Measurement of red blood cell-bound C3b and C3d using an enzyme-linked direct antiglobulin test

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Complement has a complex role in immune mediated red blood cell (RBC) destruction and usually induces extravascular hemolysis of C3b-coated RBCs by erythrophagocytosis and by acting synergistically with cell-bound immunoglobulins. A sensitive two-stage enzyme-linked direct antiglobulin test (ELDAT) was developed and used to measure RBC-bound C3b and C3d in 120 healthy adult individuals and in 60 patients suffering from a variety of conditions, including warm- and cold-type autoimmune hemolytic anemia, neoplasia, and collagen diseases. The results were compared with those of standard agglutination tests employing polyclonal and monoclonal antiglobulin reagents. Small amounts of C3b and C3d were detected on RBCs of the healthy individuals only by the ELDAT and probably reflected the continuing low-grade activation of complement necessary for the maintenance of homeostasis of a variety of physiological systems. The quantity did not vary with age or gender. In the patients, increased amounts of RBC-bound C3b and C3d were relatively common and probably resulted from autoantibody activity, immune-complexes, and nonspecific adsorption. There was no association between positive ELDAT results and the presence of active hemolysis. The ELDAT was far more sensitive than the agglutination tests for detecting RBC-bound C3b and also for C3d if the monoclonal reagent was employed. ***Immunohematology*** 1997;13:123–131.

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In patients with autoimmune hemolytic disorders, the relationship between the immunoproteins coating the red blood cells (RBCs) and the degree of in vivo hemolysis is complicated.[1-3] There was some early support for the idea that the quantity of immunoglobulin was a major determinant of the severity of hemolysis[4] and a hemolytic threshold (for IgG) was postulated.[5] However, this was not confirmed by later studies[6] and is now considered to be too simplistic a view, as autoimmune hemolysis has been found in patients in whom small quantities of RBC-bound immunoglobulins could be demonstrated[7] and who may even have had a negative direct antiglobulin test (DAT).[6,8,9] Other important factors in determining hemolytic activity are thought to include the presence and interactions of autoantibodies of more than one immunoglobulin class,[1] their IgG subclass pattern,[2] complement, and the state of activation of the effector cells involved—mainly mononuclear phagocytes, but possibly also lymphocytes and granulocytes.[3]

The role of complement in immune mediated hemolysis is complex.[3] RBC destruction by complement is usually extravascular, for although complement activation is often triggered, it rarely proceeds to completion (i.e., intravascular hemolysis). Regulatory inactivators (e.g., decay-accelerating factor and CD59) operate at several points on the complement cascade, usually at the level of C3, and result in RBCs coated with C3b or iC3b. Destruction is then by phagocytosis (or through the production of spherocytes) and occurs via binding to CR1 and CR3 receptors, mostly in liver macrophages. Many RBCs are immediately engulfed, but since binding capacity is considerably in excess of phagocytic ability, other RBCs slowly return to the circulation because of cleavage by naturally occurring regulatory factors of the fixed C3b to produce C3d,g, and C3c (the former is further reduced in vitro to C3d; the latter is lost to the plasma). Macrophages do not react efficiently with C3d,g in vivo and the RBCs may survive normally, even though the DAT is strongly positive. There is some evidence that RBC-bound complement has a quantitative effect in relation to hemolysis. This is difficult to assess in vitro as the C3b-coated RBCs have either been destroyed or the C3b converted to C3d,g. Nevertheless, it has been suggested that about 550–800 molecules of bound C3b are required to activate the hepatic clearance mechanism and, in one study, most patients with > 1,100 C3d molecules per RBC had an associated hemolytic anemia,[10] presumably because of a proportional relationship with the number

of C3b molecules originally bound to the cells. Complement also acts synergistically with immunoglobulins in producing RBC destruction.[11-13] Complement coating significantly reduced the number of IgG molecules needed to initiate antibody-dependent cellular cytotoxicity, the augmentation persisting even after degradation of C3b to C3d,[14] and marked augmentation of in vitro monocyte erythrophagocytosis of IgG-coated RBCs was observed when there was concurrent coating with complement components.[15]

A sensitive method for measuring the amount of RBC-bound C3 would be very useful, as the routine agglutination tests are not quantitative and have limited sensitivity, failing on occasions to detect the increased amounts of RBC-bound C3 related to in vivo hemolysis. This paper describes a recently developed two-stage enzyme-linked direct antiglobulin test (ELDAT) and reviews the relevant literature. The test has been used to measure C3 on RBCs in healthy participants and in patients in pathological states, comparing the sensitivity with that of agglutination methods that use polyclonal and monoclonal anti-human globulin reagents.

