

Immunology and Diseases of Connective Tissue*

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Since immune responses play a major role in the development of connective tissue diseases, it is not surprising that a number of laboratory studies reflect these responses. Prior to the 1940s when rheumatoid and LE factors became widely known, one relied mainly on erythrocyte sedimentation rate and serum electrophoresis to identify protein abnormality. Elevated sedimentation rate depends on rouleaux formation, and rouleaux formation is dependent upon large asymmetric molecules of fibrinogen and gamma globulin in plasma. The demonstration of gamma globulin has become the cornerstone of the immunologist's edifice. It is amazing to see how the subspecialty of immunology has mushroomed to involve the many facets of disease processes such as connective tissue diseases, skin diseases, gastrointestinal diseases, renal diseases, and cancer. More recently, immune deficiency diseases have included the pediatrician in the ever-enlarging field of immunology as has the modern-day discovery of human leukocyte antigen (HL-A) testing and tissue typing included the geneticist.

In this paper, I will discuss four connective tissue diseases—rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, and mixed connective tissue disease syndrome—and the role immunological processes play in their pathogenesis. I will also comment upon certain laboratory tests that use immunological methods in making a diagnosis.

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1. Rheumatoid Arthritis.

It is believed that an immune process plays a role in the perpetuation of rheumatoid arthritis and there are at least five items which may provide evidence for this hypothesis.

1. Lymphoid cell infiltration of the synovial membrane with follicle formation. Note similarity to architecture of lymph node (Figs 1A and 1B).
2. Local synthesis of IgG and rheumatoid factor by plasma cells in the synovium as demonstrated by Smiley et al.¹
3. Decreased synovial fluid complement in some cases, and in other cases decreased serum complement as demonstrated by Ruddy and Schur.^{2,3}
4. Presence of IgG and IgM and complement components in the synovial lining cells and in remote sites of tissue damage indicating that some immunological process is taking place also at distant sites.⁴
5. Presence of rheumatoid factor components and complement in leukocytes of synovial fluid cells.⁵

The current concept is that IgG is produced in rheumatoid synovial membrane by an unknown stimulus; this in turn stimulates production of IgM by plasma cells of the synovium and lymph nodes. Why IgG, which is a product of the human system, should serve as an antigen for IgM antibody production, is unknown; however, immune events may depend upon circulating complexes which may or may not be important in pathogenesis. There are some who feel that rheumatoid factor plays a protective role similar to the antistreptolysin titer in streptococcal infection,

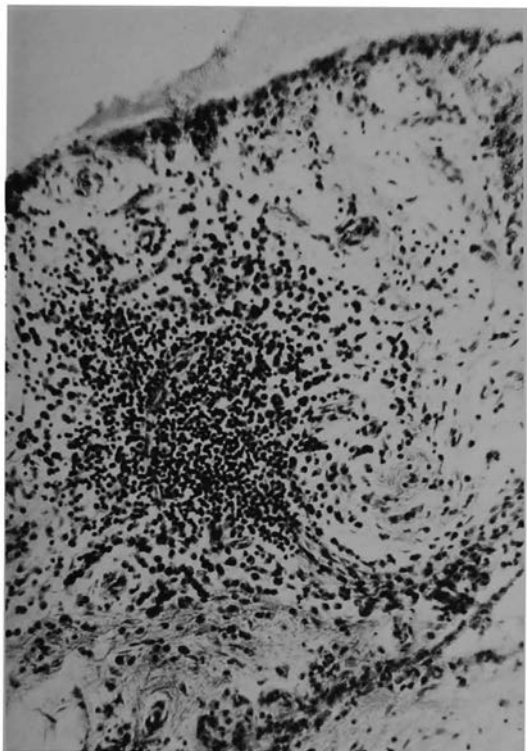


Fig 1A—Microscopic view (*medium power*) of synovial membrane showing "nesting" of lymphocytes in perivascular area of synovium.

while others feel that in certain systems it plays a part in the inflammatory mechanism. The basic pathology in these diseases seems to be vasculitis; hence the term collagen-vascular disease arose, which is a misnomer, since collagen is not the only connective tissue involved. In rheumatoid arthritis with high rheumatoid factor titers and many subcutaneous nodules, one is more likely to see those with severe disease: peripheral neuritis, vasculitis, and leg and fingertip ulcerations. In these patients one may find evidence of antigen-antibody complement complex deposition in the vessels leading to the ulcers and in the perineural vessels of the peripheral nerves (Figs 2A, 2B and Figs 3A, 3B).

