

Circulating and Intra-articular Immune Complexes in Patients with Rheumatoid Arthritis

CORRELATION OF ^{125}I -Clq BINDING ACTIVITY WITH CLINICAL AND BIOLOGICAL FEATURES OF THE DISEASE

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ABSTRACT The correlation between the incidence and level of immune complexes in serum and synovial fluid and the various clinical and biological manifestations of rheumatoid arthritis has been studied. Immune complexes were quantitated using a sensitive radioimmunoassay, the ^{125}I -Clq binding test, in unheated native sera and synovial fluids from 50 patients with seropositive (RA +) and 45 with seronegative (RA -) rheumatoid arthritis, 17 with other inflammatory arthritis, and 37 with degenerative and post-traumatic joint disease. The following observations were made: (a) when compared to the results from patients with degenerative and post-traumatic joint diseases, the ^{125}I -Clq binding activity (Clq-BA) in synovial fluid was found to be increased (by more than 2 SD) in most of the patients with RA + (80%) and RA - (71%) and in 29% of patients with other inflammatory arthritis; the serum Clq-BA was also frequently increased in both RA + (76%) and RA - (49%) patients, but only exceptionally in patients with other inflammatory arthritis (6%); (b) a significant negative correlation existed between the Clq-BA and the immunochemical C4 level in synovial fluids from patients with RA + and RA -; (c) neither the serum nor the synovial fluid Clq-BA in rheumatoid arthritis significantly correlated with the erythrocyte sedimentation rate, the clinical stage of the disease, or the IgM rheumatoid factor titer; and (d) the serum Clq-BA in patients with rheumatoid arthritis and extra-articular disease manifestations ($40 \pm 34\%$ in those with RA +, $32 \pm 29\%$ in those with RA -)

was significantly increased as compared to the serum Clq-BA in patients with joint disease alone ($24 \pm 30\%$ in those with RA +, $10 \pm 13\%$ in those with RA -). Experimental studies were carried out in order to characterize the Clq binding material in rheumatoid arthritis. This material had properties similar to immune complexes: it sedimented in a high molecular weight range on sucrose density gradients (10–30S) and lost the ability to bind Clq after reduction and alkylation, or after acid dissociation at pH 3.8, or after passage through an anti-IgG immunoabsorbant. DNase did not affect the Clq BA.

These results support the hypothesis that circulating as well as intra-articular immune complexes may play an important role in some pathogenetic aspects of rheumatoid arthritis. The ^{125}I -Clq binding test may also be of some practical clinical value in detecting patients who have a higher risk of developing vasculitis.

INTRODUCTION

The evidence for the involvement of immune complexes in the pathogenesis of rheumatoid arthritis (RA)¹ is based on the direct demonstration of such complexes in the synovium (1, 2) or in the synovial fluid (3–5), and on the observation of biological changes generally as-

¹Abbreviations used in this paper: aggHGG, aggregated human gammaglobulin; Clq-BA, ^{125}I -Clq binding activity; DJD, degenerative joint disease; IA, inflammatory arthritis; NHS, normal human serum; PEG, polyethylene glycol; RA, rheumatoid arthritis; RA+, seropositive RA; RA-, seronegative RA; RF, IgM rheumatoid factor; TR, post-traumatic joint effusions; VBS, barbital buffered saline.

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sociated with the formation of immune complexes such as an increased catabolism of complement components (6-9). Two major approaches were used in order to detect immune complexes in serum or in joint fluid. First, the existence of specific antigen-antibody systems was investigated and immunoglobulin-anti-immunoglobulin complexes were particularly demonstrated (4, 5, 10-15). Secondly, methods which are not specific for any particular antigen-antibody system were used. These studies were mostly qualitative and led to the demonstration of immune complex-like material such as cryoglobulins (16, 17), macromolecular complexes (3, 10, 11), Clq precipitins (4, 5), complement fixing material (4, 16), or of complexes reacting with monoclonal rheumatoid factor (5). Recently, quantitative investigations were carried out using radioimmunological techniques such as for binding of complexes by rheumatoid factor (18, 19) and macrophages (20). These studies have suggested a possible role of immune complexes in articular and extra-articular manifestations of RA.

The main purpose of this work was to define the correlation between the incidence and the level of immune complexes in serum and synovial fluid on one hand, and the various forms of clinical and biological manifestations of RA on the other. Immune complexes were quantitated in the sera and the synovial fluids from patients with various forms of arthritis, using a sensitive radioimmunoassay, the ^{125}I -Clq binding test, which has been previously applied to the study of patients with systemic lupus erythematosus (21) and which has been recently modified for the detection of complexes in unheated native serum or synovial fluid samples (22). These studies confirmed the presence of immune complexes in synovial fluid but also led to the demonstration of a high amount of immune complexes in serum. The Clq binding complexes have been further characterized physicochemically and immunochemically. These clinical investigations suggested that the use of such radioimmunoassays for immune complexes would be of practical value in the diagnosis and in follow up studies of RA.

METHODS

Selection of patients studied. The patients included in this investigation were followed at the Universitäts-Rheumaklinik, Kantonsspital, Zurich, Switzerland (Dr. Fehr), and at the Rheumatic Disease Unit, Northern General Hospital, Edinburgh, Scotland (Dr. McCormick). Patients were selected on the basis of availability of sufficient synovial fluid for arthrocentesis. 156 synovial fluids, 149 sera, and 115 plasma samples were obtained from 149 patients with rheumatoid and various nonrheumatoid joint diseases. Patients with RA satisfied the American Rheumatism Association's criteria for classical or definite RA (23). Clinical staging was performed according to Steinbrocker et al. (24). IgM rheumatoid factor was measured by the sensitized sheep cell agglutination technique (25). RA patients were considered

as seropositive when the rheumatoid factor titer was equal or higher than 1:64. Nonrheumatoid patients satisfied generally accepted diagnostic criteria. Synovial fluids of 15 patients who suffered from post-traumatic joint effusions (7 with meniscus lesions, 7 with luxations or closed fractures, 1 with hyarthrosis) were used as controls for the other studies.

