Clin. exp. Immunol. (1975) **19**, 131–141.

CRYOGLOBULINAEMIA IN PATIENTS WITH INFECTIOUS ENDOCARDITIS

D. HURWITZ, F. P. QUISMORIO AND G. J. FRIOU

Clinical Immunology and Rheumatic Disease Section, Department of Medicine, School of Medicine, University of Southern California, Los Angeles, California, U.S.A.

(*Received* 13 *May* 1974)

SUMMARY

Serum cryoglobulins were found in nineteen out of twenty patients with infectious endocarditis. The cryoglobulins were of the 'mixed type' consisting of IgG, IgM and IgA. C3 and fibrinogen were present in some specimens. The concentration of the cryoglobulins tended to fall with therapy and clinical improvement of the patients. Serum antibodies to the offending bacterial organism were not preferentially concentrated in the cryoglobulins. In contrast, IgM rheumatoid factor was present in the cryoglobulins, though undetectable in the corresponding serum. These findings are consistent with the view that cryoglobulins represent circulating immune complexes which may be important in the pathogenesis of immunological sequelae sometimes found in patients with infectious endocarditis.

INTRODUCTION

Infectious endocarditis has immunological sequelae that bear close resemblance to phenomena seen in immunological disorders such as systemic lupus erythematosus (SLE). Serum antiglobulins (rheumatoid factor), hypocomplementaemia, cryoglobulins and glomerulonephritis have all been reported in endocarditis patients (Williams & Kunkel, 1962; Gutman *et al.*, 1972). Cryoglobulins were first described in this condition by Wertheimer & Stein (1944) and again noted by Lerner *et al.* (1947). Dreyfuss & Librach (1952) described the presence of cold precipitable serum globulins in 84% of fifty patients with bacterial endocarditis. Cryoglobulins in several disease states are felt by many to represent immune complexes, and may mediate inflammation (Barnett *et al.*, 1970), but uncertainty exists as to the identity of antigen(s) and the antibody specificity of the immunoglobulins contained therein. Infectious endocarditis offers an excellent opportunity to study cryoglobulins because the primary etiological agent, which presumably provides the initial antigenic stimulus, is known.

Correspondence: Dr F. P. Quismorio, Clinical Immunology and Rheumatic Diseases Section, Department of Medicine, School of Medicine, University of Southern California Medical Center, Los Angeles, California 90033, U.S.A.

MATERIALS AND METHODS

Patients with infectious endocarditis (Table 1)

Twenty patients with infectious endocarditis were selected from the medical and surgical services of Los Angeles County/University of Southern California Medical Center. The diagnosis of infectious endocarditis was based on the presence of fever, organic heart murmur, and positive blood cultures.

TABLE 1. Clinical features of twenty IE patients studied

Patient	Age	Valve involved*	Predisposing factor	Organism
R.M.	40	A	I.v. drug	*Candida*
R.H.	38	A	I.v. drug	*Candida*
J.G.	32	T	I.v. drug	*Staphylococcus aureus*
C.M.	47	A	I.v. drug	*Staphylococcus aureus*
J.B.	25	A	I.v. drug	*Staphylococcus aureus*
G.W.	19	T	I.v. drug	*Staphylococcus aureus*
J.L.	48	A	I.v. drug	*Streptococcus* (group D)
E.Ba.	33	A	I.v. drug	*Streptococcus* (group D)
W.J.	50	T	I.v. drug	*Streptococcus* (group D)
F.B.	32	A	I.v. drug	*Streptococcus* (group D)
C.J.	43	A	I.v. drug	*Streptococcus* (group D)
E.Be.	40	M	I.v. drug	*Streptococcus* (group D)/*Pseudomonas*
B.L.	37	A, M	Rheumatic heart disease	*Streptococcus* (alpha)
C.C.	25	A, M	Rheumatic heart disease	*Streptococcus* (alpha)
J.S.	27	A	Rheumatic heart disease	*Streptococcus* (alpha)
M.B.	38	M	Rheumatic heart disease	*Pneumococcus* (group D)
A.A.	48	M	Rheumatic heart disease	*Haemophilus aphrophilus*
M.M.	33	A	Rheumatic heart disease	*Actinomycetes*
D.C.	22	A, M	Rheumatic heart disease	Unknown
L.H.	34	A, M	Rheumatic heart disease	Unknown

* A = aortic; T = tricuspid; M = mitral.

