Specification and quantitation of circulating immune complexes in the serum of patients with active pulmonary sarcoidosis

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Abstract

Background – Circulating immune complexes can be elevated in serum samples of patients with sarcoidosis and are associated with disease activity, but their diagnostic significance is not understood.

Methods – The different classes of circulating immune complexes containing immunoglobulin A, G, or M, and the content of complement in circulating immune complexes (polyethylene glycol precipitation) as well as levels of complement binding circulating immune complexes (complement binding assay) were determined in 19 patients with active, untreated pulmonary sarcoidosis. The results were compared with other parameters in the serum (soluble interleukin 2 receptor, angiotensin converting enzyme, immunoglobulin A, G, and M) and the bronchoalveolar lavage fluid (lymphocytes, helper cells, suppressor cells, activated T cells), and with radiological stage and functional parameters (FEV₁, vital capacity, total lung capacity, transfer coefficient (KCO), and the alveolar–arterial oxygen difference during exercise).

Results – In all patients circulating immune complexes could be detected by polyethylene glycol precipitation and were similar to control subjects. The content of C1q in circulating immune complexes was higher than in controls, yet in all but one of the cases was still within normal limits. In contrast, elevated levels of complement binding circulating immune complexes were found in 67% of the patients. No correlation was seen between circulating immune complexes and any of the other parameters in the serum, bronchoalveolar lavage fluid, or lung function values. No differences were found between radiological type I and II presentations of sarcoidosis.

Conclusions – The complement binding assay showed a much higher sensitivity for the detection of circulating immune complexes in active pulmonary sarcoidosis than the polyethylene glycol precipitation method. As there was no correlation between levels of circulating immune complexes and other parameters of the disease they are probably not useful for the assessment of disease activity.

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Sarcoidosis is a chronic multisystem disorder of unknown aetiology, characterised by various immunological processes. Although in the past several authors have reported elevated circulating immune complexes in serum samples of patients with sarcoidosis,^{1–4} the contribution of circulating immune complexes to the assessment of the disease activity is still under discussion. The uncertainty remains because of the use of various techniques for detection of circulating immune complexes, the association of the finding of circulating immune complexes with diverse parameters of the disease, and the unknown role of immune complexes in the pathogenesis of sarcoidosis.

The purpose of the present study was to evaluate the phenomenon in patients with clinically active, untreated disease combining two different quantitative methods with regard to the composition and biological functions of circulating immune complexes, thus following a WHO recommendation on their detection.⁵ The results were compared with a number of other parameters of the disease in serum and bronchoalveolar lavage fluid, with the radiographic appearances of the lung, and with lung function values.

Methods

PATIENTS

The study population consisted of five women and 14 men with a median age of 36 (range 25–72) years. Seventeen were admitted to hospital for the first time after they had developed clinical symptoms including cough, dyspnoea on exertion, or an acute illness with erythema nodosum. Two untreated patients with previously diagnosed sarcoidosis had radiological evidence of progressive disease. The diagnosis in all patients was based on the histopathological finding of epithelioid cell granulomata and characteristic chest radiographic appearance. Eight of the patients had type I disease, 10 had type II, and one type III. For the evaluation of serum circulating immune complexes 10 healthy volunteers (seven men and three women with a median age of 39.5 (range 18–65) years) served as controls.

LUNG FUNCTION

Vital capacity (VC), total lung capacity (TLC), and forced expiratory volume in one second (FEV₁) were measured by whole body plethysmography. The single breathholding method

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was used to obtain the carbon monoxide transfer factor (TLCO). Reference values of the European Community for Coal and Steel (1983) were used. Progressive incremental work rate testing was performed using a cycle ergometer. During exercise the mixed expired concentrations of oxygen and carbon dioxide, the ECG, heart rate, expiratory flow, minute ventilation, and tidal volume were continuously monitored. Arterial blood gas tensions and alveolar-arterial oxygen difference ($A-aDO_2$) were measured at rest and at several exercise levels. The $A-aDO_2$ was considered abnormal if it was above 4.7 kPa at the last step of exercise where steady state conditions were still present.

BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage fluid was obtained during fiberoptic bronchoscopy. A total of 150 ml sterile saline was instilled into a segment or subsegment of the middle lobe and recovered by gentle suction. After filtering through gauze and centrifugation the cells were washed with phosphate buffer saline (pH 7.4) and incubated with monoclonal antibodies labelled with phycoerythrin or fluorescein isothiocyanate. The following antibodies (Becton Dickinson, Heidelberg, Germany) were used: CD3 (T cells), CD4 (helper cells), CD8 (suppressor/cytotoxic cells), HLA-DR/CD3 (activated T cells). The samples were analysed with a FACScan flow cytometer (Becton Dickinson). The size of lymphocyte fraction was calculated as a percentage of total cells and the size of lymphocyte subsets as a percentage of total lymphocytes.

CIRCULATING IMMUNE COMPLEXES

Polyethylene glycol precipitation (Fresenius, Oberursel, Germany)

Serum immune complexes were precipitated at 2–8°C in the presence of polyethylene glycol (molecular weight 6000). After washing the precipitates were centrifuged and finally redissolved in a buffer. The concentration of IgG, IgA, IgM, and complement factors C3c and C1q in the precipitates was determined by radial immunodiffusion using canine antisera. The evaluation after 48 hours was facilitated using Coomassie blue for staining of precipitate rings. The accuracy of the test was examined by simultaneous evaluation of human control serum. To obtain the reference range 500 random samples from European blood donors were tested for circulating immune

complexes using this test. The upper limits of the resulting tolerance limits (two standard deviations) are listed in table 1.

Complement binding assay (Medac, Hamburg, Germany)

Monoclonal antibodies against C1q or C3d complement components are bound to the solid phase of microtitre tubes. Circulating immune complexes in the serum sample react with the solid phase anti-C1q (anti-C3d). After washing peroxidase-linked mouse anti-human IgG was added which reacts with the Fc region of human IgG in circulating immune complexes. After incubation and repeated washing the enzyme substrate (O-phenylenediamine and H_2O_2) was added which is oxidised in the presence of bound enzyme conjugate. The reaction was read in a spectrophotometer at 490 nm optical density after fixation with 4N H_2SO_4 . The WHO standard for circulating immune complex assays was used to calibrate this system. Sera from 488 healthy persons were tested to define the reference ranges; 3% of the examined persons had circulating immune complex values above the upper limits shown in table 1.

OTHER SERUM PARAMETERS

The concentration of serum IgG, IgA, and IgM was determined by laser nephelometry (Behringwerke, Marburg, Germany). The ELISA technique was used to determine serum levels of soluble interleukin 2 receptor (Immunotech, Marseille, France) and kinetic method was used to determine serum levels of angiotensin converting enzyme (Hermann Biermann, Bad Nauheim, Germany).

STATISTICAL ANALYSIS

The Mann-Whitney U test was used to compare group means. Correlations were made using the Pearson's correlation coefficient.

Results

The levels of circulating immune complexes in patients and controls are shown in table 2. In two patients circulating immune complexes could be assessed by only one of the two methods. Circulating immune complexes could be detected by polyethylene glycol precipitation in all patients, and the values were similar to the healthy controls and exceeded the upper limit of normal in only two cases for IgM-containing circulating immune complexes. The content of C1q in circulating immune complexes was significantly higher than in controls ($p < 0.05$), but remained within normal limits in all but one case. The content of C3c in circulating immune complexes was similar to the control subjects. In contrast, elevated levels of complement binding circulating immune complexes were found in 67% of the patients for both components using complement binding assay ($p < 0.01$ v controls).