Collection and storage of synovial fluid serum and plasma. Synovial fluids were collected mostly from the knee and only in some instances from the shoulder or elbow joints. The samples were immediately mixed with EDTA (20 mM, final concentration) then centrifuged at 1,500 *g* for 15 min at room temperature. The supernates were immediately frozen and stored at -70°C in portions of 0.5 ml. For the collection of serum, blood was allowed to clot at room temperature for 60 min, was then centrifuged at 1,500 *g* for 15 min and the serum was stored at -70°C . To obtain plasma blood was collected in plastic tubes containing EDTA (20 mM final concentration), centrifuged at 1,500 *g* for 15 min, and the supernate was immediately stored at -70°C .

^{125}I -Clq binding test. The basic principle of this test is outlined on Fig. 1. For the present investigation, the technique was performed as follows (22): Clq was isolated from fresh normal human serum (NHS) by the method of Volanakis and Stroud (26), but relative salt concentrations of 0.04 and 0.078 M NaCl, respectively, were attained for the first and second precipitation steps. Radioiodination of Clq with lactoperoxidase was performed according to Heusser et al. (27). The ^{125}I -Clq had a sp act of 1 $\mu\text{Ci}/\mu\text{g}$ protein, and was stored in portions of 10 μg in 100 μl of barbital buffered saline (containing Ca and Mg) (VBS) (28). On the day of testing, a portion of isolated labeled Clq was diluted in 3-5 ml VBS containing 1% (wt/vol) bovine serum albumin (A grade, Calbiochem, San Diego, Calif.) and centrifuged at 18,000 *g* for 40 min at 4°C in order to remove aggregated Clq. The supernate was used for the test. The test was carried out in duplicate, using Bio-Vial polypropylene tubes (Beckman Instruments, Inc., Palo Alto, Calif.). For testing of patients' sera, 50 μl of tested serum was mixed with 100 μl of Na_2EDTA , 0.2 M, (adjusted to pH 7.5 with NaOH) and incubated for 30 min at 37°C . Then, the mixture was transferred into an ice bath. 50 μl of the ^{125}I -Clq and 1 ml of 3% (wt/vol) PEG (polyethylene glycol) (DAB-7, mol wt 6,000, Siegfried, Zofingen, Switzerland) solution were added. PEG was dissolved in 0.1 M boric acid, 0.025 M di-sodium-tetraborate, 0.075 M NaCl, pH 8.3. After the addition of PEG, the mixture was left on ice for 60 min and centrifuged at 1,500 *g* for 20 min at 4°C . The supernate was completely discarded and the radioactivity was measured on the precipitate. Results were expressed as percent ^{125}I -Clq precipitated as compared with the radioactivity precipitated in a "trichloroacetic acid control" tube in which 1 ml 20% trichloroacetic acid was added to 100 μl of NHS mixed with 50 μl of ^{125}I -Clq.

Synovial fluids were tested as follows: 50 μl of freshly thawed synovial fluids were mixed with 100 μl of EDTA buffer containing 20% (wt/vol) NHS heated for 30 min at 56°C (NHS 56°C), incubated for 30 min, and then tested as for serum. Sucrose gradient or immunoabsorbant column fractions to be tested were also mixed with NHS 56°C in order to maintain a suitable protein concentration. 100 μl of these EDTA containing fractions were mixed with 50 μl NHS 56°C and incubated and tested as for serum. Sucrose (DAB-7, Merck A.G., Inc., Darmstadt, W. Germany) alone up to concentrations of 50% (wt/vol) in EDTA buffer did not influence the results of the test.

In some experiments, the test was performed on synovial

C1q BINDING TEST

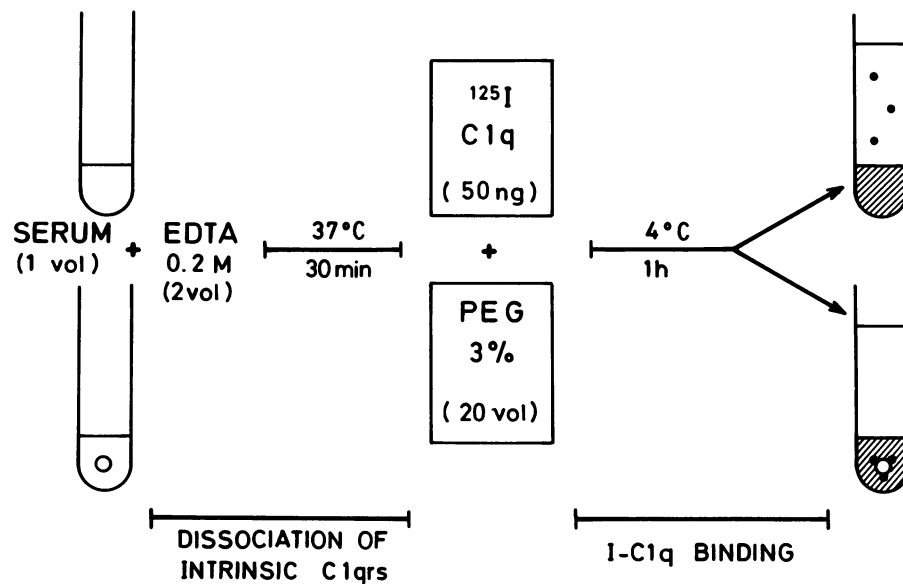


FIGURE 1 Basic principle of the ^{125}I -C1q binding test: in a first step, the tested native serum sample is incubated for 30 min at 37°C with EDTA in order to prevent the integration of ^{125}I -C1q into the intrinsic macromolecular C1qrs complex. In a second step, ^{125}I -C1q and PEG are added to this mixture which is further incubated at 4°C for 1 h. Under these conditions, free C1q (●) remains soluble while C1q bound to macromolecular complexes (○) is precipitated. The radioactivity of the precipitates is then measured.

fluids treated with 2-mercaptoethanol or with DNase; 100 μl of the mixtures were incubated with 50 μl EDTA buffer and tested as for serum.

For each test series NHS was used for negative controls and various amounts of heat aggregated human gamma-globulins (agg HGG) in NHS 56°C were used for positive controls. The agg HGG were obtained by heating human Cohn fraction II (Globuman, Berna, Berne, Switzerland) at 63°C for 20 min at a concentration of 6 mg/ml in 0.9% NaCl. This preparation was centrifuged for 15 min at 1,500 *g* and stored at -70°C. Reproducibility studies of the C1q binding test have indicated that, within duplicates (50 samples), the variation coefficient was 3.4%. When agg HGG positive controls were tested on 10 separate days, the variation coefficient was 6.2%. As shown in previous investigations (22), the minimal amount of agg HGG detected in this C1q binding test is 10 μg agg HGG/ml NHS 56°C or 30–50 μg agg HGG/ml native serum.