Materials and Methods

Studies were carried out on venous blood that had been collected using K_2EDTA as an anticoagulant from 120 healthy individuals and from 60 patients. All samples were tested within 48 hours of collection.

The healthy individuals were divided into three groups by age (20–25 years, 40–45 years, and 60–65 years); each group contained 20 males and 20 females.

In order to obtain a variety of different RBC complement levels, the patients' samples were chosen from cases referred to this Center for immunohematologic investigation of RBC autoantibody problems. The clinical and laboratory records were carefully reviewed and an assessment was made of the likelihood of in vivo hemolysis. These data included hemoglobin concentration and its rate of fall; reticulocyte count; blood and marrow film appearances; and measurement of RBC life span, serum haptoglobins, bilirubin concentration, and lactic dehydrogenase activity.

Full details of the investigations carried out have been described previously[1,2,7,16,17] and recently summarized.[18] They include DATs using heavy chain-specific reagents to detect RBC-bound IgG, IgM, and IgA by spin agglutination and by enzyme-linked methods, and examination of RBC autoantibodies in serum and eluates.

Since this study concerned C3 coating of RBCs, additional spin agglutination DATs were carried out to detect C3b and C3d. Both polyclonal and monoclonal anti-human globulin reagents were employed; the former were produced in-house at this Center, the latter were obtained from BPL Diagnostics (Elstree, UK). Antibody specificities were confirmed in pilot studies using a series of RBCs sensitized with different C3 fractions. Briefly, anti-Vel was used to produce RBCs coated with C3b; C3d,g- and C3d-coated RBCs were obtained by degradation of the C3b with complement-fresh serum in 0.05 M K_2 EDTA and by enzyme treatment, respectively. Because C3b expresses epitopes for C3c and C3d, it was detected using specific anti-C3c reagents. The polyclonal anti-C3d recognized C3b/iC3b, C3d, and C3d,g epitopes, but the monoclonal reagent did not react with C3d,g-coated RBCs. None of the reagents showed cross-reactivity with IgG, IgM, IgA, and C4.

The enzyme-linked test was specifically developed for this study and employed polyclonal C3 antisera (Dako, Glostrup, Denmark); these had the same specificities as the corresponding polyclonal agglutinating reagents (i.e., C3b was again detected using anti-C3c). In preliminary studies, the assay response was shown to be linear and the results reliable and reproducible, with intraplate and interplate coefficients of a variation of ≤ 0.07 and ≤ 0.12, respectively, for replicate samples.

The tests were performed in microtiter plates with 96 U-bottomed wells that were pretreated with bovine serum albumin to prevent nonspecific adsorption. Leaving the first two wells of the top three rows empty for blanking, 50μL of polyclonal rabbit anti-human C3c and C3d were added at their predetermined optimal dilutions to appropriate rows, along with a third row containing 50μL of wash buffer (10mL of 20% bovine serum albumin in 1L of phosphate buffered saline, pH 7.2) as a lysis control. Duplicate test samples (20μL of 25% washed RBC suspensions) were added, mixed using an agitator, covered, and incubated at 25°C in a water bath for 30 minutes. The plate was then centrifuged at 2,800 g for 30 seconds at 20°C, the supernatants were removed, and the RBCs were resuspended in 200μL of wash buffer and again centrifuged. This process was repeated until the cells had been washed x 6.

The RBCs were resuspended, and 50μL of a 1:100 dilution of sheep anti-rabbit IgG conjugated with alkaline phosphatase were added to the rows containing anti-C3c and anti-C3d reagents, while 50μL of wash buffer were added to the lysis control rows, excluding the blanking wells as previously stated. Following mixing, the plate was covered and incubated at 37°C for 30 minutes in a water bath. The RBCs were washed x 6.

