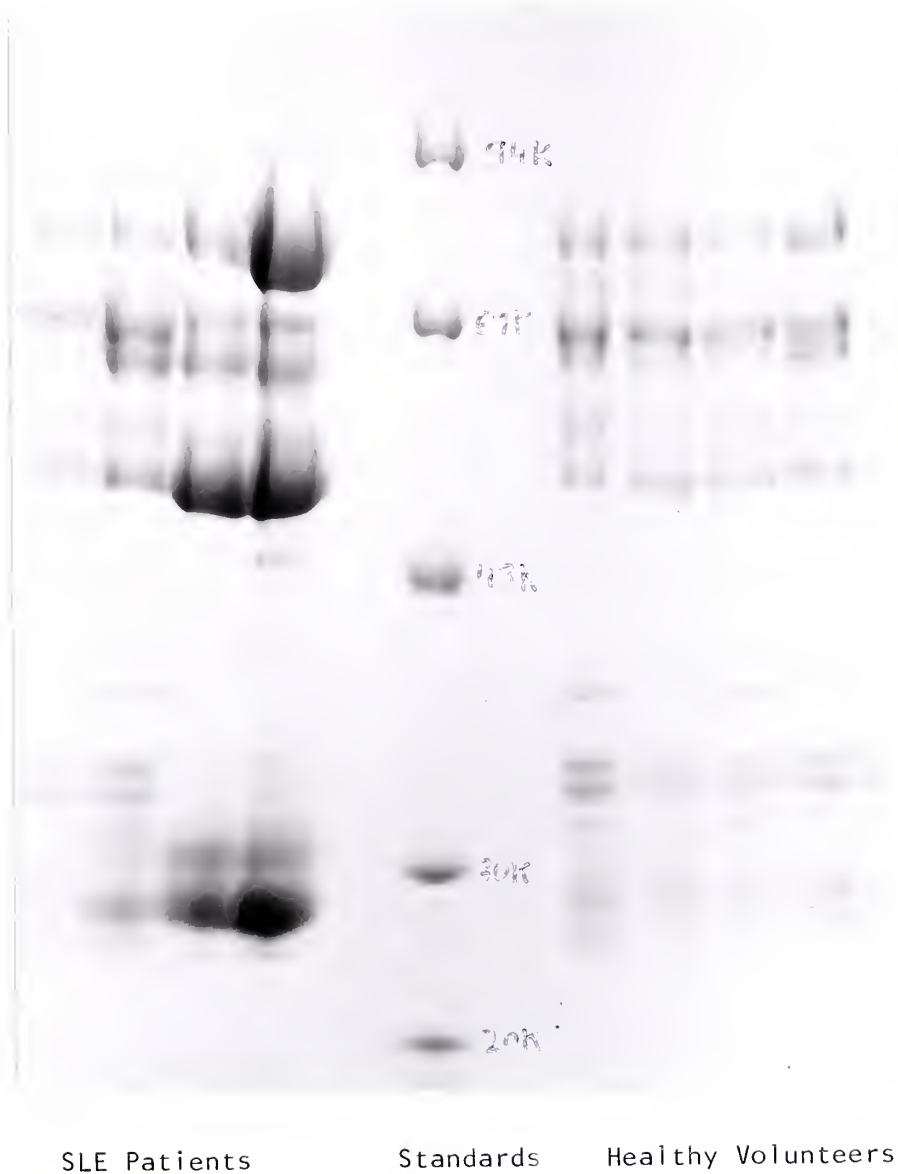SLE Patients   Standards   Healthy Volunteers

Figure 5. Comparison of protein precipitate from 1ml of SLE sera and 1ml of sera from healthy volunteers. Four patients and four volunteers were used and proteins were precipitated with PEG. Control lane contains proteins of MW 94K, 67K, 43K, 30K and 20K.

| | HEALTHY CONTROLS | | | | | | | | SLE PATIENTS | | | | |
Antisera Against	A	B	C	D	E	F	G	H	MS	SM	RC	DJ	IL
C3	+	+	+	+	−	−	+	+	+	+	−	+	−
C4	+	−	−	+	+	+	+	+	+	+	−	+	−
C5	+/−	−	−	−	−	−	−	−	−	−	−	−	−
Fibrinogen	−	−	−	−	−	−	−	−	−	−	−	−	−
Ceruloplasmin	−	−	−	−	−	−	−	−	−	−	−	−	−
IgG	+	+	+	+	−	+	+	+	+	+	−	+	+
Whole Serum	+	+	+	+	+	+	+	+	+	+	+	+	+

