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# Significance of circulating immune complexes in pulmonary tuberculosis

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SUMMARY

In the present study we have tried to demonstrate circulating immune complexes (CIC) in sera from patients with pulmonary tuberculosis (TB) by three techniques; (1) latex agglutination; (2) 3·5% PEG precipitation and determination of optical density at 280 nm and (3) RIA of CIC using bovine spermatozoa. About 40 normal control sera and 100 TB patients sera were investigated for the presence of CIC. Seventeen per cent cases of pulmonary TB were positive by latex agglutination while none of the control was positive. Levels of CIC as detected by PEG precipitation and RIA were significantly elevated in patients as compared to normal controls. While IgG, IgA and IgM were elevated in the CIC of patients, IgM immunoglobulins were detected only in patients and not in controls. Detection of CIC may at times be useful in diagnosis, prognosis and therapeutic monitoring of disease processes, but it is the characterization of immune complexes (IC) and identification of the specific components of these complexes which holds the greatest potential for better understanding of disease mechanisms. CIC were precipitated using 3·5% PEG from sera of patients suffering from TB. The specific anti-TB antibody component of complex was determined using *S. aureus* protein A as a solid phase, Anti-BCG antibody and $^{125}$I-labelled TB antigen. The specific TB antigen component of the IC was dissociated thermally from TB antibody and assayed by a radioimmunoassay technique developed in our laboratory. Patients were classified into two groups. Those whose sputum was positive for *Mycobacterium tuberculosis* by smear and/or culture and those whose sputum was negative. The TB antigen concentrations of CIC was higher $19{\cdot}1 \pm 2{\cdot}3$ ng/ml (mean +s.e.) in sputum positive cases, and $9{\cdot}9 \pm 1{\cdot}9$ ng/ml in sputum negative cases as compared to $2{\cdot}2 \pm 0{\cdot}3$ ng/ml in controls. Patient groups were significantly different from controls as well as from each other ($P < 0{\cdot}001$). Anti-TB antibody ratios were $11{\cdot}7 \pm 1{\cdot}48$, $5{\cdot}1 \pm 1{\cdot}5$ and $0{\cdot}6 \pm 0{\cdot}1$ in sputum positive, sputum negative and controls. The significance of differences between the groups was $P < 0{\cdot}001$. The effect of treatment administered over a period of 12 weeks or more was evaluated. It was observed that in patients with persistent demonstration of *M. tuberculosis* in the sputum, the TB antigen and TB antibody levels of CIC were consistently high. In patients who responded to anti-tubercular drugs the TB antigen levels decreased progressively while TB antibody levels remained high. These studies indicate that isolation and identification of specific antigen and antibody is not only useful in the separation of diseased groups from normal but is useful in evaluating therapeutic responsiveness and progress of disease.

**Keywords** pulmonary tuberculosis circulating immune complexes

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## INTRODUCTION

Immune complex (IC)-mediated injury plays a major role in the pathophysiology of diseases such as systemic lupus erythematosus, rheumatoid arthritis, various infectious diseases and malignancies. An increasing appreciation of the biological and pathological significance of circulating immune complexes (CIC) has led to the development of several techniques for identifying and measuring them. Detection of CIC may at times be useful in diagnosis, prognosis and therapeutic monitoring, but it is the characterization of IC that holds the greatest potential for better understanding of the disease mechanisms. With these considerations in view, we have studied CIC in patients suffering from pulmonary tuberculosis (TB).

We used five different techniques: (1) latex agglutination; (2) 3·5% polyethelene glycol (PEG) precipitation and determination of optical density at 280 nm; (3) RIA for CIC using bovine spermatozoa (BS) to detect CIC in serum samples; (4) the type of immunoglobulins associated with the complexes as determined by radial diffusion and (5) IC were then isolated from the serum samples, dissociated and the antigen and antibody components associated with the complexes were determined by radioimmunoassays (RIA) developed in our laboratory to detect tubular antigen and anti-tubercular antibody.

## MATERIALS AND METHODS

*Patient selection.* Sera were obtained from patients attending the Tuberculosis Clinic. These patients had undergone detailed investigations which included clinical examination, investigations of family members, radiological investigations, haematological tests like ESR, blood counts, etc. and sputum culture for *M. tuberculosis*. Patients who were being evaluated for the follow up of various regimes of anti-tubercular treatment were also studied. Sera were stored at −20°C.