Group D streptococci and *Staphylococcus aureus* were the most common organismss two cases of fungal endocarditis were seen during the period of the study. In two patient; all blood cultures were negative. Of the twenty patients, there were eight with rheumatic heart disease (RHD). The aortic valve was most frequently involved, but mitral or combined mitral and aortic involvement was common. Twelve patients had a history of intravenous drug abuse without any previous history of heart disease.

Ten patients had evidence of active renal disease at the time of study. The criteria for renal disease were one of the following: (1) red cell casts; (2) cellular casts plus haematuria of at least five RBC/high power field, or pyuria of at least eight WBC/high power field; (3) proteinuria of at least 250 mg per 24 hr. In the absence of a 24-hr specimen, 3+ protein uria in a random specimen was considered significant.

Controls

Six individuals who were known to take intravenous drugs in a pattern similar to those with endocarditis were tested for cryoglobulinaemia. All were free of systemic bacterial

infection. Two were admitted for treatment of overdose of self-administered drugs, two had hepatitis, one trauma and the other a superficial skin abcess.

Cryoprecipitates

Tubes containing venous blood were placed in a 37°C water bath immediately after being drawn and kept warm during transportation to the laboratory. Specimens were incubated for 1 hr at 37°C and then centrifuged twice at 37°C to separate the serum. Sera were then held at 4°C and the precipitate present at 72 hr washed three times in cold phosphate-buffered saline (PBS) 0·01 M, pH 7·0. For quantitation, the precipitate from 4 ml of serum, along with appropriate human IgG standard, were dissolved in 1 N NaOH and absorbance read spectrophotometrically at 280 nm. Results were expressed as milligrams %. Sera from three patients were divided into two aliquots of 4 ml each and one of the aliquots incubated at 56°C for 30 min prior to storage at 4°C. Cryoprecipitate isolated from the remainder of the serum (8–10 ml) was stored at −20°, and redissolved in 2 ml of PBS (pH 7) at 37°C for subsequent procedures.

Antibacterial antibodies

Indirect immunofluorescence (IF) was used to detect the presence of antistreptococcal (Group D) and antistaphylococcal antibodies in the cryoprecipitates and sera of patients with IE due to those organisms. Laboratory strains of these organisms were smeared on glass slides; serum or redissolved cryoprecipitate was layered over the organisms and the presence of specific antibodies was detected by fluorescein-labelled sheep anti-whole human globulin. The conjugate was tested and found to be free of antistreptococcal antibodies; however, significant titres of antistaphylococcal antibodies were present and required removal by serial absorptions with heat-killed organisms. The cryoglobulin of one additional patient, C.Co., with *Staphylococcus aureus* endocarditis, was fractionated on a sucrose density gradient and antistaphylococcal antibody titres were determined in fractions 4, 5 and 6 containing IgG.

Other procedures

Immunoglobulins and C3 complement were determined by radial immunodiffusion plates (Hyland Laboratories). Serum haemolytic complement was measured expressed as CH_{50} units (normal 480–1280 units). Antinuclear antibodies were determined by indirect IF test using rat kidney and deoxyribonucleoprotein and DNA spots as substrates (Friou, 1967). Latex fixation tests for RF were done by the tube method in serum and whole cryoprecipitates, and by slide latex (Hyland Laboratories) when testing cryoprecipitate fractions. Selected cryoprecipitates were fractionated by zone ultracentrifugation using 10–40% sucrose dissolved in glycine–HCl buffer, pH 3·2. Nine fractions were obtained through the bottom of the tube. These were dialysed against PBS before analysis.