Correction of the results for nonspecific ^{125}I -C1q precipitation was performed by using a modified Farr's formula (29). The mean values for ^{125}I -C1q precipitation observed in presence of synovial fluids from 15 patients with post-traumatic joint effusions (7.1 ± 1.8 [mean ± 1 SD]) and of sera from 30 healthy blood donors ($5.8 \pm 1.5\%$) were used for the calculation of the correction factors for synovial fluid and serum respectively. The C1q-binding activity (C1q-BA) of tested samples represents the corrected percentage of ^{125}I -C1q precipitated in these samples.

Enzymatic and chemical treatment of synovial fluids. For DNase treatment, 0.2 ml of synovial fluid without EDTA was mixed with 0.2 ml VBS containing Ca^{++} and Mg^{++} and 50 μl DNase (Deoxyribonuclease I, Worthington Biochemi-

cal Corp., Freehold, N. J.) (0.5 mg/ml) in VBS and incubated at 37°C for 3 h. Synovial fluid controls were incubated with VBS alone. For reduction and alkylation, 0.2 ml of synovial fluid was incubated with 0.2 ml of 0.4 M 2-mercaptoethanol at room temperature for 60 min and thereafter with an additional 0.2 ml of 0.66 M iodoacetamide at 4°C for 60 min. Control synovial fluids were incubated with 0.4 ml VBS. For control of the reduction and alkylation procedure, 0.2 ml NHS containing agg HGG (1 mg/ml) was treated like synovial fluid. The mixtures were dialysed for 24 h against VBS, before testing the C1q-BA.

Density gradient studies. Ultracentrifugations were performed with a SW65-Ti rotor in a Spinco L2-65B preparative ultracentrifuge (Beckman Instruments, Inc.). Linear 10–40% (wt/vol) sucrose gradients were performed with a gradient mixer simultaneously in three tubes, in either borate-NaCl buffer 0.1 M, pH 8.3 containing EDTA 0.1 M or acetate buffer 0.1 M, pH 3.8. Acid gradient fractions were neutralized by addition of 0.5 M Tris-NaOH, pH 12, and left on ice for 1 h before testing. 0.5 ml of synovial fluid, serum, or PEG fraction obtained from synovial fluid were layered, undiluted or diluted $\frac{1}{10}$ in 0.9% NaCl, on 4.5-ml gradients and centrifuged at 34,000 rpm for 18 h at 5°C. In some experiments, 1 ml of a gradient fraction, diluted one fourth in buffer, was layered on a 4-ml gradient and a second run was performed at 60,000 rpm for 5 h at 5°C. Serial fractions were collected and OD patterns were obtained with a 0.3-mm flow cell (Uvicord, LKB-Produkter, Bromma, Sweden) and a logarithmic recorder (W + W electronic A.G., Basel, Switzerland). The 19 S peak of synovial fluid or serum was used as one reference marker. ^{125}I -labeled IgG was centrifuged in control tubes and used as

7S reference marker. The apparent S values were calculated according to the method of Martin and Ames (30). In some experiments, a PEG-precipitation was done in order to concentrate the Clq binding material before the ultracentrifugations: 1 vol of synovial fluid was mixed with 4 vol of a 5% (wt/vol) PEG solution in borate-EDTA buffer, pH 8.3. The mixture was kept at 4°C for 1 h, centrifuged at 2,000 *g* for 30 min, the precipitate was homogenized and resuspended with borate-EDTA buffer in one fifth of the original volume of synovial fluid.

Immunochemical techniques. Immunoabsorption: rabbit anti-human IgG (21) or bovine serum albumin were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) according to the manufacturer's recommendation. Two identical 3 ml-(0.5 cm diameter) columns were prepared. The columns were washed with normal rabbit serum to decrease the nonspecific binding and were then equilibrated with borate-EDTA buffer, pH 8.3 at 4°C. Thereafter, 0.4 ml of Clq binding gradient fraction was applied to each column and the flow was stopped for 30 min. Serial fractions of 0.2 ml were eluted and tested for ¹²⁵I-Clq BA.

Immunoelectrophoresis: gradient fractions were tested by the method of Scheidegger (31) using commercial rabbit antiwhole human serum and rabbit antihuman IgG (γ chain) serum (Behringwerke AG., Marburg-Lahn, W. Germany).

Complement studies: these were carried out on either EDTA-synovial fluid or EDTA-plasma. Hemolytic activity was quantitated in a continuous flow system (32), Clq, C4 and C3 were quantitated by single radial immunodiffusion as described earlier (33). All values were expressed as a percentage of the values obtained with a pool of 30 blood donors, which was stored in liquid nitrogen. These values were corrected for a protein concentration of 70 mg/ml. Protein concentration was assayed by the method of Lowry et al. (34).

Statistical evaluation. Statistical evaluation was carried out according to Student's *t* test and by linear regression analysis by the method of the least squares.

RESULTS

Clq-BA in synovial fluid and serum samples. The Clq-BA of synovial fluids from 149 patients with various joint diseases were studied. The patients were classified according to the clinical diagnoses: seropositive rheumatoid arthritis (RA +), seronegative rheumatoid arthritis (RA -), various other inflammatory arthritis (IA), degenerative joint disease (DJD) and post-trau-

matic joint effusion (TR). The synovial fluids of 15 patients with TR were used for the estimation of the nonspecific ¹²⁵I-Clq precipitation. The mean values for specific Clq-BA in synovial fluids of the remaining four patient groups are listed in Table I. The following observations were made: (a) the mean Clq-BA was significantly increased in RA + and RA - as compared to DJD (*P* < 0.0005) and to IA (*P* < 0.0005); (b) there was no significant difference in synovial fluid Clq-BA between RA + and RA - (*P* < 0.1); and (c) the mean Clq-BA in IA was significantly increased when compared to DJD (*P* < 0.025). The values for Clq-BA in synovial fluids of individual patients are shown on Fig. 2. When compared to DJD the synovial fluid Clq-BA was increased by more than 2 SD in 80% of patients with RA +, 71% with RA -, 29% with IA, and 0% with DJD. With respect to the IA group, it should be mentioned that the values were increased by more than 2 SD in three out of seven patients with infectious arthritis, two out of seven patients with gout, in a single patient with chondrocalcinosis, and in one of two patients with Reiter's disease. Synovial fluids from seven patients with RA + or RA - were obtained on the same day from both knee joints. The mean Clq-BA was the same for the right side joints (49±28%) and the left side joints (49±25%), and the mean individual difference between the right and the left knee joints was 6±4% (mean±1 SD).