*Latex agglutination.* CIC and rheumatoid factor (RF) are known to agglutinate latex particles coated with human gamma-globulin (Singer *et al.*, 1956). The presence of CIC was thus observed by checking for the agglutination of latex particles. The R.A. Test kit supplied by Span Diagnostics was used. The agglutination was checked at the end of 2 min. A strongly positive result (+ + + +) was considered if within 2 min coarse agglutination of latex particles occurred. A weakly positive (+) result was considered if small clumps of agglutinated latex particles were observed.

*PEG precipitation and measurement of optical density at 280 nm.* It has been shown that 3·5% PEG precipitates about 80% of IC, aggregated forms and other macromolecular substances (Fust *et al.*, 1980). Equal volumes of sera diluted 1:10 and 7% PEG (mol. wt 6,000) were incubated overnight at room temperature. After redilution of the precipitates in 0·01 M phosphate-buffered saline pH 7·6 (PBS) the optical density was determined at 280 nm on a spectrophotometer.

*RIA of IC using BS.* BS have been known to possess Fc receptors specific for CIC and aggregated human IgG (hIgG). They were, thus, used to measure the levels of CIC from unknown samples. BS were obtained from Bombay Veterinary College. BS were washed thrice with 0·01 M PBS, pH 7·4 and stored at −20°C with equal amount of glycerine.

For actual assay, the procedure given by Witkins *et al.* (1981) was followed. hIgG at concentrations ranging from 1·0 to 100 μg/ml was used to construct a standard curve. Purified anti-human IgG was labelled with $Na^{125}I$ by chloramine-T method (Hunter & Greenwood, 1962) and used as a labelled source. Direct serum samples from controls as well as the patients were processed. Results were expressed in the form of a ratio:

$$\frac{\text{IC levels in } \mu\text{g of unknown}}{\mu\text{g of standard control}}.$$

RIA of CIC using BS was found to be reliable, reproducible and highly sensitive. As low as 1 μg/ml of hIgG was detectable. The intra-assay and interassay variations were of the order of 10% and 15%, respectively.

*Characterization of antigen/antibody in CIC.* Detailed methodology for isolation of CIC from sera, dissociation of the antigen and antibody components of the complex and determination of TB antigen and anti TB antibody concentrations of the dissociated complexes is described.

In brief, 3·5% PEG was added to sera and left overnight at room temperature to allow the precipitation of CIC to be completed. The precipitated CIC was redissolved to make up its original volume of sera used. One aliquot was assayed for the anti-TB antibody content. It was felt that since antibodies are usually multivalent, there would be sufficient binding sites available to bind radioactively labelled TB antigen used for its detection, inspite of some of the binding sites being occupied by the TB antigen of the CIC. Hence dissociation of antigen–antibody complex for identification of the antibody component was not thought to be necessary. However, for the determination of the antigen component of the complex, the technique of thermal dissociation (Tabbarah *et al.*, 1980) was employed. The complex precipitated by 3·5% PEG was heated to 90°C for 15 min. The antibody, which is a globulin, gets denatured and precipitates out, while the heat stable TB antigen can be recovered from the supernate after centrifugation. Tubercular antigen was determined by RIA from the supernate.

Anti-TB antibody was evaluated by a solid phase assay, using *Staphylococcal aureus* protein A as the solid phase. Anti-BCG antibody (Dakopats, Copenhagen, Denmark) was used as standard antibody for comparison of unknowns. The standard anti-BCG antibody in dilutions of 1 in 10,000 to 1:1,000,000 was adsorbed to *S. aureus* protein A (10% formalized suspension of *S. aureus*). The unknowns which consisted of the PEG precipitated IC from patient's sera was also adsorbed on *S. aureus*. TB antigen labelled with $^{125}I$ (Kadival *et al.*, 1982) was added to the standards and unknown samples, incubated overnight and centrifuged next morning. The pellet of *S. aureus* was washed twice with phosphate buffer (pH 7·6, 0·1 M containing 1% BSA) and counted in a gamma counter set for $^{125}I$ counting. The plot of the standard curve using co-ordinates comprizing of the various dilutions of anti-BCG antibody and counts obtained in standard tubes was constructed. The unknown antibody dilutions were read off from the standard curve. The result was expressed as a ratio of the lowest detectable standard anti-BCG antibody titre/antibody titre obtained for the unknown samples. This normalization procedure would eliminate variations in assay batches. Circulating anti-TB antibody titres were also determined by this technique except that serum was directly used for the measurement of antibody titres.