PEG   CRYO

Table 2.   Above table shows the results of a double immunodiffusion experiment. Antisera against C3, C4, C5, fibrinogen, ceruloplasmin, IgG and whole serum was diffused against cryoglobulins from eight healthy controls and five SLE patients. Cryoglobulins from controls A-E were precipitated with PEG while controls F-H were precipitated in the cold. All patient sera was precipitated with PEG.
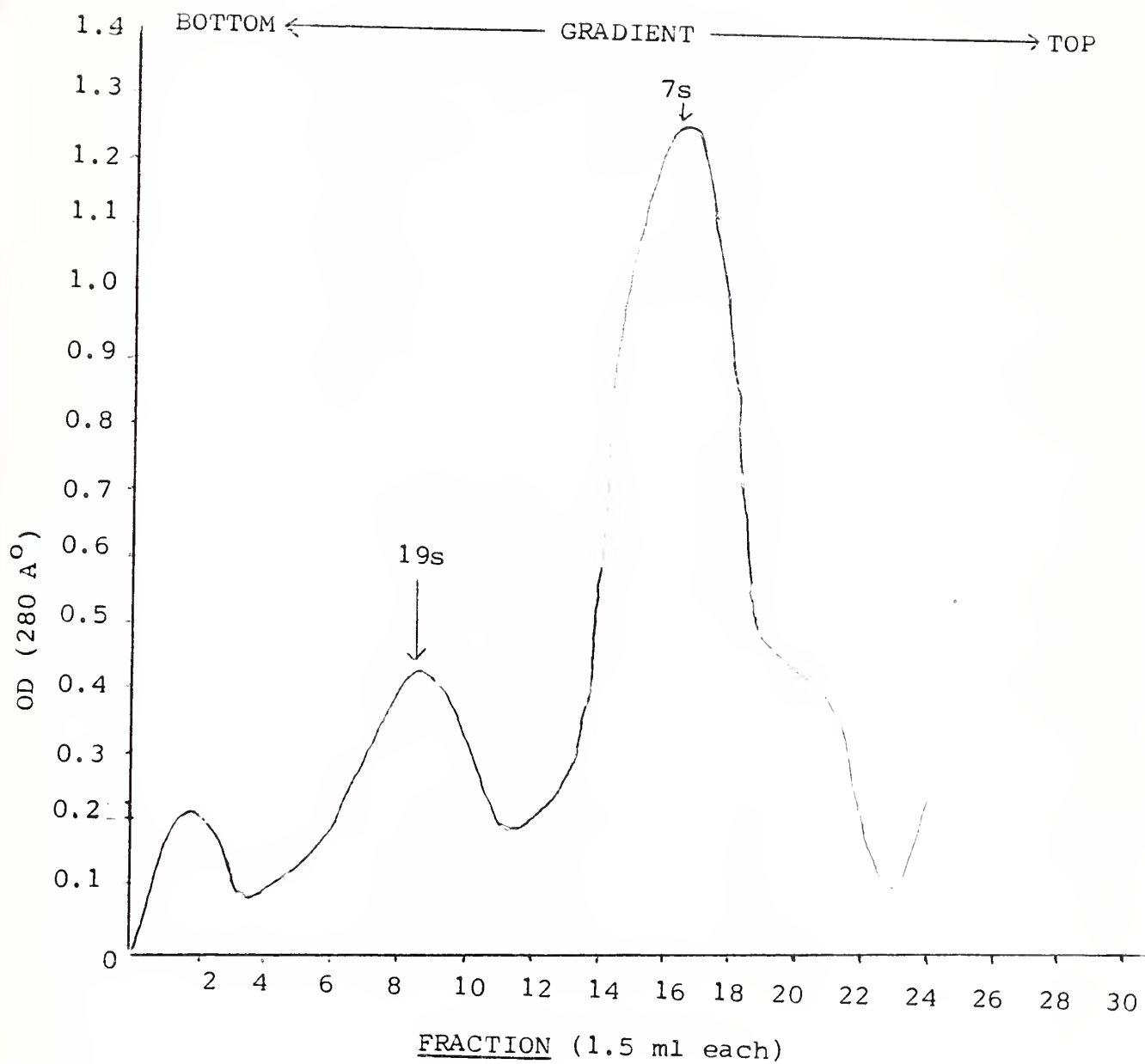
Figure 6. 10-40% sucrose density gradient of PEG cuts of SLE serum. Middle peak, centered around fraction 8, represents the immune complexes. Gradient was spun at 27K, at 4°C for 18 hours.
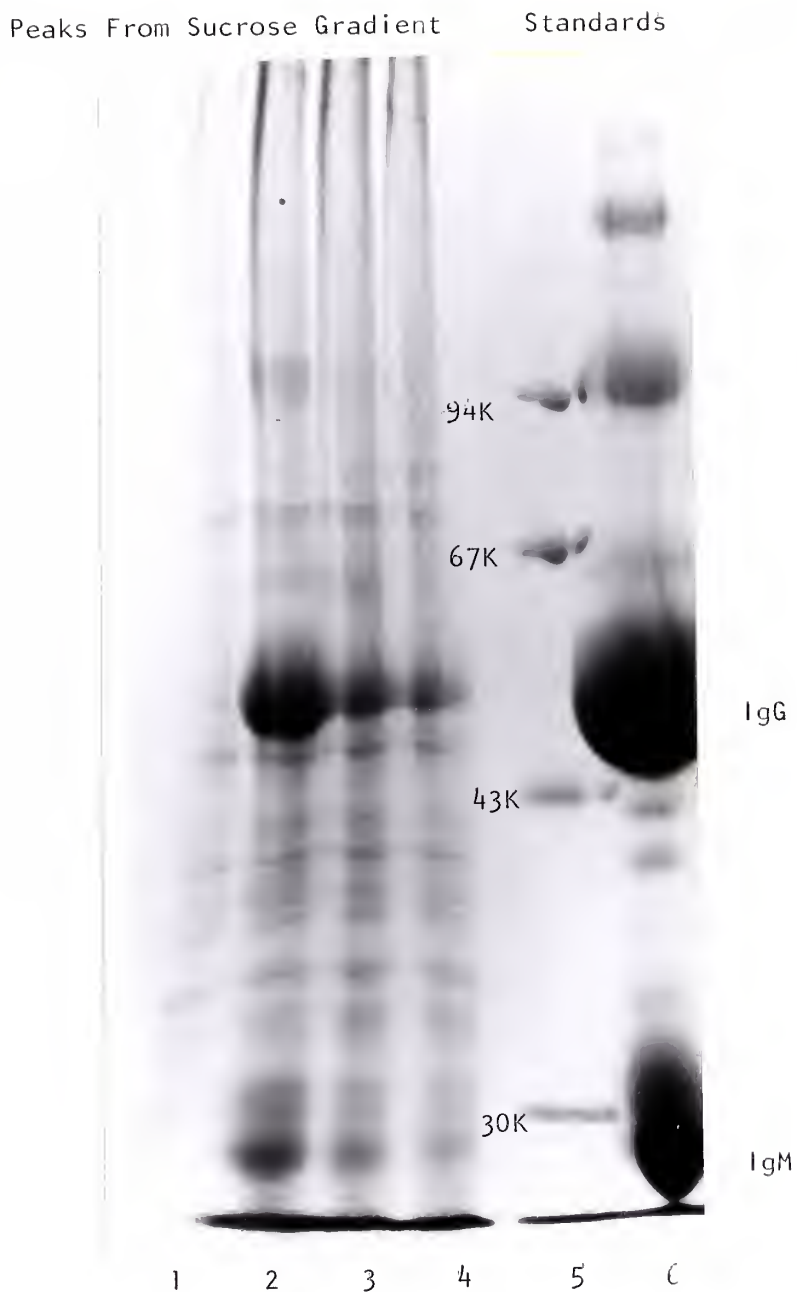
Peaks From Sucrose Gradient   Standards

