Immune Complexes: Characteristics, Clinical Correlations, and Interpretive Approaches in the Clinical Laboratory

Stephan E. Ritzmann¹ and Jerry C. Daniels²

Immune-complex-mediated injury is thought to play a role in diseases such as rheumatoid arthritis, systemic lupus erythematosus, serum sickness, various infectious diseases, and malignancies. With increased appreciation of the biological and pathological significance of circulating immune complexes, there has been an effort to develop appropriate techniques for identifying and measuring them. Common approaches exploit such phenomena as the attachment of complement components to antigen–antibody complexes, the presence of specialized receptors for immune complexes at the surface of cells, and the ability of rheumatoid factor to bind with immune complexes. This variety of assay systems for immune complexes has yielded abstruse results in numerous human pathological conditions. Unfortunately, these results seldom correlate with one another in a given disease. Thus, use of a panel of immune complex assays has been recommended. Indirect consequences of immune complex disease may still be appraised and evaluated with some confidence in clinical medicine: measurements of C3 and C4, cryoglobulins, serum viscosity, and turbidity of serum samples. Measurement of immune complexes may be useful in diagnosis, prognosis, and therapeutic monitoring, but it is the characterization of immune complexes that holds the greatest potential for better understanding of disease mechanisms.

Additional Keyphrases: rheumatoid factor • complement • cryoglobulins • systemic lupus erythematosus • infection • rheumatoid arthritis

The origin of the concept of immune complex diseases dates back 120 years to a report by Maurice Raynaud in which he detailed the clinical manifestations of patients with cyanosis, pallor, purpura, or gangrene that were aggravated by the patients’ exposure to cold (1). These conditions are known collectively as Raynaud’s syndrome or, more encompassingly, as cryopathies (2–4). They are diverse in etiology, require multifaceted diagnostic considerations, and demand individualized clinical management. The immune complexes underlying many of these disorders are protean in nature and are unpredictable with respect to their clinical implications. Cryoglobulins are the most striking physicochemical expression of circulating immune complexes.

In the 1950s, the pioneering work of Germuth (5) and Dixon et al. (6) elucidated the principal pathogenic mechanisms by which immune complexes lead to experimental serum sickness. After certain antigens such as bovine serum albumin are injected into rabbits, antibodies appear in the circulation that may consume complement and result in widespread deposition of immune complexes. Such deposition leads to characteristic clinical and pathological abnormalities (Figure 1).

Subsequently, in both experimental animals and patients, immune complex deposition was shown to lead to glomerulonephritis. The glomerular capillaries are the prime target for deposition of circulating immune complexes (CIC). These deposits are typically characterized by immunofluorescence as “lumpy-bumpy” granular deposits of IgG, IgM, C3, and C1q. The mechanism currently is considered to be responsible for 70 to 80% of cases of human glomerulonephritis (7,8). CIC are removed from the circulation mainly by deposition in the specialized vasculature of specific organs, including the kidneys, arteries, skin, lungs, joints, and choroid plexus. The mesangium of the glomerulus is noteworthy for its ability to clear macromolecular substances from the blood. It appears to be the kidney’s primary defense against CIC (10).

Nature and Function of Immune Complexes

Immune complexes are formed in the circulation or tissues as a result of interactions between endogenous or exogenous

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Fig. 1. Immunological and morphological events transpiring after the appearance of detectable antigen–antibody complexes in the circulation of a rabbit injected with ¹³¹I-labeled bovine serum albumin (250 mg/kg). There is a decrease in serum concentrations of complement. Lesions that represent the hallmark of immune-complex disease are seen: (a) vasculitis in various zones including the heart; (b) glomerulonephritis; and (c) serositis. Free antibody appears in the circulation and the inflammatory lesions of serum sickness disappear rapidly after elimination of all antigen–antibody complexes. Source: S. E. Ritzmann and J. C. Daniels, Eds., Serum Protein Abnormalities, Diagnostic and Clinical Aspects (see ref. 2); reproduced with permission.

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antigens and their corresponding antibodies (Table 1). Endogenous antigens are numerous, and they include spent proteins and tissue moieties released after injury as exemplified in myocardial infarction (11), pancreatitis (12), and trauma such as thermal burns (13). Exogenous antigens are microorganisms, plant antigens, viruses, parasites, fungi, and other foreign proteins that gain entry into the body via cutaneous, gastrointestinal (e.g., milk proteins) (14, 15), or respiratory (e.g., pollens) (16) portals. Physiologically, these immune complexes may be considered to provide a protective mechanism by maintaining the body’s homeostasis in the sense of keeping the defenses alert. But under pathological conditions, many types of CIC may be involved in autoimmune disorders (9, 17–19), infectious diseases (7, 9, 18–21), malignancies (7, 17–19, 22), and numerous other conditions (7, 17–19, 23–26). Such CIC are often found in high concentrations, concurrent with the high local concentrations in affected tissues that incriminate the CIC in the pathogenesis of tissue injury in certain of these disorders (27, 28). CIC consist of an extremely wide variety of moieties, differing qualitatively and quantitatively, which may lead to a wide variety of secondary phenomena and tissue injuries.