Freshly prepared substrate solution (200μL p-nitophenyl phosphate) were added to all the wells and mixed; the plate was covered and placed in a 20°C water bath to allow color development. After 25 minutes, the test plates were centrifuged for 1 minute at 2,800 g. From each well, 100μL of supernatant were transferred to the corresponding wells of a flat-bottomed microtiter plate containing 50μL of 3M NaOH to inhibit further color development. The absorbance (expressed in optical density units (ODU)) of each well was measured photometrically at 405nm using a Dynatech MR5000 microplate reader, subtracting the value for the lysis control. RBC counts were performed on the cell buttons in the lysis control rows in the first plate. The test result was expressed as the mean absorbance of the duplicates adjusted to a standard RBC count of 4.5×10^7.

Controls were included in each test run and consisted of pooled group O RBCs that had been trypsinized by incubation at 37°C for 30 minutes in 0.025% trypsin and thoroughly washed; this procedure removed all the complement from the RBCs, thus providing a baseline that allowed the amount of C3 on normal RBCs to be assessed. In addition, untreated pooled RBCs (normal C3 levels) and C3b-coated cells (increased levels of C3) were included in each test run.

Statistical procedures

In the healthy participants, the means and standard deviations for the corrected absorbance values were calculated for C3b and C3d for each group. The effects of age and gender on the results were examined by analysis of variance (ANOVA), the level of significance being set a priori at the 0.05 level. Any sample having a value greater than 3 standard deviations above the entire population mean was considered to have an increased amount of RBC-bound C3 and the test to show a positive result.

For the patients, population proportions were determined for the numbers of positive results with each test and for the presence of hemolysis (for the purpose of the analysis, a definite decision was made, which sometimes was difficult and based on limited data). Differences between proportions were examined for significance by the chi-square test (with Yates correction) at 1 degree of freedom.[19] As multiple comparisons (k) were being made,[20] the significance level for individual tests (α) was set so that the overall significance level (S) was < 0.05, where $\alpha = 1-(1-S)^{1/k}$. In the present study, a chi-square value of ≥ 7.88 ($p < 0.005$) was deemed necessary before individual differences were considered significant.

Results

In the case of the healthy donors, the results of the enzyme-linked tests showed that the amounts of C3b and C3d bound to the RBCs did not depend on the age group or gender of the participant (ANOVA, $p > 0.5$). For the entire population, the mean values (± standard deviation) for C3b and C3d were 0.16 ± 0.06 ODU and 0.42 ± 0.10 ODU, respectively. The levels above which the tests could be considered positive (M + 3 SDs) were therefore 0.32 ODU for C3b and 0.72 ODU for C3d; none of the healthy donors gave a positive reaction. The controls for the enzyme-linked assay always gave the correct results: The trypsinized RBCs had very low OD values, the untreated cells were within the normal range, and the C3-coated RBCs consistently gave positive results. The spin agglutination DATs for RBC-bound C3b and C3d were always negative for the healthy participants.

Of the 60 patients, 25 were male (age range 22–86 years; median 67 years) and 35 were female (age range 25–79 years; median 65 years). Some basic clinical and serologic data are shown in Table 1, together with the results of the enzyme-linked and spin agglutination DATs.

The results of the population proportion studies are given in Table 2.

Discussion

The results showed that the ELDAT was highly sensitive and, using trypsinized RBCs as a baseline control, could measure the small amounts of C3 on RBCs of healthy individuals. In addition, the method allowed normal values to be differentiated from raised levels in pathological states. The assay was simple to perform and gave quantitative results in absorbance units (ODU) over a wide range of RBC C3 coating; it was considered an improvement over earlier methods in that the incubation times were much shorter. The normal values did not vary with the age or gender of the participant (ANOVA, $p > 0.05$) and the upper limit was taken as the mean OD value + 3 standard deviations. This would exclude more than 99% of the healthy population and was thought to be in keeping with what was clinically significant.[16] Other investigators were less stringent and, for IgG, used 1.9 or 2 standard deviations above the mean.[21, 22]

Previous reports of enzyme-linked assays for measuring RBC-bound C3 included a double antibody sandwich technique[23] and an enzyme-linked antiglobulin consumption assay.[24] Both methods needed long incubation times and therefore were unsuitable for the present work. These and other studies that used radioimmune techniques confirmed the lack of an age or gender effect;