Figure 7. Acrylamide gel of peaks 1, 2, 3, and 4 which are centered around fractions 2, 8, 18, and 24, respectively, from the 10-40% sucrose density gradient (Figure 6). Lane 5 contains protein standards of MW 94K, 67K, 43K, 30K and 20K. Lane 6 contains IgG and IgM. As can be seen, peak 2 contains the immune complexes.

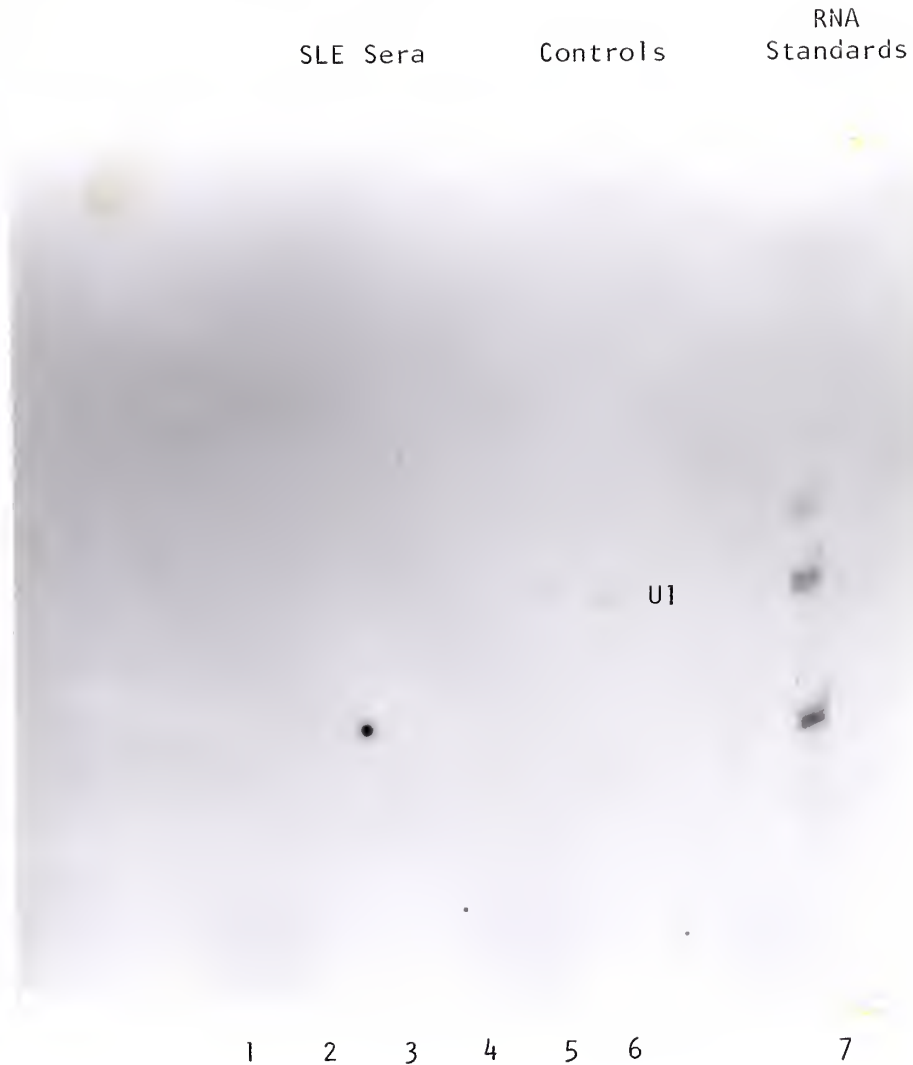**Figure 8.** Autoradiograph of Northern paper visualizes lanes 5, 6, and 7. Lanes 1-4 were to contain snRNA from immune complexes of four SLE patients. No RNA was visualizd. Lanes 5 and 6 contained Sm and RNP control immune complexes, respectively. Lane 7 shows radiolabeled RNA standards. The fact that lanes 5-7 are visualized signifies that RNA extraction, electrophoresis, transfer and hybridization were correctly performed.

VI. REFERENCES

1. Stevens, M.B. Systemic lupus erythematosus. In: The Principles and Practice of Medicine, Harvey, Johns, McKasick, Owens and Ross, eds. Appleton-Century-Crofts, New York, 1980. pp. 1115-1121.

2. Robinson, D.R. Systemic lupus erythematosus. In: Scientific American: Medicine, Rubenstein and Federman, eds. Scientific American, New York, 1981. pp. 15-1 - 15-14.

3. Fessel, J.W. Systemic lupus erythematosus in the community. Arch. Int. Med. 134:1027-35, 1974.

4. Rothfield, N.F. Systemic lupus erythematosus -- Immunologic aspects. In: Arthritis and Allied Conditions, McCarty, ed. Lea and Febiger, Philadelphia, 1979. pp. 691-714.

5. Gershon, R.K., Eardley, D.D., Naidorf, K. and Cantor, H. Association of defective feedback suppressor T cell activity with autoimmunity in NZB mice. Arthritis Rheum. 21:S180-S184 (supplement, June), 1978.