The sera from the patients who underwent synovial fluid analysis were also tested. 30 sera from healthy blood donors were used for the estimation of nonspecific ¹²⁵I-Clq precipitation in serum. The mean values for specific serum Clq-BA in the various patient groups are also listed in Table I. The following observations were made: (a) the mean Clq-BA in serum was significantly increased in RA + and in RA - when compared to DJD (*P* < 0.0005) and to IA (*P* < 0.0005); (b) the mean serum Clq-BA was significantly higher in RA + than in RA - (*P* < 0.0005). One should note that such a difference was not observed in synovial fluid; and (c) the mean serum Clq-BA was not increased in the IA

TABLE I
¹²⁵I-Clq-BA in Synovial Fluids and Sera from Patients with Various Joint Diseases

Diagnosis	No. of patients	Sex		Age	Clq-BA	
		M	F		Synovial fluid	Serum
				yr	%	%
Seropositive RA	50	20	30	55±15*	40±22*	33±29*
Seronegative RA	45	17	28	51±18	33±25	14±18
Other IA†	17	14	3	43±14	11±14	0.5±1
DJD	22	10	12	58±20	3±5	2±4

* Mean±1 SD.

† Infectious arthritis (7), Reiter's disease (2), gout (7), chondrocalcinosis (1).

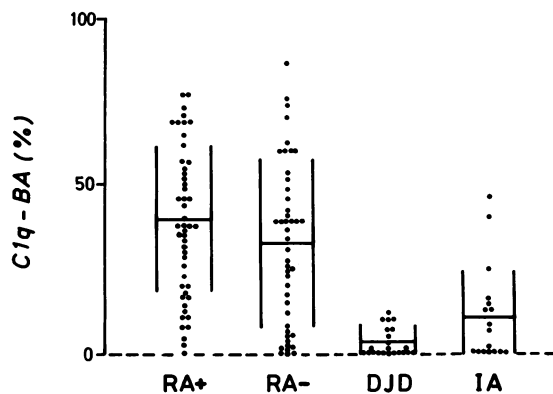


FIGURE 2 ^{125}I -Clq-BA in synovial fluid samples from patients with RA+ and RA-, DJD, and various other (see text) IA. The means (± 1 SD) of the values from each patient group are indicated.

group as compared to DJD. It is important to note therefore that only the mean Clq-BA in synovial fluid was found to be increased in IA. The values for Clq-BA in sera from the individual patients are shown on Fig. 3. When compared to DJD, the serum Clq-BA was increased by more than 2 SD in 76% of patients with RA +, 49% with RA -, 6% with IA, and 2% with DJD. With respect to IA, it should be mentioned that the serum Clq-BA was only increased in the one patient with Reiter's disease who also had an increased synovial fluid Clq-BA.

A significant correlation was found by regression analysis between the synovial fluid Clq-BA and the serum Clq-BA of patients with either RA + or RA -, and this is shown on Fig. 4.

Correlation between Clq-BA and clinical and laboratory features in RA. The Clq-BA in synovial fluids and sera from patients with RA was compared to various

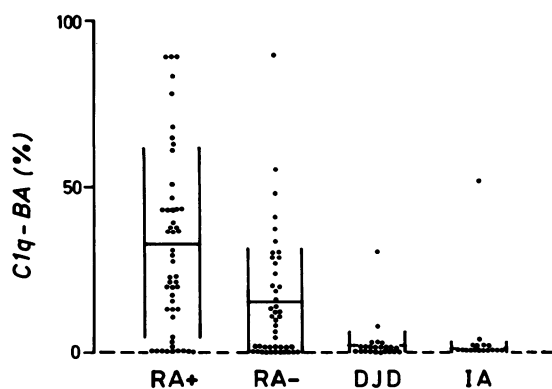


FIGURE 3 ^{125}I -Clq-BA in serum samples from patients with RA+, RA-, DJD, and various other (see text) IA. The means (± 1 SD) of the values from each patient group are indicated.

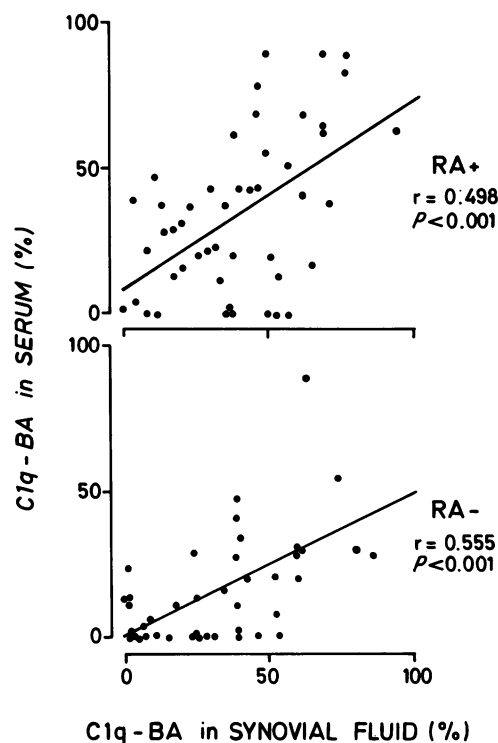


FIGURE 4 Correlation between the ^{125}I -Clq-BA in serum samples and the Clq-BA in synovial fluid samples from patients with RA+ and RA-.

clinical and laboratory parameters of RA. By linear regression analysis it appeared that the Clq-BA was not related to age, sex, or duration of the disease of individual patients nor to erythrocyte sedimentation rate, blood hemoglobin values and leukocyte counts in blood or synovial fluid. Furthermore, despite the significant difference in mean serum Clq-BA that was observed between RA + and RA -, no correlation was found between serum or synovial fluid Clq-BA and the IgM rheumatoid factor titer in individual patients with RA +. The Clq-BA could not be related to disease stages of RA + or RA -. Most of the patients had stage II or III disease. The Clq-BA in sera from three patients in stage I of RA + was $21 \pm 20\%$ (mean ± 1 SD) as compared to the serum Clq-BA of $35 \pm 33\%$ from patients in stage II to IV, and the serum Clq-BA from eight patients in stage I of RA - was $5 \pm 12\%$ as compared to the serum Clq-BA of $16 \pm 21\%$ from patients in stage II to IV. The patients were further characterized with respect to the occurrence of extra-articular disease manifestations such as subcutaneous nodules or other organ involvement (Table II). It appeared that the mean Clq-BA in serum was significantly increased in either RA + ($P < 0.05$) or RA - ($P < 0.05$) patients with extra-articular disease manifestations as compared to patients suffering