The antigen/antibody ratio in CIC ranges from marked antigen excess to antibody excess by analogy with the classic Heidelberger curve depicting the quantitative aspects of precipitating antibodies (29) (Figure 2). With increasing antigen but constant antibody concentrations, CIC-associated turbidity is maximum at the molecular equivalence ratio of antigen to antibody. With either more or less antigen, the turbidity decreases, a visible reflection of changes in the molecular arrangements of antigen–antibody complexes, which result in their increased solubility at these extreme ratios. This phenomenon explains some puzzling in vivo and in vitro observations. For instance, alterations of the antigen/antibody ratio by immunosuppressive therapy may result in the acute worsening, rather than the expected amelioration, of some immune complexes-related clinical manifestations (30). In vitro, immunoelectrophoresis precipitin patterns obtained with sera containing extremely high or low immunoglobulin concentrations and unfavorable antigen/antibody ratios are subject to loss of such precipitin lines during the washing procedure in preparation for staining.

The functions of immune complexes are intimately related to their composition, which is governed by the involved classes (e.g., IgG, IgM), subclasses (e.g., IgG1, IgG2) and types (κ- or λ-light chains) of the immunoglobulins; their affinity and epitope density; and numerous other factors such as reaction temperature, sizes of CIC, and complement fixation (19, 31).

The functions of immune complexes should be viewed in context with those of the immunoglobulins. A general function of antibody is that of a marker for antigens destined for elimination from the circulation or tissues. Subsequent to the antigen–antibody reaction, conformational changes occur in the Fc portion of the immunoglobulin, resulting in the activation of the complement cascade (Figure 3). The recognition unit for antigen–antibody immune complex is C1q of the classical complement pathway, which is subsequently activated, leading to the familiar complement cascade via C3, through the common complement assembly pathway, and terminating in the attack unit at the C9 level, resulting in cellular damage (cf. also Clin. Chem. 24: 7–22, 1978) or cytolysis in the presence of lysozyme (32) (Figure 4, left). This
classical complement pathway is activated mainly by immune complexes composed of IgG3,12 in that order, and IgM antibodies (33), whereas the IgG4 subclass and IgA, IgD, and IgE classes are inactive in this regard (34). The alternative complement pathway, however, may be activated by IgA or IgE complexes (Figure 4, right).

Immune complex functions that can be attributed to effects other than those resulting from direct elimination of desirable antigens include the enhancement or suppression of immune responses, probably via their interactions with receptors for Fc, complement, and(or) antigens on lymphocytes. Such immune modulation by immune complexes includes the inhibition of B-cells, possibly related to Mitchison's high and low zone tolerance (35), and stimulation of T-cells to produce helper or suppressor factors that modulate immune responses (9, 19, 31).

Pathophysiology of Immune Complexes

Factors determining the localization of CIC in vessel walls within a particular organ include the quantity, size, and lattice-structure of CIC (19, 31). Removal of CIC from the circulation is governed by, among other factors, their sizes. Immune complexes that are either very small or very large generally do not lead to progressive disease. The potentially very injurious large complexes are rapidly eliminated by the reticuloendothelial system; small ones do not appreciably activate the autacoids4 (i.e., the mediators and moderators of inflammation, such as histamine, prostaglandins of the E and A series, and the β-mimetic catecholamines) (36); they may circulate for long intervals without causing appreciable tissue injury. In contrast, immune complexes of intermediate size are prone to cause such tissue injuries as glomerulonephritis (37).

Some host responses to immune complexes having clinical sequelae appear to be genetically determined, because the same dose and the same antigen applied in an identical fashion will produce different responses in different animals (e.g., membranous vs proliferative glomerulonephritis) (37). By inference, similar genetic mechanisms are probably operative in humans.

The mechanisms by which tissues are injured by immune complexes are diverse and complex (37). Once an immune complex is formed, it acquires the capacity to activate a variety of autacoids, mainly through the Fc portion of the immunoglobulin molecules. Both cellular and humoral mediator systems may become involved—in particular, the complement system and, indirectly, the Hageman factor system. Liberated products of complement cleavage include anaphylatoxins peptides (C3a, C5a), which can cause basophils and mast cells to degranulate, releasing histamine and other substances that may cause anaphylaxis, and chemo-attractant agents (C5b and C3b), which bind to cells (i.e., immune adherence), causing opsonization and cell activation.

Cellular mediator systems include most blood cells because all circulating blood cells, with the possible exception of erythrocytes, possess Fc receptors. The interaction of these cells with the Fc portion of the immunoglobulins alters their cellular functions. Myeloid cells—including neutrophils, eosinophils, and basophilic leukocytes as well as platelets—become activated and release granular constituents. Macrophages are similarly activated. Tissue injury by CIC is preceded by tissue deposition of CIC (or attachment to circulating blood cells), leading to increased permeability of blood vessels by the local release of vasoactive amines. Thus, an allergic reaction occurring in the circulation may cause degranulation of basophils and mast cells, with the attendant liberation of vasoactive amines and other substances such as heparin, serotonin, and anaphylotoxin that can further augment the increased vascular permeability. This allows CIC to accumulate along the vascular basement membranes (37).

Basically, there are two types of injuries by immune complexes that depend on complement and polymorphonuclear (PMN) leukocytes, as far as the localization of the target antigen is concerned (Figure 5):

(a) Antibody reaction with tissue-fixed antigen, as exemplified in general terms by the Arthus phenomenon, and
(b) Antigen–antibody CIC, which are deposited and fixed on basement membranes and appear as the classical "lumpy-bumpy" deposition of such CIC, leading to the membranoproliferative glomerulonephritis that is a feature of systemic lupus erythematosus. Experimentally, Arthus's phenomenon can be produced by local injection of an antigen into an animal, with subsequent reaction in situ to its corresponding antibodies. Certain kidney diseases and other disorders with local formation of immune complexes may be considered to represent human analogs of the Arthus phenomenon (e.g., Goodpasture's syndrome associated with deposition of antibodies directed to the basement membrane of kidneys and lungs).

