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A SCREENING ASSAY FOR DETECTION OF
IMMUNE COMPLEXES IN SERUM

presented by

Susan Kathryn Codere

has been accepted towards fulfillment
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M. S. degree in Microbiology
and Public Health

Maria J. Patterson PhD
Major professor

Date 29 October 1979

John L. Kateley PhD.

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RAJI CELL ROSETTE INHIBITION: A SCREENING ASSAY FOR DETECTION OF IMMUNE COMPLEXES IN SERUM

By

Susan Kathryn Codere

A THESIS

Submitted to
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ABSTRACT

RAJI CELL ROSETTE INHIBITION: A SCREENING ASSAY FOR DETECTION OF IMMUNE COMPLEXES IN SERUM

Susan Kathryn Codere

The diagnosis and management of immune complex (IC) diseases has been advanced by the measurement of circulating immune complexes (CIC) in serum. At present, most clinical laboratories do not offer tests for CIC, mainly due to the complexity of available methods and the inability of one test to detect all types of IC.

The Raji cell rosette inhibition (ROS-I) assay was developed and evaluated for detection of CIC in serum. IC prepared in vitro were shown to inhibit Raji rosette formation with antibody coated (EA) and antibody and complement coated (EAC) target cells. A survey of 223 serum specimens obtained from patients with collagen vascular disorders, and healthy blood donors revealed some sera also inhibited Raji rosette formation. Sixty-three percent of sera from patients with systemic lupus erythematosus and mixed connective tissue disease, 60% with rheumatoid arthritis, and 41% with other rheumatoid disorders were positive. A comparative study of the Raji ROS-I with two other CIC assays, electrophoresis and the Raji radioimmunoassay, showed 60% and 69% correlation, respectively. Raji ROS-I is a simple and sensitive assay for the detection of CIC. It is anticipated, however, that this assay will be used as one of a panel of tests for CIC, since a single CIC assay cannot detect all varieties of IC.

DEDICATION

To Tom--thanks for standing by me and for always being there. And to all my friends in the lab--thanks for your support and for your help.

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INTRODUCTION

Immune complexes (IC) are produced when antibodies (Ab) combine with their corresponding antigen (Ag). Immune complexes play an essential role in the normal immune response by providing a mechanism for clearance and destruction of many antigens such as foreign serum proteins, drugs, microbial antigens from viruses, bacteria, and parasites, and autologous antigens.

The fate of immune complexes is dependent, in part, on the site of formation, the nature and concentration of the antigens and antibody involved, and the size of the complexes. Most IC in the circulation are readily cleared by the reticuloendothelial system (RES), particularly by liver Kupffer cells. Large complexes, usually formed in antibody excess, and IC that fix complement, are rapidly cleared from the circulation. These complexes are rarely associated with disease in contrast to smaller, soluble immune complexes usually formed in antigen excess or non-complement-fixing complexes which tend to persist in the circulation. These smaller complexes are cleared to some extent by the spleen, but often become fixed to the renal glomeruli during the filtration of blood, or in blood vessel walls or choroid plexus. Complexes formed in the extravascular spaces are not cleared as rapidly as those formed in the circulation and, thus, are more likely to be deposited in the tissues.

Under certain conditions, immune complexes may trigger a sequence of pathologic events in tissues and organs throughout the body.

Pathogenic immune complex-mediated tissue injury via plasma mediators, either by activation of the complement system or by attachment to mononuclear cells with immunoglobulin (Fc) receptors or complement receptors (C3b or C3d) has been clearly demonstrated in animal models for serum sickness (Dixon, 1963) (24). Similar glomerular, vascular, and articular lesions in human diseases are also thought to be mediated by IC. However, the pathological expression of the formation of immune complexes seems to be relatively rare in comparison with the frequent occurrence of such IC in the circulation or in extravascular spaces. Consequently, the finding of complexes in any disease does not necessarily imply that they have a pathogenic role.

Chronic immune complex-associated diseases may be classified according to the antigens involved. For example, IC associated with rheumatoid arthritis (RA; Immunoglobulin Ags.), systemic lupus erythematosus (SLE; nuclear Ags.), malignant diseases and other autoimmune disorders (cellular Ags.) involve endogenous antigens. In contrast, immune complex-associated diseases involving exogenous antigens include serum sickness (accidentally induced), diseases resulting from the inhalation or digestion of environmental antigens, and infectious diseases and their sequelae such as serum hepatitis (viral), post-streptococcal glomerulonephritis (bacterial), malaria (protozoal), and Schistosomiasis (helminthic). Irrespective of the antigen derivation of IC, there is a similarity in the pathologic tissue damage mediated by immune complex deposits.

Several approaches have been used to demonstrate the occurrence of immune complexes in human diseases; however, the two most-used

procedures include, 1) the detection of IC bound to tissues by histologic and electron microscopic techniques, and 2) serological analysis of samples from various biological fluids.

Immunofluorescence and immunoperoxidase techniques are routinely used to detect immunoglobulins and/or complement deposits in tissue sections in the absence of other plasma proteins (albumin and fibrinogen). The presence of such deposits is circumstantial evidence of immune complex involvement. Conclusive proof that immunoglobulin-containing deposits are IC requires the identification of the antigenic component in the immune complex. Elution studies have been used in some instances to identify specific antibodies (Woodroofe and Wilson, 1977) (104). Additionally, antigen identification has been accomplished by immunofluorescence studies. However, these are not standard procedures and consequently, are not routinely performed.

Due to the profound role of IC in certain diseases and the impractical nature of repeating tissue biopsies for diagnosis and monitoring patients with IC, many investigators have designed procedures to detect circulating IC (CIC). Several recent studies have compared the specificity, sensitivity, and reliability of techniques for the direct demonstration of IC in serum.

Assays for CIC may be grouped into two major categories: antigen-specific methods which permit the selective detection of IC for a single antigen; and antigen non-specific methods which are used to detect CIC independent of the nature of the antigen involved. Antigen non-specific methods can be further subdivided into procedures which identify IC either on the physical properties of the IC (size and solubility changes)

or their biological properties (interaction with complement, anti-globulins, or with cells). All of the antigen non-specific methods, however, will detect non-specifically aggregated immunoglobulins, as well as IC. The specificity of each antigen non-specific method for IC varies according to the nature of the immune complex and to the influence of interfering factors. Additionally, the difficulty in standardizing some of the required reagents (aggregated IgG, Clq, Raji cell cultures) and the complexity of some of the proposed methods (radiolabeling Clq or rheumatoid factor (RF), isolating and characterizing monoclonal RF) render some of the methods inapplicable for use in routine clinical laboratories (Lambert and Casali, 1978) (51).

In an effort to gather information on the role of IC in disease and to evaluate methods being used for their detection, the World Health Organization (WHO) organized a "Scientific Group on the Role of Immune Complexes in Disease" which met in September, 1976. Their report reviews current knowledge concerning IC and pathogenesis and makes recommendations for laboratory diagnostic tests, clinical studies and basic research in this area (WHO TRS, 1977). Later, following these recommendations, the WHO established a collaborative study to evaluate and compare the specificity and sensitivity of 18 different methods for detecting IC in serum. Results of this study indicated that the most sensitive methods for the identification of sera containing IC were the solid-phase conglutinin-binding test (KgB-SP) and the Raji cell assay (Raji-RIA), followed by the solid-phase mRF inhibition assay (mRF-I), the solid-phase (Clq-binding test (ClQ-SP), the Clq-binding assay (ClQ-BA), the Clq deviation test (ClQ-DV) and the platelet aggregation

test (PAT). Of these methods, five depend on a reactivity with complement and two depend on the recognition of immunoglobulin (Ig) aggregates by Fc receptors on platelets or by rheumatoid factor. Six of the seven recommended methods require radiolabeling of Igs, RF, or Clq by the investigator. The comparative data compiled in this study suggest that there are different types of IC depending on the disease and each method displays a particular pattern of reactivity (52).

The purpose of this research study was to review the available literature concerning the detection of immune complexes in sera and to develop an assay to detect CIC which would be simple, yet sensitive, to be offered as a routine screening assay in a clinical laboratory.

LITERATURE REVIEW

The role of immune complexes (IC) in the pathogenesis of tissue lesions was suggested as early as 1911 by Von Pirquet (97). Since that time, experimental models have been developed which clearly demonstrate the pathogenic role of immune complexes in serum sickness and their involvement in similar glomerular and vascular lesions in human diseases (Dixon, 1963) (24).

In the past several years, research in the area of immune complex-associated diseases has become very popular. Many investigators have reported new methods for the detection of immune complexes in tissues and in biological fluids and have implicated immune complexes in the pathogenesis of many diseases. In 1976, a World Health Organization Scientific Group met to discuss the role of immune complexes in disease. Their report reviews much of the current knowledge concerning immune complexes and lists methods currently used to detect IC. In addition, this report makes recommendations for future research for investigators interested in immune complexes (WHO, 1977) (105).

Immune complexes appear transiently in many infectious diseases and allergies where they play an important role in the normal immune response to such foreign antigens. They are commonly formed when antibodies are produced against antigens still persisting in the circulation or extravascular spaces or released from host cells or invading organisms. These transient IC may be responsible for some complications,

such as glomerulonephritis, in acute diseases. However, the pathogenic role of IC is probably more important in chronic diseases where the antigens involved are continually produced and released.

