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Studies on precipitating and hemagglutinating antibodies in systemic lupus erythematosus

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Abstract. We studied the precipitating and hemagglutinating autoantibodies in the sera of patients with various connective tissue diseases in general and lupus in particular. Saline soluble extract of goat thymus had adequate antigenic materials as compared to other organs. Twenty per cent of patients with systemic lupus erythematosus were positive for precipitating autoantibodies by immunodiffusion and 44% by counterimmunoelectrophoresis. Normal human subjects, nonrheumatic disease patients and patients with rheumatoid arthritis and progressive systemic sclerosis were all negative. Forty seven per cent of positive systemic lupus erythematosus sera showed two precipitin systems. Enzyme sensitivities were used as the basis of identification of most of the antigenic specificities. Passive hemagglutination was carried out to identify antibodies to non-histone nuclear protein and nuclear ribonucleoprotein antigens. Thirty eight % of systemic lupus erythematosus patients were positive by this technique. Passive hemagglutination although a highly sensitive technique could not detect antibodies against antigenic systems other than non-histone nuclear protein and nuclear ribonucleoprotein.

Keywords. Autoantibodies; lupus; autoimmunity; rheumatic diseases; hemagglutination; precipitin reaction.

Introduction

The presence of spontaneously occurring autoantibodies to many nuclear and cytoplasmic antigens is a hall mark of diseases collectively known as autoimmune diseases which include systemic lupus erythematosus (SLE), rheumatoid arthritis, progressive systemic sclerosis (PSS), dermatomyositis, Sjogren's syndrome and mixed connective tissue disease (MCTD). A variety of autoantibodies against tissue extracts have been described in the sera of these patients (Nakamura and Tan, 1978; Talal, 1978; Steensgaard and Johansen, 1980).

Abbreviations used: SLE, Systemic lupus erythematosus; PSS, progressive systemic sclerosis; MCTD, mixed connective tissue disease; Sm, non-histone nuclear protein antigen, nRP, nuclear ribonucleoprotein; CIE, counter-immunoelectrophoresis; PHA, passive hemagglutination, GTE, goat thymus extract; PBS, phosphate buffered saline, pH 7.2; ANA, antinuclear activity.

The earliest immunological characterization of nonhistone nuclear protein which was antigenic and reactive with antibodies in human sera was reported in 1966 (Tan and Kunkel, 1966). This antigen was called Sm. Because of an extremely high selectivity of Sm antibody for SLE, it has been proposed that this might be a marker antibody (Tan *et al.,* 1978). Several other nonhistone nuclear protein antigens have been reported. One of them is nuclear ribonucleoprotein (nRNP) antigen (Northway and Tan, 1972; Reichlin and Mattioli, 1972). Antibodies to nRNP are present in very high concentration in patients with MCTD, an illness in which the symptoms represent mixtures of several types of autoimmune diseases (Sharp *et al.,* 1972).

The present work describes the studies on the precipitating and hemagglutinating autoantibodies in the sera of patients with connective tissue diseases. Most of the studies were carried out with SLE sera. The work was restricted to the study of saline soluble antigens of goat thymus which contains acidic nuclear antigens, Sm, nRNP, SS-B (Alspaugh *et al.,* 1976; Akizuki *et al.,* 1977), cytoplasmic antigens (Lamon and Bennett, 1970; Cavanagh, 1977; Koffler *et al.,* 1979), and probably others which have not been characterized so far.

Immunodiffusion and counterimmunoelectrophoresis (CIE) were used to identify the precipitating systems. Enzyme sensitivities were used as the basis of their identification. Because of the availability of small amount of sera in most of the cases, the antigenic nature of a large number of the precipitin systems could not be established fully. Passive hemagglutination (PHA) was. carried out for the detection of Sm and RNP antibodies using goats thymus extract (GTE) coated formalinized sheep erythrocytes.

Materials and methods

Sera

Normal human sera was obtained from healthy individuals. Sera from non-rheumatic disease patients were obtained from various hospital out-patients. Sera of patients with SLE, rheumatoid arthritis and PSS were collected from out- and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. The samples were transported to the laboratory on ice-sodium chloride mixture and stored in small aliquots at –20°C with 0.1% sodium azide as preservative. Patients with SLE, rheumatoid arthritis and PSS had features meeting preliminary criteria of the American Rheumatism Association for these disease (1973).

