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Research Article

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Inhibition of Polymorphonuclear Leukocyte Chemiluminescence for Detection of Immune Complexes in Human Sera

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ABSTRACT An assay for the detection and quantitation of immune complexes is described. Experimental immune complexes or aggregated human gamma globulin (AHG) were incubated with polymorphonuclear leukocytes (PMN). After challenge of the PMN with opsonized zymosan, chemiluminescence was recorded in a scintillation spectrometer. A quantitative inhibition of chemiluminescence could be demonstrated by the interaction of PMN with immune complexes or AHG.

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INTRODUCTION

Immune complexes are recognized as important factors in the pathogenesis of various rheumatic and nonrheumatic diseases. Assays that detect circulating immune complexes have been developed and have been shown to be useful in the diagnosis and assessment of the clinical activities of these diseases. However, there appears to be significant variability in the results obtained by the various assays presently available (1). The variability of results encountered with these assays included lack of specificity and sensitivity, unavailability and difficulties in maintenance of reference cell lines, and interference from substances such as endotoxins, DNA, and heparin. Therefore, these assays have remained experimental tools, and it has not been practical for clinical laboratories to perform these assays.

Immune complexes have been demonstrated on the surface of polymorphonuclear leukocytes (2, 3). These complexes may produce inhibition of the killing of *Escherichia coli* by polymorphonuclear leukocytes (PMN)[1] (1) and impair neutrophil bactericidal activity of *Staphylococcus aureus* (4). Using the concept that the microbicidal function of neutrophils will be depressed after interaction with immune complexes, we have used inhibition of PMN chemiluminescence to develop a simple and quantitative assay for the detection of immune complexes in human sera. In experiments using aggregated human gamma globulin, a direct correlation between percent inhibition in chemiluminescence and the amount of aggregated human gamma globulin exposed to the PMN was demonstrated, and a quantitative inhibition curve could be shown. Application of this method to sera specimens from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and vasculitis demonstrated the presence of circulating immune complexes.

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[1] *Abbreviations used in this paper*: AHG, aggregated human gamma globulin; BSA, bovine serum albumin; CL, chemiluminescence; NHS, normal human serum; PMN, polymorphonuclear leukocytes; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

METHODS

Materials

Medium. RPMI 1640 without phenol red, supplemented with 100 U/ml of penicillin and 50 μg/ml of streptomycin (Associated Biomedic Systems, Inc., Buffalo, N. Y.) was used.

Complement source. Fresh normal human sera or pooled normal human sera stored at −70°C and thawed once was used as a complement source in all experiments.

Complement determination. Total hemolytic complement (CH_{50}) was determined by the method of Mayer (5).

Zymosan preparation. Zymosan A (from S. cervisiae yeast, Sigma Chemical Co., St. Louis, Mo.) was suspended in RPMI 1640 to produce a stock solution of 10 mg/ml.

Antisera. The immunoglobulin (Ig)G fraction of rabbit antiserum to bovine serum albumin (BSA) was obtained from Pel-Freeze Biologicals, Inc., Rogers, Ark.

Leukocyte preparation. Blood was obtained from healthy adult donors by venipuncture. Leukocyte suspensions were prepared by standard techniques as described by Allen (6), and adjusted to a final concentration of 5×10^6 cells/ml.

Preparation of aggregated human gamma globulin (AHG). Azide-free human IgG (Cohn fraction II, Pentex Biochemical, Kankakee, Ill.) was heat-aggregated in a water bath at 63°C for 30 min. Each concentration of aggregates used in the experiments was prepared from a single batch and stored in 1-ml aliquots.

Sucrose density gradient ultracentrifugation. AHG was fractionated on a linear 10–40% sucrose density gradient in phosphate-buffered saline, pH 7.2. A 300-μl sample (3 mg/ml) of AHG was layered on 5 ml of sucrose using a Buchler automatic density gradient maker (Buchler Instruments Inc., Fort Lee, N. J.). Ultracentrifugation was performed in an SW 50.1 rotor at 100,000 *g* for 16 h at 4°C in an L2 centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Human hemoglobin, human 7S IgG, and human thyroglobulin were used as 4.5, 7, and 19S markers, respectively. 25 drop fractions were collected by piercing the cellulose nitrate tubes from below. Two 25-drop fractions were pooled and dialzyed overnight in phosphate-buffered saline, pH 7.2. These pools were then used in the chemiluminescence inhibition experiments.