Clinical Correlation of Circulating Immune Complexes

In general, the correlation between results obtained by the various tests for immune complexes, measurements of CIC concentrations, and immune complex-related clinical manifestations is an uncertain one (38, 39). Nevertheless, notable exceptions do exist, reflecting an impressive correlation be-

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4 From the Greek autōs (self) and a'kô (medicinal agent).
cumulating information about CIC, should prompt the clinical laboratory to provide some basic, albeit indirect, CIC assays, either itself or via reference laboratories.

**Assays of Circulating Immune Complexes**

The plethora of experimental and clinical data incriminating immune complexes in the etiology and pathogenesis of more than 100 diseases (41) was in contrast to the paucity of reliable diagnostic assays for the detection, quantitation, and characterization of CIC. But now there are more than 40 such assays advocated for use in the clinical laboratory, many of them pioneered by Theofilopoulos and Dixon (9, 19, 22, 31, 42). Table 2 gives a partial list of them. Other assays are immunofluorescent and immunoperoxidase techniques for tissue-bound immune complexes (43, 44) and those for CIC. Tests for CIC are based on physical, chemical, immunological, and cellular properties of immune complexes. The techniques include ultracentrifugation, cryoprecipitation, precipitation with polyethylene glycol, agglutination or precipitin assays with the aid of monoclonal rheumatoid factors, C1q binding by the Fc receptors on antibodies, conglutinin radioimmunoassay or enzyme immunoassay by C3b or C3d binding, or the newer solid-phase F(ab')2 anti-C3 assay, platelet aggregation, inhibition of phagocytosis by macrophages, and the Raji cell test, which is based on the interaction of complement-fixing immune complexes with complement receptors on the surface of cultured Raji B-cells (42, 45) (Figure 6). Most of these tests are antigen nonspecific and are geared for detection and semiquantitation of CIC rather than for the tedious

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**Table 2. Partial List of Immune Complex Assay Techniques**

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<tr>
<th>Technique</th>
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<tr>
<td>Analytical ultracentrifugation</td>
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<tr>
<td>Sucrose density-gradient centrifugation</td>
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<td>Gel filtration</td>
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<tr>
<td>Ultrafiltration</td>
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<tr>
<td>Electrophoresis</td>
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<td>Precipitation with polyethylene glycol</td>
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<td>Cryoprecipitation</td>
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<td>Complement techniques</td>
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<td>Microcomplement consumption test</td>
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<td>Assays based on interactions with purified C1q</td>
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<tr>
<td>C1q precipitation in gels</td>
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<td>C1q binding tests</td>
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<tr>
<td>C1q solid-phase assays</td>
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<tr>
<td>C1q deviation test</td>
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<tr>
<td>Assays of breakdown products of C3 and C1</td>
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<tr>
<td>C3 precipitation assay</td>
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<tr>
<td>Conglutinin radioimmunoassay</td>
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<tr>
<td>Antiglobulin techniques</td>
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<tr>
<td>Rheumatoid factor tests</td>
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<tr>
<td>Other antiglobulin tests</td>
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<tr>
<td>Cellular techniques</td>
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<td>Platelet aggregation test</td>
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<tr>
<td>Inhibition of antibody-dependent cytotoxicity</td>
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<td>Intracytoplasmic staining of polymorphonuclear leukocytes</td>
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<td>Release of enzymes from eosinophils and mast cells</td>
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<td>Macrophage inhibition assay</td>
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<td>Rosette inhibition tests</td>
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<tr>
<td>Raji cell assay</td>
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<td>L1210 murine leukemia cell assay</td>
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<td>Binding to staphylococcal protein A</td>
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</table>

Adapted from Theofilopoulos (19).
Receptors Used for Antigen-Nonspecific Detection of ICs

**HUMORAL RECEPTORS**

- Rheumatoid Factor
- Conglutinin

**CELLULAR RECEPTORS**

- Phagocytized Complexes
- 8 Lymphocyte (Raj cell)
- Macrophage
- C3b Receptor
- Platelet
- Red Cell

**Immune Complexes**

- C1q

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Fig. 6. Receptors used for antigen-nonspecific detection of immune complexes

Source: ref. 17; reproduced with permission

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The task of routinely identifying the putative antigens in the immune complexes. In exceptional cases, however, antigens can be identified by some tests in clinically suspected situations, such as the hepatitis A or B viral antigens (7, 9, 18, 19, 21, 31).

Many of these tests are impeded either by low sensitivity or specificity, or a host of interfering factors, or lack of reference preparations, or economic aspects, or some combination of these (46). For instance, rheumatoid factor-dependent tests are interfered with by intrinsic rheumatoid factor; C1q by free DNA, bacterial endotoxin, and other serum proteins; and the Raji cell test by antilymphocyte antibodies in patients with certain autoimmune disorders (45), as well as by its relatively high cost.

The principles of some of these assays for CIC is summarized in the following paragraphs.

**Ultracentrifugation**

This rather insensitive approach is applicable for the detection of low, intermediate (7–19 S), and high (>19 S) CIC when their proportion exceeds 5% of total serum proteins (Table 3—CIC types I, II, III, which are reflected either as cryoglobulins, increased viscosity, or simply by turbidity) (47).

**Precipitation with Polyethylene Glycol (PEG) (38)**

High concentrations (e.g., 200 g/L) of this water-soluble polysaccharide polymer precipitate most serum proteins, but low concentrations (e.g., 20 to 40 g/L) preferentially precipitate large CIC without, for all practical purposes, affecting any other immunoglobulins or other serum proteins. This selective effect may be due to steric exclusion of immune complexes from the domain of the polymer (48).