TABLE II
Correlation between Serum and Synovial Fluid ¹²⁵I-Clq-BA and the Occurrence of Extra-articular Disease Manifestations and other Clinical Features in Patients with RA

Diagnosis	No. of patients	Sex		Age	Disease duration	ESR*	Clq-BA	Serum
		M	F				Synovial fluid	
				yr	yr	mm/h	%	%
RA +: no extra-articular manifestations	24	13	11	54 ± 16†	9 ± 6†	50 ± 28†	37 ± 29†	24 ± 30†
with extra-articular manifestations	26	17	9	56 ± 18	14 ± 7	47 ± 18	43 ± 26	40 ± 34
— nodules only	21	14	7	57 ± 18	16 ± 6	42 ± 17	42 ± 25	40 ± 33
— other extra-articular manifestations§	5	3	2	52 ± 17	6 ± 3	71 ± 10	46 ± 21	41 ± 28
RA —: no extra-articular manifestations	37	23	14	50 ± 19	11 ± 9	32 ± 24	33 ± 25	10 ± 13
with extra-articular manifestations	8	5	3	52 ± 15	8 ± 11	48 ± 29	44 ± 27	32 ± 29

* ESR, erythrocyte sedimentation rate.

† Mean ± 1 SD.

§ Erythema nodosum (1), polyneuritis (1), pulmonary fibrosis (1), endocarditis (1), lymphadenopathy (1).

|| Sjögren syndrome (2), Raynaud phenomenon (2), pleural effusion (1), episcleritis (1), lymphadenopathy (2).

from joint disease alone, whereas no significant difference was found with respect to synovial fluid Clq-BA. There was no significant difference in the mean serum Clq-BA between RA + patients with nodules and RA + patients with other organ involvement, whereas in RA — where extra-articular symptoms occurred less frequently, nodules were not observed. Moreover, it appeared that there was no significant difference ($P > 0.1$) in mean serum Clq-BA between those RA + and RA — patients who presented extra-articular disease manifestations, whereas RA + patients with joint disease alone had a higher mean serum Clq-BA ($P < 0.025$) than RA — patients with joint disease alone. When compared to DJD, the Clq-BA was found to be increased by more than 2 SD in synovial fluids of most of the patients with RA and nodules or other organ involvement (92% in RA +; 88% in RA —) but also frequently in patients with RA and joint disease alone (67% in RA +; 64% in RA —). The serum Clq-BA was increased by more than 2 SD in most of the patients with RA and nodules or other organ involvement (88% in RA +; 75% in RA —), and in 63% of patients with RA + and joint disease alone, but only in 38% of patients with RA — and joint disease alone.

Correlation between Clq-BA and complement levels. Complement studies were performed in order to allow for a correlation of the measured Clq-BA with complement level in the various patient groups. Total hemolytic complement activity, intrinsic Clq, C4 and C3 were measured in synovial fluids and plasma of 40 patients with RA +, 30 patients with RA —, 15 patients with IA, and 30 patients with DJD, or TR. The mean values found in synovial fluids were compared to the Clq-BA. It appeared that a high mean Clq-BA was associated with a significant decrease of the mean hemolytic complement activity ($P < 0.025$) and C4 ($P < 0.0005$) in RA + and with a significant decrease of C4 in RA — ($P < 0.0005$).

In contrast, a low mean Clq-BA was associated with normal (DJD and TR) or even increased (IA) complement levels. Linear regression analysis gave significant negative correlations for Clq-BA and C4 levels in synovial fluids of patients with both RA + ($P < 0.001$) and RA — ($P < 0.01$) (Fig. 5). No significant correlations were found for Clq-BA and intrinsic Clq, C3, and hemolytic complement activity, respectively. The hemolytic complement activity, intrinsic Clq, C4 and C3 were also measured in the plasma of the same patients who underwent synovial fluid complement analysis. With the exception of C4, the mean values of the different patient groups were within the normal range. However, when compared to the C4 level of the DJD and TR group ($100 \pm 23\%$; mean ± 1 SD), C4 was significantly decreased in the RA + group ($85 \pm 37\%$, $P < 0.005$), whereas it was not significantly decreased in the RA — group ($95 \pm 34\%$, $P > 0.1$) and was slightly increased in the IA group ($107 \pm 30\%$). Therefore, in serum and plasma, respectively, a high mean Clq-BA was associated with a decreased mean C4 level in the RA + group, but by linear regression analysis no significant correlation was found.

Characterization of the Clq binding material in RA. A series of experiments was performed in order to further characterize the Clq binding material in RA. For these studies, synovial fluids and sera from patients with RA were selected for their high Clq-BA and subjected to various treatments. It has been shown by others that synovial fluid from patients with RA may contain free DNA and anti-DNA antibodies (16, 35). It was therefore relevant to test if DNA was involved in the Clq-BA of synovial fluids from RA patients. For this purpose, synovial fluids from 8 patients with RA + and 4 with RA — were incubated with either DNase or buffer for controls. This treatment with DNase did not produce any significant reduction in Clq-BA of the synovial