Immune complex-associated diseases may be classified according to the antigens involved. Table 1 lists some diseases associated with immune complexes. In Tables 2 and 3 the same diseases are listed along with the antigens and antibodies involved, the associated pathology, and the methods used to detect IC in the respective diseases.

Several approaches have been used to demonstrate immune complexes in human diseases. In general, these methods can be divided into two groups, 1) methods for the detection of IC bound to tissues, and 2) methods for the detection of IC in biological fluids (Table 4).

In Tables 5, 6, and 7 the groups are further subdivided into specific assays. Eighteen of the antigen-nonspecific methods for detecting CIC were recently evaluated by the World Health Organization (Lambert et al., 1978) (52). Results of this study indicate that the most sensitive methods for the discrimination of sera containing immunoglobulin aggregates from normal sera were the KgB-SP, Raji-RIA, mRF-1, ClQ-SP, ClQ-BA, ClQ-DV, and the PAT. Three of these seven assays, the Raji cell radioimmunoassay, the solid-phase Clq-binding test, and the Clq-binding assay, are widely used and potentially applicable for specialized clinical investigation.

The Raji cell radioimmune assay (Theofilopoulos and Dixon, 1976) (92) employs cells from the Raji lymphoblastoid cell line which exhibit receptors for IgG Fc of low avidity and large numbers of receptors for C3b, C3d, and Clq. For the assay, Raji cells are incubated with serum

TABLE 1. SOME DISEASES ASSOCIATED WITH CIRCULATING IMMUNE COMPLEXES

INVOLVING ENDOGENOUS ANTIGENS
Immunoglobulin Antigens
Rheumatoid Arthritis
Mixed Cryoglobulin Diseases
Hypergammaglobulinaemic Purpura
Nuclear Antigens
Systemic Lupus Erythematosus
Specific Cellular Antigens
Tumors
Autoimmune Disorders
INVOLVING EXOGENOUS ANTIGENS
Iatrogenic Antigens
Serum Sickness
Drug Allergy
Environmental Antigens
Inhaled--Extrinsic Allergic Alveolitis
Ingested--Dermatitis Herpetiformis
Antigens from Infectious Organisms
Viral--Hepatitis
--Dengue Hemorrhagic Fever
Bacterial--Post-Streptococcal Glomerulonephritis
--Leprosy
Protozoan--Malaria
--Trypanosomiasis
Helminthic--Schistosomiasis
--Onchocerciasis
INVOLVING UNKNOWN ANTIGENS
Chronic Immune Complex Glomerulonephritis
Vasculitis

TABLE 2. IMMUNE COMPLEX DISEASES ASSOCIATED WITH ENDOGENOUS ANTIGENS

Disease Associated with IC	Antibodies and Antigens Involved	Pathology	Characteristics	References
Rheumatoid Arthritis	IgM or IgG Anti-IgG	Joint - PMNs w/ IC in fluid Mononuclear infiltrate Producing antibodies Vasculitis, Eosinophilia Subcutaneous nodules No Nephritis Normal Serum C' Levels	Joint - In vitro anti-C' activity In vivo C' activation Precipitate w/ C1q alone Serum - Not directly precipitated by C1q, need PEG[1] No intense C' activation	27 30 55 101 102 103
Mixed Cryoglobulin Diseases	IgM Anti-IgG Monoclonal Cryoprecipitation	Vasculitis Nephritis Primary or Secondary to lymphoproliferative disease Sjogren's syndrome Mild liver abnormalities	Kidney deposits - IgG, IgM, C' Intense C' activation Monoclonal IgM Anti-IgG w/ cold enhanced activity w/ IgG	27 61
Hypergamma-globulinaemic Purpura	IgG Anti-IgG	No nephritis or frank vasculitis; Petechiae 50% of cases secondary to Sjogren's syndrome with other autoantibodies	Very high levels of CIC Monoclonal IgG Anti-IgG	49
Systemic Lupus Erythematosus	Antinuclear antibodies (ANA) Anti-DNA,-Sm, (RNP)[1] Anti-lymphocyte membrane Ag. Antiglobulins	Nephritis - Glomerular Basement Membrane (GBM) Vasculitis - Skin Arthritis	Nephritis well correlated w/ CIC levels and C' consumption Alternating phases of DNA and Anti-DNA in circulation IC vary in size Some cryoprecipitation CIC poorly reactive w/mRF[1]	2 9 18 48 53 88 104 108
Malignancies	Carcinoembryonic Ag (CEA) - Anti-CEA Others		Modulate immune effector mechanisms against tumor cells	5, 6 7, 19 81, 93

[1] PEG - polyethylene glycol, Sm - Smith, RNP - ribonucleoprotein, mRF - monoclonal rheumatoid factor

TABLE 3. IMMUNE COMPLEX DISEASES ASSOCIATED WITH EXOGENOUS ANTIGENS

Disease Associated with IC	Antibodies and Antigens Involved	Pathology	Characteristics	References
Serum Sickness and Drug Allergies	Anti-serum proteins -penicillin and other drugs -bee stings	Arthus and Anaphylactic reactions	Serum proteins - long in vivo survival IC form after primary antibody production	105
Seasonal Alveolitis and Nephritis Reactions to Ingested Ags. Gluten-sensitive enteropathy	Anti-fungal spores -plant antigens -drugs -food antigens	IgE responses and Anaphylactic reactions Alveolitis Nephritis Penicillamine nephritis Dermatitis Herpetiformis	IgE-containing IC in kidney and lung IgA-containing IC in skin	78 84 62
Hepatitis	Anti-Hepatitis B surface antigen (HBs) Anti-Hepatitis B core (HBc)	Hepatitis - destruction of hepatocytes w/ membrane-bound HBs Polyarteritis nodosa - IC w/ HBs in serum and lesions	Antibody-C', Ab-K cell cell-mediated killing Increase in free HBs blocks effect	4 94
Dengue Hemorrhagic Fever	Anti-Dengue antigen	Acute febrile illness Previous infection Hypovolemic shock Nephritis Skin rashes	Massive serum C' activation Second infection - increased viral replication w/in monocytes Platelets release vasoactive amines Kidney - IgG, IgM, C3 Skin - Dengue Ag., IgM, C3	10, 13 68 83, 91
Poststreptococcal Glomerulonephritis	Anti-streptococcal Ag. crossreacts with glomerular basement membrane (GBM)	Nephritis Streptococcal components crossreact w/ human GBM antigens	Kidney IgG, IgM, C', and streptococcal antigens IgG cryoglobulins	58 106
Subacute Bacterial Endocarditis	Anti-streptococcal Ags. crossreact w/ cardiac tissue and GBM	Nephritis Vasculitis	Renal glomeruli - Antibody and C' deposits	8

TABLE 3. (Cont'd.)

Disease Associated with IC	Antibodies and Antigens Involved	Pathology	Characteristics	References
Leprosy	Anti-*Mycobacterium* antibodies	Tuberculoid - Lepromatous - Erythema Nodosum Leprosum	↑ CMI[1] ↓ Ab. few CIC ↓ CMI ↑ Ab. many CIC Subcutaneous nodules Immunoglobulin, C' deposits C3 split products	12 31 64 80 98
Malaria	Anti-malarial antibody	*Plasmodium falciparum* renal lesions - transient, respond to therapy *Plasmodium malariae* (quartan) renal lesions - chronic, progressive, poor response to therapy Variation in size and pattern of granular deposits	Soluble antigens; free or IgM-bound Granular deposits - Immunoglobulin, C', Ag. In serum - Immunoglobulin, C', Ag. Coarse - IgM and/or IgG Fine - IgG_2	40 41 42 56 57
Trypanosomiasis	Anti-parasite surface antigen	Generalized vasculitis Brain, heart, and kidney lesions Mesangial proliferation and thickening in GBM	Variation in surface antigens Alternating antigen and antibody excess Local IC formation DNA antibodies, ↓C', ↑ Immunoconglutinin Specific IgM and IgG anti-parasite antibodies	105
Schistosomiasis	Anti-parasite Ags. (gut associated, membrane, and egg antigens)	IC may interfere w/ protective immune mechanisms Inhibit eosinophil damage to Schistosomula Renal lesions	Soluble antigens and CIC Fc receptors on eosinophils bind IC Immunoglobulin and C' deposits	42 75
Onchocerciasis	Antibodies to dead parasites	Microfilaria present in skin and other tissues	CIC w/ IgM, IgG, C' ↓ C1q, C3, C4, ↑ Immunoconglutinin	105