**Complement Consumption Tests (49)**

In these screening tests, incubation of complement-binding CIC with standard amounts of complement allows quantitation of complement-consumption, or "anti-complementary" activity, based on lysis of added sensitized erythrocytes.

**C1q Tests**

C1q reacts specifically with the Fc portion of complexed IgG, IgM. Several variations are in use:

- **C1q precipitin test.** One of the first practical tests for CIC detection was pioneered by Agnello 10 years ago (41, 50). A positive precipitin test on Ouchterlony plates is the result of C1q interaction with antigen–antibody CIC. It preferentially detects large immune complexes. Its major limitation is low sensitivity, because other serum factors also can activate complement.

- **C1q binding tests (51).** Purified C1q is labeled with 125I and allowed to react with CIC; the resulting complex precipitates in the presence of 50 g of polyethylene glycol per liter. The trichloroacetic-precipitable radioactivity reflects CIC concentrations. This sensitive assay preferentially directs macro-CIC containing IgG.

- **C1q deviation and inhibition tests (52).** These sensitive but somewhat nonspecific radioimmunoassay tests measure the inhibitory activity of CIC on binding of 125I-labeled C1q to sensitized sheep erythrocytes. Results are expressed in terms of the percentage inhibition of radioactive uptake as compared with that for controls.

- **C1q solid-phase tests (53).** C1q fixed to polystyrene tubes binds CIC. This binding activity is demonstrated by competitive binding with 125I-labeled aggregated IgG (radioimmunoassay) or enzyme-labeled aggregated IgG (enzyme immunoassay). All C1q tests preferentially detect macro-CIC; they are subject to interference (9) by free DNA, bacterial endotoxins, various polyanions, C-reactive protein, heparin, and viruses, as well as by heat-aggregated IgG.

**Rheumatoid Factor (RF) Tests**

RF possesses specificity for the Cγ2 and Cγ3 domains on the Fc fragment of human IgG (54). This combination may form immune complexes in serum or joint fluid, either by reaction with autologous IgG or by self-aggregation (55). Monoclonal RF (from patients with monoclonal gammopathy of IgM class with high RF activity; "category II" CIC diseases) displays a higher affinity for immune complexes—IgG–Fc or

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5 Reference preparations of aggregated IgG and of preformed immune complexes (tetanus toxoid antigen–antibody complexes) are now available on request from Dr. Y. Nydegger, Service de Transfusion CRS, Laboratoire Central, Wankderstraate 10, 3000 Berne 22, Switzerland.
### Table 3. Classification of Cryoglobulins *a* in 86 Cases *b*

<table>
<thead>
<tr>
<th>Type I cryoglobulins—pure monoclonal immunoglobulins (~25%)</th>
<th>Nature of Immunoglobulins, and frequency</th>
<th>Diseases</th>
<th>Predominant disease manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I cryoglobulins—pure monoclonal immunoglobulins (~25%)</td>
<td>IgM, 11 cases; IgG, 7 cases; IgA, 2 cases; Bence Jones protein, 1 case</td>
<td>Waldenström's macroglobulinemia, multiple myeloma, B-cell lymphomas, other hematological malignancies, cold agglutinin disease, others</td>
<td>Cryopathy purpura, urticaria, acrocyanosis and gangrene, (40%), Raynaud's phenomenon (40%), hemorrhages, renal manifestations</td>
</tr>
<tr>
<td>Type II cryoglobulins—mixed cryoglobulins [one component may be monoclonal immunoglobulins] (~25%)</td>
<td>IgM–IgG, 19 cases; IgG–IgG, 2 cases; IgA–IgG, 1 case</td>
<td>Waldenström's macroglobulinemia, multiple myeloma, B-cell lymphomas (CLL, lymphocytic lymphomas), Sjögren's syndrome, rheumatoid arthritis, others</td>
<td>Purpura (60%), gangrene, acrocyanosis and gangrene Raynaud's phenomenon (40%), renal manifestations (35%), arthralgia</td>
</tr>
<tr>
<td>Type III cryoglobulins—polyclonal cryoglobulins [single or mixed cryoglobulins] (~50%)</td>
<td>One or more polyclonal immunoglobulins, or other proteins (e.g., C3, lipoproteins), 43 cases</td>
<td>&quot;Idiopathic&quot; cryoglobulinemia (~50%), B-cell lymphomas (CLL, lymphocytic lymphomas), other hematological malignancies, systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, periarthritis nodosa, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, bacterial, viral infections, others</td>
<td>Purpura (70%), Raynaud's phenomenon (60%), arthralgia (58%), neurologic manifestations</td>
</tr>
</tbody>
</table>

*a* The same classification pertains also to circulating immune complexes that are manifested by increased serum viscosity or simply by turbidity. *b* Modified and reproduced with permission: S. E. Ritzmann and J. C. Daniels, Eds., in *Serum Protein Abnormalities, Diagnostic and Clinical Aspects*, Little, Brown and Co., Boston, MA, 1975. Additional data are included from Brouet et al. (26). CLL, chronic lymphocytic leukemia.