RESULTS

Cryoprecipitate concentrations

Nineteen out of twenty patients studied had a cryoprecipitate in their initial serum specimen. The mean protein concentration was 9·8 mg% and ranged from 1 to 43 mg%. These values were compared to twenty-five consecutive SLE sera with cryoprecipitates studied

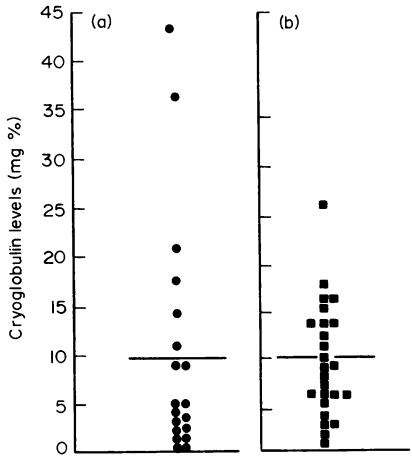

FIG. 1. Serum cryoglobulin levels in patients with (a) infectious endocarditis and (b) systemic lupus erythematosus.

in our laboratory. The SLE cryoprecipitates ranged from 2·0 to 25·5 mg% and also had a mean of 9·8 mg%. The drug abuse patients were compared to the RHD group; the former had a mean cryoprecipitate concentration of 10·8 mg%, the latter 8·3 mg%, a difference which was not significant.

Of the six controls, two had serum cryoprecipitates of 3 mg% and 2·5 mg% respectively, one with hepatitis and the other with a fractured hip without apparent infection.

Composition of cryoprecipitates

The cryoprecipitates from seventeen patients were tested by immunodiffusion using monospecific antisera to IgG, IgA, C3 and fibrinogen. The results are shown in Table 2.

TABLE 2. Composition of cryoprecipitates

Patient	IgG	IgM	IgA	C3	Fibrinogen
R.M.	+	+	+	−	+
J.G.	+	+	+	−	+
A.A.	+	+	−	−	−
C.M.	+	+	+	+	−
J.L.	+	+	−	+	−
D.C.	+	+	−	−	−
E.Ba.	+	+	+	+	−
W.J.	+	+	+	−	−
B.L.	+	+	+	−	−
F.B.	+	+	−	−	−
M.M.	+	+	+	−	−
L.H.	+	+	+	−	−
M.B.	+	+	+	+	−
E.Be.	+	+	+	−	−
G.W.	+	−	−	−	−
J.B.	−	−	−	−	−
C.J.	+	+	−	−	−

Mixed cryoglobulins were found to be present in all but two cases, one IgG cryoglobulin was found, and one cryoprecipitate had no detectable immunoglobulins. Of the fifteen mixed cryoglobulins, ten contained IgG and IgA, and five contained IgG and IgM. Four of the cryoprecipitates also contained C3 and two contained fibrinogen.

Rheumatoid factor (Table 3)

The tube latex fixation test was titred from dilutions of 1:5 to detect lower concentrations of RF. Two patients had RF in the serum, one to a titre of 1:320 and the other only to 1:5. However, twelve out of fifteen cryoprecipitates tested were found to have RF in titres ranging from 1:5 to 1:80. Surprisingly, the patient with a serum RF titre of 1:320 had no detectable RF in his cryoglobulin.

TABLE 3. Rheumatoid factor titre in infectious endocarditis serum and cryoprecipitates

Patient	Serum	Cryoprecipitate*
E.Be.	1:320	0
C.M.	0	0
J.B.	0	0
G.W.	0	1:5
A.A.	0	1:5
M.M.	0	1:5
E.Ba.	0	1:10
J.L.	0	1:10
C.J.	0	1:10
J.G.	0	1:10
M.B.	0	1:10
D.C.	0	1:20
B.L.	0	1:20
F.B.	0	1:40
W.J.	0	1:80

* Cryoprecipitate was isolated from 8–10 ml of serum and redissolved in 2 ml of PBS.

Serial studies

Serial determination of the cryoprecipitates were done in eight patients (Fig. 2). In general, levels tended to fall with clinical improvement. Patients J.G., A.A., J.L. and D.C. showed a steady clinical improvement with a fall in cryoglobulins. C.M. developed fever after his initial specimen had been obtained, and may have had Gram-negative bacteraemia superimposed on his staphylococcal endocarditis. At this time the cryoglobulin level rose. This complication resolved with appropriate therapy and a drop in the cryoglobulin level was noted. Patient E.Ba. developed a splenic abscess in the 4th week and underwent splenectomy, after which he had a cryoglobulin concentration of only 5 mg%. Cryoglobulins subsequently rose with continuing infection in spite of antibiotic therapy. Patient E.Be. had a progressive fall in cryoglobulin levels in spite of a downhill course with positive blood