[1] CMI - Cell-mediated immunity

TABLE 4. METHODS FOR DETECTION OF IMMUNE COMPLEXES

I. BOUND TO TISSUES

A. Demonstration of Immunoglobulin Deposits (w or w/o C')

1. Immunofluorescence - fresh tissue
2. Immunoperoxidase - fixed tissue (or fresh)
3. Electron microscopy

B. Demonstration of Specific Antibody

1. Elution of Ab. from tissues, Reaction w/ specific Ag.

C. Demonstration of Specific Antigen

1. Immunofluorescence
2. Electron microscopy

II. IN BIOLOGICAL FLUIDS

A. Antigen-Specific Methods

1. Electron Microscopy
2. Counter-Immunoelectrophoresis
3. Immunoglobulin precipitation
4. Antigen precipitation

B. Antigen-Nonspecific Methods

1. Based on physical properties
   a. Based on size changes
   b. Based on solubility changes
2. Based on biological properties
   a. Interaction with complement
   b. Interaction with antiglobulins
   c. Interaction with cellular receptors
3. Indirect serological evidence

TABLE 5. METHODS FOR DETECTING IC BOUND TO TISSUES

Method	Abbreviation	References
A. Demonstration of Ig Deposits		
1. Immunofluorescence	Im. Fl.	86,87,99
2. Immunoperoxidase	Im. Px.	15,77
3. Electron microscopy	E-M	4
B. Demonstration of Specific Antibody		
1. Elution of Ab. from tissues	ELUT	48
C. Demonstration of Specific Antigen		
1. Immunofluorescence	Im. Fl.	99
2. Electron Microscopy	E-M	4

TABLE 6. ANTIGEN-SPECIFIC METHODS FOR DETECTING IC IN BIOLOGICAL FLUIDS

Method	Abbreviation	References
A. Electron Microscopy	E-M	4
B. Counter Immunoelectrophoresis	CIE	45
C. Immunoglobulin Precipitation	Ig. PPT	69
D. Antigen Precipitation	Ag. PPT	35,43

TABLE 7. ANTIGEN NON-SPECIFIC METHODS FOR DETECTING IC IN BIOLOGICAL FLUIDS

Method	Abbreviation	References
I. BASED ON PHYSICAL PROPERTIES		
A. Based on Size Changes		
1. Combined Fractionation & Immunochemical Analysis		27,33,49,85
Gradients and Filtrations		9,12,66
2. Analytical Ultracentrifugation	UC	5,6,101
B. Based on Solubility Changes		
1. Cryoprecipitation; Precipitation of Cryoglobulin	CRYO	16
2. Precipitation in Polyethylene Glycol (PEG)	PEG	14,22,23,112
II. BASED ON BIOLOGICAL PROPERTIES		
A. Interaction with Complement		
1. Binding of Clq		
Clq Binding Assay	ClQ-BA	71,107,109
Solid Phase Clq RIA	ClQ-SP	36,37
Clq Agarose Precipitation Test	ClQ-PTT	1
2. Inhibition of Clq		
Clq Deviation Test	ClQ-DV	29,60,83
Clq Latex Agglutination Inhibition Test	ClQ-LI	17,54
Clq Binding Inhibition RIA	ClQ-RI	30
Measurement of Anti-C' Activity	AC	65
3. Reactivity with Conglutinin		
Solid Phase Conglutinin Binding Assay	KgB-SP	20,25

continued

TABLE 7 (Cont'd.)

Method	Abbreviation	References
II. BASED ON BIOLOGICAL PROPERTIES (Cont'd.)		
B. Interaction with Antiglobulins		
1. With Monoclonal Rheumatoid Factor (mRF)		
mRF Binding Inhibition RIA		
with Solid Phase RF	mRF-I	55
with Soluble RF	mRF-RI	30
mRF Agarose Precipitation Test	mRF-PPT	1,102
2. With Polyclonal Rheumatoid Factor (pRF)		
pRF inhibition RIA	pRF-I	21
pRF Latex Agglutination Inhibition Assay	pRF-LI	17,54
C. Interaction with Cellular Receptors		
1. Complement Receptors		
Raji Cell RIA	Raji-RIA	91,92
Raji Cell Fluorescence	Raji-FL	89,95
EAC Lymphocyte Rosette Formation Inhibition	ROS-I	26,46,82
2. Fc Receptors		
Platelet Aggregation Test		
on Native Serum	PAT-1	67
on Heated Serum	PAT-2	73
Macrophage Uptake Inhibition Test	MUI	47,63,72
K-cell Cytotoxicity Inhibition Test	KIT	59,79
Neutrophil Inhibition Test	NIT	
Neutrophil-dependent Red Cell Browning Assay	BET	
Rat Spleen Leucocyte RIA	RSL	3
D. Indirect Serological Evidence		

samples to allow IgG-containing complexes which have bound complement to bind to C3b receptors. Cell-bound IgG is then measured using radiolabeled anti-human IgG. Results are evaluated using a standard curve prepared using normal human serum (NHS) and aggregated human gammaglobulin (AHG) in varying amounts. The Raji cell assay is sensitive and quantitative, but detects only IgG-containing IC and involves radiolabeled anti-human IgG which is not commercially available at present. Other IC which fix complement, but do not contain IgG, would also bind to Raji cells; however, these IC would not be detected since they would not bind the labeled anti-human IgG.

In the solid-phase Clq binding test (Hay, Nineham and Roitt, 1976) (36,37), EDTA-treated serum samples are incubated in plastic tubes coated with Clq. IC bind to the solid phase Clq by virtue of the affinity of immunoglobulin Fc regions for Clq. The amount of IC bound to Clq is determined using a radiolabeled anti-human IgG. This method, like all methods involving Clq, has the disadvantage that only complement-fixing classes will bind to the Clq. Additionally, only bound IC containing IgG will react with the labeled anti-human IgG. In addition to requiring the iodination of anti-human IgG, this assay also requires the purification of Clq.

The Clq binding assay (Nydegger, 1974; Zubler et al., 1976; and Zubler and Lambert, 1976) (71,108,109) measures the binding of radiolabeled Clq to IC. EDTA-treated serum is incubated with ^{125}I-Clq and polyethylene glycol (PEG). Complement-fixing IC bind to Clq and are precipitated by the PEG while free Clq remains soluble. The Clq binding activity is the percentage of protein-bound radioactivity which is

precipitated after centrifugation. This assay also detects only IC which fix complement, but differs from the Raji cell assay and the solid-phase Clq binding test in that it does not employ anti-human IgG. Therefore, this assay detects any IC which react with the Clq. However, complexes already saturated with Clq in vivo may not be detectable by any Clq assays unless EDTA or heat treatment removed previously bound Clq.

In addition to the Clq purification necessary for the ClQ-SP test, the ClQ-BA also requires labeling of the Clq with ^{125}I or other isotopes, making this test less adaptable to the routine clinical laboratory setting.

An additional technique evaluated in the WHO study which has particular relevance to the present study was the inhibition of complement-dependent lymphocyte rosette formation. Ezer and Hayward isolated B lymphocytes from human adenoid tissue and evaluated complement-dependent rosette formation by these cells following their incubation in sera from healthy controls or patient populations (26).

More recently, Kammer and Schur (46) used human peripheral blood lymphocytes and HEAC (erythrocytes coated with antibody and complement) and EA rosette inhibition to test for CIC. In both procedures, HEAC rosette inhibition occurred when C3b-bound complexes attached to lymphocytes bearing C3b receptors (HEAC rosette inhibition).

The Schur modification includes inhibition of rosette formation with HEAC by C'-bound IC, as well as inhibition of rosette formation with EA cells by complexes which do not bind C'. This modified method detects IC of the IgG and IgM classes with or without bound C'; however,

with CIC involving IgA or IgE, only complexes with C' fixed by the alternative pathway would be detected.

The assay developed for this study combines concepts employed by the Raji cell assay, as well as those of the rosette inhibition assay, since Raji cells are used as the rosetting cells and IC binding both Fc and C3b receptors are detected.

MATERIALS AND METHODS

Serum

A total of 223 sera included in this study were obtained from healthy laboratory personnel; individuals donating a unit of blood for transfusion; patients with rheumatoid disorders including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA); patients on whom kidney, muscle, or skin biopsies were performed; and other patients on whom specimens were submitted to the Immunology Laboratory, E. W. Sparrow Hospital, for routine analysis.

Venous whole blood was collected in vacutainer tubes and allowed to clot at room temperature (22° C). After 30 minutes the clotted blood was placed on ice for an additional 30 minutes. Serum was separated from the clot by centrifugation at 3500 x g at 0° C in an RC-3 refrigerated centrifuge (Dupont-Sorvall, Wilmington, DE). Serum was stored at -70° C in 200 µl and 1 ml aliquots until tested.

Raji Cells

Raji cells, a lymphoblastoid cell line derived from Burkitt's lymphoma, were purchased from the American Type Culture Collection (Rockville, MD). Raji cell cultures were initiated from an aliquot of cells frozen in DMSO-supplemented tissue culture medium. Frozen cells were thawed at 37° C, washed twice by centrifugation with RPMI 1629 (GIBCO, Grand Island, NY) at 37°C in 5% CO_2. A continuous supply of cells was maintained by passing an aliquot of cells every third day.

For the assay, Raji cells were harvested 72 to 96 hours after passage, washed two times in RPMI, and counted in a hemocytometer. Cell viability was determined by trypan blue exclusion. An average viability was 94%.