Table 1. Classification of 60 patients based on disease association and serological findings

			Results of direct antiglobulin tests*							
		Warm/cold/	Immunoglobulins	C3b			C3d			
Condition	Patient number	mixed type autoantibodies	Enzyme-linked	Enzyme-linked	Polyclonal agglutination	Monoclonal agglutination	Enzyme-linked	Polyclonal agglutination	Monoclonal agglutination	Hemolysis present
Idiopathic										
	1	warm	IgG	0.39	0	0	0.57	0	0	yes
	2	warm	IgG	0.30	0	0	0.57	0	0	mild
	3	mixed	IgG	0.23	0	0	1.41	+	0	mild
	4	warm	IgG	0.41	0	0	0.68	0	0	yes
	5	warm	IgG	0.49	0	0	2.21	++	w	mild
	6	warm	IgG+IgM	0.68	0	0	2.04	++++	w	yes
	7	warm	IgG+IgM	0.68	w	w	2.73	++++	++	yes
	8	cold	IgG+IgA	1.22	w	0	3.02	+++	++	yes
	9	cold	IgG+IgA	1.18	w	w	3.85	++++	++	yes
Neoplasia										
Chronic lymphocytic leukemia	10	warm	negative	0.16	0	0	1.04	+	0	mild
Non-Hodgkin's lymphoma	11	none	negative	0.28	0	0	0.59	0	0	no
Myeloma and monoclonal gammapathy	12	none	IgM	0.21	0	0	0.51	0	0	no
	13	none	IgG	0.23	0	0	1.22	+	0	no
	14	none	IgG	0.21	0	0	0.55	0	0	yes
	15	none	IgG	0.32	0	0	1.14	0	0	no
	16	warm	IgG	0.51	0	0	2.36	++	w	yes
Carcinoma	17	warm	IgG	0.23	0	0	1.57	+	w	mild
	18	none	IgG+IgM	0.18	0	0	1.50	+	0	no
	19	warm	negative	0.27	0	0	1.30	+	0	no
	20	none	negative	0.22	0	0	0.72	0	0	no
	21	none	negative	0.26	0	0	1.79	0	0	yes
Myelodysplastic syndromes	22	warm	IgG	0.29	0	0	0.39	0	0	yes
	23	none	IgG	0.44	0	0	0.59	0	0	no
	24	none	IgG	0.20	0	0	0.77	w	0	no
	25	warm	IgG	0.20	0	0	0.45	0	0	mild
	26	warm	IgG	0.37	0	0	1.98	+++	0	no
	27	none	IgG	0.15	0	0	1.17	w	0	no
	28	none	IgG	0.39	0	0	1.16	w	0	no
	29	warm	IgG	0.33	0	0	1.07	w	0	mild
	30	none	IgM	0.32	0	0	0.90	w	0	no
	31	none	IgG+IgA	0.18	0	0	1.17	+	0	no
	32	none	IgA	0.38	0	0	1.12	w	0	no
	33	mixed	IgG+IgM+IgA	0.45	0	0	2.73	++	0	yes
Other neoplasms	34	none	IgG+IgA	0.23	0	0	2.06	++	0	no
	35	mixed	IgG+IgM	0.63	0	0	2.70	++	0	yes
Collagen diseases										
Rheumatoid arthritis	36	none	negative	0.24	0	0	1.94	+++	0	no
	37	warm	IgG+IgA	0.33	0	0	1.25	w	0	no
	38	warm	IgG+IgA	0.19	0	0	0.76	0	0	no
	39	warm	IgG	0.15	0	0	0.58	0	0	no
	40	warm	IgG+IgM	0.15	0	0	2.96	+++	w	yes
Sjögren's syndrome	41	none	IgG	0.50	0	0	1.40	+	0	no
Pregnancy	42	mixed	negative	0.27	0	0	0.99	w	0	no
	43	warm	IgG+IgA	0.32	0	0	1.54	+	0	no
	44	cold	IgM	2.56	+	w	3.11	++++	+	yes

Table 1 continued.