Chemicals

Ribonucleic acid, DNA, ribonucleas A, trypsin and agarose were purchased from Sigma Chemical Company (USA). Pronase was obtained from E. Merck (Germany). All the other chemicals were of analytical grade.

Preparation of antigen

Acetone dried powder of various organs from goat and rabbit was prepared according to the method of Horecke (1955). The reconstitution of acetone

powder was done as described by Kurata and Tan (1976). Acetone powder (60-80 mg) was mixed with 1 ml of phosphate buffered saline (PBS; pH 7.2) for 4 h at 4°C with slow continuous stirring. The resulting solution was centrifuged and supernatant stored in small aliquots at –20°C. Protein content of the supernatant was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as Standard. DNA was estimated by the method of Burton (1956) while RNA was estimated by orcinol reaction (Mejbaum, 1939).

Incubation of goat thymus extract

Goat thymus extract (GTE) was the most reactive antigen. To simulate the experimental conditions, the effect of incubation of GTE on stability of protein and RNA antigens from the extract was tested before addition of the test serum. GTE was incubated in 1 ml aliquots for different time intervals at 37°C in a thermostatic water bath upto 24 hours. The incubation was stopped by immersing the tubes in ice bath. Effect on RNA and protein content was followed after incubation by the estimation of RNA and free amino acids after each time interval.

Enzymatic digestion of GTE

The extract was treated with ribonuclease, trypsin and pronase according to the method of Kurata and Tan (1976). The ratio of enzyme to substrate by weight was 1 : 10 for ribonuclease and 1:50 for trypsin and pronase. Digestion was carried out for 1 h at 37°C. Appropriate controls were included in each set of experiments. The digest was centrifuged and supernatant used for further studies. RNA digestion was followed by orcinol reaction, and protein digestion by estimating free amino acids liberated (Moore and Stein, 1948).

Immunodiffusion

The precipitin reactions were performed by Ouchterlony double diffusion (Tan and Kunkel, 1966) in 0.4% agarose.

Counterimmunoelectrophoresis

CIE was carried out in 0.6% agarose using microscopic slides (Kurata and Tan, 1976).

Passive hemagglutination

Microhemagglutination test of Nakamura *et al.* (1978) with slight modification was used for the detection of antibodies to Sm and RNP antigen. GTE was used as antigen and formalinized sheep erythrocytes were, employed as the passive carriers.

Results

Extraction of various tissue acetone powders with PBS resulted in preparation with a preponderance of proteins. The average ratio of proteins, RNA and DNA in different saline soluble extracts of thymus acetone powder was 4: 0.25: 0.01.

In immunodiffusion experiments the precipitin reactions require at least 24 h of incubation at room temperature. It was ensured that during this period of

incubation the antigenocity of the preparations used was not affected. In order to see the effect of keeping the GTE at room temperature on the stability of protein and RNA antigens, the extracts was incubated at 37°C for different time intervals. Protein and RNA concentrations were followed after each time interval. The results indicate that there is almost complete destruction of RNA within 24 h. However, no appreciable effect on protein content was observed as indicated by a negligible increase of amino acids.

Serological characteristics of patients with various rheumatic diseases were followed by immunodiffusion, CIE and PHA.

Immunodiffusion

Using GTE as a source of antigens the precipitin reaction was negative with sera from normal human subjects and from non-rheumatic disease patients by immunodiffusion. Ten out of 50 patients (20%) with SLE showed precipitating auto-antibodies by immunodiffusion. Patients with rheumatoid arthritis and PSS were negative for precipitating autoantibodies against GTE antigens.

Counterimmunoelectrophoresis

Twenty two of 50 SLE patients (44%) had precipitating autoantibodies in their sera when the reaction was carried out by CIE using GTE as antigenic source. Normal human subjects, non-rheumatic disease patients, patients with rheumatoid arthritis and PSS were all negative.

None of the sera had precipitating autoantibodies when goat and rabbit brain acetone powder extract in PBS was used as antigen in immunodiffusion and CIE. Rabbit and goat kidney extract gave positive precipitin lines by CIE with only a few sera. The titer was considerably low as compared to GTE as antigen. Goat and rabbit heart extracts gave negative results with all the sera positive with GTE. However, liver acetone powder extracts of these animals gave similar results as with GTE.