Preparation of Aggregated Human Gammaglobulin

Cohn fraction II (Sigma Chemical Co., St. Louis, MO) was used to obtain aggregated IgG following a technique reported by Theofilopoulos and Dixon (90). One gram of Cohn fraction II was dissolved in 10 ml PBS, pH 7.2, and centrifuged at 146,000 x g for 90 min. in a Sorvall Ultracentrifuge. The upper third of the supernatant fluid was separated and used as the source of aggregated IgG. The protein concentration, determined by the Biuret method, was 8.2 g/dl. The immunoglobulin solution was heated in a 63° C water bath for 30 min. and stored in 25 µl aliquots at 4° C.

Preparation of Hypertet-Tetanus Toxoid Immune Complexes

One ml (400 Units) of Tetanus Immune Globulin (Cutter Laboratories, Berkeley, CA) was mixed with 1 ml (200 Units) of tetanus toxoid (Michigan Department of Public Health, Lansing, MI) at 4° C. After a one-week incubation period, the tetanus-anti-tetanus complexes were centrifuged at 1800 x g for 20 min, in a Sorvall RC-3 refrigerated centrifuge. The supernatant was removed and stored at 4° C. Immune complexes with mouse complement were prepared by incubating 0.25 ml aggregated IgG or 0.25 ml Hypertet-toxoid complexes with equal amounts of mouse serum for one hour at 37° C. Mouse serum was obtained from adult ICR female mice. The serum was prepared in a manner which would maintain its complement activity.

Indicator Cells for the Raji Cell Rosette Assays

Human O erythrocytes were washed 4X with RPMI and resuspended to 2.5%, in RPMI. One hundred µl of rabbit serum with anti-human erythrocyte antibody were incubated with 2 ml (1:20 v/v) of the erythrocyte suspension for 40 min. at 37° C. Following incubation, the erythrocytes were pelleted by centrifugation, the supernatant removed, and 200 µl of mouse serum added. The cell pellet was resuspended in the mouse serum and the mixture incubated for 30 min. at 37° C. The complement-sensitized erythrocyte-antibody complexes (HEAC) were washed 7X with cold RPMI and resuspended to 5%. Antibody-coated erythrocytes (EA) also were used in this study. Sheep erythrocytes sensitized with hyperimmune (IgG) rabbit anti-SRBC were purchased from Cordis Laboratories (Miami, FL) for the assay. EA cells were washed 5X with RPMI and resuspended to 5% (v/v) concentration. Target cells were used within one week (HEAC) or two weeks (EA) from date of preparation (11,28).

Raji Cell Rosette Inhibition (ROS-I) Assay for Detecting Immune Complexes in Human Sera

Fifty µl of washed Raji cells at a concentration of 15 x 10^6 cells per ml were incubated with 100 µl of human sera from healthy volunteers or hospitalized patients for one hour at 4° C. The Raji cell viability exceeded 90%. After the incubation of Raji cells and serum, the cells were washed twice with RPMI at 130 x g in an IEC Clinical Centrifuge (Damon, Needham Hgts., MA) and resuspended to 2 x 10^6 cells per ml. HEAC and/or EA target cells (0.1 ml of 0.5% v/v in RPMI 1629 medium) and 0.1 ml Raji cell suspension (2 x 10^6 cells/ml in RPMI) were mixed in

10 x 75 mm glass tubes. The Raji cell-target cell suspensions were centrifuged for 8 min. at 120 x g in a Sorvall GLC-2 centrifuge. Following centrifugation, the tubes were placed in the refrigerator at 4-6° C. Raji cells and HEAC target cell mixtures were incubated for 30 min. while Raji cells with EA target cell mixtures were incubated for 12 hr. At the time of assay, the Raji cell-target cell pellets were resuspended in 0.05% (wt/vol) trypan blue. The viability of Raji cells was determined microscopically by trypan blue exclusion (> 85% excluded the dye). Calculation of Raji cell rosette formation and rosette inhibition was based on 200 viable Raji cells. Generally, 75 to 150 Raji cells formed rosettes with HEAC cells and 90 to 130 formed rosettes with EA cells.

Cytochalasin B

A 100 µg/ml stock solution of Cytochalasin B (Sigma Chemical Co., St. Louis, MO) was prepared in RPMI. One ml aliquots of stock solution were frozen at -70° C. The stock was diluted 1:20 with RPMI to yield a 5 µg/ml working solution.

Colchicine

A 50 µg/ml working solution of colchicine (Sigma Chemical Co., St. Louis, MO) was made by adding 1 mg colchicine to 20 ml RPMI 1929 medium.

RESULTS

Raji Cell Culture Requirements

The recommended fetal calf serum (FCS) concentration for Raji cells was 10% (v/v). Experiments were undertaken to determine whether a 1% or 2% FCS concentration would be adequate for immune complex evaluation. The number and viability of Raji cells cultured in 1%, 2%, or 10% FCS were evaluated on days 3 and 4 of culture. Additionally, Raji cell rosette formation with EA target cells, with and without pretreatment with normal serum or aggregated IgG, was performed on the third day of culture.

The Raji cells cultured in 10% FCS for 3 days had three to four times the number of cells as cultures supplemented with 1% or 2% FCS. For Raji cells cultured four days, an even greater difference was noted (Table 8). Satisfactory cell viability (> 90% V) was maintained in three-day cultures with either 2% or 10% FCS concentrations; however, for cells cultured for four days, only cells incubated in 10% FCS yielded viabilities greater than 90%.

Portions of Raji cell cultures supplemented with 2% or 10% FCS were adjusted to 15 x 10^6 cells/ml for use in the Raji cell ROS-I assay. Raji cells (50 µl) were incubated with RPMI, aggregated IgG, or 100 µl of normal serum at 4° C for 60 minutes. EA rosette formation was greatest in Raji cells cultured in 10% FCS (Table 9). Figure 1 shows representative Raji cells with EA rosettes.

TABLE 8. INFLUENCE OF FETAL CALF SERUM CONCENTRATION ON RAJI CELL NUMBER AND VIABILITY

FCS Concentration	Cell counts x 10^{-6} /ml	
	Day 3	Day 4
1%	3.2 (87)[a]	5.0 (84)[a]
2%	6.5 (94)	5.0 (85)
10%	16.7 (95)	41.0 (96)

[a]Number in parentheses indicates the percentage of viable cells in 200 cells counted using trypan blue dye exclusion.

TABLE 9. INFLUENCE OF FETAL CALF SERUM CONCENTRATION ON RAJI CELL EA ROSETTE FORMATION

Raji Cell Pretreatment	Percent (%) EA Rosette Forming Cells		
	1% FCS	2% FCS	10% FCS
None	18 (83)[a]	23 (90)	34 (90)
RPMI	---	30 (83)	41 (93)
Agg IgG	---	1 (83)	2 (83)
NS-01	---	16 (81)	42 (91)
NS-07	---	21 (86)	40 (68)

[a]Cell viability evaluated by trypan blue dye exclusion.

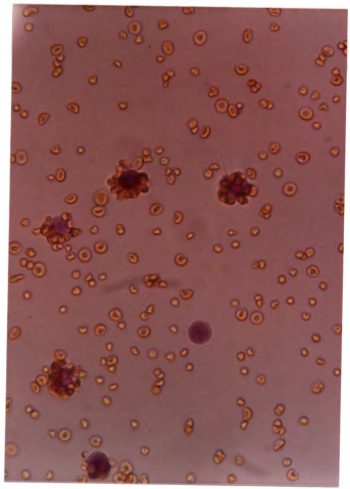

Figure 1. Raji cells with EA rosettes.

The influence of time and temperature on Raji cell viability was evaluated by a vital dye staining procedure. For the assay, 7.5 x 10^6 Raji cells in 50 µl RPMI 1629 were incubated at room temperature (22° C) or in the refrigerator (4° C) with either RPMI (100 µl) or NHS (100 µl or 200 µl). Four incubation periods, ranging from 10 minutes to 120 minutes, were evaluated (Table 10). Preliminary experiments revealed that serum interfered with the trypan blue diffusion into non-viable Raji cells, and thus, Raji cells were washed once in RPMI to remove serum prior to viability determinations. The viability of Raji cells was not influenced by the incubation temperature; however, cells incubated in NHS were consistently less viable, albeit only slightly, compared with cells incubated in RPMI.

Influence of Colchicine and Cytochalasin B on Raji Cells

Microtubules and microfilaments within the cell play an important role in the movement of surface receptors and surface immunoglobulins in lymphocytes (i.e., capping and patching). A similar phenomenon may occur in Raji cells. Cytochalasin B and colchicine are known inhibitors of microfilament and microtubule function, respectively (96). Treatment of Raji cells with cytochalasin B or colchicine may therefore result in higher percentages of rosette forming Raji cells.