Results of bronchoalveolar lavage and other

Table 1 Sensitivity threshold and upper normal limits ($\mu\text{g/ml}$) of complement binding circulating immune complexes (CIC) and circulating immune complexes containing IgG, IgA, and IgM antibodies and complement components C1q and C3c.

	Method	Sensitivity	Upper normal limit
IgA-containing CIC	PEGP	2	25
IgG-containing CIC	PEGP	4	110
IgM-containing CIC	PEGP	3	84
C1q-containing CIC	PEGP	2	88
C3c-containing CIC	PEGP	1	29
C1q-binding CIC	CBA	14	34
C3d-binding CIC	CBA	4	8

PEGP = polyethylene glycol precipitation; CBA = complement binding assay.

Table 2 Results of quantitative assessment ($\mu\text{g/ml}$) of circulating immune complexes (CIC) containing IgA, IgM, IgG, C1q, or C3c ($n=18$) and complement (C1q and C3d component) binding circulating immune complexes ($n=18$) in sera of patients with sarcoidosis and controls ($n=10$)

	Method	Median	Minimum	Maximum
Sarcoidosis:				
IgA-containing CIC	PEGP	4	<2	9
IgG-containing CIC	PEGP	<4	<4	94
IgM-containing CIC	PEGP	30	15	193
C1q-containing CIC	PEGP	28	<2	126
C3c-containing CIC	PEGP	2	<1	18
C1q-binding CIC	CBA	41	<14	67
C3d-binding CIC	CBA	13.5	<4	20
Controls:				
IgA-containing CIC	PEGP	3	<2	7
IgG-containing CIC	PEGP	<4	<4	69
IgM-containing CIC	PEGP	15	<3	67
C1q-containing CIC	PEGP	<2	<2	126
C3c-containing CIC	PEGP	<1	<1	9
C1q-binding CIC	CBA	<14	<14	29
C3d-binding CIC	CBA	<4	<4	4

PEGP = polyethylene glycol precipitation; CBA = complement binding assay

serum parameters are shown in tables 3 and 4. The mean vital capacity was 103% (range 75–126%) and the total lung capacity was 91% (64–128%) of the predicted value. The mean FEV₁ was 81% (65–93%) of vital capacity. The mean TLCO was 84.5% (52–123%) of the predicted value, and corrected for the alveolar volume (Kco) the mean value was 78% (49–108%). The mean A-aDo₂ during exercise was 4.4 kPa (1.9–9.0 kPa).

No correlation was found between different fractions of circulating immune complexes and any of the other parameters in serum, bronchoalveolar lavage fluid, or lung function tests. No significant differences in the levels of circulating immune complexes were found between types I ($n=8$) and II ($n=10$) as defined by radiology.

Discussion

The formation of immune complexes is part of the normal response to infectious agents or

foreign substances. Whereas in most infectious or neoplastic diseases they can occur as a concomitant phenomenon without pathophysiological significance, in some chronic inflammatory diseases they may have a more important role. Immune complexes only induce immune complex disease when they are deposited in vessel walls and generate inflammation – for example, systemic lupus erythematosus, serum sickness, glomerulonephritis, or rheumatoid arthritis – and are usually eliminated by the mononuclear phagocytic system.⁸

The pathogenetic relevance of immune complexes depends on their size and composition which determines how fast they can be eliminated from the circulation and whether they are able to activate the complement cascade and cellular immune system. IgG-containing immune complexes bind to Fc receptors on phagocytic cells, and immune complexes containing IgG or IgM can activate complement thus inducing chemotaxis.⁸ It therefore follows that methods of assessing circulating immune complexes should consider their physicochemical properties and biological functions. No fewer than 18 techniques were evaluated in a WHO study which recommended that one should always use two methods as different assays quite obviously measure different fractions of immune complexes and can vary considerably in their sensitivity.⁵