6. Gibofsky, A., Winchester, R., Hansen, J., Peterroyo, M., Dupont, B., Paget, S., Lahita, R., Halper, J., Fotino, M., Yunis, E. and Kunkel, H.G. Contrasting patterns of newer histocompatibility determinants in patients with rheumatoid arthritis and systemic lupus erythematosus. Arthritis Rheum. 21:S134-S138 (supplement, June), 1978.

7. Christian, L.L. Clues from genetic and epidemiologic studies. Arthritis Rheum. 21:S130-S133 (supplement, June), 1978.

8. Agnello, V. Association of systemic lupus erythematosus and SLE like syndromes with hereditary and acquired complement deficiency states. Arthritis Rheum. 21:S146-S152 (supplement, June), 1978.

9. Mellors, R.C. and Mellors, J.W. Type C RNA virus expression in SLE: New Zealand mouse model and human disease. Arthritis Rheum. 21:S68-S75 (supplement, June) 1978.

10. Phillips, P.E. Type C oncorna virus studies in systemic lupus erythematosus. Arthritis Rheum. 21:S76-S81 (supplement, June), 1978.

11. Schur, P.H. Systemic lupus erythematosus. In: Textbook of Medicine, Wyngaarden and Smith, eds. W.B. Saunders, Philadelphia, 1982. pp. 1852-1856.

12. Tan, E.M., Schur, P.H., Carr, R.I. and Kunkel, H.G. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J. Clin. Invest. 45:1732-1740, 1966.

13. Koffler, D., Schur, P.H. and Kunkel, H.G. Immunological studies concerning the nephritis of systemic lupus erythematosus. J. Exp. Med. 126:607-623, 1967.

14. Rothfield, N.F. Systemic lupus erythematosus. In: Rheumatic Diseases: Diagnosis and Management, Katz, W.A. ed., J.B. Lippincott, Philadelphia, 1977. pp. 756-775.

15. Hargraves, M.M., Richmond, H. and Morton, R. Presentation of two bone marrow elements: The "tart" cell and the "LE" cell. Proc. Staff Meet. Mayo Clin. 23:25-28, 1948.

16. Holman, H.R. and Kunkel, H.G. Affinity between the lupus erythematosus serum factor and cell nuclei and nucleoprotein. Science 126:162, 1957.

17. Seligmann, M. and Milgram, F. Mise en evidence par la fixation du complement de la reaction entre acide desoxyribonucleique et serum de malades atteints de lupus erythemateux dissemine. Comp. Rend. Acad. Sci. 245:1472, 1957.

18. Ceppellin, R., Polli, E. and Celada, F. A DNA-reacting factor in serum of a patient with lupus erythematosus diffusus. Proc. Soc. Expec. Biol. Med. 96:572, 1957.

19. Robbins, W.C., Holman, H.R., Deicher, H. and Kunkel, H.G. Complement fixation with cell nuclei and DNA in lupus erythematosus. Proc. Soc. Exper. Biol. Med. 96:575, 1957.

20. Friou, G.J., Finch, S.C. and Detre, K.D. Anti-DNA immunoglobulin from lupus erythematosus serum demonstrated with fluorescent antibody. J. Immunol. 80:324, 1958.

21. Deicher, H.R.G., Holman, H.R. and Kunkel, H.G. The precipitin reaction between DNA and a serum factor in systemic lupus erythematosus. J. Exp. Med. 109:97-114, 1959.

22. Atchley, W.A. An immunoelectrophoretic analysis of antigens reacting with lupus serum. Arthritis Rheum. 4:471, 1961.

23. Winfield, J.B., Koffler, D., and Kunkel, H.G. Specific concentration of polynucleotide immune complexes in the cryoprecipitates of patients with systemic lupus erythematosus. J. Clin. Invest. 56:563-570, 1975.

24. Stollar, D., Levine, L., Lehrer, H.I. and van Vunakis, H. The antigenic d eterminants of denatured DNA reactive with lupus erythematosus serum. Proc. Natl. Acad. Sci. USA 48:874-880, 1962.

25. Koffler, D. and Kunkel, H.G. Antibodies to polynucleotides in human sera: Antigenic specificity and relation to disease. J. Exp. Med. 134:294-312, 1971.

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

27. Tan, E.M. Special antibodies for the study of systemic lupus erythematosus: An analysis. Arthritis Rheum. 25:753-756, 1982.