Studies were undertaken to determine the influence of cytochalasin B and colchicine on Raji cell number, viability, and rosette formation. Three-day cultures of Raji cells were washed once with RPMI 1629, resuspended in RPMI, divided into three equal volumes and incubated with 20 ml of RPMI, or 50 µg/ml colchicine in RPMI, or 5 µg/ml cytochalasin B

TABLE 10. INFLUENCE OF TIME, TEMPERATURE AND SERUM CONCENTRATION ON RAJI CELL VIABILITY

Time of Incubation (min.)	Incubation Temperature	Percent (%) Viability		
		RPMI	NHS (100 µl)	NHS (200 µl)
10	22°C	92	--	100
	4°C	95	98	100
30[a]	22°C	96	92	
	4°C	96	91	
60[a]	22°C	96	88	
	4°C	96	88	
120[a]	22°C	92	89	86
	4°C	90	89	88

[a]Washed 1X after incubation.

in RPMI for 10 minutes at 20° C. Following incubation, the cells were washed once with RPMI and resuspended in equal volumes of RPMI. Cell counts, viability, and rosette formation with EA and HEAC target cells were then determined (Table 11). The cell counts and viabilities were similar for RPMI and colchicine-treated Raji cells, while the cytochalasin B had a slight cytotoxic effect on the cells. The percentage of HEAC and EA target cells were comparable for all three groups and thus neither cytochalasin B nor colchicine increased rosette forming cells (RFC).

Collection and Storage of Serum for Raji Rosette Inhibition Assay

Since current methods of immune complex detection cannot differentiate between non-specifically aggregated immunoglobulins and true IC, it was important to determine the influence of repeated freezing and thawing of normal serum on rosette formation and Raji cell viability.

Venous blood from a normal serum donor was incubated at room temperature for 30 minutes and then centrifuged at 3500 x g for 15 minutes at 4° C in an RC-3 refrigerated centrifuge. The serum was divided into six aliquots. The serum aliquots were frozen and thawed from one to six times, in a dry ice-ethanol mixture. Serum samples were tested for ROS-I. The number of RFC when incubated with RPMI, aggregated IgG, Hypertet-toxoid complexes, and NHS frozen and thawed one through six times are shown in Table 12.

Three cycles of rapid freezing and thawing of serum appeared to have little effect on rosette formation. Thereafter, however, ROS-I increased although Raji cell viability was not affected by freezing and

TABLE 11. INFLUENCE OF COLCHICINE AND CYTOCHALASIN B ON RAJI CELL VIABILITY AND ROSETTE FORMATION

Raji Cell Treatment	Percent (%) Rosette-Forming Cells	
	HEAC	EA
RPMI	152 (5)[a]	117
Colchicine (50 μg/ml)	156 (93)	104
Cytochalasin B (5 μg/ml)	163 (93)	97

[a]Percent viable cells determined by trypan blue dye exclusion.

TABLE 12. INFLUENCE OF FROZEN AND THAWED NORMAL HUMAN SERUM ON RAJI EA ROSETTE FORMING CELLS

Freeze-Thaw Cycle	EA RFC/200 Raji Cells	Percent Inhibition
RPMI Control	78	--
1	79	--
2	76	(4)[a]
3	76	(4)
4	67	(15)
5	63	(20)
6	55	(30)

[a]Numbers in parentheses represent percent inhibition of rosette formation compared to sera frozen and thawed once.

thawing. To assure optimal results from patient sera, specimens were aliquoted and thawed only once immediately before testing.

Determination of Normal Range for Raji Cell Rosette Inhibition

The normal range for Raji Cell ROS-I was determined by computing the average of the RFC obtained for Raji cells incubated with the negative control sera (NHS) used. Three or four negative control sera were run concurrently with positive controls and patient sera. The number of RFC per 200 viable Raji cells counted was recorded and the average of the three or four values for the negative controls was designated zero percent inhibition. All patient values were compared to the amount of rosette inhibition exhibited by normal sera.

Four examples of baseline determination for HEAC and EA rosettes are given in Figure 2. Representative assays performed between March 30, 1979 and May 24, 1979 are presented. The mean values are also presented and vary for HEAC rosettes from 94 to 150 RFC, and 102 to 133 RFC for EA rosettes.

The daily variation in baseline values for 20 HEAC assays and 28 EA assays is depicted in Figure 3. The baseline HEAC rosette values ranged from 76 to 154 RFC, and baseline EA rosette values ranged from 62 to 156 RFC.

Utilization of Normal Sera to Calculate Percent Inhibition and Establish a Normal Range in the Assay

Sera from twelve healthy individuals were selected as negative control specimens. The serum was aliquoted and stored at -70° C.

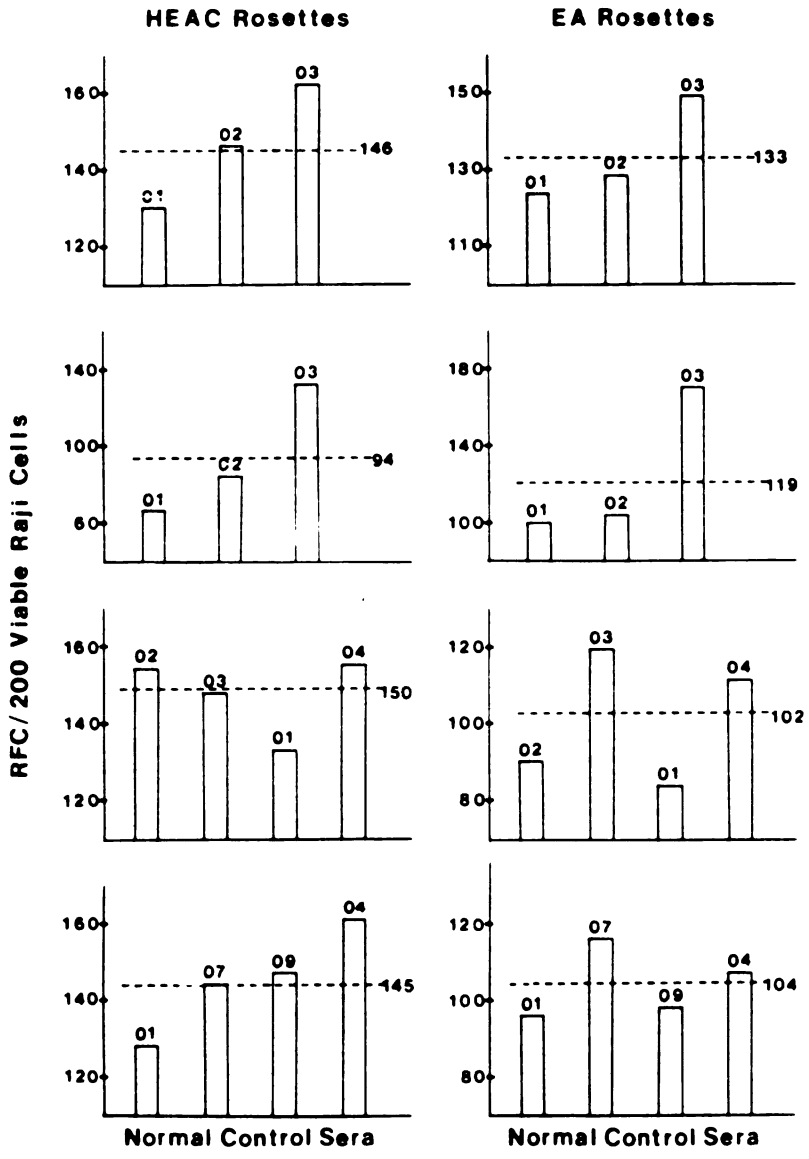

Figure 2. Baseline determination for Raji rosette inhibition: Four examples using 3 to 4 NHS per assay. Mean value indicated by broken line.

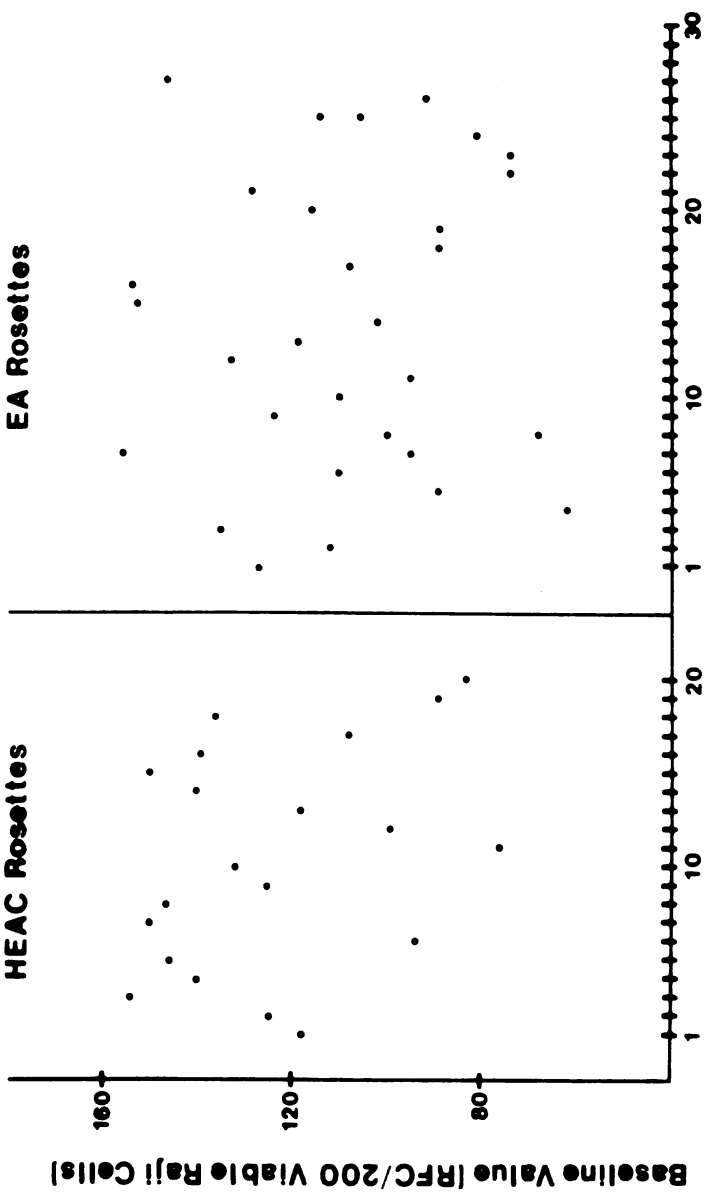

Figure 3. Daily variation in baseline HEAC and EA values used to determine Raji Rosette inhibition.