Several authors have tried to assess the clinical significance of elevated levels of circulating immune complexes in sarcoidosis in comparison with other parameters of the disease. Biglino *et al* used the conglutinin binding assay and found elevated levels of circulating immune complexes in 19 of 22 cases with a positive gallium-67 lung scan compared with six of 15 patients with a negative scan.³ Rooney *et al* demonstrated elevated levels of circulating immune complexes by the Raji cell assay in 11 of 14 patients with sarcoidosis who had both pulmonary and non-pulmonary features and a recent history of deterioration, some of whom were receiving treatment with systemic steroids. In contrast, only one of 17 treated or untreated patients considered stable was found to have an elevated level of circulating immune complexes.¹ Kuehn *et al* used the non-quantitative method of immunofluorescence microscopy to detect circulating immune complexes after phagocytosis by granulocytes and found that they could be detected more frequently in patients with progressive disease.² Of more interest, Saint-Remy *et al* found that levels of circulating immune complexes obtained by conglutinin binding assay were higher in patients with disease of more recent onset than in those with chronic disease. However, this finding was not reproducible when polyethylene glycol precipitation was used in the same patient group.⁴ These studies suggest that elevated levels of circulating immune complexes are more frequently seen in active and, particularly, acute disease.

In our study we used polyethylene glycol precipitation to identify different fractions of circulating immune complexes containing IgG, IgM, and IgA, as well as complement

Table 3 Lymphocyte fraction (% of total cells) and lymphocyte subsets (% of total lymphocytes) in bronchoalveolar lavage fluid of patients with sarcoidosis

	n	Median	Range
Lymphocytes	19	37	12–89
CD3+	18	87.5	64–96
HLA-DR+/CD3+	17	57	29–85
CD4+	19	66	25–88
CD8+	19	12	6–40
CD4+/CD8+ ratio	19	5.4	0.6–14.7

Table 4 Serum parameters in patients with sarcoidosis ($n=19$)

	Median	Range	Upper normal limit	% cases with elevated values
ACE (U/ml)	50	24–82	29	95
sIL-2R (pmol/l)	143	34–365	115	58
IgA (mg/dl)	346	90–644	280	32
IgG (mg/dl)	1660	1250–2230	1600	58
IgM (mg/dl)	286	44–751	280	53

ACE = angiotensin converting enzyme; sIL-2R = soluble interleukin 2 receptor

factors of the classic (C1q) and alternative (C3c) pathway. There was a tendency to higher levels of IgG-, IgM-, and IgA- containing classes of circulating immune complexes in the patient group, but values exceeded upper limits of normal in only two cases for IgM-containing circulating immune complexes. Thus, not a single fraction of circulating immune complexes appeared to be responsible for the finding of elevated levels described in the previously mentioned studies. Using the much more sensitive complement binding assay abnormal levels of circulating immune complexes were found in two thirds of our patients for both complement factors C1q and C3d, indicating that the immune complexes are able to bind complement and hence could be pathogenic by inducing a complement cascade in vivo.

All our patients had clinical signs of active disease which was reflected in the frequent finding of elevated serum levels of angiotensin converting enzyme and soluble interleukin 2 receptor. However, correlation tests did not reveal a relation between the different fractions of circulating immune complexes and any of the other parameters in serum, and activation markers in bronchoalveolar lavage fluid such as CD4+/CD8+ ratio and HLA-DR expression of T lymphocytes⁹ did not correlate with levels of circulating immune complexes. Serum immunoglobulins IgG, IgM, and IgA were also frequently elevated, indicating B lymphocyte activation at sites of active disease where the immunoglobulins are secreted.¹⁰ Their production is polyclonal and obviously non-specific;¹¹ the same could very well be true for the formation of immune complexes.

In spite of the frequent finding of elevated

levels of circulating immune complexes in the serum of patients with sarcoidosis, we doubt that their evaluation can make a useful contribution to the clinical management of the disease. In contrast to other useful laboratory tests – particularly the varying sensitivity and precision of techniques used to measure circulating immune complexes – there remains an unsolved methodical problem which should always be considered before the interpretation of these test results in clinical management.

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