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Assessment of three simple techniques for the screening of circulating immune complexes: Correlation with a parameter of complement consumption

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Abstract. The sera of 36 normal controls, 45 patients with various diseases and 11 pregnant women were screened for circulating immune complexes using three relatively simple and inexpensive techniques. These included inhibition of agglutination of IgG coated latex particles with a serum having rheumatoid factor activity, polyethylene glycol precipitation and anti-complementary activity test. The circulating immune complexes were detected in a significantly higher proportion of patients as compared to normal controls. In the patients, the presence of circulating immune complexes did not always correlate with clinically detectable immunoinflammatory tissue damage indicating that pathogenic as well as nonpathogenic immune complexes were being detected by the above mentioned techniques. The alpha-1-antitrypsin/C3 ratio, however, correlated well with clinically apparent immuno-inflammation.

Keywords. Circulating immune complexes; polyethylene glycol; anti-complementary activity; alpha-l-antitrypsin/C3; aggregated human IgG; latex agglutination inhibition.

Introduction

The presence of circulating immune complexes is being shown to be important for diagnosis and prognosis in an increasing number of clinical conditions (Cochrane and Koffler, 1973; Cochrane and Dixon, 1976). A number of methods are available and are extensively used for the detection of circulating immune-complexes (Zubler and Lambert, 1977). Some of them are based upon the direct detection of immune-complexes and include both the antigen specific and antigen nonspecific methods. Other methods are indirect and dependent upon physico-chemical and/ or biological properties characteristic of immune-complexes and not found in uncomplexed antigen or antibody. Because of the large number of methods available the selection of a particular technique suitable for clinical investigation of immune complexes is often difficult. Ideally, the method should be highly specific and sensitive for circulating immune complexes. Also, for the test to be clinically meaningful, it must preferentially detect the harmful pathogenic immune complexes. An additional prerequisite would be that the technique should be simple.

The present study describes the application of 3 relatively simple techniques for detecting circulating immune complex in healthy and disease conditions. In addition, a method for detecting complement consumption dependent upon immunologically mediated (type III hypersensitivity) inflammation was also investigated. The results indicate that these four techniques in combination may be suitable for most laboratories in this country.

Materials and methods

Subjects

Thirty six normal healthy controls from amongst the faculty members, resident staff and laboratory persons with no history of any recent illness were included in the present work. Their ages ranged from 18 years to 40 years. The patient group included those with infection (upper respiratory tract infections) systemic connective tissue diseases (systemic lupus erythematosus, progressive systemic sclerosis, rheumatoid arthritis), and malignancies (leukemias and carcinoma breast). A few patients with idiopathic thrombocytopenic purpura and 11 women in first trimester of normal pregnancy were also studied.

Diagnosis was made by standard clinical and investigative parameters. Only proven cases of respective conditions were included in the study.

Collection of serum

The blood was collected in fasting state from all the subjects. The serum was kept at —20°C till tested (not more than 6 weeks). However, for anticomplementary activity, the sera were usually tested within a few days, but not later than 2 weeks after collection.

Preparation of aggregated human IgG

Standard DEAE–cellulose ion exchange chromatography was used for the purification of IgG from normal human serum (Cann *et al.,* 1971). Phosphate buffer, 0.05M, pH 8 was used for the chromatography. The purity of IgG was tested by the standard microslide technique of Immunoelectrophoresis using anti-whole human serum raised in a rabbit. Purified IgG gave a single are on Immunoelectrophoresis indicating its purity. The aggregation was carried out by heating a 6 mg/ml solution of IgG at 63°C for 10 min. The aggregated human IgG was used as a positive control.

IgG-coated latex agglutination inhibition test

The technique of Lurhuma *et al.* (1976) was used. In brief, it consisted of using the serum of a seropositive rheumatoid arthritis patient with known titre of rheumatoid factor as an indicator. This was mixed with the serum to be tested for circulating immune complex and then mixed with IgG-coated particles (latex reagent). Allowance was made for dilution factors. Reduction in the capacity of rheumatoid arthrites serum (indicator) to cause agglutination of IgG-coated latex particles by the serum under test, was considered as indicative of the presence of circulating immune complex. Aggregated human IgG was used as a positive control.