Baseline, zero percent inhibition, was calculated for each assay by determining the mean number of RFC for three or four negative control sera. The percent inhibition for aggregated IgG and Hypertet-toxoid complexes (positive controls), negative control specimens, and patient sera was calculated as a percentage of the baseline value. A representative example of the calculation appears in Table 13. The baseline value, the mean of four negative control sera, for EA rosettes was 102 RFC/200 viable Raji cells. Raji cells incubated with aggregated IgG and Hypertet-toxoid complexes exhibited 45 and 40 EA-RFC, respectively. Serum from patient EX-01 incubated with Raji cells resulted in 72 EA-RFC or 30% inhibition (1 - 72/102 = 0.3).

Ninety-three assays performed using serum from 12 negative controls exhibited values ranging from zero percent to 29 percent inhibition. Seventy-eight specimens (84%) exhibited values between zero and nine percent rosette inhibition. Fifteen specimens (16%) resulted in 10 to 29 percent rosette inhibition; however, only four of 93 specimens exceeded 15 percent as shown in Table 14.

Serum samples were classified as negative, borderline positive or positive for immune complexes according to the following definitions: Negative: less than 10% inhibition of rosette formation; Borderline Positive: 10 to 15% inhibition; Positive: greater than 15% inhibition.

Raji Rosette Inhibition Using Serum from Healthy Blood Donors

Normal serum was collected from individuals donating units of blood. An aliquot of the serum drawn for routine blood work was stored at -70° C and evaluated with positive and negative control specimens and

TABLE 13. CALCULATION OF EA ROSETTE INHIBITION

Treatment of Raji Cells	EA-RFC	Percent Inhibition
NS-01	94	8
NS-03	118	0
NS-02	96	6
NS-04	101	0
	Baseline value: $\bar{x}$ = 102	
Agg.IgG	45	56
Hyp/tox	40	61
Ex-01	72	30

TABLE 14. RAJI ROSETTE INHIBITION USING SERUM FROM 12 NEGATIVE CONTROLS TO ESTABLISH THE NORMAL RANGE FOR THE ASSAY

Negative Control	Total No. Runs	Negative 0-9% ROS-I	Borderline 10-15% ROS-I	Positive >15% ROS-I	Percent (%) Inhibition Range	Percent (%) Inhibition Average
NS-01	36	26	6	4	0-29	7.5
NS-02	20	17	3	0	0-15	4.9
NS-03	8	7	1	0	0-13	1.8
NS-04	8	8	0	0	0- 2	0.3
NS-05	6	5	1	0	0-13	3.4
NS-06	2	2	0	0	3- 7	5.0
NS-07	2	2	0	0	0	0
NS-08	1	1	0	0	0	0
NS-09	4	4	0	0	0- 8	2.0
NS-10	2	2	0	0	0- 6	3.0
NS-11	2	2	0	0	0	0
NS-12	2	2	0	0	0	0
Total	93	78	11	4		
Percent of Total		84%	12%	4%		

patient sera. In all, 31 blood donors were tested by the Raji cell ROS-I assay. Results appear in Table 15. Seventeen (55%) had negative ROS-I values with both HEAC and EA target cells. Six (19%) fell into the borderline category with values for one or both target cell types in the 10 to 15% inhibition range, or one value less than 10% and the other value greater than 20% inhibition. Eight (26%) were positive for both cell types with the exception of one whose values were eight percent inhibition with HEAC cells and 29 percent inhibition with EA cells. Four of eight positive sera were reassayed to confirm the positive results. None of the eight positive sera were positive for rheumatoid factor by the latex bead agglutination slide test. All eight were also negative for antinuclear antibody by indirect immunofluorescence.

Raji Rosette Inhibition Using Serum from Patients Submitted for Diagnostic Tissue Biopsies

Sera from 24 patients on whom kidney, muscle or skin biopsies were performed were collected and stored at -70° C. As shown in Table 16, sera from 10 patients with immune complexes exhibited by immunofluorescence were positive for Raji ROS-I.

The values ranged from 11 percent (borderline positive) to 83 percent. Sera from nine patients positive for immune complexes by immunofluorescence were negative by rosette inhibition. Five of the 24 patients were negative for immune complexes by immunofluorescence. Four of the five negatives were also negative for ROS-I. There was no apparent correlation between the value of Raji cell ROS-I and the presence of immune complexes in the kidney, skin or muscle.

TABLE 15. HEALTHY BLOOD DONORS EVALUATED FOR CIRCULATING IMMUNE COMPLEXES BY RAJI ROSETTE INHIBITION

Raji ROS-I	Number of Sera	Percent (%) Inhibition Range of Values HEAC	EA	Average
Negative	17	0-10	0-5	0.8
Borderline	6	0-18	0-18	12.2
Positive	8	8-58	19-53	36.5

TABLE 16. KIDNEY, SKIN AND MUSCLE BIOPSY PATIENTS EVALUATED FOR IMMUNE COMPLEXES BY TISSUE IMMUNOFLUORESCENCE AND RAJI ROSETTE INHIBITION

ROS-I Greater Than 10%				ROS-I Less Than 10%			
Patient	Diagnosis	Biopsy pos. for	Percent ROS-I	Patient	Diagnosis	Biopsy pos. for	Percent ROS-I
		Biopsy Patients Positive by Immunofluorescence					
BX-01	GN-SLE[b]	M,G,C3	21	BX-11	GN-SLE	M,G,Clq,C3,C4	0
BX-02	SLE	M,G,A,Clq,C3,C4	32	BX-12	MP-GN[b] chemical	M,G,A,Clq,C3,C4	2
BX-03	Alports Disease	M,G,Clq,C3	59	BX-13	Goodpastures	M,G,A,Clq,C3,C4	0
BX-04	IgA nephropathy	M,A,C3	28	BX-14	SLE	M,C3 skin[a]	0
BX-05	Vasculitis	C3,(G,M) skin[a]	83	BX-15	MP-GN	M,G,C3,Clq	0
BX-06	MCD-Proteinuria[b]	M only	12	BX-16	MP-GN	M,G,C3,Clq	0
BX-07	Polymyositis	M,C3,Clq muscle[a]	11	BX-17	Focal segmental sclerosis (drug)	M,G,C3	7
BX-08	M-GN-SLE[b]	M,G,C3	21	BX-18	Henöch-Schönlein purpura	M,A,C3	0
BX-09	SLE	M,G,C3,Clq	61	BX-19	Mesangial IgA nephropathy	M,A	0
BX-10	SLE	M,C3	43				
		Biopsy Patients Negative by Immunofluorescence					
BX-20	Hypersensitivity Alveolitis		31	BX-21	Vasculitis	skin[a]	0
				BX-22	PS-GN[b]	C3 only	0
				BX-23	Amyloidosis	k lt. ch.	0
				BX-24	Scleroderma	C3 only	3

[a]Biopsies are kidney unless otherwise labeled.

[b]GN--glomerulonephritis, MCD--minimal change disease, M--membranous, MP--membranoproliferative, PS--post-streptococcal.

Raji Rosette Inhibition Using Serum from Patients with Collagen Vascular Disorders

Serum was obtained from 11 patients diagnosed with systemic lupus erythematosus (SLE) or mixed connective tissue disease (MCTD). Results of Raji cell ROS-I assay, antinuclear antibody and other pertinent data are recorded in Table 17. ROS-I was positive in five of 11 (45%), with a range of 21 to 82 percent inhibition, borderline in two (18%) and negative in four (36%). There was no apparent correlation between Raji cell ROS-I value and antinuclear antibody titer, percent DNA binding, extractable nuclear antigen titer or complement level.

Raji Rosette Inhibition Using Serum from Patients with Rheumatoid Arthritis

Serum was obtained from ten rheumatoid arthritis patients having RA latex titers exceeding 1:80. Results of Raji cell ROS-I assay, rheumatoid factor titer and other pertinent data are recorded in Table 18. ROS-I values were positive in five of 10 (50%) with a range of 22 to 70 percent inhibition, borderline in one (10%) and negative in four (40%). All five CIC positive sera had RF values of 160 or greater, but there was no direct correlation between RF titer and percent inhibition as the four CIC negative sera also had RF values greater than 160.