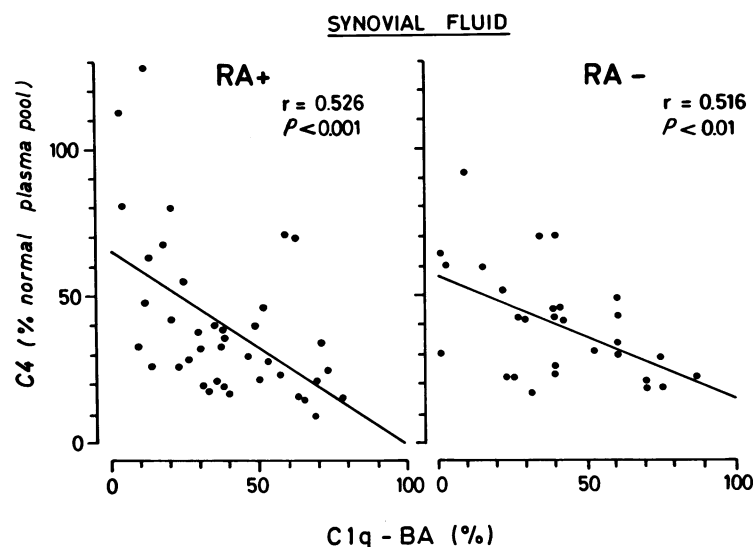


FIGURE 5 Correlation between C4 levels and ^{125}I -Clq-BA in synovial fluid samples from patients with RA+ and RA-.

fluids tested (Table III). The 12 synovial fluids with elevated Clq-BA were also subjected to reduction with 0.2 M 2-mercaptoethanol and alkylation with iodoacetamide. This treatment completely abolished the Clq-BA of the synovial fluids (Table III). The same treatment led to a decrease in Clq-BA of agg HGG in NHS 56°C (0.5 mg/ml) from 50 to 17%.

In order to investigate the size of Clq binding material, synovial fluids and sera from 20 patients with RA+ and RA-, exhibiting a high Clq-BA, were subjected to ultracentrifugation analysis, using sucrose density gradients (10–40% wt/vol). For comparison, the respective materials from 10 patients with DJD and from 4 blood donors (NHS) were also analyzed. After ultracentrifugation, the Clq-BA was measured in each of seven serial gradient fractions. In experiments using synovial fluids from RA patients, Clq-BA was always encountered in gradient fractions one to four from the

bottom of the tubes containing about 10–34S material. The highest Clq-BA was most often found in fractions three and four (10–22S), independent of whether the synovial fluids came from patients with RA+ or RA-. However, different sedimentation patterns of Clq-BA could be observed and a selection of representative results is shown on Fig. 6. Out of 20 samples, soluble Clq-BA was found in all but one case. In this case, the whole Clq-BA was recovered in the cryoprecipitate which was obtained when the synovial fluid was left for 24 h at 4°C and centrifuged at 1,500 *g* for 20 min. In contrast to the experiments with RA synovial fluids, the maximal Clq-BA in any gradient fraction obtained with synovial fluids from patients with DJD was only 2.7%.

After ultracentrifugation of the serum samples on sucrose density gradients, the maximal Clq-BA obtained from either RA+ or RA- sera occurred in 10–22S fractions. The maximal Clq-BA obtained from DJD sera or NHS in any of the gradient fractions was only 2%. Representative results obtained from three sera (1 RA+, 1 RA-, 1 NHS) are shown on Fig. 7. In a further ultracentrifugation experiment, 2 RA+ and 2 RA- synovial fluids were centrifuged in parallel runs on sucrose gradients either at pH 3.8 or at pH 8.3. After the run, the acid fractions were neutralized and the Clq binding test was performed on the fractions from both gradients. Whereas soluble Clq-BA was found in fractions one to four of the normal gradient, no Clq-BA could be detected in any fraction from the acid gradient.

Clq binding material in synovial fluids and sera from patients with RA+, or RA- was partially purified and concentrated using PEG, and then fractionated on su-

TABLE III
Effect of Pretreatment with DNase or 2-Mercaptoethanol on ^{125}I -Clq-BA in Synovial Fluids from Patients with RA

Pretreatment	Clq-BA*	Δ †
DNase	48 ± 24	
Controls‡	51 ± 23	1 ± 3
2-mercaptoethanol (0.2 M)	0 ± 3	
Controls‡	32 ± 17	31 ± 18

* Clq-BA in 8 RA+ and 4 RA- synovial fluids (% mean ± 1 SD).

† Individual difference (mean ± 1 SD).

‡ Same dilution as pretreated samples.

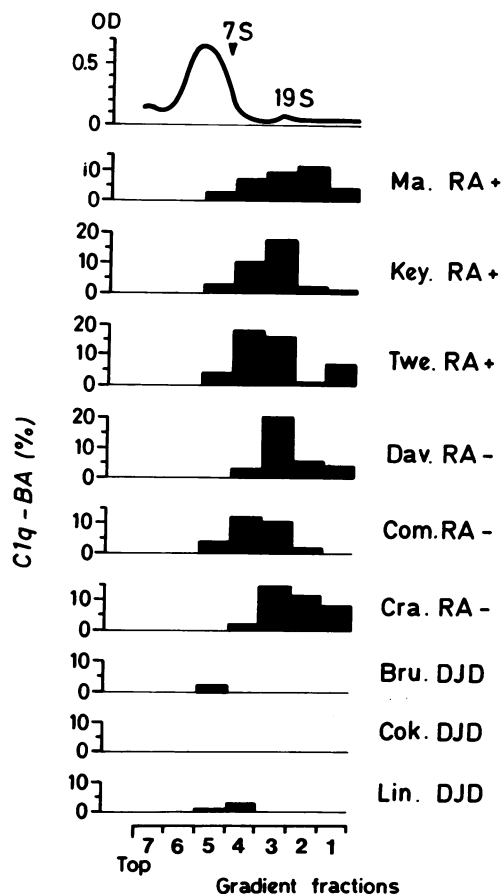


FIGURE 6 ^{125}I -Clq-BA measured in individual sucrose gradient fractions obtained by separation of synovial fluids from patients with RA+ and RA- and DJD: on top of the figure the OD pattern obtained with a 1:10 diluted synovial fluid and the positions of the 7S and 19S markers are indicated, Clq-BA is represented by the black columns on the lower part of the figure.

crose density gradients. This procedure led to a three to four times increased Clq-BA in 14–24S gradient fractions when compared to the Clq-BA in respective gradient fractions obtained by centrifugation of the original untreated synovial fluids. A representative experiment is shown on Fig. 8. Two experiments were performed with the 14–24S concentrated Clq binding material from 2 RA+ and 2 RA- synovial fluids: first, the influence of acidification was investigated by an experiment involving parallel ultracentrifugation runs either at pH 8.3 or at pH 3.8. The OD patterns from these two gradients were compared (Fig. 9). The OD pattern at pH 8.3 resulted in two peaks of similar size, one in the 19S region and another one in the 14–15S region. In contrast, the OD pattern at acid pH resulted in a major peak in the 7S region and a small peak in the 19S region. IgG was quantitated in the gradient frac-

tions by double immunodiffusion. At pH 8.3 maximum IgG was found to be located in the 14S peak, whereas at pH 3.8 the maximum was found to be located in the 7S peak. Immunoelectrophoretic analysis of this 7S peak fraction with antiserum to total human serum revealed only IgG. Secondly, the concentrated Clq binding material was left to react in an immunoabsorbant system. Small columns of CNBr-sepharose 4B coupled with rabbit antihuman IgG or with bovine serum albumin were used. Concentrated Clq binding material from RA synovial fluid was passed through these columns and the Clq-BA of the effluents were compared. Clq-BA was still observed in the effluent of the bovine serum albumin sepharose columns, while the effluent of the anti-IgG sepharose columns no longer exhibited Clq binding activity.