28. Stollar, D. and Levine, L. Antibodies to denatured deoxyribonucleic acid in lupus erythematosus serum. IV. Evidence for purine determinants in DNA. Arch. Biochem. Biophys. 101:417, 1963.

29. Holman, H.R. Partial purification and characterization of an extractible nuclear antigen which reacts with SLE sera. Ann. N.Y. Acad. Sci. 124:800-806, 1965.

30. Tan, E.M. and Kunkel, H.G. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. J. Immunol. 96:464-471, 1966.

31. Lerner, M.R. and Steitz, J.A. Antibodies to small nuclear RNAs complexes with proteins are produced by patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA 76:5495-5499, 1979.

32. Ro-Chai, T.S. and Busch, H. The Cell Nucleus, Vol. 3, Busch, H., ed. Academic Press, N.Y., 1974. pp. 151-208.

33. Northway, J.D. and Tan, E.M. Differentiation of antinuclear antibodies giving speckled staining patterns in immunofluorescence. Clin. Immunol. Immunopathol. 1:140-154, 1972.

34. Mattioli, M. and Reichlin, M. Characterization of a soluble nuclear ribonucleoprotein antigen reactive with SLE sera. J. Immunol. 107:1281-1290, 1971.

35. Lerner, E.A., Lerner, M.R., Hardin, J.A., Janeway, C.A. Jr. and Steitz, J.A. Deciphering the mysteries of RNA-containing lupus antigens. Arthritis Rheum. 25:761-766, 1982.

36. McGilvery, R.W. Biochemistry: A Functional Approach, 3rd edn., W.B. Saunders, Philadelphia, 1983. pp. 58-79.

37. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. Are snRNPs involved in splicing? Nature 283:220-224, 1980.

38. Rogers, J. and Wall, R. A mechanism for RNA splicing. Proc. Natl. Acad. Sci. USA 777:1877, 1980.

39. Reddy, R., Ro-choi, T.S., Henning, D. and Busch, H. Primary sequence of U_1 nuclear ribonucleic acid of Novikoff hepatoma ascites cells. J. Biol. Chem. 249:6486-6494, 1974.

40. Shibata, H., Ro-Choi, T.S., Reddy, R., Choi, Y.C., Henning, D. and Busch. H. The primary nucleotide sequence of nuclear U_2 ribonucleic acid. J. Biol. Chem. 250:3909-3920, 1975.

41. Zieve, G. and Penman, S. Small RNA species of the HeLa cell: Metabolism and subcellular localization. Cell 8:19-31, 1976.

42. Yang, V.W., Lerner, M.R., Steitz, J.A. and Flint, S.J. A small ribonucleoprotein is required for splicing of adenoviral early RNA sequences. Proc. Natl. Acad. Sci. USA 78:1371-1375, 1981.

43. Gottlieb, E. Personal communication.

44. Clark, G.M., Reichlin, M. and Tomasi, T.B. Characterization of a soluble cytoplasmic antigen reactive with sera from patients with systemic lupus erythematosus. J. Immunol. 102:117-122, 1968.

45. Maddison, P.J., Provost, T.T. and Reichlin, M. Serological findings in patients with "ANA negative" systemic lupus erythematosus. Medicine 60:87-94, 1981.

46. Reichlin, M. Clinical and immunological significance of antibodies to Ro and La in systemic lupus erythematosus. Arthritis Rheum. 25:767-772, 1982.

47. Alspaugh, M.A. and Madison, P.J. Resolution of the identity of certain antigen-antibody systems in systemic lupus erythematosus and Sjogren's syndrome: An interlaboratory collaboration. Arthritis Rheum. 19:216-222, 1976.

48. Notman, D.D., Kurata, N. and Tan, E.M. Profiles of antinuclear antibodies in systemic rheumatic disease. Ann. Intern. Med. 83:464-469, 1975.

49. Franco, H.L., Weston, W.L., Peebles, C., Forstot, S.L. and Phanuphol, P. Autoantibodies directed against sicca syndrome antigens in the neonatal lupus syndrome. J. Amer. Acad. Derm. 4:67-77, 1981.

50. Weston, W.L., Harmon, C., Peebles, C., Manchester, D., Franco, H.L., Huff, J.C. and Norris, D.A. A serological marker for neonatal lupus. Brit. J. Derm. 107:377-382, 1982.

51. Kephart, D.C., Hood, A.F. and Provost, T.T. Neonatal lupus erythematosus: New serologic findings. J. Invest. Derm. 77:331-333, 1981.

