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Physiological aspects of circulating immune complexes in the normal population

CARMEN E. CONTRERAS, ANA OROZCO, P. SÁNCHEZ, G. ORTEGA & N. E. BIANCO *Unidad de Inmunologia Clinica, Instituto Anatomopatologico, F. de Medicina U.C.V. Unidad Piloto del Centro Nacional de Referencia en Inmunologia Clinica, S.A.S. Venezuela*

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SUMMARY

Circulating immune complexes (CIC) have been investigated in 100 normal subjects; the RIA-Raji and the C1q-BA conventional methods, as well as a new solid phase microassay utilizing purified C1q and the systematic search of cryoprecipitates were employed. CIC serum levels did not differ in regards to sex; in relation to age, values for C1q-BA were identical in subjects from 0 to 60 years and also in those beyond age 60; the differences encountered by RIA-Raji or by the C1q-SP microassay in these two main groups were not statistically significant. Cryoprecipitates were present in 100% of tne 68 examined subjects. Immunoglobulins (G, A and M), anti-nucleic acid (DNA and Poly A) and CIC (by the three methods) were present in the cryoprecipitates while lymphocytotoxins, rheumatoid factor and C3 were undetectable; protein content of the cryoprecipitates increased significantly with age, reaching a normal superior limit of 0·52 mg/ml beyond age 30. These findings further support the role played by CIC in normal immune response and may help in the understanding of the physiopathology of clinical conditions associated with immune complexes.

INTRODUCTION

Circulating immune complexes (CIC) have been linked to the physiopathology of an increasing number of human (Williams, 1980; Zubler & Lambert, 1978; Theofilopoulos & Dixon, 1980) and experimentally induced diseases (June *et al.*, 1979; Contreras *et al.*, 1980); however, it should be stressed that CIC represent a normal and effective immunological effector mechanism for antigenic clearance. During the last few years, several methods have been utilized to monitor CIC in many clinical conditions (Lambert *et al.*, 1978; Haakenstad & Mannix, 1979); even though normal values of CIC are given in each report, very little is known about the incidence and characteristics of CIC in normal subjects at risk.

In a prospective protocol, we have examined this aspect in 100 normal subjects, establishing mean values and normal superior limits of CIC by four distinct methods and reporting the prevalence and composition of cryoprecipitates in normal population.

MATERIALS AND METHODS

Selection of healthy normal population. One hundred normal subjects were selected after a

Correspondence: Carmen E. Contreras, Unidad de Inmunologia Clinica, Apartado 50647, Correos de Sabana Grande, Caracas, Venezuela.

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careful clinical history and physical examination; particular interest was placed in ruling out all possible diseases associated with CIC; routine laboratory and serial stool examinations were also performed; 40 were females and 60 were males. The selected group of healthy controls was divided in four subgroups according to age. Seven out of the 100 specimens were cord blood which were obtained from healthy newborns as well.

Processing of serum samples. Fifty millilitres of venous blood was drawn and allowed to clot at 37 C for 2 hr; the specimens were centrifuged at 1,500 *g* for 30 min at room temperature; the serum was distributed in five aliquots (1 ml). An additional 10 ml aliquot (pyrex sterile tubes) was placed at 4 C to induce cryoprecipitation.

Cryoprecipitation. After 7 days at 4°C, the 10 ml aliquots were centrifuged at 7,000 rpm for 30 min at 4 C; the supernatant was discarded and 1 ml of cold phosphate-buffered saline (PBS, 0·01 M, pH 7·6) was added; after 1 hr at 4 C the tubes were centrifuged at 7,000 rpm for 30 min at 4°C; the supernatant was discarded and four washes were performed consecutively with 30 ml of distilled water each time (7,000 rpm, 30 min, 4°C). Finally, 1 ml of cold PBS was added, the specimens were stored at 4 C; before analysis, they were placed at 37°C for 1 hr to allow resolubilization.

Preparation of standard reagents. Two different types of human gamma globulins (Cohn fraction II) were tested; one obtained from Pentex Miles Lab prepared in PBS (20 mg/ml) and ultracentrifuged at 150,000 *g* for 90 min (Beckman Ultracentrifuge, T-385 rotor); the upper third of the supernate (deaggregated or 7S IgG) was removed and the protein concentration was determined at 280 nm; the other obtained from Globuman, Bern, Switzerland, at a final concentration of 3 mg/ml prepared in normal human serum (NHS). Aggregation of both types was performed by heating at 63°C for 30 min.

Antisera against human IgG was purchased from Atlantic Antibodies, Scarborough, Maine, USA (anti-human IgG fraction, Fc piece-specific). The specificity of the antisera was tested by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965), immunoelectrophoresis (Grabar & Williams, 1953) and Ouchterlony (1962) tests. Radioiodination of the specific antisera was performed with ^{125}I according to the procedure of McConahey & Dixon (1966). The specific activity of the ^{125}I-anti-human IgG was 0·2 μCi c.p.m./μg of protein.