			Results of direct antiglobulin tests*							
		Warm/cold/	Immunoglobulins	C3b			C3d			
Condition	Patient number	mixed type autoantibodies	Enzyme-linked	Enzyme-linked	Polyclonal agglutination	Monoclonal agglutination	Enzyme-linked	Polyclonal agglutination	Monoclonal agglutination	Hemolysis present
Miscellaneous conditions										
Sickle cell disease	45	warm	IgG	0.20	0	0	0.89	0	0	no
ß thalassaemia	46	none	IgG	0.15	w	0	1.35	+	0	no
Crohn's disease	47	warm	negative	0.12	0	0	0.53	0	0	no
Gilbert's syndrome	48	none	IgM	0.15	0	0	0.44	0	0	yes
Ulcerative colitis	49	warm	IgG+IgA	0.18	0	0	0.71	0	0	no
Anemia of unknown origin	50	warm	IgG+IgA	0.29	0	0	0.48	0	0	no
	51	none	negative	0.32	0	0	1.70	+	0	no
	52	warm	IgG	0.90	0	0	1.47	0	0	no
No details given	53	cold	IgM+IgA	0.22	0	0	0.42	0	0	yes
	54	none	negative	0.19	0	0	0.74	0	0	no
	55	cold	negative	0.24	0	0	1.04	w	0	no
	56	none	IgM	0.26	0	0	1.06	w	0	yes
	57	warm	IgG+IgA	0.18	0	0	0.64	w	0	no
	58	cold	IgM	0.20	0	0	3.08	++++	+	yes
	59	warm	IgG	0.56	0	0	2.56	++	0	no
	60	warm	IgG	0.40	0	0	1.68	w	0	no

*Enzyme-linked method: results given in ODU for a standard red cell count of 4.5×10^7; values underlined denote a positive result, i.e., are more than 3 standard deviations above the normal mean value. Spin agglutination methods (polyclonal and monoclonal agglutination): 0 = negative result, w = positive result detectable microscopically, + to ++++ = strength of macroscopic reaction

results were expressed in molecules per cell rather than in absorbance units,[23-28] but there was noticeable variation between the different investigations that perhaps reflected the specificity of the reagents used.[29] In addition, any apparent advantage of presenting results in absolute values may be false, as the figures were based on some questionable methodological assumptions. For example, in the enzyme-linked assays that measured C3 release by lysis,[23,24] errors could have occurred because a significant portion of C3 remained bound to the RBC debris or the complement antigens were altered or lost. Also, if standards were made in simple buffers[23] and compared with test material in lysates, differences in milieu could have produced nonspecific effects that modified the kinetics of antigen:antibody reactions.[30] In the studies that used radioimmunoassay,[25-27] a binding ratio for the C3:anti-C3 reaction had to be determined. With monoclonal antibodies, this was assumed to be 1:1,[25-27] but later work showed that binding ratios varied with the amount of immunoprotein bound to the RBCs, even with monoclonal reagents.[31]

Several possible reasons have been put forward to explain why RBCs of healthy individuals are coated with small amounts of C3. These RBCs probably reflect a continuing low-grade activation of complement[32,33] either in response to the immunologic stresses encountered in everyday life or in the spontaneous activation of the alternative pathway, which is part of the normal physiological mechanism necessary for the maintenance of homeostasis of a variety of physiological systems. For example, it is thought that old or damaged RBCs are removed from circulation by the mononuclear phagocyte system via selective binding of IgG autoantibodies directed against a neo- or senescent-cell antigen, which is formed by oxidative modification of a band 3 protein.[34,35] However, concerns have been raised that the low amounts of IgG on old RBCs are insufficient to mediate effective erythrophagocytosis, and it has been suggested that the role of IgG in this situation is to activate complement, which becomes the main effector of erythrocyte sequestration.[36,37] In addition, a major function of RBCs is the provision of a transport and processing system during the elimination of a wide variety of substances from the peripheral blood, including immune-complexes, bacteria,