Passive hemagglutination

Nine out of 24 SLE sera (38%) were positive by PHA with GTE coated sheep erythrocytes. The titer of the reaction ranged from 1:128 to 1:16384. Three types of reactions were observed (table 1). Three positive sera had same or almost same titer with untreated and RNase treated antigen coated erythrocytes and represented the reaction due to anti-Sm antibodies. Another three showed a considerable decrease in the titer and indicated antibodies predominantly to RNP antigen. The remaining three showed a moderate decrease in the titer and probably represented antibody activity against both Sm and RNP antigens. All the normal human sera studied had titer of less than 1:8.

Table 2 shows the serological findings of groups of SLE patients tested by immunodifussion and CIE. Forty seven % patients had two precipitin systems and 53% showed a single line with GTE as antigen. Out of 8 patients with two precipitin lines, four (DK, MK, RK and JS) had a weak precipitating system sensitive to RNase, trypsin and pronase, while the other system was resistant to RNase but

Table 1. Passive hemagglutination with sera of SLE patients.

Patient	Titer untreated	Titer RNase treated
PM	1:16384	1:8192
PN	1:128	1:128
JS	1:512	1:512
AS	1:8192	1:512
SH	1:8192	1:512
ST	1:8192	1:512
DK	1:4096	1:1024
MK	1:8192	1:1024
RK	1:2048	1:512

Formalinized tanned sheep erythrocytes were coated with GTE at a protein concentration of 2 mg/0.1 ml packed erythrocytes for 1 h at 37°C. RNase digestion was performed at a concentration of 1 mg RNase/0.05 ml packed antigen coated erythrocytes. One % cell suspension in 0.07% bovine serum albumin was used in agglutination reaction.

Table 2. Serological findings of some SLE patients.

Patients	Precipitin titer	No. of precipitin lines	Sensitivity of lines to			Possible identification of system(s) A/B
			RNase A/B	Trypsin A/B	Pronase A/B	
MK, RK	1:16−1:32	Two A and B	+/−	+/+	+/+	RNP/Sm
DK, JS						
AW, ST	1:4−1:8	„	+/−	+/−	+/−	RNP/SS-B
PS	1:16	„	−/−	+/+	+/+	?
SS	1:16	„	+/+	+/+	+/+	?
KR, JP PM, PN	1:16−1:32	One	−	−	−	SS-B
AS, RD	1:8	„	+	+	+	RNP
UR, VN, PT	1:8−1:32	„	−	+	+	Sm

Precipitin reaction was carried out by immunodiffusion and CIE using GTE as antigen. The details are given in text. Enzyme digestion was performed at 37°C for 1 h. The ratio of enzyme to substrate (w/w) was 1:10 for RNase and 1:50 for trypsin and pronase. The precipitin lines were designated as "A" and "B" for convenience.

sensitive to trypsin and pronase. These represent Sm and RNP systems respectively. The hemagglutination titer of two of these patients MK and RK was 1:8192 and 1:2048 which reduced to 1:1024 and 1:512 respectively when RNase treated antigen coated cells were used. This type of reaction is indicative of

the occurrence of antibodies to Sm and RNP antigen simultaneously. Two patients AW and ST had two precipitin systems one of which was sensitive and another resistant to all the enzyme treatments. Another patient PS showed 2 lines both of which were resistant to RNase but sensitive to trypsin and pronase.

Of nine patients with a single precipitin line, four (KR, JP, PM and PN) had system resistant to RNase, trypsin and pronase. Sera KR, JP and PM showed immunological identity by immunodiffusion (figure 1). Two sera PM and PN were

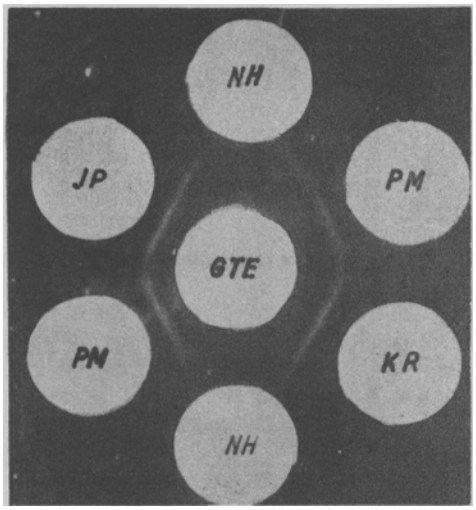

Figure 1. Immunodiffusion reaction of SLE sera KR, JP and PM with goat thymus extract. Both KR and JP showed complete immunological identity with PM. NH represents serum from a normal individual.