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**Fig. 7.** Schematized are steps in the Raji cell radioimmunoassay (left) and in the solid-phase conglutinin and F(ab′)2 anti-C3 solid-phase assays (right)

The Raji cell is a lymphoblastoid cell of B-lymphocyte derivation, having many receptors for complement and few for IgG Fc. Because it lacks surface Ig, any Ig on the Raji cell surface after it binds immune complexes will be derived from the complexes. One can measure the complexes by incubating the cells with serum containing immune complexes, adding radiolabeled antihuman IgG antibody, and then quantifying the surface radioactivity of the Raji cells. Complement-fixing immune complexes can also be detected by either conglutinin or anti-C3 bound to a solid matrix. The bound immune complexes are then "counted" with radiiodinated or enzyme-linked anti-lg or with labeled staphylococcal protein A. Source: same as Fig. 6

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aggregated normal human IgG than does polyclonal RF (from patients with "category III" CIC diseases) (56). Two main varieties of the RF tests are applicable:

RF precipitin test (56,57). This test is analogous to the C1q precipitin test, except that purified monoclonal RF is substituted for C1q. Unlike the C1q test, it detects both small and large CIC. But like the C1q precipitin test, it is of low sensitivity and specificity.

RF competitive inhibition tests (58). In contrast to the RF precipitin test, in this radioimmunoassay the competitive inhibition of binding of aggregated human globulin is very sensitive, 5–10 mg of aggregated human globulin per liter. With this test, small IgG–CIC can be detected, and the test is independent of the complement-fixing properties of CIC. High concentrations of IgG or high intrinsic RF activities cause interference.

Solid-Phase F(ab')2 Anti-C3 Assay (42) (Figure 7, right)

This recently developed assay is based on the fact that complement-fixing CIC binding with anti-C3 is quantitated with either radiolabeled or enzyme-linked anti-IgG (radioimmunoassay or enzyme immunoassay); or with radiolabeled staphylococcal protein A, which also binds to the Fc region of G immunoglobulins 1, 2, and 4, but not to IgG3 (59) or to some IgA or IgM. This test is comparatively simple, sensitive, and specific.

Conglutinin Binding Test (42) (Figure 7, right)

In this test, also developed by Theofilopoulos et al. (42), conglutinin, an unusual nonimmunoglobulin protein found only in the serum of certain members of the bovine family, is used. It binds to immune complexes via specific fragments of activated C3 (i.e., C3b or C3d). Serum is incubated in conglutinin-coated polypropylene tubes to allow C3-associated CIC to bind to solid-phase conglutinin. Then the amount of immune complexes that have reacted with conglutinin is measured, after incubation with radiolabeled (radioimmunoassay) or enzyme-labeled (enzyme immunoassay) anti-IgG. With this relatively simple but highly specific and sensitive assay, macro-CIC are preferentially detected, but only those containing C3b or C3d. The rheumatoid factor reacts, however, with immune complexes that activate either the classical or the alternative complement pathways, or both.

Cell Surface Tests (60) (Figure 6)

Current cell-surface-receptor tests are all based on cell-surface receptors for the Fc portion and various complement fragments on B-cells, macrophages, polymorphonuclear leukocytes, platelets, and others (Figure 6). Such tests include:

Platelet aggregation test. Platelets aggregate as a result of the interaction of their surface Fc receptors with IgG immune complexes (61). These tests are sensitive but laborious and subject to interference by anti-platelet antibodies, myxoviruses, and enzymes (leading to false-positive results) and by RF and complement-fixing CIC (which may lead to false-negative results).

Inhibition of phagocytosis by peritoneal macrophages. In this somewhat cumbersome test (62), CIC compete with radiolabeled aggregated immunoglobulin for binding to Fc receptor sites on guinea pig peritoneal macrophages, leading to inhibition of phagocytosis of radiolabeled aggregated immunoglobulin added to the macrophages.

Antibody-dependent cellular cytotoxicity test. This represents another competitive assay in which immune complexes interact with Fc receptors on effector cells, resulting in inhibition of antibody-dependent lysis of target cells (63).

Raji cell assay. Raji cells, cloned from a patient named Raji who had Burkitt's lymphoma, are of continuous lymphoblastoid B-cell lineage (42). They lack surface immunoglobulins but have receptors for C3b, C3d, and the Fc portion of the IgG (high-affinity Fc receptors for binding complement and low-affinity Fc-receptors for binding immunoglobulins); therefore, any IgG bound to the Raji cells is assumed to be due to the immune complexes. Serum is incubated with the cells, and the mixture is washed and then exposed to 125I-labeled rabbit anti-human IgG (Figure 7). The amount of radioactivity reflects the amount of human immunoglobulin fixed to the cell, and therefore, the amount of the immune complexes. This sensitive test (~6 μg of aggregated human globulins per milliliter) detects CIC of various sizes but preferentially macro-immune complexes; it can also be used for antigen identification in these complexes on cell surfaces, with use of radiolabeled or fluoresceinated antisera.

Sources of error for all cellular tests include interference by isologous anticellular IgG antibodies, such as auto-antilymphocyte antibodies found frequently in patients with autoimmune disorders (7, 9).

In general terms, false-negative findings may result from sampling blood at less than optimal times. False-positive results can be induced with most tests (whether cellular or noncellular) by IgG aggregated by repeated freezing and thawing, by heat inactivation, or by improper sample storage. These considerations underscore the importance of appropriate sample handling and shipping to reference laboratories.

At present, there are no simple assays for CIC with IgA, IgD, or IgE antibodies.

In a collaborative World Health Organization study, 18 techniques were compared for their sensitivity, specificity, and reproducibility for detecting CIC (39). The most sensitive techniques were the conglutinin binding test and the Raji cell test, followed by the RF inhibition assay, the solid-phase C1q-binding and the C1q deviation test. Reproducibility appeared superior for humoral as compared with cellular assays, except for the Raji cell test, which depends on a constant source of similar or identical cells. As expected, specificity was limited by the multiplicity of CIC and the restricted nature of the CIC detectable by a given test. Therefore, negative results in one assay did not necessarily rule out the presence of CIC detectable by another test system. For instance, the Raji cell test shows higher values for CIC in systemic lupus erythematosus than in rheumatoid arthritis, whereas the opposite results are obtained with the C1q binding assay or the RF inhibition assay.