52. Sharp, G.C., Irvin, W.S., Tan, E.M., Gould, G. and Holman, H.R. Mixed connective tissue disease -- an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractible nuclear antigen (ENA). Am. J. Med. 52:148-159, 1972.

53. Sharp, G.C., Irvin, W.S., May, C.M., Holman, H.R., McDuffie,
     F.C., Hess, F.V. and Schmid, F.R. Association of autoantibodies
     to ribonucleoprotein and Sm antigens with mixed connective tissue
     disease, systemic lupus erythematosus and other rheumatic
     diseases. N. Engl. J. Med. 259:1149-1154, 1976.

54. Leibfarth, J.H. and Persellin, R.H. Characteristics of patients
     with serum antibodies to extractible nuclear antigens. Arthritis
     Rheum. 19:851-856, 1976.

55. Parker, M.D. Ribonucleoprotein antibodies: Frequencies and
     clinical significance is systemic lupus erythematosus,
     scleroderma, and mixed connective tissue disease. J. Lab. Clin.
     Med. 82:769-775, 1973.

56. Wolfe, J.F., Kingsland, L., Lindberg, D.A. and Sharp, G.C.
     Disease pattern in patients with antibodies only to nuclear
     ribonucleoprotein (RNP). Clin. Res. 25:488A, 1977.

57. Winn, D.H., Gerhardt, D., Winship, D. and Sharp, G.C. Esophageal
     function in steroid treated patients with mixed connective tissue
     disease 9MCTD). Clin. Res. 24:545A, 1976.

58. Winn, D.M., Wolfe, J.F., Lindberg, D.A., Fristoe, F.H.,
     Kingsland, L. and Sharp, G.C. Identification of a clinical
     subset of systemic lupus erythematosus by antibodies to the Sm
     antigen. Arthritis Rheum. 22:1334-1337, 1979.

59. Lerner, A.B., and Watson, C.J. Studies of cryoglobulins. I.
     Unusual purpura associated with the presence of a high
     concentration of cryoglobulin. Amer. J. Med. Sci. 214:410-415,
     1947.

60. Lerner, A.B., Barnum, C.P. and Watson, C.J. Studies of
     cryoglobulins. II. The spontaneous precipitation of proteins
     from serum at 5°C in various disease states. Amer. J. Med. Sci.
     214-416, 1947.

61. Brouet, J.-C., Clauvel, J.-P., Danon, F., Klein, M. and
     Seligmann, M. Biologic and clinical significance of
     cryoglobulins. A report of 86 cases. Am. J. Med. 57:775-788,
     1974.

62. Levo, Y. Nature of cryoglobulinemia. Lancet i:285-286, 1980.

63. Levo, Y. Clinical, biological and biochemical aspects of
     cryoglobulins. Int. J. Derm. 20:590-591, 1981.

64. Zinneman, M.M., Levi, D. and Seal, U.S. On the nature of
     cryoglobulins. J. Immunol. 100:594-603, 1968.

65. McIntosh, R.M., Kaufman, D.B. and Kulvinskas, C. Cryoglobulins: II. The biologic and chemical properties of cryoglobulins in acute poststreptococcal glomerulonephritis. Int. Arch. Allergy Appl. Immunol. 41:700-715, 1971.

66. Hickman, S. and Kornfeld, S. Effect of tunicamycin on IgM, IgA, and IgG secretion by mouse plasmacytome cells. J. Immunol. 121:990-996, 1978.

67. Buxbaum, J. The biosynthesis, assembly and secretion of immunoglobulins. Sem. Hematol. 10:33-52, 1973.

68. Erickson, B.W., Gerber, Jenson, B., Wang, A.-C. and Litman, G.W. Molecular basis for the temperature dependent insolubility of cryoglobulins. XI. Sequence comparison of the heavy chain variable regions of the human cryoimmunoglobulins McE and Hil by metric analysis. Molec. Immunol. 19:357-365, 1982.

69. Christian, C.L., Hatfield, W.B. and Chase, P.H. Systemic lupus erythematosus. Cryoprecipitation of sera. J. Clin. Invest. 42:823-829, 1963.

70. Mosesson, M.W., Chen, A.B. and Huseby, R.M. The cold-insoluble globulin of human plasma: Studies of its essential structural features. Biochim. Biophys. Acta 386:509-524, 1975.