There are a number of immunological tests available to make a diagnosis of rheumatoid arthritis. The three currently in use at the Medical College of Virginia are the slide latex test, sensitized human cell test (SHC), and the sensitized sheep cell test (SSC). The latex test is the most sensitive and the sheep cell

test is the most specific for the presence of rheumatoid factor. Sometimes there are false-positive latex tests in older patients and in patients with large amounts of gamma globulin present. The SSC may be positive in low titers and in other connective tissue diseases. In general, all three tests employ the same principle. The particle, whether it be latex, human red cells, or sheep cells, is coated with IgG from one source or another, which will agglutinate in the presence of rheumatoid factor. If the sheep cell test is positive in a significant titer, the chances are over 90% that the patient has rheumatoid arthritis. It should be remembered that a large number of patients do not show rheumatoid factor, and these patients are classified as seronegative rheumatoid arthritis patients (Fig 4 and Fig 5).

We can identify rheumatoid factor in serum, demonstrate that it is produced by plasma cells in the synovium, and can state that in certain cases of rheumatoid arthritis it plays a pathogenetic role.

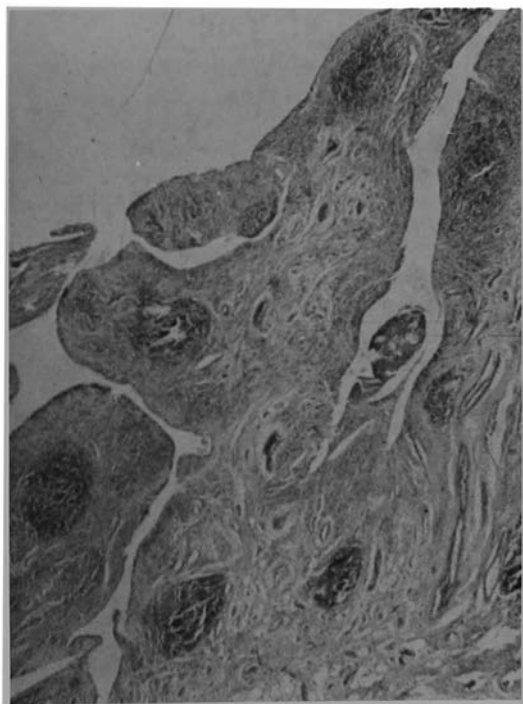


Fig 1B—View (*lower power*) showing follicle formation within the synovium. Note similarity to microscopic picture of lymph node. This is thought to be the site of rheumatoid factor synthesis.

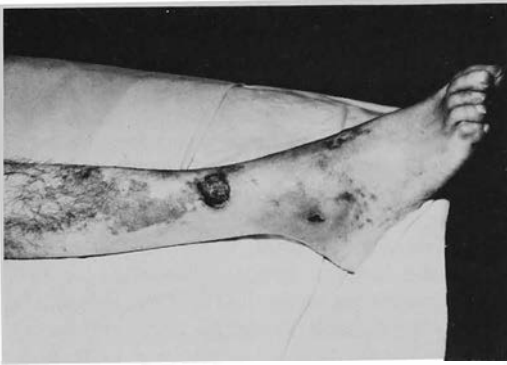


Fig 2A—Indolent leg ulcer in a 64-year-old male with rheumatoid arthritis which ultimately required below-the-knee amputation.

II. Ankylosing Spondylitis.

We generally categorize this as a separate disease entity from rheumatoid arthritis, although it has many similarities to that disease. There are, however, five specific differences:

1. Sex and age of onset—more common in young males.
2. Spine and girdle joints involved as opposed to peripheral joints, although peripheral joints may be involved in ankylosing spondylitis.
3. Pathology is that of ankylosis, rather than bone destruction as seen in peripheral arthritis.
4. Absence of rheumatoid factor.
5. Presence of a high incidence of HL-A B-27 antigen.