Circulating immune complexes (CIC). CIC were searched by the Raji radioimmunoassay method (RIA-Raji) of Theofilopoulos, Wilson & Dixon (1976), by the C1q-BA method of Zubler *et al.* (1976) and by a microassay adaptation of C1q solid phase method (Hay, Nineham & Roitt, 1976), designated herein as MAC1q-SP. It was performed as follows: 200 μl of C1q solution (6·25 mg ml in carbonate buffer 0·05 M, pH 9·6) were dispensed in flat-bottomed polystyrene wells (Cooke Laboratory products, No. M179A, Alexandria, Virginia) contained in styrofoam holders. After 3 hr incubation at 37°C and three washes with cold BBS containing 0·05% Tween (BBS-T), the wells were filled with 200 μl of 0·1% gelatin solution and incubated 2 hr at room temperature. Following three washes with BBS-T, the wells were set in duplicates with 200 μl of the serum samples (1:20) which was previously incubated with 0·2 M EDTA, pH 8·3. After 2 hr at room temperature, the wells were washed again and 200 μl of ^{125}I-anti-human IgG (1 μg/ml) were added. The unbound antibody was removed after 3 hr by further washes and the wells were counted on a gamma scintillation counter.

The amount of radiolabelled antibody was referred to a standard curve of antibody uptake by C1q coated wells, incubated with 200 μl of a 1:20 final dilution of various amounts (from 30 μg to 1·5 ng) of AHG prepared in fresh normal human serum. The amount of complexes in each serum tested was expressed as μg equivalents of AHG per millilitre of serum.

Analysis of cryoprecipitates. Cryoprecipitates were analysed as follows: protein content estimated by spectrophotometry at 280 nm (E_{cm}% 14·5 for human IgG); the presence of rheumatoid factor (Singer & Plotz, 1956), IgG, IgA, IgM, C3 (Mancini *et al.*, 1965) (LC-Partigen, Behring), anti-DNA and RNA (Attias, Sylvester & Talal, 1973), and lymphocytotoxins (Terasaki, Mottironi & Barnett, 1970) was also investigated. To detect CIC in the cryoprecipitate, a fresh CIC-free pool of NHS was employed utilizing optimal serum dilutions for each method.

Statistical methods. The upper limit of the normal range (normal superior limit) was calculated as the 90th percentile value (non-parametric statistical procedure) for each method. Spearman's rank order analysis and Student's *t*-test were also performed.

RESULTS

Normal population studied

The 100 selected subjects were divided into four main groups, depending on age; the first group included seven cord bloods and 15 subjects between 5 and 15 years; the other three groups were group 2: 16–30 years ($n=34$), group 3: 31–60 years ($n=24$) and group 4: 61–92 years ($n=20$).

CIC serum levels

CIC serum levels were analysed in regards to sex (Table 1) and age (Fig. 1). We found no difference between males and females; on the other hand, even though CIC levels tend to increase in group 4 (RIA-Raji and MAC1q-SP) the differences were not significant. We wanted to explore further the difference noted before and after 60 years of age; 90 of the selected subjects were divided into two groups; group A from 0 to 60 years and group B from 61 to 92 years. The results obtained by the three methods were compared (Table 2). The differences observed in CIC levels by RIA-Raji and MAC1q-SP were not statisticaly significant.

Normal superior limits of serum CIC

Once mean values for CIC in the studied population were established, the normal superior limits for the obtained values were calculated (Table 3); the upper limit of the 90th percentile, a non-parametric statistical procedure was utilized (Reed, Henry & Mason, 1971), discarding the extreme values by r10 calculations as proposed by Dixon (1953). The differences between both groups, observed in the results obtained by MAC1q-SP and RIA-Raji were not significant (Student's *t*-test).

Table 1. CIC serum levels in normal population

Population (sex)	Number	C1q-BA (%)	MAC1q-SP (μg AHG/ml)	RIA-Raji (μg AHG/ml)
		mean ± s.d.		
Female	40	2·1 ± 0·8	22·1 ± 27·2	17·5 ± 18·7
Male	60	2·4 ± 1·4	18·3 ± 15·1	19·7 ± 24
Total	100	2·2 ± 0·9	16·2 ± 10·2	19·2 ± 17·8

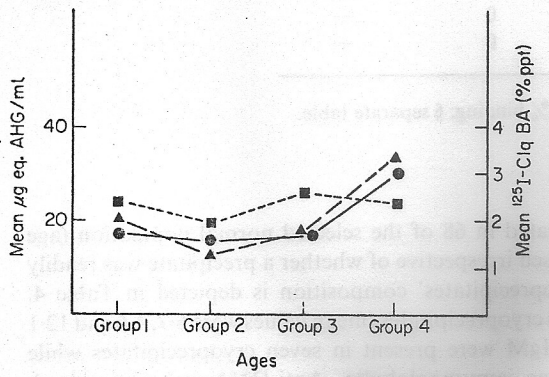

Fig. 1.