71. Mosher, D.F. Cross-linking of cold insoluble globulin by fibrin-stabilizing factor. J. Biol. Chem. 250:6614-6621, 1975.

72. Hynes, R.O. and Destree, A. Extensive disulfide bonding at the mammalian cell surface. Proc. Natl. Acad. Sci. USA 74:2855-2859, 1977.

73. Mosesson, M.W. Structure of human plasma cold-insoluble globulin and the mechanism of its precipitation in the cold with heparin or fibrin-fibrinogen complexes. Ann. N.Y. Acad. Sci. 312:11-30, 1978.

74. Ruoslahti, E. and Vaheri, A. Interaction of soluble fibroblast surface antigen with fibrinogen and fibrin. J. Exp. Med. 141:497-501, 1975.

75. Beaulieu, A.D., Vallet, J.P., Strevey, J., Kimura, A. and Wigzell, H. Fibronectin is partly responsible for the formation of cryoprecipitates in synovial fluids of rheumatoid arthritis patients. Arthritis Rheum. 23:653, 1980 (Abstract).

76. Levo, Y. Presence of fibronectin in cold precipitates of patients with cryoglobulinemia. Int. Arch. Allergy Appl. Immunol. 68:179-181, 1982.

77. Hardin, J.A. Cryoprecipitatogogue from normal serum: A mechanism for cryoprecipitation of immune complexes. Proc. Natl. Acad. Sci. USA 78:4562-4565, 1981.

78. Hardin, J.A., Steere, A.C. and Malawista, S.E. Immune complexes and the evolution of Lyme arthritis: Dissemination and localization of abnormal C1q binding activity. New Eng. J. Med. 301:1358-1363, 1979.

79. Mattioli, M. and Reichlin, M. Heterogeneity of RNA protein antigens reactive with sera of patients with systemic lupus erythematosus. Arthritis Rheum. 17:421-429, 1974.

80. Singsen, B.H., Swanson, V.L., Bernstein, B.H., Heuser, E.T., Hanson, V. and Landing, B.H. A histologic evaluation of mixed connective tissue disease in childhood. Am. J. Med. 68:710-717, 1980.

81. Mattioli, M. and Reichlin, M. Physical association of two nuclear antigens and mutual occurrence of their antibodies: The relationship of the Sm and RNA-protein (Mo) systems in SLE sera. J. Immunol. 110:1318-1324, 1973.

82. Cohen, A.S., Reynolds, W.E., Franklyn, E.C., Kulka, S.P., Ropes, M.W., Shulman, L.E. and Wallace, S.L. Bull. Rheum. Dis. 21:643-648, 1971.

83. Creighton, W.D., Lambert, P.H. and Miescher, P.A. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. J. Immunol. 111:1219-1227, 1973.

84. O'Farrell, P.H. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021, 1975.

85. O'Farrell, P.Z. and Goodman, H.M. Resolution of simian virus 40 proteins in whole cell extracts by two-dimentional electrophoresis: Heterogeneity of the major rapsid protein. Cell 9:289-298, 1976.

86. Merril, C.R., Goldman, D., Sedman, S.A., Ebert, M.H. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211:1437-1438, 1981.

87. Ouchterlony, O. Progress in Allergy, Vol. 6, Kallos, P. and Waksman, B.H., eds. Karger, New York, 1962. p. 30.

88. Rosenblum, K. Intravenous vs. intraportal transplantation of islet tissue from adult donors into syngeneic diabetic rat recipients. M.D. Thesis, Yale University, 1984.

89. Zubler, R.H., Nydegger, U., Perrin, L.H., Fehr, K., McCormick, J., Lambert, P.H. and Miescher, P.A. Circulating and intra-articular immune complexes in patients with rheumatoid arthritis. J. Clin. Invest. 57:1308-1319, 1976.

90. Kessler, S.W. Cell membrane antigen isolation with the
     staphylococcal protein A-antibody adsorbent. J. Immunol.
     117:1483-1490, 1980.

91. Alwine, S.C., Kemp, D.J., Parker, B.A., Reiser, J., Renart, J.,
     Stark, G.R. and Wahl, G.M. Detection of specific RNAs or
     specific fragments of DNA by fractionation in gels and transfer
     to diazobenzyloxymethyl paper. Methods Enzymol. 68:220-242,
     1979.

92. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. Labeling
     deoxyribonucleic acid to high specific activity in vitro by nick
     translation with DNA polymerase I. J. Mol. Biol. 113:237-251,
     1977.

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