TB antigen was determined using *S. aureus* protein A as a solid phase. Anti-BCG antibody (Dakopatts) in dilution 1:2500,000 (antibody dilution determined by optimization) was adsorbed to *S. aureus*. TB antigen isolated from *M. tuberculosis* H37Rv sonicates in concentrations of 2·5–200 ng/ml was added in the standard tubes (Kadival *et al.*, 1982). In unknown tubes, the dissociated complex which was thermally treated to denature antibody part of the complex and containing antigen was used for measurement of antigen. TB $^{125}I$-antigen containing about 6,000–8,000 ct/min was added to standards and unknown, incubated at room temperature overnight and centrifuged next day. After two or three washings of the pellet of *S. aureus*, the tubes were counted in a gamma counter. Counts in standard tubes was plotted against concentration of stable TB antigen added and unknown antigen concentrations were determined. The sensitivity of the assay system was 1 ng/ml with intra- and interassay variations ranging between 10–15%. Results were expressed as ng/ml of TB antigen.

## RESULTS

The patients were classified in two groups: (1) group 1—radiologically positive with tubercular lesions in the lung and AFB positive by smear and/or culture for *M. tuberculosis* and (2) group 2—radiologically positive with tubercular lesions in the lung but AFB negative by smear and/or culture for *M. tuberculosis*.

Table 1 indicates the levels of CIC obtained by three techniques, as well as the levels of CIC antibody (i.e. IgG, IgA or IgM) in both the groups. Twenty-one per cent of cases studied in group 1 were positive, and in group 2, 13·5% cases were positive by latex agglutination. The PEG detection of CIC was OD $0·62 \pm 0·006$, and OD $0·53 \pm 0·007$ in group 1 and group 2, respectively, which was

**Table 1.** CIC detected by different techniques & the type of immunoglobulins in TB patients classified as: (1) sputum +ve and (2) sputum −ve groups

| TB patients | Latex agglutination (%+ve) | 3·5% PEG precipitation & OD at 280 nm | BS RIA ratio of unknown value / standard value | IgG | IgA | IgM |
|---|---|---|---|---|---|---|
| | | mean+s.e. | | | | |
| Group 1 X-ray TB lesions culture for *M. tuberculosis* positive ($n=57$) | 21 | 0·62* +0·006 | 0·104* +0·002 | 898·6* +8·5 | 30·5* +0·34 | 35·1* 0+0·27 |
| Group 2 X-ray TB lesions culture for *M. tuberculosis* negative ($n=37$) | 13·5 | 0·53* +0·007 | 0·072† +0·003 | 890·4* +10·4 | 25·9* +0·5 | 19·2* +0·5 |
| Controls ($n=40$) | None | 0·18 +0·002 | 0·024 +0·001 | 556·12 +4·52 | 8·13 +0·12 | UD |

UD = undetectable.
* $P<0·0005$ as compared to the normal controls.
† $P=0·0025$ as compared to the normal controls.

higher ($P<0·005$) than the controls (OD $0·18\pm0·002$). Group 1 values of CIC were higher ($P<0·0125$) than group 2.

RIA of CIC using BS followed a similar pattern with values of $0·10\pm0·002$ and $0·07\pm0·003$ in group 1 and group 2, respectively. While the control values were much lower ($0·02\pm0·001$; $P<0·0005$ in group 1 and $P<0·0025$ in group 2). Again group 1 had higher levels ($P<0·05$) than group 2. IgM was significantly higher again in group 1 ($35·1\pm0·274$ mg) than in group 2 ($19·2\pm0·511$ mg%; $P<0·0005$). This suggests that there is an association of IgM with the severity and the progress of the disease.