Raji Rosette Inhibition Using Serum from Patients with Rheumatoid Disorders

An additional 32 sera specimens were collected from patients with rheumatoid disorders. The sera were grouped according to results from antinuclear antibody (ANA) and rheumatoid factor (RF) tests into: 10 ANA-positive sera with titers ranging from 20 to 1,280, 10 RF-positive

TABLE 17. COLLAGEN VASCULAR DISEASE PATIENTS EVALUATED FOR IMMUNE COMPLEXES BY RAJI ROSETTE INHIBITION

Patient	Percent (%) ROS-I	Anti-Nuclear Antibody[a]	Percent (%) DNA Binding	Extractable Nuclear Antigen[b]	Complement[a]	Other Studies[d]
CV-01	11	2560-H	44	neg.	D	RF - 80
CV-02	0		19			Biopsy Pos., Raji-RIA -
CV-03	0	320-H	9	160	N	RF - 1280
CV-04	21	neg.	neg.		N	Biopsy Pos.
CV-05	21	320-H	neg.	10,000Sm	D	Biopsy Pos.
CV-06	43	80-H	53	80	D	Biopsy Pos.
CV-07	82	5120-S	neg.	neg.	D(N)	RF - 1280 Raji-RIA +
CV-08	61	neg.		720,480RNP 640Sm	N	RF - 640
CV-09	14	20,480-S	56	40,960Sm	N	
CV-10	0	20,480-H	95		D	Biopsy Pos., Raji-RIA +
CV-11	9	20,480-S	neg.	20,480RNP		Raji-RIA +

[a]Numbers represent ANA titer; H--homogenous pattern; S--speckled pattern.

[b]Numbers represent ENA titer; Sm--ribonuclease-resistant antigen; RNP--ribonuclease-sensitive antigen.

[c]D--decreased, N--normal.

[d]RF--Latex particle rheumatoid factor titer.

TABLE 18. RHEUMATOID ARTHRITIS PATIENTS EVALUATED FOR IMMUNE COMPLEXES BY RAJI ROSETTE INHIBITION

Patient Serum	Percent ROS-I	Rheumatoid Factor Titer	Other Laboratory Results
RA-01	70	1280	
RA-02	64	5120	cryoglobulins
RA-03	46	160	ANA negative
RA-04	0	1280	
RA-05	0	320	ANA negative
RA-06	22	320	ANA negative
RA-07	56	320	
RA-08	0	2560	
RA-09	0	640	ANA negative
RA-10	13	80	ANA 80-S, Dec.C', ssDNA +, ENA -

sera with titers ranging from 20 to 160 (three sera were positive for both tests), and 15 which were negative for both ANA and RF or on which results for one or both tests were not available. The Raji cell ROS-I assay was performed on all 32 specimens (see Table 19). Nine of the 32 sera (28%) were positive, with a range of 18 to 83 percent inhibition, four (13%) were borderline and 19 (59%) were negative. For the nine sera positive for Raji ROS-I, four were ANA positive while five were negative for both ANA and RF.

Raji Rosette Inhibition Using Serum from Patients with Digestive Tract Cancers

Serum was collected from six patients with gastrointestinal tract cancer submitted for carcinoembryonic antigen (CEA) evaluation. All exhibited elevated CEA values (greater than 10 ng/ml) ranging from 11.3 to 1,202 ng/ml. Two of the six positive CEA sera were positive for CIC (29 and 76 percent inhibition), while the remaining four sera were negative (zero to six percent inhibition).

Raji Rosette Inhibition Using Miscellaneous Patient Sera

Eleven serum specimens from patients with various other diagnoses were also evaluated for CIC by the Raji cell ROS-I assay. Four were positive with values ranging from 18 to 54 percent inhibition. The four were: 1) a kidney transplant donor (18%), 2) a Hepatitis B hyperimmune globulin recipient (34%), 3) a multiple sclerosis patient (43%) and a pregnant woman on whom amniocentesis was performed (54%). The other sera were negative for CIC (see Table 20).

TABLE 19. PATIENTS WITH RHEUMATOID DISORDERS EVALUATED FOR IMMUNE COMPLEXES BY RAJI ROSETTE INHIBITION

Raji ROS-I Value	ANA Pos.	RF Pos.	ANA, RF Neg.	Total
Positive	4	0	5	9 (28%)
Borderline	1	0	3	4 (13%)
Negative	5	10	7	19 (59%)

TABLE 20. MISCELLANEOUS PATIENT SERA EVALUATED FOR IMMUNE COMPLEXES BY RAJI ROSETTE INHIBITION

Patient Serum	Diagnosis	Percent ROS-I
MS-01	Histoplasmosis	0
MS-02	Multiple granulomata	0
MS-03	Kidney transplant	0
MS-04	Transplant donor	6
MS-05	Transplant donor	18
MS-06	Multiple Sclerosis	43
MS-07	Multiple Sclerosis	0
MS-08	Hepatitis exposure	34
MS-09	Hepatitis exposure	0
MS-10	Hepatitis	0
MS-11	Amniocentesis	54

Comparison of Three Techniques for Evaluation of Circulating Immune Complexes

Sera from 12 patients on whom kidney, skin or muscle biopsies were performed, were collected and stored at -70° C. Eight of these sera and five other sera from patients whose clinical diagnosis would suggest the presence of CIC were sent to Scripps Immunology Reference Laboratory in LaJolla, California for evaluation of CIC by the Raji-RIA. Fifteen sera were also sent to Dr. R. H. Kelly, Department of Pathology, University of Pittsburgh School of Medicine for evaluation of CIC by a zone electrophoresis assay. Six sera sent to Dr. Kelly were also evaluated at Scripps. All of the aforementioned sera were evaluated for CIC by the Raji cell rosette inhibition assay. Comparative results from the three assays appear in Tables 21 and 22. Results from nine of 13 specimens (69%) evaluated by Raji radioimmune assay and Raji ROS-I correlated, Table 22 A. Results from six of ten specimens (60%) evaluated by electrophoresis and Raji ROS-I correlated, Table 22 B. Three of six results obtained by Raji-RIA and electrophoresis correlated, Table 22 C.

TABLE 21. COMPARISON OF THREE TECHNIQUES FOR EVALUATION OF CIRCULATING IMMUNE COMPLEXES

Patient Serum	Diagnosis[a]	Biopsy[b] Immunofluorescence Positive for	Raji-ROS-I Percent Inhibition	Raji-RIA µg AHG Equivalents/ml[c]	Electrophoresis[d]
BX-11	GN-SLE	M,G,Clq,C3,C4	0	0	Positive (Ag excess)
BX-01	GN-SLE	M,G,C3	21	240	--
BX-12	MP-Gn, chemical	M,G,A,Clq,C3,C4	2	0	--
BX-20	Alveolitis	negative	31	0	Negative
RD-01		--	11	14	Negative
BX-21	Vasculitis	negative (skin)	0	0	--
RD-02	Collagen vascular	--	4	0	--
BX-02	SLE	M(G,A),Clq,C3,C4	32	--	Positive (Ab excess)
BX-13	Goodpasture's	M,G,A,Clq,C3,C4	0	0	--
BX-22	Poststrepto-coccal-GN	C3 only	0	0	--
BX-03	Alport's Ds.	M,G,Clq,C3	59	0	Positive (Ag excess)
BX-14	SLE	M,C3 (skin)	0	450	--
BX-04	IgA nephropathy	M,A,C3	28	--	Positive (Ab excess)
BX-05	Vasculitis	C3, (M,G) (skin)	83	--	Positive (Ag excess)
CV-11	SLE	--	9	49	Positive (Ag excess)
RD-03	SLE	--	21	--	Positive (Ag excess)
CV-07	SLE	--	82	120	Positive (Ag excess)

[a]GN--Glomerulonephritis, MP--membranoproliferative; [b]Biopsies are of kidney unless otherwise labeled; [c]0-12 µg AHG equivalents/ml-normal; assay performed at Scripps Reference Laboratory, LaJolla, Cal.; [d]Non-fasting serum samples; Ag--Antigen; Ab--Antibody; zone electrophoresis assay performed at University of Pittsburgh.