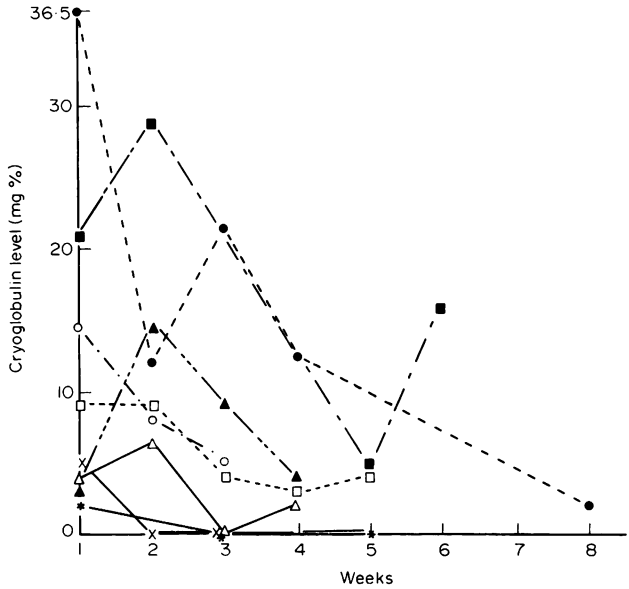

FIG. 2. Serial levels of serum cryoglobulins in eight patients with infectious endocarditis. (●) A.A. (■) E.Ba. (○) E.Be. (▲) C.M. (□) D.C. (△) J.G. (×) G.W. (*) J.L.

cultures, and deteriorating cardiac status requiring emergency mitral valve replacement. Similarly, G.W. had falling levels in spite of continuing positive blood cultures.

Fractionation

Cryoglobulins from two patients were fractionated on sucrose density gradients by ultracentrifugation. Fig. 3 shows a representative sample from patient J.G. Protein peaks

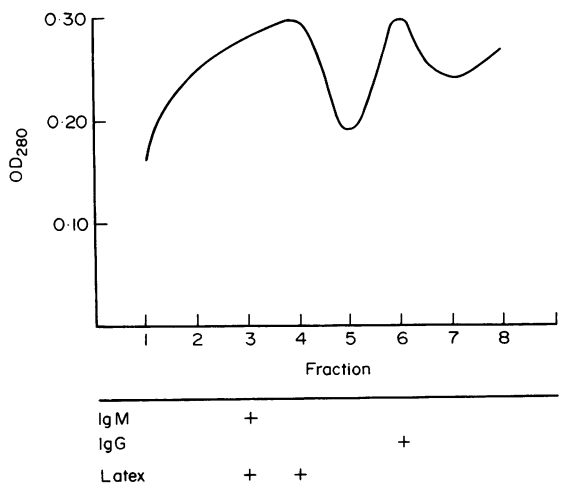

FIG. 3. Protein concentration of nine fractions of a cryoglobulin from a patient with infectious endocarditis separated by zone ultracentrifugation in sucrose gradients. Fraction 3 contained IgM and fraction 6 contained IgG. Rheumatoid factor activity was present in fractions 3 and 4.

were seen in fractions 3 and 4 and fraction 6. The two peaks contained IgM and IgG respectively and rheumatoid factor was found in the IgM peak.

Effect of heat on cryoprecipitation

In three patients, paired specimens were compared as to the effect of incubation at 56°C for 30 min on cryoprecipitation. As can be seen from Table 4 heating resulted in a decrease

TABLE 4. Effect of heating serum to 56°C on cryoprecipitation

Patient	Unheated (mg%)	Heated (mg%)
E.Ba.	29	11
J.B.	43	28·5
E.Be.	8	2·5

in the amount of cryoprecipitate in all three cases. This suggests the participation of heat-labile factors in cryoprecipitation.

Antibacterial antibody

Antibacterial antibody to the specific infecting organism was searched for in the sera and whole cryoglobulins of three patients with staphylococcal endocarditis and four with Group D streptococcal endocarditis, as well as the serum and fractionated cryoglobulin on one patient with staphylococcal endocarditis. Results are shown in Table 5. In general, antibody

TABLE 5. Titre of antibacterial antibodies in sera and cryoprecipitate of infectious endocarditis patients