The bewildering array of diverse properties of CIC in the general patient population precludes reliance on a single CIC assay for satisfactory diagnostic purposes. Until a single test with such qualifications becomes available, a panel of several tests appears advisable, such as a C1q assay, a conglutinin test, and a cellular technique.

Clinicians are becoming increasingly aware of the deceptive role of CIC as masqueraders and imitators (64) and, consequently, of the need for objective and clinically relevant tests for CIC. However, the somewhat unpredictable and not uniformly satisfactory correlation between results of CIC assay and clinical manifestations has prompted numerous investigators to caution against over-interpretation of such results (37, 38, 65). For instance, Wiggins and Cochrane (37) state that the "mere detection of immune complexes does not necessarily imply that they are important in the pathogenesis. Nevertheless, the potential for their pathogenesis has been established, and their detection in the circulation could alert the physician to seek evidence of their phlogogenic effects."
Table 4. Simplified Approach to Detection And Characterization of Circulating Immune Complexes (CIC) in Serum

<table>
<thead>
<tr>
<th>Indirect assays</th>
<th>Direct assays</th>
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<tr>
<td>Serum protein electrophoresis</td>
<td>1. Cryoglobulins: 4 °C overnight (or for three days*), usually reversible on warming to 37 °C (cryocrit*)</td>
</tr>
<tr>
<td>1. Cellulose acetate electrophoresis—abnormal patterns: plateau formation, banding patterns; asymmetries, smearing, etc.; polyclonal gammopathy patterns (common variety, β-γ bridging, pseudo-M-protein)</td>
<td>2. Increased viscosity: shake test (Ostwald’s viscosimeter*)</td>
</tr>
<tr>
<td>2. High-resolution agarose electrophoresis—abnormal patterns: Same as 1 above</td>
<td>3. Turbidity: 4 °C overnight, usually decreasing on warming to 37 °C (DD: Airfuge—R/O lipoproteins*)</td>
</tr>
<tr>
<td>Immunelectrophoresis (IEP)</td>
<td>4. Rheumatoid factor assays (e.g., latex titer)</td>
</tr>
<tr>
<td>Agarose IEP: 2-Mercaptoethanol depolymerization; CIC Categories I, II, III</td>
<td>5. Analytical ultracentrifugation*</td>
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<tr>
<td>* indicates ideal conditions for serum collected in red-top Vacutainer Tubes at 37 °C. Other methods indicated are practical routine approaches.</td>
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</table>

Others have advocated the use of ancillary information, such as data on complement concentrations for monitoring patients with CIC disease (65), rather than the CIC assays described, at least until the biological significance of CIC is more satisfactorily explored (66).

Because the variability of the CIC now prevents reliance on a single assay (67), it indeed appears that such ancillary assays lend themselves to the screening, recognition, characterization, and monitoring of CIC in the general clinical laboratory, at least as the initial diagnostic approach to CIC. Once established or suspected, the more specific tests described above can be applied for confirmation and quantitative analysis. Proposed ancillary tests (see Table 4) include pattern analysis of serum protein electrophoretograms and evaluation for unusual γ-globulin configurations; detection of cryoglobulins and their counterparts, namely, increased serum viscosity and turbidity at low temperature; high RF activity; decreased complement concentrations (especially C3, C4, and Factor B); and a host of other nonspecific manifestations, such as double rings on radial immunodiffusion, “broad” precipitates on immunelectrophoresis that migrate cathodically, etc.

Ancillary Presumptive Tests for CIC

Cryoglobulins, Increased Serum Viscosity, and Serum Turbidity

These three variables of CIC in serum have in common the fact that they are exaggerated by low temperatures. They represent different aspects of the CIC. Consequently, cryoglobulins, especially of types II and III (26), may be viewed as representing only the tip of the iceberg of CIC, increased serum viscosity or simply turbidity being of equal significance for reflecting CIC.

Cryoglobulins. Cryoglobulins may precipitate or gel; rarely, they crystallize. The amount of cryoglobulin generated is maximized by refrigeration for several hours to several days. Usually, but not invariably, these effects are reversible upon warming the specimen to 37 °C. Cold-insolubility of CIC increases from antigen excess to equivalence or slight antibody excess (68). Collection and processing of blood specimens at 37 °C is generally advocated, but experience has shown that these precautions are rarely necessary. Satisfactory quantitation can be provided by determining the cryocrit and expressing the amount of cryoglobulins in volume percent; cryoglobulins can be characterized by use of “whole” or supernatant serum, without resorting to cumbersome isolation procedures. Clinically, cryoglobulinemia may be associated with cryopathic manifestations of cyanosis, gangrene, purpura, and other such symptoms of circulation disturbance (2–4).

Increased serum viscosity. Serum viscosity (ηrel) may be gauged by the experienced examiner by simply tilting and shaking the test tube and observing the fluidity of the serum sample; confirmation and grading of suspected increased viscosity is afforded by more precise measurement with an Ostwald’s viscosimeter (2). The serum viscosity is expressed relative to that of water at 37 °C, or lower temperatures. Normal values are 1.6–2.0 ηrel 37 °C. Values exceeding 4.0 are often associated with clinical manifestations of the hyperviscosity syndrome, characterized by bleeding, telangiectasia, loss of hearing, and others. Plasmapheresis with the removal of large amounts of highly viscous immunoglobulins and CIC constitutes an effective first therapy.