Polyethylene glycol precipitation test

The technique of Haskova *et al.* (1978) was used Two ml of borate buffer (pH 8.4, 0.1 M) and 2 ml of buffered polyethylene glycol (PEG) 6000 solution (4.16%) of PEG in borate buffer) (Sigma Chemical Co., St. Louis, Missouri, USA) were separately added to 0.22 ml of test serum pre-diluted to 1:3 with borate buffer (so as to obtain a final concentration of 3.75% PEG and 1:30 of serum). After standard mixing in a "Vortex mixer" and 60 min incubation at room temperature the difference in the light absorbance of the two samples (serum in borate buffer and serum in phosphate buffered saline) at 450 nm using 1 cm quartz cuvettes was measured with a Carl-Zeiss, spectrophotometer. Aggregated human IgG and borate buffer were used as positive and negative controls respectively.

Test for anti -complementary activity

Anticomplementary activity of the serum was assayed by the laboratory bench complement fixation test (Public Health Monograph, 1965) and by the technique of Verrier-Jones and Cumming (1977). Sheep red blood cells and hyperimmune sera raised in rabbits against sheep red blood cells were used as the hemolytic system. The optimal sensitizing dose of the hemolysin and the 50% hemolytic dose of the guinea pig complement were predetermined.

Serum stored at—20°C was inactivated at 56°C for 30 min. Two fold dilutions of the sera were made in microtitre plates in 25 µl of buffer and 50 µl of complement containing 5 CH_{50} units was added to each well. Control wells contained buffer alone with 5, 2.5 and 1.25 CH_{50} units of the complement. Aggregated human IgG was used as a positive control. The plates were sealed with transparent non-toxic tapes and kept at 4°C overnight. Sensitized cells were prepared by mixing thoroughly 3% sheep red blood cells with the optimal sensitizing dose of hemolysin and incubating at 37°C. After 15 min 50 µl of sensitized sheep red blood cells were added to each well and the plates were incubated at 37°C for 1 h. Colour standards (representing 0-100% lysis) were prepared and distributed in separate wells. The plates were centrifuged and the results were interpreted as follows.

Test serum which lysed all the red cells (100%) was scored as negative and where no lysis occurred (0%) as a positive indicator of anti-complement activity. The intermediary reactions, as compared with colour standards, were scored as 25 %, 50 % or 75%. Fifty per cent lysis was taken as the end point. The reciprocal of the highest dilution giving a 50% lysis was taken as the titre of the anti-complement activity of the serum.

Determination of alpha-1 -antitrypsin/C3 ratio (α_1-AT/C3)

Principle and technique: As the estimation of serum C3 may not always indicate complement utilization, Lurhuma *et al.* (1976) suggested the determination of another acute phase reactant, viz. alpha- 1-antitrypsin. If the inflammation is immunological in origin where C3 is being utilized, then the rise in C3 will be less in comparison to rise in α_1-AT. This would alter the ratio which would rise as against the ratio seen in normals or in acute inflammations not mediated immunologically. Therefore in the present study the α_1–AT/C3 ratio was

estimated. Estimation of serum C3 and α_1-AT was done by the standard single radial immunodiffusion technique of Mancini *et al.* (1965) with minor modifications, as suggested by Fahey and Mckelvey (1965). The anti serum to C3 was locally prepared (Malaviya, 1972). The anti serum to α_1-AT was obtained commercially (Meloy Lab., Springfield, Virginia, USA).

Results

Sensitivity of the tests

Latex inhibition test: Using aggregated human IgG at 1 mg/ml concentration the test gave a positive result upto a concentration of 330 µg/ml while the dilution of the test sample was 1:3.

Polyethylene glycol precipitation test

In a serial dilution test of aggregated human IgG (1 mg/ml), it was found to give a positive test upto a concentration of 40 µg.

Anti-complementary activity

Using aggregated human IgG in doubling dilution with a starting concentration of 1 mg/ml, the test was positive upto a titer of 1:64. Thus, this test was able to detect aggregated human IgG upto 1 µg per ml dilution.

Circulating immune complexes in controls

Of the 36 persons studied, 29 (80.5%) were negative by all the test while none were positive by all the tests. Of the 7 who were positive by one or 2 tests the titre was very low and in the anti-complementary activity test only 2 persons showed a titre of 1:8 (table 1).

Table 1. Circulating immune complexes in subjects studies.

Subjects (number)	All 3 tests positive	Only 1 or 2 tests positive				All tests negative
		Latex + PEG	Latex + ACA	PEG + ACA	Single test only	
Controls (36)	0	1	2	3	ACA-1	29
Collagen disease (19)	10	1	2	0	ACA-1 Latex-1	4
Idiopathic thrombocytopenia purpura (5)	1	0	1	1	ACA-1	1
Malignancy (14)	2	0	0	0	ACA-1	11
Upper respiratory infections (7)	2	2	0	0	0	
Normal pregnancy (11)	1	0	1	0	ACA-2 PEG-1	6

Latex, IgG coated latex agglutination inhibition test; ACA, anti-complementary activit, test; PEG, polyethylene glycol precipitation test.