DISCUSSION

The occurrence of immune complexes during the course of RA has been previously demonstrated by analytical studies of synovial tissue, synovial fluid, and serum samples (1–5, 10–15). These investigations have been conducted mainly on a qualitative basis. Macromolecular complexes containing immunoglobulins have been detected in synovial fluids and serum and, in some instances, their size has been characterized (3–5, 10–15).

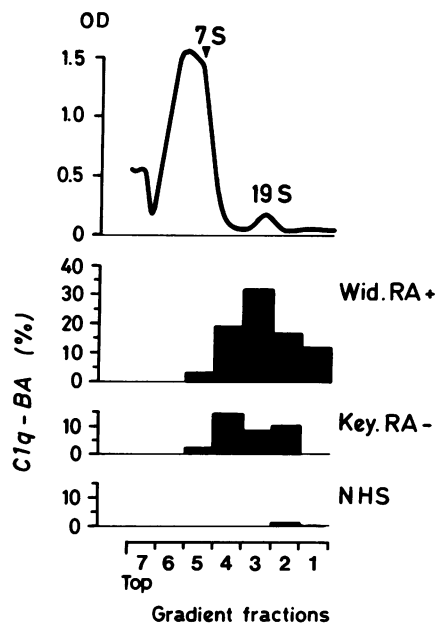


FIGURE 7 ^{125}I -Clq-BA measured in individual sucrose gradient fractions obtained by separation of sera from a patient with RA+ and one with RA- and from a healthy blood donor (NHS): on top of the figure the OD pattern obtained with a 1:10 diluted serum and the positions of the 7S and 19S markers are indicated, Clq-BA is represented by the black columns on the lower part of the figure.

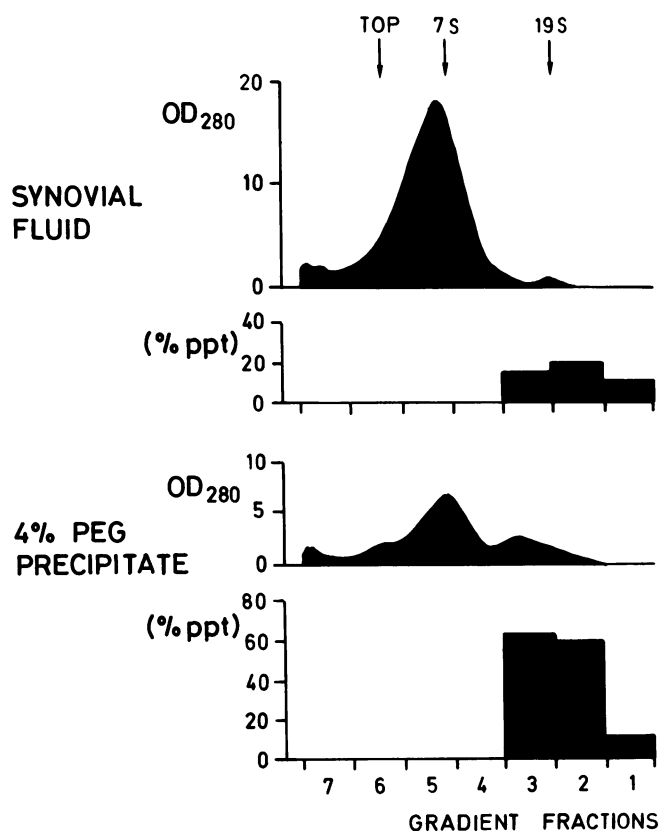


FIGURE 8 Concentration and partial purification of the Clq binding material from the synovial fluid of a patient with RA+: the curves represent the OD pattern obtained after sucrose gradient separation either of synovial fluid (upper part of the figure) or of a concentrated fraction obtained after incubation of that synovial fluid with PEG, (4% final concentration) (lower part of the figure). The black columns represent the percent specific ^{125}I -Clq precipitation (% ppt) in individual gradient fractions obtained from either sample.

The purpose of the present study was to investigate on a quantitative basis the incidence of immune complexes in serum and in synovial fluids from patients with RA, using a newly developed methodology which allowed for a sensitive detection of such immune complexes. As with the Clq precipitation test described by Agnello et al. (36), the radioimmunoassay used in this investigation is based on the higher avidity of Clq for aggregated or complexed immunoglobulins as compared with monomeric immunoglobulins (37). However, the sensitivity of the method has been increased by a direct measurement of the binding of Clq by immune complexes rather than by observing a secondary precipitation of those complexes by Clq. This procedure also largely avoided the possible interference of some substances which can nonspecifically fix Clq, such as DNA, bacterial lipopolysaccharides (22), or calcium-dependent C reactive protein complexes (38). However, one cannot a priori rule out the possibility that other substances with similar properties may influence these studies.