Preparation of immune complexes. A quantitative precipitin curve was constructed for the BSA-anti-BSA system and equivalence determined. Increasing amounts of BSA (5–400 μg) were added to a constant amount of anti-BSA antibody (310 μg). These solutions were incubated for 1 h at 37°C and overnight at 4°C. Precipitates formed were dissolved in 0.1 M NaOH, and the protein was quantitated by spectrophotometry at 280 nm. Supernates from antigen excess to antibody excess were used in the chemiluminescence inhibition assay.

PMN chemiluminescence (CL) inhibition assay for the detection of immune complexes in human sera. Sera (1000 μl) to be assayed for immune complexes were incubated with 25 μl normal human sera (NHS) for 30 min in a shaker water bath at 37°C. 5×10^6 PMN was added to this mixture, and the incubation continued for another 30 min. The cells were centrifuged at 300 *g* for 5 min at 22°C, and the supernate decanted. The pellet was washed once with RPMI 1640, centrifuged at 300 *g* for 5 min and the supernate discarded. The leukocyte pellet was resuspended in RPMI 1640 to a volume of 4.5 ml in a siliconized dark-adapted vial. CL was then measured in a scintillation spectrometer as described by Allen et al. (6, 7).

Briefly, after completing two preliminary cycles to obtain background counts, 2.0 mg/ml of opsonized zymosan was added to each vial after a 1-min delay. Each vial was counted for 1 min, and counting was continued until a minimum of 10 cycles were completed. CL was measured with a Beckman scintillation spectrometer, model L230 (Beckman Instruments, Inc., Fullerton, Calif.) operated in the out-of-coincidence mode at room temperature (22°C). For continuous recording, the summation signal was fed into a Beckman Ratemeter and then to a Beckman Electronik Strip Chart Recorder. All additions to the siliconized vials were done under actinic light.

The CL, as total counts over a stated time interval, was calculated from measurements of chemiluminescent intensity (counts per minute) at 10 different 1-min counting periods. The time between counts was the time required for one cycle of the sample train (6 min). The percent inhibition of CL produced by experimental sera was calculated by the equation:

$$\% \text{ inhibition} = \frac{\text{counts (untreated)} - \text{counts (experimental sera treated)}}{\text{counts (untreated)}} \times 100.$$

The percent inhibition was then referred to a standard curve obtained by incubating increased amounts of AHG (10–10,000 μg) with NHS and control neutrophils, challenging the cells with opsonized zymosan, and measuring CL. The amount of immune complexes in each serum tested was expressed as micrograms AHG equivalent per milliliter of serum.

Experimental sera. Serum samples were collected from 50 patients. The clinical diagnosis included: 15 with SLE, 30 with RA, and 5 with vasculitis. Controls were 30 healthy laboratory and hospital personnel, and 30 hospitalized patients with no suspected immune complex disease. All serum samples were collected by venipuncture and stored at −70°C until used in the assay. Assay determinations were done in duplicate, and many specimens were repeated on two separate occasions.

RESULTS

Inhibition of CL AHG. AHG, which possesses many properties of immune complexes (8), was used in the initial experiments to determine its effects on CL. Increasing amounts of AHG (0.01–10 mg/ml) were incubated with NHS and 5×10^6 PMN/ml, and CL was measured after challenge with opsonized zymosan. Controls were PMN obtained from healthy donors, not incubated with AHG, but challenged with opsonized zymosan. Fig. 1 is a representative dose-response curve demonstrating that the inhibition in CL is directly proportional to the quantity of AHG added to the neutrophils. Using the area under each curve, the percent inhibition of integral CL response was calculated from the mean of triplicate samples of AHG done on the same day according to the following equation: $y = a + b \times \log [x]$ where y = % inhibition of CL, a = 5.45, b = 14.90, and x = micrograms AHG per milliliter (Fig. 2). The mean percent inhibition ±2 SD of 10 μg/ml was 21.33±1.8; 100 μg/ml was 36.28±1.93; 1,000 μg/ml was 48.93±2.69; and 10,000 μg/ml was 65.91±4.23.