Alpha-1 antitrypsin/C3 ratio in 36 normal controls

The determination of alpha-1 antitrypsin/C3 ratio in 36 normal controls showed a mean ratio of 0.72 with S.D. of ±0.315. Thus, the ratio of 1.35 (mean plus 2 S.D.) was taken as the upper limit of normal ratio. Any value above it was considered as indicative of abnormal complement consumption *in vivo.*

As shown in table 2; none of the controls had any abnormality in alpha-1 antitrypsin/C3 ratio, thus showing the absence of any complement consumption.

Table 2. Correlation of circulating immune complex with α_1-AT/C3 ratio.

	α_1-AT/C3 upto 1.35	Ratio more than 1.35	P
Controls			
CIC+	7	0	<0.05
CIC−	29	0	
Collagen diseases			
CIC+	1	9	<0.001
CIC−	9	0	
I.T.P.			
CIC+	0	3	>0.1
CIC−	2	0	
Malignancy			
CIC+	2	1	>1.8
CIC−	10	1	
U.R.I.			
CIC+	0	4	>0.02
CIC−	3	0	
Pregnancy			
CIC+	5	0	0
CIC−	6	0	

CIC, Circulating immune complex; ITP, idopathic thrombocytopenic purpura; URI, upper respiratory infection; α_1-AT, alpha1-antitrypsin.

Circulating immune complexes in diseases (table 1)

In collagen diseases: There were a total of 19 patients (10 with rheumatoid arthritis, 6 with systemic lupus erythematosus and 3 with progressive systemic sclerosis) in this group. Only 4 (21%) of these patients were negative by all tests. All these 4 patients were in complete remission. Of the 10 subjects positive by all tests 5 were rheumatoid arthritis, 3 were systemic lupus erythematosus and 2 were progressive systemic sclerosis. All these patients were in severe relapse. Of the 5 subjects who showed only 1 or 2 of the 3 tests positive for circulating immune complex, those with either anti-complementary activity and/or latex inhibition tests positive were in acute relapse. In contrast only one patient with positive

polyethylene glycol and latex inhibition test but negative anti-complementary activity, was in remission.

The results for the presence of circulating immune complex correlated well with the complement consumption as tested by alpha- l-antitrypsin/C3 ratio (table 2).

Circulating immune complex in other conditions: In 5 patients with idiopathic thrombocytopenic purpura, circulating immune complex was shown in 4 (80%) subjects. Again the positivity correlated well with complement consumption tested by alpha-l-antitrypsin/C3 ratio (table 2).

Very few patients with malignancy (breast cancer, 1 out of 7 and leukemia, 2 out 7) showed circulating immune complex (table 1). There was no complement utilisation detectable in these patients (table 2).

Minor illness studied included 7 patients with upper respiratory tract infection. Four of these patients showed circulating immune complex out of whom 2 patients were positive by all the 3 tests (table 1). The same 4 patients also showed significant increase in alpha-l-antitrypsin/C3 ratio, indicating complement consumption (table 2).

Eleven cases of pregnancy were also studied. Five amongst these were positive for circulating immune complex but only 1 was positive by all the 3 tests. The alpha-l-antitrypsin/C3 ratio was normal in all these subjects indicating non complement utilizing type of circulating immune complex, probably nonpathogenic in nature.

Correlation of clinical activity with circulating immune complex and alpha -1-antitrypsin/C3 ratio in patients and controls (table 3)

The presence of circulating immune complex generally correlated well with disease activity. On the other hand, elevated alpha-l-antitrypsin/C3 ratio

Table 3. Correlation of clinical condition with circulating immune complex and α_1-AT/C3 ratio.

Clinical state (number)		ACA positive alone or with other tests	PEG and/or latex only or with ACA also positive	α_1-AT/C3 ratio	
				>1.35	<1.35
Group* I (33)		21	21	30	3
Group* II (59)		11	7	2	57
	X^2	16.95	24.40	67.65	
	P	<0.001	<0.001	<0.001	

* Group I includes cases who were clinically active or in relapse or severe or, in case of carcinoma breast, preoperative cases or cases more than clinical state II. Group II includes normal healthy controls, normal pregnancy, cases of different diseases which were in remission or they were inactive or, in case of carcinoma breast, post-operative cases or early case up to clinical stage II only.

correlated even better with disease activity and severity. Patients with collagen diseases showed a trend such that whenever anti-complementary activity was present in combination or alone, invariably alpha-l-antitrypsin/C3 ratio was also elevated. This may indicate that anti complementary activity test detects pathogenic circulating immune complex more often. But it would require the examination of a large number of patients to prove it.