Table 2 shows that TB antigen concentrations of CIC was higher $19·1\pm2·3$ ng/ml (mean ±s.e.) in sputum positive cases, and $9·9\pm1·9$ ng/ml in sputum negative cases as compared to $2·2\pm0·3$ ng/ml in controls. Patient groups were significantly ($P<0·001$) different from controls as well as from each other (Student's *t*-test).

The anti-TB antibody ratios in the complexes were $11·7\pm1·48$, $5·1\pm1·5$ and $0·6\pm0·1$ in sputum positive, sputum negative and controls. The significance between the groups was ($P<0·0001$). Circulating anti-tubercular antibody ratios were $145·5\pm62·2$, $72·5\pm34$ and $1·4\pm0·2$ for the three respective groups with a significant difference ($P<0·001$) between each group.

Table 3 is an analysis of the effect of anti-tubercular treatment on the various parameters discussed earlier. Patients were further subdivided into untreated cases, treated for less than 12 weeks and those treated for 12 weeks to 24 weeks.

Table 3 shows that when sputum remains positive for *M. tuberculosis* inspite of administrationn of anti-tubercular treatment for more than 12 weeks, there is no significant change in the levels of antigen or antibody of CIC.

In the group where sputum was negative for culture of *M. tuberculosis* the antigen levels of CIC were as high as $14·8\pm5·8$ ng/ml in untreated cases at the beginning of the study. However, as

**Table 2.** Anti-tubercular antibody and TB antigen levels of dissociated IC

| Disease state | TB antigen detected from CIC (ng/ml) (mean ± s.e.) | TB antibody detected from CIC. Ratio of titres control/sample (mean ± s.e.) | Circulating total TB antibody from serum. Ratio of titres control/sample (mean ± s.e.) |
|---|---|---|---|
| Pulmonary TB sputum +ve for *M. tuberculosis* X-ray lesions seen ($n=57$) | 19·1 ± 2·3 | 11·7 ± 1·48 | 145·5 ± 62·2 |
| Pulmonary TB sputum −ve for *M. tuberculosis* X-ray lesions seen ($n=37$) | 9·9 ± 1·9† | 5·1 ± 1·5† | 72·5 ± 34·7‡ |
| Controls ($n=40$) | 2·2 ± 0·3* | 0·6 ± 0·1* | 1·4 ± 0·2* |

* $P<0·001$ controls significantly different from sputum +ve and sputum −ve cases.
† $P<0·001$ sputum +ve and sputum −ve cases.
‡ $P<0·01$ sputum +ve and sputum −ve cases.

**Table 3.** Evaluation of anti-tubercular treatment on the antigen and antibody of dissociated IC

| | Untreated cases | | Anti-TB treated for less than 12 weeks | | Anti-TB treatment given for 12 weeks–2 years | |
|---|---|---|---|---|---|---|
| | CIC antigen (ng/ml) | CIC antibody (ratio titre control/sample) | CIC antigen (ng/ml) | CIC antibody (ratio titre control/sample) | CIC antigen (ng/ml) | CIC antibody (ratio of titre control/sample) |
| | mean ± s.e. | | | | | |
| TB sputum +ve for *M. tuberculosis* X-ray lesions seen | ($n=16$) 19·2 ± 5·4 | ($n=16$) 8·3 ± 4·1 | ($n=14$) 18·2 ± 2·1 | ($n=14$) 9·5 ± 5·3 | ($n=23$) 22·1 ± 6·0‡ | ($n=27$) 14·8 ± 5·7 |
| TB sputum −ve for *M. tuberculosis* X-ray lesions seen | ($n=8$)† 14·8 ± 5·8 | ($n=8$) 1·1 ± 0·4 | ($n=13$)§ 11·4 ± 4·2 | ($n=13$) 4·9 ± 2·6 | ($n=16$) 6·2 ± 1·7 | ($n=16$)¶ 7·85 ± 3·1 |
| Controls ($n=40$) | 2·2 ± 0·3* | 0·6 ± 0·1* | — | — | — | — |