positive by hemagglutination with a titer of 1:128 and 1:16384 with untreated erythrocytes and 1:128 and 1:8192 with RNase treated cells. JP and KR were negative by PHA although positive by immunodiffusion and CIE. Sera AS and RD gave a single precipitin line by immunodiffusion and CIE. The reaction was sensitive to RNase, trypsin and pronase.

Discussion

Antibodies against saline soluble mammalian antigens are of considerable clinical interest because they comprise as much as 20% of the total serum immunoglobulins in some patients with connective tissue disease (Maddison and Reichlin, 1977). These antibodies have been associated with disease subgroups with characteristic features and prognosis (Sharp *et al.,* 1972, Notman *et al.,* 1975; Farber and Bole, 1976).

Saline extracts of thymus acetone powder had adequate antigenic material reactive with antibodies from the sera of patients with SLE. This is in agreement with other studies where thymus extracts from other mammalian sources have been used for antinuclear antibody (ANA) assay (Northway and Tan, 1972; Keiser

and Weinstein, 1980). Our findings that only thymus, liver and kidney antigens reacted with SLE sera whereas antigens from heart and brain extracts were unreactive is indicative of differential distribution of various antigenic materials in the various organs of the animals. Thymus and liver are tissues which have rapid generation cycles and may probably contain many of the antigens which are absent in other organs like brain and heart which are least regenerating. This, however, does not exclude the possibility of the presence of small amounts of active antigens in these organs.

In the present study 20% of patients with SLE were positive for precipitating autoantibodies by immunodiffusion where as 44% were positive by CIE. This clearly indicates the greater sensitivity of the CIE technique over immunodiffusion. Our results are consistent with the previous report (Parker, 1973; Kurata and Tan, 1976) with respect to the sensitivity for the detection of ANA.

Using enzyme digestion studies we have demonstrated an incidence of 53% for ribonuclease sensitive system in CIE positive SLE sera which is somewhat higher than previously reported (Parker, 1973). This difference could essentially be due to combined detection of nRNP and other RNase sensitive systems including ribosomal RNP. Forty seven % of positive SLE sera had RNase resistant and trypsin sensitive systems and would probably represent Sm system although there are controversies regarding the enzyme sensitivity of this antigen (Dorsch *et al.,* 1979).

Thirty six % of our positive sera had RNase, trypsin and pronase resistant system. Whether the system represents the ill characterized SS-B or Ha or a new unknown system remains to be investigated. The association of RNase sensitive system with another system resistant to RNase probably represents the Sm-RNP whose close association has been reported by many workers (Mattioli and Reichlin, 1973; Lerner and Steitz, 1979; Waelti and Hess 1980). However, the association of RNase sensitive system with another system resistant to RNase and trypsin reported in this study is not clear. Miyawaki *et al.* (1978) described two antigens MU and TM in their studies with connective tissue disease patients. MU was sensitive to the treatment with RNase and trypsin while TM was resistant to such treatments. Later studies by Miyachi and Tan (1979) found that MU system represented rRNP. The association of RNase sensitive system with RNase and trypsin resistant system seen in present study might be rRNP equivalent of Miyachi and Tan (1979) and TM equivalent of Miyawaki *et al.* (1978).

PHA was used for distinguishing between antibodies to Sm, RNP and Sm-RNP. The technique was reproducible and almost 100 times more sensitive than precipitin reaction. An important observation in our hemagglutination reaction was that some of the SLE sera were negative for agglutination although they showed a positive precipitin reaction with immunodiffusion and CIE. Akizuki *et al.* (1977), and Keiser and Weinstein (1980) have described similar results where only Sm and RNP antigens could be detected by hemagglutination and sera positive for other extractable nuclear antigens by immunoprecipitation were

negative by PHA. Thus although a highly sensitive technique, PHA cannot be used for the detection of antinuclear antibodies other than Sm and RNP by the method described by Nakamura *et al.* (1978).

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