Discussion

The techniques chosen for the present work fulfil several of the prerequisites mentioned earlier. Thus the techniques are simple, fairly sensitive, give a satisfactory results with the time-honoured 'positive control' (i.e. aggregated human IgG), require little sophistication and do not involve expensive imported reagents. They seem to have a fair degree of correlation among themselves. Moreover, the presence of circulating immune complex in different conditions correlated with the severity of clinical state. However, this correlation was not absolute, several normal healthy controls and pregnant women also showed circulating immune complexes by one or more of the techniques used.

Using different techniques, several authors have reported varying proportions of normal individuals having circulating immune complexes, the figures being comparable to the present work (Lurhuma *et al.,* 1976; Theofilopoulos *et al.,* 1977). The possible presence of subclinical infection, presence of food antigen-antibody complexes, presence of some degree of aggregation of immunoglobulins, have all been implicated as the possible cause of circulating immune complex being detected in otherwise normal individuals.

The presence of circulating immune complex in normal pregnancy is a controversial issue. Thus, some workers found circulating immune complex in all normal pregnancy (Lambart and Houba, 1974) while others did not find circulating immune complex in any of the pregnant women (Gleicher *et al.,*1978). Later, McLaughlin *et al.* (1979) found circulating immune complex by the latex-inhibition technique but not by direct Clq binding. These workers reported 9 out of 12 pre-eclamptic pregnancies to be positive for circulating immune complex as against only 2 out of 12 normal pregnancies. Using the 3 tests mentioned above, the present work detected circulating immune complex in less than half of the pregnancies.

Circulating immune complexes were detected in a majority of patients with collagen vascular diseases. This is in agreement with the vast literature already available on the subject (Luthra *et al.,* 1975); Davis *et al.,* 1977; Levinsky *et al.,* 1977; Malaviya *et al.,* 1980). Similarly, circulating immune complex are well documented in upper respiratory infection (Schwenk and Baenkler, 1979) as was also seen in the present work. Similarly, the circulating immune complexes were found in a comparable proportion of ITP cases in the present study as reported by Lurhuma *et al.* (1976). However, the main discrepancy was seen in cases with leukemia and breast carcinoma. Thus, Carpentier *et al.* (1977), Hoffken *et al.*

(1978) and Theofilopoulos *et al.* (1977) have reported a high proportion of such pateints with circulating immune complex. However, the present work failed to detect circulating immune complex in a high proportion of these patients. The most likely explanation could be that the techniques used in the present work may not be sensitive enough to detect the special types of complexes found in malignancies.

In situations where circulating immune complexes are detected without obvious immuno-inflammatory tissue damage, it would be of interest to correlate its presence with a parameter indicating the presence of immune-complex mediated immunoinflammation. A simple and easy way to test it is to study the ratio of alpha 1-antitrypsin/C3. The study of this ratio showed that in obvious pathological states (e.g. active systemic lupus erythematosus, acute rheumatoid arthritis etc.) the presence of circulating immune complex correlated well with abnormally high alpha l-antitrypsin/C3 ratio. But subject without any obvious pathological state with detectable circulating immune complex, did not show abnormally high alpha-1-antitrypsin/C3 ratio.

It would thus appear that the 3 tests chosen to detect circulating immune complexes are moderately sensitive. They were successful in detecting 'pathogenic' immune complexes in the majority of active cases of collagen diseases which as a group was considered a prototype of immune-complex disease (Gleicher *et al.,* 1978; McLaughlin *et al.,* 1979).

The findings of the present work also show that firstly, as reported from various laboratories (Report of WHO Scientific Group, 1977), circulating immune complexes are detectable in diverse clinical conditions. It was seen most often in collagen vascular diseases. However, a significant proportion of other conditions also showed circulating immune complexes. Secondly, a combination of simple techniques like latex inhibition, polyethylene glycol precipitation and anti-complementary activity, may be able to detect almost all varieties of circulating immune complexes. Thirdly, the screening for the alpha-l-antitrypsin/C3 ratio in the serum appeared to be a good indicator of immuno-inflammatory tissue damage due to circulating immune complexes.

In conclusion, it is recommended that laboratories with modest facilities, interested in clinical work related to immune complexes, may use these techniques with a fair degree of confidence. Also, for detecting pathogenic immune complexes capable of tissue damage through complement consumption (type III hyper-sensitivity) the alpha-l-AT/C3 ratio is possibly an excellent test.

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