* $P<0·001$—Controls different from sputum +ve and sputum −ve cases.
† $P<0·01$—difference between sputum −ve untreated and treated more than 12 weeks (CIC) antigen.
‡ $P<0·01$—difference between sputum +ve and sputum −ve cases treated for more than 12 weeks (CIC) antigen.
§ $P<0·05$—difference (CIC antibody) between sputum −ve cases untreated and treated for less than 12 weeks.
¶ $P<0·05$—difference (CIC antibody) between sputum −ve cases given treatment for $<12$ weeks and $>12$ weeks.

treatment progressed the TB antigen levels fell progressively from 14·8 ± 5·8 ng/ml to 11·4 ± 4·2 ng/ml after 12 weeks treatment and there was still further reduction to 6·2 ± 1·7 ng/ml after several more weeks of treatment. Comparison of the antigen levels after prolonged treatment in sputum positive and negative cases showed a significant difference ($P < 0·001$) between the levels of 22·1 ± 6 ng/ml and 6·2 + 1·7 ng/ml in the two groups. Anti-TB antibody of CIC in both sputum positive and negative groups rose steadily over the period of study.

Table 4 is an analysis to show the prevalence of antigen and/or antibody in the CIC of the two groups of patients. In sputum positive cases 56% had both TB antigen and TB antibody present in CIC, 21% only detectable antigen and 19% only antibody while in 4% neither antigen nor antibody could be demonstrable in the CIC. In sputum negative cases 36% showed both antigen and antibody in the CIC while 6% showed only antigen, 25% only antibody and 17% were negative for both.

**Table 4.** Percentage positivity of TB antigen and antibody in dissociated IC

| Diseases state | Both TB antigen + antibody detected in CIC (% of cases) | Only TB antigen detected in CIC (%) | Only TB antibody antibody detected in CIC (%) | Both TB antigen + TB antibody not detected (%) |
|---|---|---|---|---|
| Pulmonary TB sputum +ve for *M. tuberculosis* X-ray lesions seen. | 56 | 21 | 19 | 4 |
| Sputum −ve for *M. tuberculosis* X-ray lesions seen | 36 | 6 | 25 | 17 |

## DISCUSSION

In general, the relation between demonstration of CIC and related clinical manifestations is uncertain. In disorders like lupus erythematosus, it is reported that CIC may disappear in remission, and monitoring CIC concentrations permits evaluation of effectiveness of treatment (Theofilopolous, 1980). In bacterial endocarditis, (Kauffman, Thompson & Valantijim, 1981), an excellent correlation has been established between CIC levels, duration of disease, extravalvular manifestations and therapeutic response. High, persistent levels of CIC are an indication of failure to respond to antibiotics. Similarly in certain malignancies (Barnett, Khutson & Abrass, 1979) CIC concentrations may have prognostic significance and may be used to judge the efficacy of surgery and/or radiological treatment. At times increased levels may herald onset of metastasis.

The development and applications of rapidly accumulating information on CIC is confined to a large extent to autoimmune diseases and malignancies. Very little information is available as to what happens to CIC in mycobacterial diseases. Using methods based on complement-mediated and receptor-mediated binding techniques (Geniteau *et al.*, 1981) it has been shown that while complement-mediated complexes were more marked in lepromatous and reactional leprosy, receptor-mediated complexes were predominant in tuberculoid leprosy. Other studies (Sehgal & Kumar, 1981) using platelet aggregation tests indicated that CIC were present in a large percentage of cases of leprosy but there was no diagnostic or prognostic significance in relation to the disease. In patients suffering from *M. tuberculosis* infections (Carr *et al.*, 1980) it was shown that in 68% of cases with active disease, CIC levels measured by CIq binding activity were elevated and there was a fall in the CIq binding activity with treatment. However, 15% of bacteriologically cured, 22% of non-tubercular patients with chronic obstructive lung disease and 3% of normals also had high

levels of CIC. Elevated levels of CIC in non-TB patients suggests that CIC may be antigen–antibody complexes of other than bacterial origin, probably due to the presence of anti-lung antibodies and others.

PEG (3·5%) precipitation and determination of optical density (OD) at 280 nm indicates the presence of smaller, more soluble complexes. These soluble complexes circulate freely and are believed to be capable of being precipitated *in situ*, start a series of inflammatory reactions by activating the complement series leading finally to be destruction of the target tissues. Elevated levels of these soluble complexes were obtained in patients suffering from tuberculosis as compared to controls.