Effect of AHG size on detection by PMN CL inhibi-

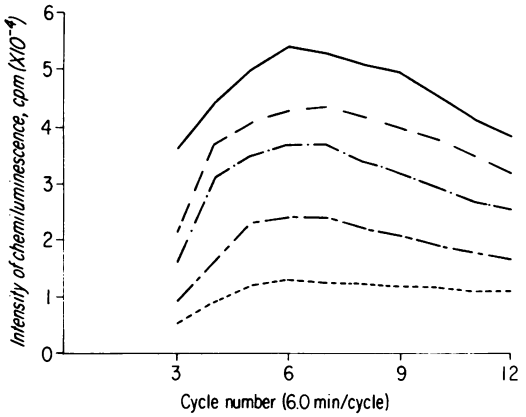

FIGURE 1 Dose-response curve of the inhibition of CL by AHG. PMN (5×10^6) without AHG (–), 10 μg AHG (– –), 100 μg AHG (– · –), 1,000 μg AHG (— – —), and 10,000 μg AHG (--). CL intensity was determined as described in Methods.

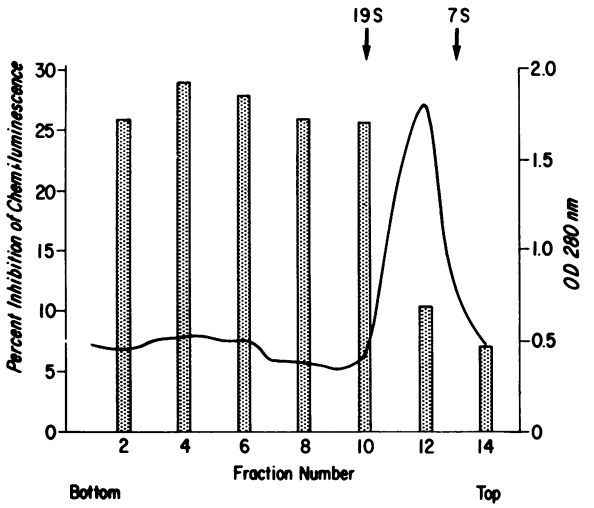

FIGURE 3 Sucrose density gradient fractionation of AHG. 14 fractions of dialyzed AHG were collected and every two fractions were pooled. CL was determined and the percent inhibitions are shown by the bars with the positions of the 19 and 7S markers. The curve represents the protein concentration of each fraction determined at an OD of 280 nm.

tion assay. To determine the effect of different size aggregates on the PMN CL inhibition assay, inhibition of CL was determined for the pooled fractions of AHG obtained by sucrose density gradient ultracentrifugation. As demonstrated in Fig. 3, inhibition in CL was produced by intermediate size complexes. Complexes 19S or greater in size produced more inhibition than aggregates < 19S. The protein concentration was determined for each fraction by optical spectrophotometry at 280 nm, and as demonstrated in Fig. 3, the inhibition is related to the activity of the complexes.

Effect of complement on binding AHG and BSA-anti-BSA complexes to PMN. To assess the role of complement binding immune complexes to PMN, we depleted complement by heating NHS to 56°C for 30 min. The serum was used in the first incubation step in the PMN CL inhibition assay. Controls were PMN incubated with NHS as a source of complement. As shown in Table I, AHG and BSA-anti-BSA complexes produced more inhibition in chemiluminescence when complement was available to bind the particles to the PMN.

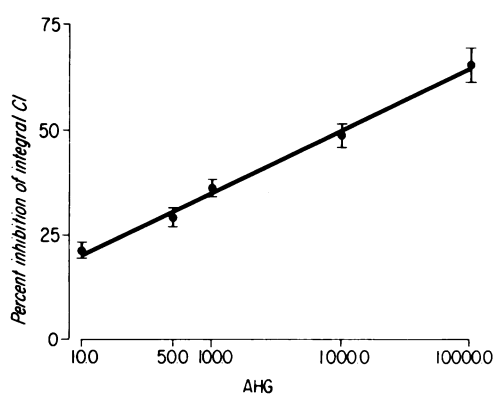

FIGURE 2 Standard curve of PMN-CL inhibition. Each point represents the mean±2 SD of the percent inhibition of CL when triplicate samples of AHG (micrograms per milliliter) were incubated with PMN (5×10^6) and challenged with opsonized zymosan.

Detection of experimental immune complexes. To determine which types of immune complexes bind most efficiently to PMN, BSA-anti-BSA complexes from antigen excess to antibody excess were used in the PMN CL inhibition assay. The precipitin curve for the BSA-anti-BSA complexes is shown in Fig. 4. Undiluted supernates obtained from each point of the precipitin curve were used in the PMN CL inhibition assay, and the greatest amount of inhibition in CL was produced by complexes formed in antigen excess. As demonstrated in Table II, representative fractions from antigen excess, equivalence, and antibody excess were used in the PMN CL inhibition assay. As with previous supernates, complexes formed in antigen excess produced the most inhibition in CL.