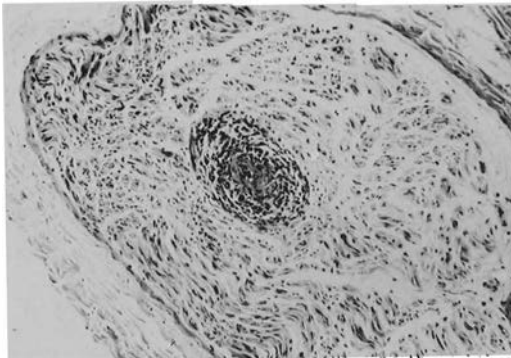


Fig 2B—Area of vasculitis (*medium power*) in the center of a nerve trunk of the lower extremity. Note perivascular changes in the nutrient artery of the trunk.



Fig 3A—Digital vasculitis in a patient with rheumatoid arthritis with gangrenous changes.

HL-A stands for Human Leukocyte Antigen, and B-27 refers to its genetic locus on the chromosome. All of this is an outgrowth of tissue typing necessary in renal transplantation. We have known for some time that ankylosing spondylitis patients have a tendency to familial clustering, and there has been a low incidence of spondylitis in American and African blacks. In 1949, Toone reported studies from the McGuire Veteran's Hospital indicating the paucity of blacks who suffered from ankylosing spondylitis.⁶ Baum later confirmed this in a larger group of patients from a Veteran's cooperative study (Table 1).⁷ Schlosstein et al, and Brewerton et al, had found that there was a high correlation between the presence of HL-A B-27 antigen in ankylosing spondylitis—as high as 88% to 96%—and normal (8% in a controlled Caucasian population) (Table 2).^{8,9} There

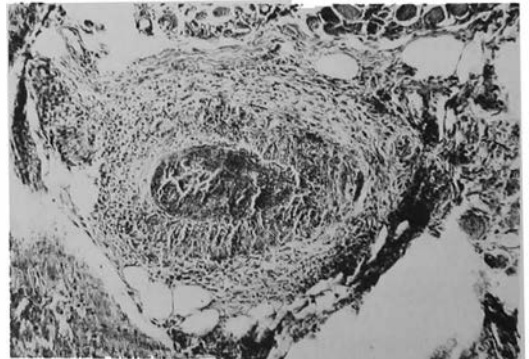


Fig 3B—Vasculitis and perivasculitis (*medium power*) of the necrotizing variety showing obliteration of the artery of the foot.

Rheumatoid Factor Formation

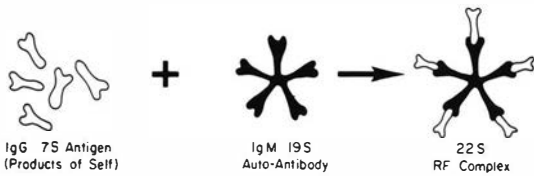


Fig 4—Rheumatoid factor formation. Rheumatoid factors are antibodies which react with other immunoglobulins to form a rheumatoid factor complex in serum. 7S refers to sedimentation constant in the ultracentrifugation analysis (Svedberg units).

are other diseases which have B-27 as a genetic marker. These include Reiter's syndrome, acute anterior uveitis, psoriasis with spondylitis, and others.

To better understand the immunological processes in developing a test such as the lymphocyte microcytotoxicity test (LMCT), it is important to understand the mechanics of the HL-A testing procedure. The LMCT is performed approximately as demonstrated in Figure 6.

1. Specific HL-A B-27 antiserum from multiparous women, or from those who have undergone numerous transfusions as a source of developing B-27 antibodies, is used.
2. The patient's lymphocytes to be tested for B-27 antigen are prepared and mixed with the antisera and rabbit complement in the chamber wells.

Test For Rheumatoid Factor

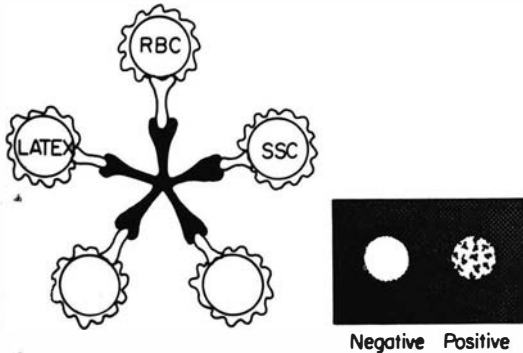


Fig 5—Test for rheumatoid factor. Rheumatoid factor complex will agglutinate either latex particles, human RBC or sheep RBC when sensitized with IgG. In the square at lower right the well on the left shows a negative test and on the right a positive test with latex particles.