Patient	Infecting organism	Antibacterial antibody titre	
		Serum	Cryoprecipitate
E.Ba.	*Streptococcus* (group D)	1:16384	1:16
W.J.	*Streptococcus* (group D)	1:4096	1:2
J.L.	*Streptococcus* (group D)	1:4096	0
F.B.	*Streptococcus* (group D)	1:128	0
J.G.	*Staphylococcus aureus*	1:512	1:64
C.J.	*Staphylococcus aureus*	1:16384	1:64
G.W.	*Staphylococcus aureus*	1:32768	1:2
C.C.	*Staphylococcus aureus*	1:131072	1:128 (4)* 1:1024 (5)* 1:256 (6)*

* Numbers in parentheses refer to sucrose gradient zonal ultracentrifugation fractions of cryoprecipitate containing IgG.

titres against the infecting organism were much lower in the cryoglobulin that in the respective serum. Two cryoprecipitates had no detectable antibody, and two had a titre of only 1:2.

To determine if there were preferential concentrations of antibacterial antibody activity

in the cryoglobulins, IgG concentrations were determined in the dissolved cryoglobulins and divided by the reciprocal of antibody titres to determine the minimum concentration of IgG with antibody activity. A similar determination was made for the serum, and comparison was possible in the five patients shown in Table 6 who had measurable immuno-

TABLE 6. Minimum concentration of IgG (mg%) in the serum and cryoglobulin with antibacterial antibody activity

Patient	Serum IgG	Cryoglobulin IgG
E.Ba.	0·076	2·688
W.J.	0·857	13·50
C.M.	0·305	1·484
J.G.	4·88	0·343

globulin levels. Only one patient, J.G., had evidence of preferential antibody concentration in the cryoprecipitate as compared to the serum. In this instance only, the cryoglobulin demonstrated higher antibody activity per milligram of IgG than the corresponding serum. Similar calculations were done for IgM and IgA, where these immunoglobulins were measurable, with identical results.

Other correlations

Cryoglobulin levels did not correlate with the type of infecting organism, serum complement, both C3 and CH_{50}, or quantitative immunoglobulins. There was no relationship between initial cryoglobulin levels and eventual clinical outcome. The cryoglobulin level in the group with renal disease (mean = 9·2 mg%) did not differ from that of the group without renal disease (mean = 10·3 mg%).

DISCUSSION

We have confirmed earlier reports on the occurrence of cryoglobulinaemia in infectious endocarditis. Our prevalence of 90% is similar to the earlier study of Dreyfuss & Librach (1952) where the prevalence of cryoglobulinaemia was 84%. These findings indicate that cryoglobulinaemia is a very common finding in this disease. We have demonstrated that these cryoglobulins are of the mixed type, i.e. the cryoglobulins contain two or more immunoglobulin classes. Similar mixed cryoglobulins have been found in other immunological disorders such as SLE (Barnett *et al.*, 1970).

Infectious endocarditis resembles SLE in that immune complex-mediated inflammation appears to be present to both diseases. Studies by Williams & Kunkel (1962) documented the presence of serum RF in 50% of their cases and serum immunoconglutinin in most of their cases. These abnormalities indicated that circulating immune complexes might be present during the active phase of the disease. Of particular interest in their study was the presence of low serum haemolytic complement in infectious endocarditis patients with nephritis. It was later shown that this renal lesion was characterized by subepithelial deposits,

seen by electron microscopy, and granular deposits of IgG and β1c globulins in the basement membrane as seen by immunofluorescence (Gutman *et al.*, 1972, Messner *et al.*, 1968, Tu, Shearn & Lee, 1969). This pathological picture in association with hypocomplementaemia is consistent with an immune complex type of nephritis, a process believed to occur in SLE.

Circulating immune complexes, as such, have not been identified in infectious endocarditis. It would be logical to assume that such complexes, if they exist, are composed of antigens of the infecting organism, and antibody directed towards that organism. Recent studies have demonstrated deposition of specific antibacterial antibody in a nephritic kidney of a patient with subacute bacterial endocarditis (Levy & Hong, 1973) and deposition of bacterial antigen in the kidney of a patient with shunt nephritis (Kaufman & McIntosh, 1971), a disease analogous to those studied here. In neither case were circulating immune complexes of bacterial antigen and antibody demonstrated.