Detection of immune complexes in human serum. Because PMN CL could be inhibited by BSA-anti-BSA complexes and AHG, this assay was used for the detection and quantitation of immune complexes in human sera. The amount of immune complexes from experimental samples was calculated from a standard curve of AHG and expressed as micrograms AHG equivalent per milliliter serum.

Sera obtained from 50 patients were assayed for immune complexes by the PMN CL inhibition assay.

TABLE I
Effect of Complement on Inhibition of PMN CL

Conditions	Percent Inhibition CL*
AHG (100 μg/ml)	
With complement	36.30±2.0
Without complement‡	29.20±4.0
BSA-anti-BSA§	
With complement	28.80±1.6
Without complement	13.80±5.4

* Mean±2 SD of triplicate samples.
‡ NHS heat inactivated to 56°C for 30 min.
§ 300 μg BSA and 310 μg anti-BSA tested at 1:5 dilution.

TABLE II
Percent Inhibition of CL by BSA-anti-BSA Complexes

Percent inhibition of CL*		
Antigen excess‡	Equivalence§	Antibody excess‖
40.5‡	20.5	5.0

* Means of duplicate samples diluted 1:5.
‡ 300 μg BSA–310 μg anti-BSA.
§ 80 μg BSA–310 μg anti-BSA.
‖ 5 μg BSA–310 μg anti-BSA.

Using this technique, >15 μg AHG eq/ml were detected in the sera of 9/15 patients with SLE, 18/30 patients with RA, and 2/5 patients with vasculitis. In contrast, no healthy controls had > 5±2 μg AHG eq/ml of serum (upper limit of normal), and only 3/30 hospitalized patients without suspected immune complex disease had > 10 μg AHG eq/ml of serum (Table III).

In the sera of patients with SLE, the PMN CL inhibition assay detected immune complexes with a range of 23 to 1,200 μg AHG eq/ml. One patient had > 1,000 μg AHG eq/ml on two separate determinations. Total hemolytic complement was measured in these sera (Table IV), and the degree of complement depression correlated well with the immune complex detection in six of nine patients. However, in some patients immune complex detection did not correlate with complement depression. Thus, detection of immune complexes may be the initial manifestation of disease exacerbation.

This assay detected immune complexes in the sera from 18 of 30 RA patients with a range of 23 to 1,500 μg AHG eq/ml serum. Two patients had very high levels of immune complexes, one with > 1,000 AHG eq/ml, another with 800 μg AHG eq/ml. Patients with extraarticular manifestations of RA, or those with very active disease, as supported by an increased erythrocyte sedimentation rate, activity index, and advanced disease by physical exam, were generally positive for immune complexes. These patients also had a greater quantity of complexes detected in their sera than patients with mildly active or inactive disease.

Of the five patients with vasculitis, one had long-standing RA, with intermittent lower leg ulcers and palpable purpura. At the time of examination, his ulcers were resolving and 100 μg AHG eq/ml was detected in his serum. Four patients had a diagnosis of idiopathic cutaneous vasculitis. One patient demonstrated 45 μg AHG eq/ml serum and had a history of amaurosis fugax, an orchiectomy, a mild hemiplegia, and intermittent leg ulcers. The other three patients had palpable purpura with leg ulcers, but immune complexes were not detected on repeat examinations.

For comparison of this assay with another method used to detect immune complexes, sera from 16 patients with active RA were studied by both the Raji cell assay (kindly performed by Dr. R. Gupta, Denver,

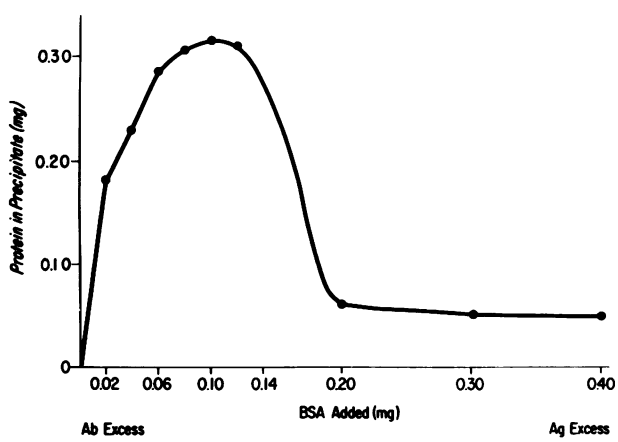

FIGURE 4 Precipitation curve of rabbit anti-BSA with BSA. Each point represents the amount of protein in the precipitates quantified at an absorbance of 280 nm. Ab, antibody; Ag, antigen.