3. The addition of trypan blue dye will stain the interior of lymphocytes which have been lysed by the antigen-antibody complement reaction. These lymphocytes are then counted under inverted phase microscopy to determine if the number of lysed cells is sufficient to make a specific identification of B-27 antigen.

One may conclude then that the HL-A B-27 antigen is a genetic marker for the development of certain diseases in which spondylitis seems to be a common denominator. However, the part this marker plays in the pathogenesis of the disease is unknown.

III. Systemic Lupus Erythematosus (SLE).

There is no other connective tissue disease which has evoked more interest among immunologists than lupus; it might be labeled "the immunologist's delight." The ability to diagnose this disease stemmed from the Hargraves' LE cell test in 1948 through various patterns of antinuclear antibody (ANA) testing, through the LE band test, and through the "lumpy-bumpy" deposits in the glomeruli.

If one understands the basic concept of production of experimental serum sickness, which was so well demonstrated by Dr. Frank Dixon with the formation of immune complexes, one might understand more clearly the pathogenesis of such diseases as glomerulonephritis, rheumatic fever, SLE, and perhaps even rheumatoid arthritis (Fig 7).¹⁰ The parallel between these findings in experimental serum sickness and human SLE and the occurrence of a wide range of autoantibodies against nuclear and tissue antigens, provides strong support for an immunological process in SLE. These antibodies include anti-DNA antibodies, anti-DNA histone antibodies, anti-ribonucleoprotein (RNP) antibodies, anti-Smith (Sm) antibodies, and a host of others. The demonstration of DNA and anti-DNA antibodies in the

TABLE 1 Racial Aspects of Ankylosing Spondylitis*	
1.	Random study McGuire VA Hospital: 26 white, 3 black.—Toone 1949
2.	Combined VA Hospital study, 301 patients: 10% black.—Baum 1971
3.	HL-A 27 absent in Black Africans.
4.	HL-A 27 only 4% in Black Americans.
5.	HL-A 27 present in 8 of 10 Black American spondylitics.







*The above figures demonstrate the relationship of HL-A 27 and ankylosing spondylitis on Black Americans.

TABLE 2
HL-A 27 in Rheumatic Disease Patients*

Condition	Presence of HL-A 27	Percentage	Source
Normal	119/1456	8%	Russell, 1972 Brewerton, 1973 Schlosstein, 1973 White, 1972
Rheumatoid arthritis	10/119	8%	Schlosstein, 1973
Gout	6/66	9%	Schlosstein, 1973
Ankylosing spondylitis	35/40	88%	Schlosstein, 1973
	72/75	96%	Brewerton, 1973
Reiter's syndrome	25/33	76%	Brewerton, 1973
Acute anterior uveitis	26/50	52%	Brewerton, 1973
Psoriasis	9/156	6%	White, 1972
	6/44	14%	Russell, 1972
Psoriasis/spondylitis	10/14	71%	Metzger, 1974

* A compilation of rheumatic diseases and HL-A 27 shows its strong association with ankylosing spondylitis, Reiter's syndrome, and psoriatic spondylitis.

B-27 ASSAY

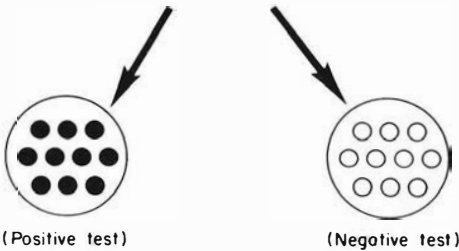
	#1	#2	#3
Undiluted B-27 antisera			
Diluted B-27 antisera			

+

Lymphocytes to be tested

+

Trypan Blue



human kidney in SLE reinforces the concept that SLE is an example of an immune-complex deposition disease involving autoantibodies.^{11,12}