The results obtained confirm the existence of large amounts of immune complex-like material in synovial fluids from patients with RA. Indeed, the Clq-BA was significantly increased in those fluids when compared to the Clq-BA observed in samples from patients with DJD or with TR. The physicochemical characterization of the Clq binding material showed that it was similar to the 10–30S macromolecular complexes observed in such fluids by ultracentrifugation analysis (3–5, 14). Furthermore, the fact that this material lost its ability to bind Clq after acid dissociation or reduction-alkylation (39), or after passage through an anti-IgG immunoabsorbant, demonstrates that it has properties similar to that of immune complexes. DNA or DNA-anti-DNA complexes do not contribute to this binding of Clq since DNase treatment did not reduce the Clq-BA of synovial fluids. The average Clq-BA observed in synovial fluids from patients with RA (37%) corresponds to that obtained when heat-aggregated immunoglobulin is added to native normal serum at a concen-

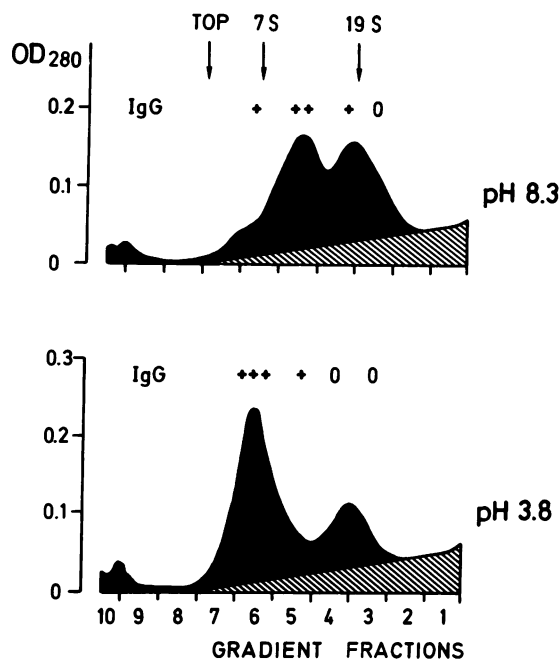


FIGURE 9 Acid dissociation of Clq binding material: the curves represent OD pattern obtained after separation of concentrated and partially purified Clq binding material recovered from the synovial fluid of a patient with RA+ on sucrose gradients at either pH 8.3 or pH 3.8. The hatched area represents the OD of a control tube containing only sucrose. The top of the 10–40% sucrose gradient, the positions of the 7S and 19S markers and the relative concentration of IgG in various gradient fractions are indicated on the figure.

tration of about 0.5 mg/ml (22). However, this value should be considered as approximate, since the size and the nature of the detected complexes influence their efficiency to bind Clq (37). It has been found previously that IgG-anti-IgG complexes can exhibit a higher Clq-BA than a similar amount of heat-aggregated IgG (21).

The participation of 19S rheumatoid factor (RF) in the Clq binding complexes seemed relatively limited since (a) fluids from patients with RA– exhibited similar levels of Clq-BA to those from patients with RA+, and (b) the maximum Clq binding material by sucrose density gradient analysis was most frequently observed in the 14–15S range. An increased Clq-BA was also observed in synovial fluids from some patients with various IA. Although bacterial antigen-antibody complexes may be responsible for this binding in infectious arthritis, the occurrence of an increased Clq-BA in gout or chondrocalcinosis may correspond to a non-specific aggregation of IgG in synovial fluids.

The investigation of immune complexes in serum from patients with RA led to some discrepancies according to various methods which had been previously used.

While 57% of RA+ reacted with monoclonal RF, none of these sera precipitated with Clq in agarose (5). Recently, using a radioimmunoassay with monoclonal RF, Luthra, et al. (19) detected complexes in 24% of RA sera while in a similar assay, but using polyclonal RF, none of the RA sera studied exhibited a positive reaction (18). Cryoglobulins were also found in 25% of sera (17). In the present study, Clq binding material was found in 76% of RA+ sera and in 49% of RA– sera. The differences observed between the results obtained in these various studies probably indicate differences in the sensitivity of the methods used for the particular complexes present in RA sera or in the susceptibility of each technique to the interfering effect of RF. This was apparent when the method using the inhibition of the uptake of labeled agg HGG by macrophages was applied to RA sera (20).

Using the Clq binding test, the main finding was that an increased Clq-BA in serum was observed frequently in both RA+ and RA– patients but occurred only exceptionally in other IA. This result suggests that RA is a disease associated with circulating as well as articular immune complexes, while other IA may be associated with complexes formed in the joint spaces but rarely with circulating complexes. The size of the material binding Clq in RA sera is similar to that of the complexes which were detected with monoclonal rheumatoid factors (5). It corresponds mostly to 15–20S intermediate complexes and may represent IgG-anti-IgG complexes. However, some larger complexes were also found in these serum samples. One cannot exclude the possibility that other antigen-antibody systems may be also responsible for the increase in Clq-BA in RA. The criteria for seropositivity with respect to rheumatoid factor was a positive sheep cell agglutination titer of 1:64 or higher. This is arbitrary and therefore some seronegative patients' sera may indeed have had a low IgM RF agglutinating activity. Moreover, it is well known that such "seronegative" patients usually exhibit the presence of IgG RF.

The studies of the correlation between Clq-BA and other biological features of RA indicated that the presence of immune complexes is not directly related to the concentration of RF in serum. Furthermore, nonspecific changes in the serum protein profile characterizing the inflammatory stimulus in RA were clearly not interfering with the Clq binding test. However, there was a negative correlation between the level of C4 in synovial fluid and the Clq-BA indicating that the Clq binding material may be responsible for the activation of complement in the joint spaces and may play an important role in some pathogenic aspects of the disease. Such significant correlation could not be demonstrated in serum samples and this may reflect a better efficiency of

modulating mechanisms such as that due to complement inhibitors in circulating blood rather than in the articular cavity which is a relatively closed anatomical space. In addition, an increased synthesis of complement components, which is frequently associated with inflammatory syndromes (40) may mask the hypercatabolism of these complement components as suggested by the results of catabolic studies (41, 42). However, it is likely that the binding of Clq is not necessarily followed by an intense activation of the whole complement sequence.

The significant correlation between the values of Clq-BA in serum and the presence of extraarticular manifestations of RA is indeed suggestive of the role of circulating immune complexes in the pathogenesis of these lesions. It confirms previous elution and immunofluorescence studies demonstrating the deposition of immunoglobulins and complement in rheumatoid nodules (1), nerve lesions (43), and other types of vasculitis (44). The role of IgM RF in these manifestations is not clear, but it is possible that circulating intermediate complexes may have an enhanced pathogenicity when IgM RF is available in the medium. IgM RF might also bind to intermediate complexes already localized in some interstitial tissues. Furthermore, the finding of a high Clq-BA in serum from patients with RA may be an indicator of a higher risk of those patients to develop vasculitis and may be considered of some importance for the monitoring of the treatment of the disease. Long-term follow up studies should provide further information on that particular interpretation.

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