Table 2. Results of population proportion studies in the 60 patients

Populations tested	Results of direct antiglobulin tests: Positive	Negative	Chi-square	p
Red cell C3b				
ELDAT	22	38		
			12.23	< 0.001
Polyclonal agglutination	5	55		
			0.13	> 0.05
Monoclonal agglutination	3	57		
Red cell C3d				
ELDAT	43	17		
			0.61	> 0.05
Polyclonal agglutination	38	22		
			27.4	< 0.001
Monoclonal agglutination	9	51		
Hemolysis/no hemolysis				
C3b ELDAT				
hemolysis	12	16		
			0.44	> 0.05
no hemolysis	10	22		
C3d ELDAT				
hemolysis	19	9		
			0.11	> 0.05
no hemolysis	24	8		

viruses, and abnormal cells. Circulating immune complexes, for instance, activate complement and the antigen-antibody-C3 unit binds to RBCs via CR1 receptors.[38-41] C3 bound to normal RBCs may also result from nonspecific adsorption from the plasma[42] or it may be a response to activation of other proteolytic pathways (e.g., coagulation, fibrinolysis, or kallikrein-kinin).[43]

In the 60 patients studied, significant differences were found in the number of positive results obtained for C3b using the enzyme-linked and agglutinating DATs (see Table 2), showing that the latter were clearly less sensitive than the ELDAT. For C3d, there were no significant differences comparing the ELDAT and polyclonal agglutination test, but significant differences were present between the results for the different agglutinating reagents (see Table 2). The polyclonal agglutinating reagent was very good and gave similar findings to the ELDAT (see Tables 1 and 2); this was not surprising because both methods employed antibodies that recognized similar epitopes. The sensitivity of the monoclonal anti-C3d was poor (see Table 1) and pilot studies had shown that the reagent did not react with C3d,g-coated RBCs, which implied that it recognized an epitope only on C3d. Its value for routine use is considered wholly to be its potency. However, since C3d,g is the major in vivo breakdown product of C3, the limited specificity of this reagent must be viewed as a disadvantage.[44,45]

The ELDAT is more objective than agglutination methods and therefore is very useful for assessing the sensitivity and specificity of anti-complement reagents. For example, many patients had a weakly positive DAT result with the polyclonal agglutinating anti-C3d, and, using the ELDAT, we demonstrated that these reactions were in fact due to slightly raised levels of cell-bound C3d (see Table 1), thus confirming the sensitivity and specificity of the agglutination reagent.

With the ELDAT, 22 of the 60 patients had increased amounts of RBC-bound C3b and 43 had raised levels of C3d (see Table 1). These findings were not unexpected and presumably reflected the sensitivity of the assay and, particularly in the case of C3b, the fact that the blood samples had been specifically selected from patients with warm and cold autoantibodies. The increased levels of C3b (see Table 1) indicated that the RBCs were being detected before they could be bound to mononuclear phagocytes and before the C3b could be degraded to C3d,g.[46] This most likely resulted from either ongoing hemolysis and immediate complement activation or to a slower activation and decay process, probably due to bacterial or viral infection. Nearly all patients with increased levels of C3b also had raised amounts of C3d; those few individuals with positive reactions in the spin agglutination DATs for C3b all had absorbance values of > 1.3 ODU for C3d in the enzyme-linked test (see Table 1). Raised levels of C3d did not correlate with any particular clinical

condition but rather seemed to parallel the severity of the illness.

There are many reasons for finding complement on RBCs; previous studies noted that increased RBC-bound complement was relatively common in hospitalized patients if sensitive detection methods were used.[25,26,29] In the majority of cases, the reason for the increased level was unclear,[25] but autoimmune hemolytic anemia was unusual.[29] Patients with moderately raised RBC C3d tended to have diseases in which complement was thought to be activated;[10] in patients with systemic lupus erythematosus, rheumatoid arthritis, and other collagen diseases, the increased levels could have been due to immune complexes[13,47] or to an association with immunoglobulins nonspecifically adsorbed onto the RBCs.[48] Relatively few of our patients suffered from collagen diseases (see Table 1). Patients with markedly elevated C3d and a positive agglutination DAT usually had autoimmune hemolytic anemia; and in individual cases, C3d levels correlated with both the severity of the disease and the response to treatment.[10]

The presence of complement on RBCs was not a predictor of hemolysis (see Table 2), a finding that was consistent with previous studies.[10,25] However, the present series of patients was relatively small and was selected to provide cases with a wide range of RBC-bound complement levels rather than cohorts with and without active hemolysis. Further work is clearly necessary to examine the complex interrelationships between complement levels and RBC destruction, perhaps using cold hemagglutin disease as a model, because immunoglobulin probably has only a minor role in the destruction mechanism. The quantitative ELDAT method should prove a very useful tool in this respect.

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