Prior to the widespread use of antinuclear antibody testing for the diagnosis of SLE, one relied primarily on the demonstration of hematoxylin bodies in fixed tissue, or the presence of LE cells in the serum. Hematoxylin bodies are globular masses of nuclear material which stain blue with hematoxylin and eosin (H & E) stain and are histochemically identical with the inclusion body of an LE cell. Severe vasculitis is the hallmark of SLE, and fibrinoid deposits in vessels have been shown to be composed chiefly of DNA, anti-DNA, and complement components. The use of various immunological tests to determine the presence of ANA have taken the place of the LE cell test in making the diagnosis of SLE. The method used here is the indirect immunofluorescent antibody test, which employs mouse liver cells as the source of nuclear antigen. If antinuclear antibodies are present, various patterns of immunofluorescence will appear, depending on what type of antinuclear antibody is present in the patient's serum. The patient's serum to be tested is added to the mouse liver cells and will adhere to the nuclear antigen even when washed. When anti-human gamma globulin, which is commercially prepared and stained with fluorescein is added, a bright apple-green fluorescence will appear in the preparation when viewed under ultraviolet light (Fig 8). We have come to recognize four different fluorescent staining patterns, which may help in differentiating different patterns of disease expression in lupus. These include the homogeneous or diffuse pattern, indicating antibodies to

Fig 6—Lymphocyte microcytotoxicity test for B-27 antigen. Lymphocytes to be tested for B-27 antigen are added to each of three donor wells of diluted and undiluted specific B-27 antisera. With addition of complement and trypan blue dye, cells will lyse and stain positively with blue dye if B-27 antigen is present.

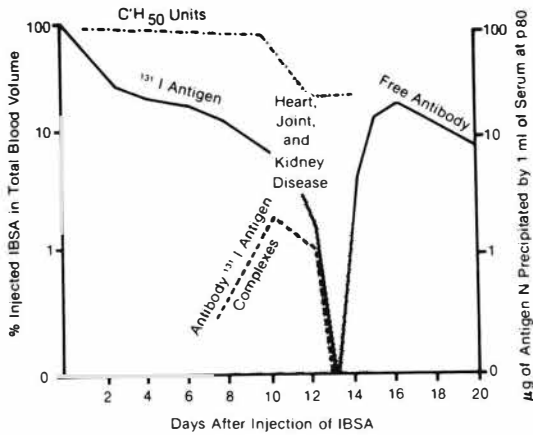


Fig 7—Circulating BSA-anti-BSA complexes: development of lesions. Correlation between formation of immune complexes with bovine serum albumin and appearance of lesions of heart, joints, and kidneys of rabbits with experimental serum sickness induced by injection of tagged BSA.

single- or double-stranded DNA; the peripheral or ring pattern, usually indicating the presence of antibodies to double-stranded DNA with active lupus present, usually with renal involvement; the nucleolar pattern, in which the nucleoli of the mouse liver cells take the stain, can be found in patients with systemic sclerosis or Sjögren's syndrome; and the speckled pattern, which stains for ribonucleoprotein and has a fibrillar appearance in the nucleus and is found in mixed connective tissue disease syndrome. It should be emphasized that these staining patterns and the statements regarding them are generalizations and should not be interpreted as explicit evidence in these cases. Sometimes we see a mixture of patterns, which may confuse the issue (Fig 9).

In some cases of SLE, where a drug such as hydralazine or procainamide is suspected in producing the LE phenomenon and antinuclear antibodies, a hemagglutination test or agar gel precipitation test using single- or double-stranded DNA may be used. Antibodies to single-stranded DNA are usually present in drug-induced SLE, whereas antibodies to double-stranded DNA may indicate active lupus with renal involvement. The ANA test is superior to the LE cell test in making a diagnosis in SLE because only 75% to 90% of patients with active SLE will have positive LE cell tests. Almost all patients with lupus will have a positive ANA test.

The conclusion here is that SLE is the immunol-

ogist's disease—many types of autoantibodies are present, which can be demonstrated by various immunodiffusion and immunofluorescent techniques. These autoantibodies are thought to play a specific role in the pathogenesis of SLE, particularly where there is renal involvement.

IV. Mixed Connective Tissue Disease (MCTD).

The introduction of the term *speckled pattern* of ANA immunofluorescence and the presence of ribonucleoprotein and Smith antigens opens the door to mixed connective tissue disease syndrome.