TABLE III
PMN-CL Inhibition Assay for Immune Complexes in Human Sera

Diagnosis	No. positive patients	AHG	
		Mean	Range
	%	μg eq/ml	
SLE	9/15 (60)	123	23–1,200*
RA	18/30 (60)	129	17–1,500*
Vasculitis	2/5	72	45–100*
Hospitalized patients	3/30 (10)	4	0–50‡
Normal	0/30	2	0–7‡

* Mean and range for positive patients.
‡ Mean and range for all subjects.

TABLE IV
PMN-CL Inhibition Assay for Immune Complexes in Patients with SLE

Patient	AHG	CH_{50}*
	μg eq/ml	
NJ	45	35
MM	23	50
TW	1200	24
GD	32	55
PL	5	80
MS	71	35
MM	32	115
BS	200	80
LW	7	70
TP	0	70
SP	197	30
MJ	34	95
JB	9.5	70
PC	0	15
AG	0	124

* Total hemolytic complement N1 60–150 U.

Col.) (9, 10) and the PMN CL inhibition assay (Table V). Of 16 patients, 14 were positive by the Raji cell assay, and 12 were positive by the PMN CL inhibition assay. 11 sera were positive by both methods, whereas 2 were negative by both assays. Two sera were positive by the Raji cell assay and negative by the PMN CL inhibition assay. One patient was positive by the PMN CL inhibition assay, but negative with the Raji cell assay. Of the 11 patients with detectable immune complexes by both assays, the quantity was similar in 6.

DISCUSSION

The results of our study indicate that PMN can be used in a CL assay for the detection of immune complexes. Under normal conditions, phagocytosis of particles by neutrophils results in metabolic alterations such as increased hexose monophosphate shunt metabolism of glucose, activation of an oxidase system, and increased oxygen consumption. A portion of these metabolic reactions results in generation of electronically excited oxidation products. Relaxation of these products to ground state results in light emission or CL (7).

TABLE V
Immune Complex Detection by Raji Cell and PMN-CL Inhibition Assays

	Raji cell $n = 16$	PMN-CL inhibition $n = 16$	Raji cell and PMN-CL inhibition $n = 16$
Positive	14	12	11
Negative	2	4	2

This assay is based on a decrease in the CL response produced by neutrophils that have been preincubated with sera containing immune complexes and then challenged with opsonized zymosan. Previous investigators (11–17) have reported that normal PMN that were exposed to immune complexes, immunoglobulin aggregates, or zymosan particles, released lysosomal enzymes. PMN membrane receptors were stimulated with initiation of biochemical events. These events occur with phagocytosis of the particles. However, they may occur independent of phagocytosis, as a consequence of leakage of enzymes into the extracellular environments when incompletely closed phagosomes fuse with lysosomal granules. Thus, in our assay, the decreased CL response produced by PMN preincubated with immune complexes may be secondary to a depressed microbicidal function of the PMN produced during their initial encounter with immune complexes.

To determine the size of complexes detected by the PMN CL inhibition assay, sucrose density gradient studies were performed using AHG. Sucrose density gradient fractionation of AHG demonstrated that intermediate-sized complexes and complexes 19S or greater were best detected by the PMN CL inhibition assay. Protein quantitation of each fraction demonstrated that inhibition was related to the activity of the complexes, and although there was some inhibition of the 7S fractions, these contained a significantly greater amount of protein. This is similar to the Raji cell (10) and monoclonal rheumatoid factor assays (18) which also detect immune complexes of intermediate and large molecular size.

Additional studies to determine the type of immune complexes detected demonstrated that complexes formed at equivalence to antigen excess produced the most inhibition in CL. This observation is similar to the fluid phase Clq binding assay which also detects immune complexes formed at equivalence to antigen excess. However, this feature is in contrast to the Raji cell and the solid phase Clq binding assays, which are more sensitive for the detection of immune complexes formed in antibody excess (19).