TABLE 22. COMPARISON OF THREE ASSAYS FOR EVALUATION OF CIRCULATING IMMUNE COMPLEXES

A.	Raji ROS-I Value		
	Negative	Borderline	Positive
	8	1	4
Raji-RIA Negative	6	0	2
Borderline	0	1	0
Positive	2	0	2

B.	Raji ROS-I Value		
	Negative	Borderline	Positive
	2	1	7
Zone Electrophoresis			
Negative	0	1	1
Positive -			
Antigen excess	2	0	4
Antibody excess	0	0	2

C.	Raji-RIA Value		
	Negative	Borderline	Positive
Zone Electrophoresis			
Negative	1	1	0
Positive			
Antigen excess	2	0	2
Antibody excess	0	0	0

DISCUSSION

The Raji cell ROS-I assay developed for this study has proved useful in CIC detection. Raji cells have receptors for complement and for the Fc portion of IgG and will spontaneously bind antibody sensitized erythrocytes (EA) via Fc Receptors, as well as complement coated human erythrocyte target cells (HEAC) via complement (C3b) receptors. Rosette formation with either EA or HEAC target cells was observed in only 50 to 70% of the Raji cells. Theoretically, 90 to 100% of the cells should exhibit Fc and C3b receptors, and therefore, nearly all Raji cells should bind EA and HEAC target cells. Possible explanations for the lower than anticipated number of Raji rosette forming cells include: 1) Raji cells used in the assay are at different stages of development so some may not exhibit Fc and C3b receptors, or may have fewer receptors than cells at maturity, 2) receptors may be present on cells but are masked by products of cell metabolism or ingredients in the culture medium, or 3) receptors are present in a fluid membrane and are only available for rosette formation in 70% of the cells. Incubation of Raji cells with colchicine and cytochalasin B, known inhibitors of microtubule and microfilament function, respectively, did not enhance rosette formation, a result which would be expected if not all cells exhibit Fc and C3b receptors due to variations in the maturation of Raji cells in culture. A previous report, however, showed that receptors for Fc, C3b and C3d are expressed equally well throughout the Raji cell cycle

(91, 92). It is known that isolates of Raji cells vary and, perhaps, the cell line used in this study differed with respect to concentration and density of Fc and C3b receptors during the cell cycle. It is noteworthy that some investigators have not been able to duplicate exactly the Raji-RIA technique as reported by Theofilopoulos (33). The explanation generally offered has been the variation in receptor concentration on the Raji cells.

Results of ROS-I assays performed on 93 normal serum specimens used as negative controls revealed that 78 (84%) were negative, 11 (12%) were borderline, and 4 (4%) were positive. The 16% positive value for normal sera compares well with the Raji-RIA assay in which 10% of normal serum specimens are positive for CIC.

Serum from patients with SLE, RA, and other disorders reported by others (52, 100, 105, 109) to exhibit CIC were evaluated in this study. Some sera in each patient population were positive for CIC, while others were negative. In patients with diagnostic tissue biopsies, the Raji ROS-I was positive in 10 of 19 patients. All patients, however, exhibited either immunoglobulin and/or complement deposits, presumably immune complexes, by immunofluorescence. Other investigators (100) have reported the absence of CIC in patients with positive biopsies. As indicated by the WHO study (52), IC are usually present in the circulation of patients with acute glomerulonephritis, but may not be detected in patients with more indolent forms of IC nephritis. Both acute and indolent disorders, however, can have IC deposits in renal tissues. Additionally, IC may not always be detected when anticipated since complexes may be present in the circulation early in the disease course,

or only intermittently. Such observations thus question the current usefulness of CIC assays. This paradox may be explained, perhaps, by the sensitivity or specificity of the assays available. Moreover, it has been suggested that some patients may develop IC nephritis in the absence of an unusual amount of CIC material via an inappropriate processing of normal amounts of IC encountered during the course of day-to-day immunologic surveillance (100).

Systemic lupus erythematosus is generally considered as the model IC disease. In this study, serum specimens from 12 patients with SLE were evaluated for CIC by the Raji ROS-I assay. Kidney biopsies performed on six of these patients revealed immunoglobulin and complement deposits by immunofluorescence studies. Seven of 12 (59%) were positive, two (17%) were borderline positive, and three (25%) were negative. Two of the three negative sera were from patients with positive biopsies. Other investigators have noted that while DNA-anti-DNA complexes represent an important fraction of the glomerular deposits in SLE (48), such complexes are usually not detectable in the circulation (110). This may reflect a particular mechanism for the localization of IC in renal glomeruli. It has also been shown that there is a high affinity of DNA for the collagen component of the glomerular basement membrane (GBM), and that under certain circumstances, free DNA can bind in vivo to renal glomeruli (51). Such bound DNA can react later with circulating anti-DNA antibodies, resulting in a local formation of IC at the site of the GBM, in the absence of circulating DNA-anti-DNA complexes. The clinical relevance of the detection of IC in SLE is unclear at present; however, a few studies indicate the level of CIC fluctuates with disease activity

(18, 111, 112). Elevated levels are usually reported in association with the acute phase of an exacerbation of the disease. Correlations between immune complex levels in SLE and plasma concentration of complement breakdown products have also been reported (51). Studies to evaluate the role of CIC in the pathogenesis of other IC associated diseases are currently being performed in other laboratories (51, 76, 81, 93, 100).

The nature of the IC detected in the serum and synovial fluid of patients with rheumatoid arthritis is still incompletely defined. Although complexes of 19S RF with IgG, and of 7S anti-IgG with IgG have been identified (27, 49), their relative importance is unknown and the possibility that other antigen-antibody systems may be involved remains open.

With most IC detection methods, the incidence of IC in synovial fluid appears relatively high in all types of RA patients, but elevated values are also reported in other forms of inflammatory arthritis (30, 107). The incidence and the level of IC in serum from these same patients, however, may vary considerably according to the method used, as shown by the WHO study (52). Results of the WHO study emphasize that CIC in RA patients may have limited biological activities, and cannot be similarly detected by methods based on the use of different biological recognition units. Such findings may explain why 50% (five of ten) of sera from RA patients were positive for CIC by the Raji ROS-I assay.

A comparison study to correlate the results of the Raji ROS-I assay with Raji-RIA and zone electrophoresis assays indicated that the sensitivity and specificity of these three assays were quite variable.

This finding was not unexpected since the WHO study, designed to evaluate and compare IC methods, showed a marked difference in sensitivity and specificity in the 18 assays evaluated. The WHO study noted further that a single IC assay was not sufficient to detect all types of IC. Immune complex assays dependent on biological properties such as binding of IC to cell receptors or Clq binding showed marked variations in sensitivity and specificity. One of the most sensitive assays in the WHO study, the Raji-RIA assay, detected only 70% of IC sera. Some of the variability in IC assays is dependent on the underlying principle of the test. Clq-dependent assays detect only complexes which activate complement by the classical pathway (containing IgM or IgG) and do not detect IC containing only IgA, IgD, or IgE. Assays dependent on lymphocyte Fc receptors detect only complexes composed of IgG. False positive results can also occur in immune complex assays due to interfering factors such as aggregated immunoglobulin, rheumatoid factors, and substances which react with Clq such as DNA, heparin, and endotoxin. For the Raji assays anti-lymphocyte antibodies can be a potential source of error. Activated complement components may also give false positive results in the Raji ROS-I assay.

Increased specificity for all CIC assays has been discussed in the literature, but as yet, little progress has been made in this area (100, 109). This objective could be obtained by developing methods for the isolation of IC, dissociation of Ag-Ab complexes, and identification of the antigens involved. A more reliable comparison of sensitivities of various assays would be possible if reference standards for IC detection were made available. Reference standards should include stable

antigen-antibody complexes of varying sizes and aggregated immunoglobulin preparations. When such reference materials are available, individual laboratories will be able to run comparison studies to determine which method or panel of assays would best suit their needs in terms of sensitivity and specificity for the types of IC to be detected in various diseases.

A considerable range in the background level of rosette formation was noted in the Raji ROS-I assay. This variability may be due to differences in Fc and C3b receptor densities on the Raji cells at different stages of the cell cycle, variability in target cell preparations and differences in the negative control sera used to determine the baseline value.

Although there was a daily variation in the baseline values, serial assays on sera exhibited reproducible rosette inhibition. Moreover, CIC in some sera were detected by three different procedures. These findings indicate that the Raji ROS-I assay can detect CIC.

Other investigators have used the principle of rosette inhibition to detect CIC. Ezer and Hayward (26) used adenoid tissue and EAC rosette inhibition while Kammer and Schur (46) used peripheral blood lymphocytes and EA and EAC rosette inhibition. The Raji ROS-I assay has advantages over other rosette inhibition methods in that it utilizes a continuous cell line with a greater percentage of total cells exhibiting Fc and C3b receptors in higher density. A comparative study to evaluate rosette inhibition for CIC detection using Raji cells, peripheral blood lymphocytes and adenoid tissue will be undertaken in the future.

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APPENDIX

APPENDIX

Zone Electrophoresis Assay for Circulating Immune Complexes

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The zone electrophoresis assay for detecting CIC is an adaptation of the standard zone electrophoresis in agarose gel procedure reported by Jeppsson (J. O. Jeppsson, C. B. Laurell, and B. Franzen, Clin. Chem. 25:629-638, 1979) and modified by Kelly (R. H. Kelly, M. A. Scholl, V. S. Harvey, A. G. Devenyi, and T. J. Hardy, Fed. Proc. 38:1358, 1979). Dr. Kelly's modification utilizes a lower ionic strength buffer to allow for greater resolution of protein bands near the cathode. The test is based on the observation that antigen-antibody complexes have a net surface charge that differs from either component alone. This allows for the detection of separate bands of free antigen, antigen-excess IC, antibody-excess IC, and free antibody on stained gels. Most CIC are detected as broad rectangular bands with well-defined edges in the gamma region. The specific class of antibody involved can be determined by immunofixation procedures.

The assay is extremely sensitive, detecting IC in 70-80% of normals if serum specimens are collected within two hours postprandially. This is apparently due to food antigen-antibody complexes present in the circulation. On fasting specimens, the assay is positive for 15% of

normal serum specimens. For patient sera, the test is considered positive for CIC when the C3 level (β_2 band) is decreased in addition to a prominent IC band.

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