The clinical picture of MCTD reveals it as a syndrome consisting of many of the features of the connective tissue disorders including rheumatoid arthritis with arthritis and arthralgias and rheumatoid factor, SLE with skin rashes and speckled pattern of antinuclear antibody immunofluorescence, progressive systemic sclerosis with Raynaud's phenomenon and thickening of the skin, and polymyositis with

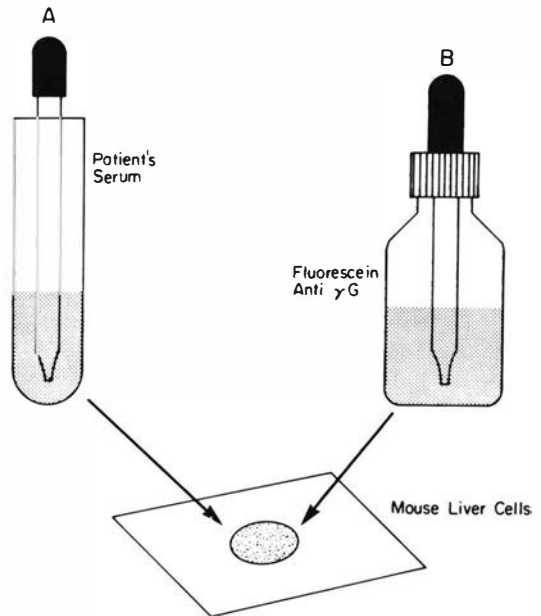


Fig 8—Indirect immunofluorescent staining for antinuclear antibodies. Mouse liver cells are used as the substrate for source of nuclear antigen. The patients' serum (A) containing antinuclear antibodies binds to the nuclei leaving a coating. After washing, the preparation is incubated with an anti-human gamma globulin (B) which binds only at the site where antinuclear antibody is bound. Nuclei exposed to SLE serum will fluoresce when examined under the fluorescence microscope.

NUCLEAR PATTERNS OF ANA

<u>ANTIGEN</u>	<u>STAINING PATTERN</u>	<u>CLINICAL CORRELATION</u>
Histone Component Deoxyribonucleoprotein	Homogenous or Diffuse	SLE RA OTHERS
DNA	Peripheral or Ring	Active Lupus usually with nephritis
RNA	Nucleolar	SCLERODERMA SJOGREN'S SLE
Ribonucleoprotein RNP	Speckled	MCTD

Fig 9— Different patterns of ANA testing. Antigen, pattern of fluorescent staining, and clinical correlation in the four types of nuclear antibody tests.

myopathy and muscle enzyme changes. In other words, it is a “mishmash” of the connective tissue diseases, but generally patients are thought to have a better prognosis with this syndrome and as a rule do not have severe renal or central nervous system (CNS) involvement as those with systemic lupus.

Concerning the diagnosis of MCTD, Gordon Sharp at the University of Missouri, who has done much work in this disease, feels that a diagnosis can be made if the clinical picture fits and one can demonstrate antibodies to extractable nuclear antigen (ENA) which are RNase sensitive.¹³ Extractable nuclear antigen is prepared from calf thymus cells containing mainly RNP and Sm antigens. This test is performed either by an immunodiffusion technique or by hemagglutination method, to demonstrate anti-

bodies to RNP and Sm antigens. RNP can then be removed from either test system by the addition of RNase, leaving only Sm antigen if it is present. If a precipitin band remains in the immunodiffusion dish after RNase is added, this is Sm antigen antibody band, which may be present in SLE and may indicate a poorer prognosis than in those patients with MCTD (Fig 10). The hemagglutination test for RNP and Sm antigen is very similar to the technique employed in the tanned sheep cell test in rheumatoid factor. ENA is added to tanned sheep cells to coat the cells with RNP and Sm antigens. When the patient's serum containing antibodies to ENA is added, agglutination results, indicating a positive test. If after RNase is added there is no agglutination and the sheep cells fall to the bottom of the tube, this in-