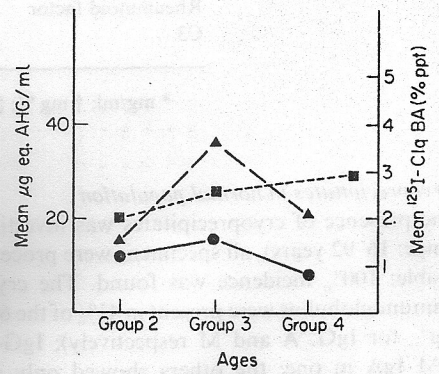

Fig. 2.

Fig. 1. CIC serum levels according to age. (▲ = RIA-Raji; ● = MAC1q-SP; ■ = C1q-BA).

Fig. 2. Levels of CIC in cryoprecipitates according to age. (▲ = RIA-Raji; ● = MAC1q-SP; ■ = C1q-BA).

Table 2. CIC serum levels in normal population

Groups	Number	C1q-BA (%)	MAC1q-SP (μg AHG/ml)	RIA-Raji (μg AHG/ml)
		mean ± s.d.		
A (0–60 years)	70	2·2 ± 0·8	16·5 ± 8·5	16·7 ± 11·3
B (61–92 years)	20	2·0 ± 0·8	21·7 ± 13·9*	25·4 ± 20·5*

* Differences not significant.

Table 3. Normal superior limits of serum CIC*

Groups	C1q-BA (%)	MAC1q-SP (μg AHG/ml)	RIA-Raji (μg AHG/ml)
A (0–60 years)	3·5	18·6	39·2
B (61–92 years)	3·6	23·1†	58·0‡
Total (0–92 years)	3·6	35	38·0

* Upper limit of 90th percentile; † $t = 0{\cdot}73$; ‡ $t = 1{\cdot}45$.

Table 4. Composition of cryoprecipitates in 68 normal subjects

Parameter	Mean ± s.d. ($n = 68$)	Normal superior limit
Protein content*	0·37 ± 0·40	§
IgG†	6·7 ± 2·6	—
IgA†	4·6 ± 0	—
IgM†	12·1 ± 12·2	—
Anti-DNA‡	0·9 ± 0·5	1·5
Anti-poly A‡	2·2 ± 1·3	3·8
CIC–RIA-Raji	20·8 ± 18·5	55
CIC–MAC1q-SP	8·5 ± 6·5	25
CIC–C1q-BA	1·8 ± 1·0	4·2
Lymphocytoxins	0	—
Rheumatoid factor	0	—
C3	0	—

* mg/ml; † mg %; ‡ % binding; § separate table.

Cryoprecipitates in normal population

The presence of cryoprecipitates was investigated in 68 of the selected normal population (age range: 16–92 years), all specimens were processed irrespective of whether a precipitate was readily visible; 100% incidence was found. The cryoprecipitates' composition is depicted in Table 4. Immunoglobulins were present in 41% of the 68 cryoprecipitates (mean values were 6·7, 4·6 and 12·1 mg% for IgG, A and M respectively); IgG–IgM were present in seven cryoprecipitates while IgM IgA in one; the others showed only one immunoglobulin. Anti-DNA and anti-poly A antibodies were detected in 32% and 17% respectively of the total cryoprecipitates analysed. CIC

Table 5. Protein content (mg/ml) of cryoprecipitates

Groups	Mean ± s.d.	Normal superior limit
C (16–30 years)	0·092 ± 0·052	0·12
D (31–92 years)	0·43 ± 0·25*	0·52

* ($P < 0·001$).

were present in the cryoprecipitates of the three age groups (Fig. 2), while lymphocytotoxins, rheumatoid factor and C3 were undetectable. As far as protein content, mean values in the 68 cryoprecipitates was 0·37 mg/ml; as protein content was higher in subjects beyond age 30, the whole group was divided into two subgroups: group C (age 16–30 years) and group D (age 31–92 years). As can be seen in Table 5, the mean value for protein content of the cryoprecipiates of subjects of group D was statistically higher ($P < 0·001$) than those of group C with a normal superior limit of 0·52 mg/ml.