The sensitivity of the PMN CL inhibition assay is the same as that of the Raji cell assay (10). In our assay, 7 μg AHG equivalence, which is the sensitivity limit of the Raji cell assay, produced 15% inhibition in CL. The variation in CL produced by normal controls in our lab was 6±4%. This assay is more sensitive than the C1q precipitation in gel (20) and radiolabeled C1q polyethylene glycol precipitation methods (21) which cannot detect AHG at concentrations <50 μg/ml. Thus the PMN CL inhibition assay offers sensitivity in detecting immune complexes that is similar to or greater than assays previously reported.

The effect of complement on the binding of immune

complexes to the PMN has been demonstrated in this study. Henson (11) reported that human PMN have receptors for the Fc fragment of immune complexes, C3b, and immunoglobulin aggregates. Also, other studies (22–25) suggest that the C3b and Fc receptors of neutrophils have separate but complementary functions. The C3b receptor primarily promotes the attachment of particles to the neutrophil, while the Fc receptor serves to initiate ingestion of particles. Our studies with AHG and BSA-anti-BSA support the concept of C3b serving to enhance binding of immune complexes to neutrophils. This was demonstrated by an increased inhibition of CL when complement was available to bind AHG or BSA-anti-BSA complexes in the first step of the assay.

Our results using AHG and BSA-anti-BSA complexes, encouraged the application of this assay for the detection of immune complexes in human diseases. Previous studies have reported a high incidence of circulating immune complexes in SLE (10, 21, 26–28). Immune complexes in sera from 9/15 patients with SLE were detected by the PMN CL inhibition assay. This is less than the results reported using the Raji cell assay (10). However, the Raji cell assay tested 92 serial monthly samples from 10 patients. This assay detected immune complexes in random single serum specimens from 15 patients. Our results are higher than those reported using monoclonal rheumatoid factor for the demonstration of immune complexes (18, 29) but similar to the results using staphylococci containing protein A (26) and the ^{125}I-C1q binding assays (21, 27) in the detection of immune complexes. These differences may be secondary to the patient population studied, variations in assay sensitivities, differences in immune complex size and composition, and variation in reference curves.

Immune complexes have also been detected frequently in the sera and synovial fluid of patients with RA (18, 29–32). In this series of 30 patients with RA, 18/30 had immune complexes detected by the PMN CL inhibition assay. This is higher than previous results using monoclonal rheumatoid factor for the detection of immune complexes (18, 29) but lower than a recent report by a group using three different assays (32) (C1q binding, monoclonal rheumatoid factor, and Raji cell). Again, these percent differences may be secondary to the population studied or variability in the tests themselves or reference standards.

In the small group of patients with idiopathic vasculitis, immune complexes were detected in only one subject. This low percent is similar to the Raji cell assay (10) in which 3/8 patients with idiopathic vasculitis had elevated levels of immune complexes. Furthermore, investigators recently reported a lower percentage of detectable immune complexes in lymphocytic vasculitis as compared with leukocytoclastic vasculitis (33). Thus, it is possible that factors other than immune complex deposition are responsible for vascular damage. Moreover, either the nature of the immune complexes or rapid clearance of complexes from the circulation with deposition in the vessel walls could limit the detection of immune complexes by the PMN CL inhibition assay.

Previous assays for the detection of immune complexes have various limitations. Methods using C1q are limited by interaction from DNA, lipopolysaccharides, and heparin (1, 19). Assays using polyclonal rheumatoid factor are unable to quantitate immune complexes in sera containing rheumatoid factors (1). Polyethylene glycol precipitation is less sensitive and may detect spontaneous aggregation of IgG (19). Antilymphocyte antibodies that are prevalent in SLE (34) may be limiting factors in the Raji cell assay. Moreover, because the Raji cell assay employs ^{125}I radiolabeled anti-human IgG, it does not detect immune complexes formed by other immunoglobulin aggregates.

Antineutrophil antibodies, which have been reported in Felty's syndrome (35), could possibly produce a false positive result in the PMN CL assay. Also, interference from circulating bacterial endotoxins could produce inhibition in CL. However, neither of these limitations have been tested using the PMN CL inhibition assay. Finally, as in previous assays, the PMN CL inhibition assay is unable to determine the antigen component of immune complexes.

At present, numerous assays are available for the detection of immune complexes in human sera. The PMN CL inhibition assay described in this report is simple, quantitative, reproducible, and readily performed. It is inexpensive and does not require cumbersome isolation of cells nor the use of radiolabeled substances. Further efforts are being directed at application of this assay in the detection of immune complexes in various other rheumatic and nonrheumatic diseases.

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