ENA Test

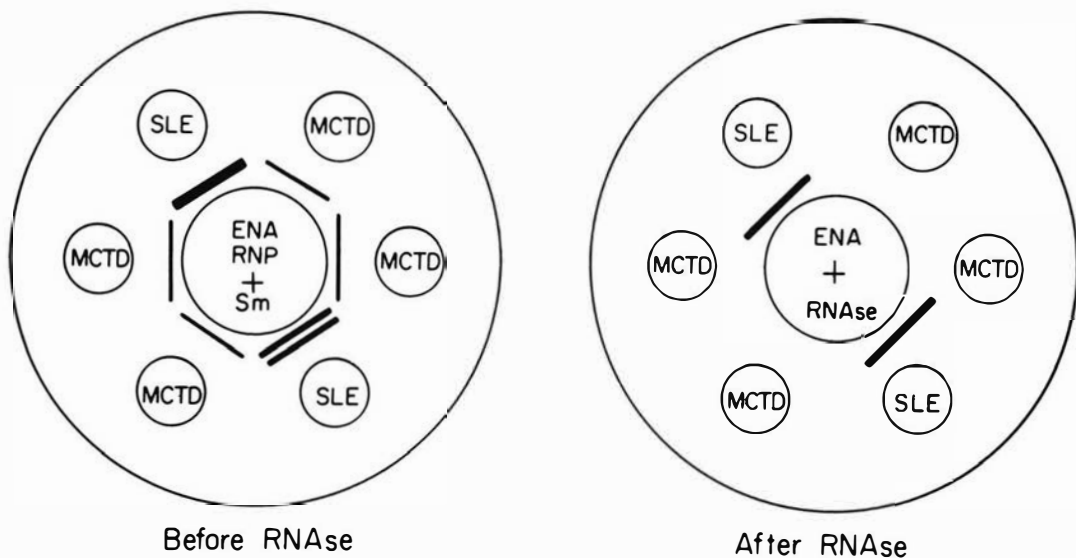


Fig 10—Extractable nuclear antigen test (ENA): Precipitating Antibodies to RNP and Sm Antigens. ENA (RNP and Sm antigens) in center well of agar gel before RNase show precipitin bands with both MCTD and SLE sera. After RNase is added to center well in figure on right bands for MCTD sera are no longer present, leaving only Sm precipitin bands for SLE sera.

HEMAGGLUTINATION TEST FOR RNP AND Sm ANTIGENS

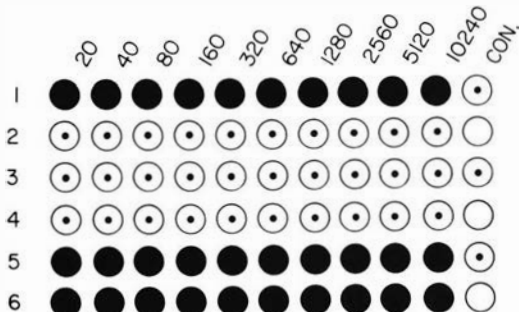


Fig 11—Hemagglutination test for RNP and Sm Antigens. Row 1 represents a positive ENA agglutination in a titer over 100,000 for MCTD; Row 2 demonstrates the effect after RNase has been added, identifying an RNase-sensitive antibody, and the red cells fall to the bottom of the well, concluding that this serum contains RNP antibodies found in MCTD; Rows 3 and 4 are sera from a normal individual with negative ENA tests before and after RNase; Rows 5 and 6 represent SLE sera which contain antibodies to ENA which are RNase-resistant, indicating no RNP to be present and Sm antigen accounting for the persistent agglutination. Control sera are in the column on the right.

indicates that only RNP antibodies for MCTD are present. If on the other hand the agglutination remains after RNase is added, this would indicate other antibodies, such as those to Sm antigen, are present, as is seen in SLE. Titers over 1:10,000 are thought to be significant in the interpretation of this test (Fig 11).

For a diagnosis of mixed connective tissue disease, high titers of hemagglutination antibodies to ENA and no antibodies to ENA after RNase is added are required. A speckled pattern on fluorescent ANA test is another indication for the diagnosis.

In a group of 100 patients studied by Sharp et al, 74% had RNase-sensitive ENA by hemagglutination or immunodiffusion techniques, and had mixed connective tissue disease.¹³ Of the 26% who were RNase-resistant ENA patients, a large majority had SLE. This group then was composed of those patients in whom the immunodiffusion and hemagglutination tests showed the presence of antibodies to Sm antigen.

One can conclude, therefore, that those patients who demonstrated antibodies to ENA, which are

RNAse-sensitive (RNP antibody), may have MCTD and are less likely to have renal and CNS involvement and have a better prognosis.

Summary.

1. Rheumatoid factor can be identified in the sera of certain patients with rheumatoid arthritis, is produced in synovial cells, and may have a role in pathogenesis in *some* patients.
2. HL-A B-27 antigen is a genetic marker for development of certain diseases in which spondylitis is a common denominator; its role in pathogenesis is unknown.
3. Systemic lupus erythematosus is an example of antigen-antibody complement complex deposition disease. Different types of antinuclear-antibody tests are associated with different patterns of disease expression.
4. Patients with antibody to the ribonucleoprotein component of extractable nuclear antigen may have mixed connective tissue disease and are less likely to have severe renal and CNS involvement.

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