Increased serum turbidity. Basically, increased serum turbidity may be caused by bacterial growth, hyperlipoproteinemia, or CIC. Figure 8 depicts a practical method for differentiation. The usual centrifugal procedure, with a clinical centrifuge, clears sera from particulate matter. High-speed centrifugation in an Airfuge at 208 kPa (30 psi) and ~165,000 × g for 10 min usually clears turbidity due to lipoproteins. No clearing under these conditions is presumptive evidence of CIC. As is the case in cryoglobulins and increased viscosity,
serum turbidity due to CIC is aggravated by refrigeration and usually diminished by warming to 37 °C.

The mere detection of cryoglobulinemia, increased serum viscosity, or CIC-related serum turbidity should not be a diagnostic endpoint in itself. Rather, it should prompt immunological characterization and, whenever feasible, the appropriate clinical correlation and followup. To facilitate this approach, one needs an all-encompassing immuno diagnostic and clinical classification of cryoglobulins and, by extension, of increased serum viscosity and CIC-caused turbidity. The classification of Brouet et al. (26) satisfies these criteria. It is based on the analysis of the immunochemical nature of cryoglobulins from 86 patients and their correlation with the corresponding clinical manifestations (Table 3). Other studies (2, 69) have corroborated this approach.

Briefly, Category I cryoglobulins (Figure 9) consist of monoclonal immunoglobulins, mainly IgM, usually without recognizable immunoglobulin antigen targets, usually IgG. Clinically, monoclonal B-cell disorders of the symptomatic variety (e.g., multiple myeloma, macroglobulinemia Waldenström, and chronic lymphocytic leukemia) or the asymptomatic benign forms are associated with this category of cryoglobulins.

Category II and III cryoglobulins are mixed cryoglobulins in that both the antibody and antigen components forming the CIC are required for cryoglobulin production; neither component by itself will result in cryoglobulin formation. Category II CIC (Figure 10) are due to monoclonal immunoglobulins and polyclonal immunoglobulins or other proteins. Most frequently encountered are monoclonal IgM antibodies to autologous polyclonal IgG (i.e., RF), but nonimmunoglobulin autologous antigens (e.g., lipoproteins and albumin) (Figure 10c), are also seen, as well as allogeneic or heterologous antigens (e.g., porcine, caprine, and bovine meat- and milk-protein antigens), especially in patients with selective IgA deficiency (15). Category III CIC (Figure 11) consist of polyclonal antibodies and polyclonal antigens. The most frequently encountered antibodies are of the IgM and IgG classes and the antigens of the IgG class.

Clinically, categories II and III are distinct in that category II is associated with the same disorders encountered in category I, as expected by the presence of the monoclonal components signifying a B-cell monoclonal gammopathy (70). Additionally, however, some patients with Sjögren's syndrome and related autoimmune disorders are also encountered in category II, because they may convert from the ordinary polyclonal category III to a monoclonal IgM or IgA gammopathy (71–73). Category III-associated disorders include a wide spectrum of autoimmune diseases [e.g., systemic lupus erythematosus, rheumatoid arthritis, hypergammaglobulinemic purpura (74)]: of bacterial infections (e.g., bacterial endocarditis, syphilis, and leprosy), viral (e.g., hepatitis B infections, infectious mononucleosis, and cytomegalovirus disease) and parasitic disorders [e.g., schistosomiasis (75), visceral larva migrans (89)]: malignancies (e.g., melanoma); and others (7, 9, 17, 18, 67). In about 50% of the diseases in this category, the underlying cause remains elusive (idiopathic cryoglobulinemia). Type III CIC diseases are often complicated by vasculitis and glomerulonephritis (62).

The relative incidences of CIC of categories I, II, and III are about 25, 25, and 50%, respectively. The concentrations of cryoglobulins are highest in category I (>10 g/L) and lowest in category III (<1 g/L in 90% of cases). The storage interval required for maximum manifestation of cryoglobulins at 4 °C is shortest for category I (hours), longest for category III (one to three days).

Diagnostic examples of these three categories of CIC are presented in Figures 9–11.

Increased rheumatoid factor activity. An extraordinary proportion of cases of cryoglobulinemia [especially of type III (25)], increased serum viscosity, and CIC-caused serum turbidity also display increased rheumatoid factor activity (76). Reactions of IgM, IgG, or IgA antibodies with autologous IgG by their Fc fragments result in the formation of RF-related CIC. Latex tests detect mainly IgM antibodies. Human IgG as substrate appears more sensitive but less specific than the IgG from rabbit or goat (71). Therefore, human IgG is preferable for CIC screening purposes; horse and rabbit IgG, however, may be more sensitive than human IgG for detecting IgG–RF (71).

The detection of high RF activity establishes, by definition, the presence of CIC. Numerous disorders, as well as a certain percentage of healthy persons (77), display RF activity (Table 5). Second-generation RF assays, including radioimmunoassay (71), enzyme-labeled immunosorbent assays (ELISA) (77), and nephelometry (48), allow a more satisfactory quantitative approach to RF.