DISCUSSION

The synthesis of immune complexes represents one of the most useful effector mechanisms for antigenic clearance. Its interaction with fluid phase and cell membrane recognition systems provides an efficient means of antigenic destruction. Physiologically, CIC may be formed by specific antibodies reacting with an ingested, or inhaled antigen or by antigenic penetration through any blood vessel source. In addition, CIC may also originate from reacting with autologous constituents or from idiotype–anti-idiotype reactions (Williams, 1980; Haakenstad & Mannix, 1979; Nyddeger, Kazatchkine & Lambert, 1980). Recently, the interesting observations of Nussenzweig (1980) suggested that complement induces the solubilization of CIC, which comes from the intercalation of the complement peptides into the formed lattice. The end result would favour the participation of cell surface receptors and the final antigenic degradation.

On the other hand, immune complexes may alter significantly the host immune response and/or may induce tissue injury (Williams, 1980; Theofilopoulos & Dixon, 1980; Haakenstad & Mannix, 1979; Pérez-Rojas *et al.*, 1978; Arango *et al.*, 1981; Blanca *et al.*, 1981). Certainly, the availability of new methods (Lambert *et al.*, 1978) has allowed the detection and monitoring of CIC in numerous pathological conditions.

Nevertheless, immune complexes in the normal host have received little attention. The little available data is contradictory, mainly based on estimates of CIC in normals, when established or new methods are applied or else when cryoprecipitation is considered (Williams, 1980; Lambert *et al.*, 1978; Weisman & Zvaifler, 1975). We have approached certain aspects of the physiology of CIC by carefully screening 100 normal individuals, including seven cord blood specimens and 20 subjects over age 60. To study CIC in the selected groups, four methods were employed: cryoprecipitation which is felt to induce the precipitation out of solution of relatively insoluble CIC (Williams, 1980; Clauvel, Klein & Seligman, 1974; McIntosh, Kaufman & Kulvinskas, 1970), the Raji cell radioimmunoassay (Theofilopoulos & Dixon, 1980) which will bind CIC (11–19S) mainly through complement receptors; the C1q-BA of Zubler *et al.* (1976), which combine interaction of the CIC with C1q and precipitation with polyethyleneglycol, detecting small size complexes (9–11S) and a microassay adaptation of C1q-solid phase method (Hay *et al.*, 1976).

As may be seen in our results, 100% incidence of CIC may be detected under normal conditions by all methods employed, levels of serum CIC did not differ by sex or age, the tendency in the aged (group 4) was to show increased serum levels; however, the difference compared with the other

tested groups was not significant. Nevertheless, we explored this aspect by dividing the whole group into those with age range from 0 to 60 years and the subjects over 60 (60–92 years). Even though the mean by RIA-Raji and by MAC1q-SP were greater in the group over 60 years, the differences were not significant. By applying non-parametrical statistical procedures (upper limit of 90th percentile), we estimated the normal superior limit (NSL) of CIC serum levels for the three selected methods. Thus, the available NSL may allow a better distinction between normal or elevated levels of serum CIC in a given patient or in clinical conditions, mediated by immune complexes.

Cryoprecipitates were found in 100% of the 68 screened sera. Contrary to others (Weissman & Zvaifler, 1975) we find no difficulty in solubilizing the precipitate after 7 days at 4°C. The protein concentration showed a mean value of 0·37 mg/ml which is higher than those observed by Cream (1972). Further, beyond age 30, protein content increased significantly, with a mean value of 0·43 and a NSL of 0·52 mg/ml. Immunoglobulins were detectable in 41%, being eight out of 26 of mixed nature (seven IgG–IgM and one IgM–IgA). Weissman & Zvaifler (1975) found that three out of 14 normals showed cryoprecipitates with small amounts of IgG and protein concentration in the range of 0·2 mg ml.

In relation to anti-nucleic acid activity, we found that 32% of the cryoprecipitates showed anti-double stranded DNA and 17% anti-RNA, both in low levels (mean values of 0·9 and 2·2% of binding respectively).

CIC were detected in 100% of our 68 cryoprecipitates by the three employed methods. Further, NSL were higher in the cryoprecipitates than those established in serum. We were unable to detect rheumatoid factor or C3, although Weissman & Zvaifler (1975) found that in three cryoprecipitates from 14 normal subjects, C3 and rheumatoid factors were present. Also, no lymphocytotoxins were detected in the 68 cryoprecipitates.

In this investigation, we have shown that CIC are universally present in normal subjects. Moreover, cryoprecipitation seems to be also a physiological phenomenon; the immune complex nature of the cryoprecipitates may be suggested since CIC were readily detectable. We have no clear explanation for the presence of anti-double stranded nucleic acid antibodies in the cryoprecipitates of the population studied.

CIC plays an important role in normal mediation of most immune responses, modulating perhaps through positive or negative signals numerous effector mechanisms; our findings offer new evidence of the significance of CIC in normal conditions, which in turn should help in the understanding of the role played by CIC in human diseases.

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