RIA using BS demonstrates the ability of CIC to interact with Fc receptors present on BS. High levels of CIC were detected by BS RIA (Table 1). The possibility of these Fc receptor binding CIC, suggest that they may adhere to the Fc receptors of various cells like macrophages, platelets and others. Once they are deposited, CIC are known to bring about pathophysiological changes in the affected tissue, releasing altered autologous antigens and finally contributing to the necrosis of the target tissue. A high incidence of anti-nuclear antibodies was reported in TB by Lindquist, Coleman & Osterland (1970).

All the three types of immunoglobulins i.e. IgG, IgA and IgM were elvated in TB patients suggesting the participation of three different type of immunoglobulins in the formation of CIC. Amongst these IgM in CIC of TB patients was significantly increased and was undetectable in controls. Hence the presence of IgM was a good marker of the presence of infection.

Mere detection of CIC does not give any information of the origin of the specific type of antigen and antibody involved. It would be of interest to identify the antigenic components and its corresponding antibody. Anti-tubercular antibody and tubercular antigen components specific to *M. tuberculosis* and *M. bovis* with little or negligible cross-reactivity with other mycobacteria (Kadival *et al.*, 1982) were demonstrated in CIC of patients and not in controls. Hence a major portion of CIC are composed of antigens and antibodies specific to the infective agent.

It was seen in our study that in patients suffering from pulmonary TB, the TB antigen and TB antibody levels in CIC were higher than controls. It was necessary to distinguish two groups of patients, one with extensive cavitary, open TB with excretion of bacilli in the sputum, and those with radiologically evident disease but where no bacilli could be demonstrated in the sputum. Patients whose sputum was positive for *M. tuberculosis* on culture had higher levels of antigen and antibody than patients who were sputum negative. The circulating anti-TB antibody levels were also higher in the patients suffering from TB. The higher antigen levels in sputum positive cases as compared to sputum negative cases is probably due to a difference in antigenic load in the two groups. It was presumed that as treatment becomes effective, there is bacterial clearance and hence detectable antigens should fall significantly. This presumption was further corroborated by the study of the effect of treatment on the antigen and antibody levels. In the group of patients who persisted in showing the presence of *M. tuberculosis* in sputum, inspite of drug treatment, the antigen and antibody levels of CIC remained elevated even after 12 or more weeks of treatment. The test could detect the presence of CIC antigen in patients with bacterial excretion in the sputum, in other words, there is a persistent anti-genaemia in this group of patients. The reason for this observation could be due to drug resistance or inadequate and irregular treatment. The sputum negative (also included patients initially sputum positive and those who became sputum negative with treatment) showed interesting and predictable pattern of changes. There was a gradual decrease in the concentrations of TB antigen and an elevation of TB antibody levels in CIC, as treatment progressed over a period of time, indicating a good therapeutic response. The response to treatment was also evident by clinical, radiological and haematological improvement.

Our studies also showed that in sputum positive cases only 4% of cases showed undetectable antigen or antibody in CIC suggesting a low false negative rate. The detectability of TB antigen and/or antibody in CIC would depend on the relative concentrations of antigen or antibody in circulation. During the phase of antigen excess with lower antibody levels, only antigen would be detectable. During the phase of antigen/antibody equivalence, both antigen and antibody would be demonstrable in the CIC, while during the phase of antibody escess, only antibody could be

detectable in the complexes. The variability of detection of antigen/antibody in the present study needs further evaluation.

These studies indicate that mere detection of CIC in the sera of patients is not of much diagnostic or prognostic value, hence isolation and identification of specific antigen and antibody is not only useful in the separation of diseased group from normal but is useful in evaluating therapeutic responsiveness and progress of disease.

There is much that needs to be still considered. Is the detection of CIC useful in other conditions of TB infection like renal, bone CNS involvement? What is the role of CIC and the tissue concentrations of these complexes and its significance in pathophysiology of disease? Are mycobacteria purely intracellular and if so, there should be no detectable antigen in circulation? In future, all these doubts and queries will be found to have suitable answers.

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