Decreased serum-complement concentrations. Complement-dependent immune complexes may consume complement components of the classical (e.g., C1q, C4) or alternative (e.g., Factor B) pathways, or of both (C3), and result in their decreased concentrations in serum in spite of increased synthesis (78). For instance, serum from patients with the hepatitis-arthritis syndrome may mimic the immunological events demonstrated by Dixon in his rabbit model for experimental serum sickness (Figure 1). Such patients often do have temporarily lowered complement concentrations, owing to consumption of complement by the HBs-antigen–HBs-anti-
Unusual patterns on serum electropherograms. During recent years, it has become evident that certain unusual γ-globulin configurations on serum protein electrophoresis correlate with the presence of CIC. These consist variously of plateau formation (Figure 11), banding patterns, block formation, or unusual forms of asymmetries (Figure 10a,b,c). These patterns are found on both cellulose acetate and high-resolution agarose electropherograms, but not necessarily by both methods on the same serum. It appears as if the former technique is somewhat less sensitive. However, the latter results in a break-up and disappearance of some CIC. Cawley et al. (80) described a special "backfire" rocket electrophoresis, and Kelly et al. (87) have recently reported an agarose electrophoresis pattern suggestive of CIC. During the last few years, we have found the ancillary serum protein electrophoresis parameters to be extremely useful, especially if performed in conjunction with the other tests mentioned in this section.

Immunoelectrophoresis (IEP) (82, 83). IEP without subsequent staining (i.e., wet analysis) may provide useful, albeit nonspecific, evidence of CIC. An example is the "milky" trailing from the point of application towards the cathode, a phenomenon that can also be produced by M-proteins (Figures 9, 10a). Parallel, double IgG precipitin lines (Figure 11)
Table 5. Partial List of Diseases Often Associated with High Rheumatoid Factor

<table>
<thead>
<tr>
<th>Chronic inflammatory diseases</th>
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<tbody>
<tr>
<td>Tuberculosis</td>
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<tr>
<td>Leprosy</td>
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<tr>
<td>Yaws</td>
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<tr>
<td>Syphilis</td>
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<tr>
<td>Brucellosis</td>
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<tr>
<td>Subacute bacterial endocarditis</td>
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<tr>
<td>Salmonellosis</td>
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<table>
<thead>
<tr>
<th>Neoplasms</th>
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</thead>
<tbody>
<tr>
<td>After irradiation or chemotherapy</td>
</tr>
<tr>
<td>Other hyperglobulinemic states</td>
</tr>
<tr>
<td>Hypergammaglobulinemic purpura</td>
</tr>
<tr>
<td>Cryoglobulinemia</td>
</tr>
<tr>
<td>Chronic liver disease</td>
</tr>
<tr>
<td>Sarcoidosis</td>
</tr>
<tr>
<td>Other chronic pulmonary diseases</td>
</tr>
</tbody>
</table>

are highly suggestive of CIC. Occasionally, precipitin lines, e.g., IgG (λ) M-protein, may be fused with other serum protein precipitin lines, e.g., albumin (Figure 10c). IEP analysis can be performed satisfactorily on either "whole" or supernatant serum without examination of isolated cryoglobulins. Cryoglobulins, especially those of Category II, may elude detection or typing for κ- or λ-light chains on routine IEP, but prior depolymerization by exposure to 2-mercaptoethanol (83, 84), or simply by fourfold dilution of the serum with 0.14 mol/L saline, usually allows their characterization as true monoclonal IgM, IgA, or IgG. Cryoglobulins of high relative molecular mass may not enter the agarose gel, yielding misleading negative results. Depolymerization with 2-mercaptoethanol or temperature equilibration of reagents to at least room temperature, or both, may be required under these circumstances. Circumstantial evidence for CIC may be provided by the appearance of double or triple rings on radial immunodiffusion plates when serum is assayed for immunoglobulins. However, M-proteins may simulate such patterns (84).

Conclusions and Projections

The increasing realization of the clinical significance of CIC in health and disease is confounded by the methodological limitations, owing to the extreme complexity, heterogeneity, and diversity of CIC. Thus a series of tests must be available if the clinical laboratory is to provide a satisfactory service during this time of transition.

The numerous assays becoming available for the detection and quantitation of CIC currently are restricted to larger hospitals and special reference laboratories. The ancillary tests appear to serve as useful and practical screening tests in most hospital laboratories. They often do allow the characterization of CIC and their categorization into clinically correlative entities. Clearly, simpler and more specific and sensitive techniques—and, above all, more encompassing tests for a wide variety of CIC—are urgently needed.

Identification of the exogenous and endogenous antigens involved is both imperative for the basic understanding of the roles of CIC and potentially elusive because some CIC may be composed of antibodies "called forth by the primary disease state and self-reacting anti-idiotypic antibody directed at combining sites of the original disease-associated antibody" (18), raising the disturbing specter of an inherent elusiveness of certain antigenic culprits reminiscent of Heisenberg's principle of indeterminacy.

Given today's ability to visualize immunoglobulins (85, 86), C1q (87), and CIC (88) with the electron microscope, Mark Twain might not have speculated on the past and future length of the Mississippi River as determined by cutoffs of river loops, nor expressed his delightful lamentation that there is something fascinating about science. One gets such wholesale returns of conjecture out of such trifling investment of facts.

Instead, he might wryly have surmised the future of CIC

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Fig. 11. Circulating immune complexes: Category III, associated with fulminant vasculitis

Note plateau formation of γ-globulin fraction on cellulose acetate electrophoresis, but multiple bands on agarose electrophoresis, double and parallel IgG precipitin lines on IEP, and intermediate (7–19 s) complexes on analytical ultracentrifugation, reflecting the presence of low-molecular-mass IgG immune complexes. RF titer 1:640
assays to be visual, direct, and simple with the aid of dedicated electronic microscopic equipment.

References

82. Ritzmann, S. E., and Lawrence, M., Qualitative immunoelectrophoresis in Serum Protein Abnormalities, Diagnostic and Clinical Aspects (see ref. 2), pp 27–55.