Mixed cryoglobulins, because they contain immunoglobulins, rheumatoid factor, and complement components are felt to be immune complexes (Hanauer & Christian, 1967; Barnett *et al.*, 1970; Christian, Hatfield & Chase, 1963) and they represent a ready source of circulating immune complexes because of the ease with which they can be isolated. Evidence exists that mixed cryoglobulins can provoke an inflammatory reaction *in vivo*. Cryoglobulins from patients with post-streptococcal nephritis when injected intradermally into guinea-pigs caused local inflammation and when injected intravenously into rabbits produced a mild nephritis, that was shown to be different from foreign protein nephritis (McIntosh *et al.*, 1970, 1971). The induction of purpuric skin lesions in a patient with IgA–IgG cryoglobulinaemia by injection of the cryoglobulin intradermally has been reported (Whitsed & Penny, 1971) offering additional proof of the inflammatory activity and immune complex nature of those proteins.

Our investigation failed to establish a significant role for antibacterial antibody in the formation of cryoglobulins. While we found antibacterial antibody activity in a number of the cryoglobulins examined, the activity was generally of low titre and comparison to serum titres revealed a lack of preferential concentration of antibody activity in the cryoglobulins. If these cryoglobulins are immune complexes, then the antigen is presumably something other than the infecting organism. The presence of rheumatoid factor in the cryoglobulins is consistent with the possibility that altered IgG is the antigen. It is conceivable that the infecting organism may in some way cause an alteration of host IgG. Zinneman *et al.* (1968) in a study of a patient with IgG–IgM cryoglobulinaemia demonstrated that the IgG component was deficient in sialic acid and by this depletion rendered the IgG autoantigenic.

Initial cryoglobulin level did not correlate with disease outcome, since patients with both low and high initial levels succumbed to their disease. Though levels generally paralleled the course of the disease, it is possible that this immunological abnormality was a secondary phenomenon and not of direct importance to the disease process. The mean cryoglobulin concentration in our patients with nephritis was not higher than in those patients without evidence of renal disease. Urinalysis is an insensitive indicator of renal involvement, and it is conceivable that subclinical renal disease may be present in patients with a normal urinalysis. Morel-Maroger (1972) studied nine patients with *Streptococcus viridans* endocarditis, eight of whom had normal urinalyses. On renal biopsy, these patients showed prominent interstitial nephritis and mild focal segmental glomerulonephritis with granular

deposition of C3 on the basement membrane, and mesangial deposits of IgG and IgM. This suggests a universal presence of renal disease, often subclinical, in infectious endocarditis. It is possible that deposition of mixed cryoglobulins in the glomerular basement membrane might be partly responsible for the renal lesions. In SLE, rheumatoid factor found in the serum cryoprecipitate have also been demonstrated in the renal glomeruli of the same patient (Agnello *et al.*, 1971).

We have no explanation for the discrepancy in prevalence of serum rheumatoid factor in our study and those which have been previously reported (Williams & Kunkel, 1962; Messner *et al.*, 1968). We found a positive latex fixation test in only two patients, for a prevalence of 10% as compared to a prevalence of 50% in those other studies. Prevalence in the latter series was clearly related to length of disease and the frequency of positive latex fixation tests rising after 6 weeks. Many of our patients did seem to have their endocarditis for at least that length of time. Our patients differed from those in previous studies in that the underlying condition in more than half of our patients was intravenous drug use.

It is of interest to consider our results in relation to the etiology of idiopathic immune complex disorders such as SLE and rheumatoid arthritis (RA). Certain similarities of these disorders to known persistent infectious diseases, such as infectious endocarditis, has prompted speculation that they may be the result of chronic infection with a heretofore undiscovered agent, presumably a virus. We have studied the presence of antibodies to a number of common viruses in the synovial fluid cryoprecipitates in RA and serum cryoprecipitates in SLE and compared their titre to that in the corresponding serum (Cremer *et al.*, 1970). We found no evidence of selective concentration of antibodies in the RA synovial fluid cryoprecipitates. No antiviral antibodies were found in the SLE sera cryoprecipitates despite serum antibody titres comparable to those seen in RA.

ACKNOWLEDGEMENTS

We are indebted to Drs Sol Bernstein and Marv Bernstein for allowing us to study their patients. The technical assistance of Mrs Joan Albers and secretarial help of Mrs Harriet Sample are greatly appreciated. The work was supported in part by grants from the Arthritis Foundation and NIH, grant number 52-2120-6852.

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