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Study of the Optimal Reaction Conditions for Assay of the Mouse Alternative Complement Pathway

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The optimal reaction conditions for hemolytic assay of alternative complement pathway activity in mouse serum were investigated. A microtiter system was used, in which a number of 7.5×10^6 rabbit erythrocytes per test well appeared to be optimal. Rabbit erythrocytes were superior as target cells over erythrocytes from a number of other animal species. The optimal conditions were as follows: an incubation temperature of 39°C, an ionic strength of about 200 mM, and a magnesium concentration of 2.5 mM. Incubation during 60 min was not sufficient for an end-point titration. Addition of 1 mg of zymosan A per test well, however, enhanced and accelerated the hemolytic activity of mouse serum via the alternative pathway resulting in a maximum value after 45 min. This, most probably, proceeded by a mechanism involving the formation of a zymosan-C5-convertase and bystander lysis of the target cells. In contrast to the normal alternative pathway assay the zymosan-potentiated test did, most probably, not involve natural antibodies. Cobra venom factor was more efficient in enhancing the sensitivity of the assay for the mouse alternative complement pathway than zymosan. This makes this factor very useful for testing C-poor body fluids.

Key words: *cobra venom factor – complement, mouse – zymosan – alternative pathway*

Introduction

Mouse serum has an only partially understood defect in classical complement (C) pathway activation as observed in the lysis of sensitized sheep erythrocytes in vitro (Brown, 1943; Rosenberg and Tachibana, 1962; Atkinson et al., 1980; Van Amerongen et al., 1982). Such erythrocytes are efficiently lysed by human and guinea pig serum and currently in use for assay of the classical C pathway (Mayer, 1961). Also the C-dependent, 'acute' rejection of skin allografts in mice is reduced (Berden et al., 1978b).

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Shortly after the discovery that human serum is able to lyse rabbit erythrocytes (RaE) via the alternative C pathway (ACP) (Platts-Mills and Ishizaka, 1974), however, it became clear that mouse serum is also able to do so and that this phenomenon can be used for assay of the ACP (Joiner et al., 1979; Van Dijk et al., 1980; Miletic et al., 1982; Klerx et al., 1983).

Although the different authors mentioned describe some technical aspects of the mouse ACP assay, no formal optimalization studies as for the human ACP (Joiner et al., 1983) have been published. This paper concentrates on a number of variables with respect to mouse ACP activation including the identity and number of target cells, the incubation temperature and time, the ionic strength and the magnesium concentration. Since hemolysis of RaE via the mouse ACP does not reach its end-point in 1 h (Joiner et al., 1979), the influence of additional ACP activators on the kinetics of mouse serum-induced lysis of RaE was investigated.

Materials and Methods

Blood donors

F1 (BALB/c × Swiss inbred) mice, bred and maintained in our institute, were the regular donors of mouse serum. In 1 experiment also members of the parent strains and C3H/HeJ and C3HeB/FeJ mice, obtained from the Jackson Laboratories (Bar Harbor, ME, U.S.A.), and DBA/2 mice from Bomholtgard (Ry, Denmark) were used. Only male mice were used at an age between 10 and 15 weeks.

Female F1 (New Zealand White × Flamish Giant) rabbits were obtained from the Central Institute for Breeding of Laboratory Animals (CPB, Zeist, The Netherlands) and used as donors of RaE when 3–5 kg of weight.

Sera

Mice were bled by orbital puncture under ether narcosis. After clotting and storage of the blood for 1.5 h at 20°C, serum was separated by centrifugation, and used immediately. Pooled sera of at least 10 animals were used throughout the experiments.

Buffers

Unless otherwise stated, a buffer containing 5 mM veronal, 150 mM NaCl, 8 mM ethyleneglycol-bis(2-aminoethyl)tetraacetic acid (EGTA) and 2.5 mM magnesium was used (EGTA-VB; calculated ionic strength 205 mM). In the ionic strength experiment mixtures of EGTA-VB and EGTA-VGB, containing 10% of glucose instead of 150 mM NaCl (ionic strength 55 mM) and hypertonic EGTA-VB were used.

Erythrocytes

Blood withdrawn from the ear artery of a rabbit was diluted 1 : 1 in Alsever's old (citrate) solution and used as source of RaE. The cells were washed 3 times with 0.16 M sodium iodide to remove nonspecifically adsorbed serum proteins (Najjar, 1974;

Fyhrquist and Wallenius, 1975; Van Dijk et al., 1980a), suspended to a concentration of 2% (v/v) in EGTA-VB, and photometrically adjusted to a concentration of 1.5×18^8 per ml.

The origin of erythrocytes of other species was as described earlier (Van Dijk et al., 1983).

Neuraminidase treatment of erythrocytes

Three times washed erythrocytes were treated with 'test'-neuraminidase (from *Vibrio cholerae*, 1 U/ml, Behringwerke AG, Marburg, F.R.G.) by mixing 200 μl of packed cells with 800 μl of the neuraminidase and incubation at 37°C for 2 h (Rademaker et al., 1981). Before use in the C assay the cells were washed 3 times with saline and resuspended in EGTA-VB.

Hemolytic complement assay

The assay to test mouse serum ACP activity was as recently described by Klerx et al. (1983). In brief: in microtiter dishes with U-shaped wells (Greiner Labortechnik, Nürtingen, F.R.G. no. 650101) logarithmical (171 μl/271 μl = $10^{-0.2}$) serum dilutions of 100 μl were prepared using a multichannel pipette and, unless otherwise stated, normal EGTA-VB. After addition of 50 μl of target cells (normally 1.5×10^8 of RaE per ml EGTA-VB) and mixing on a plate shaker, the dishes were covered with transparent plastic tape and incubated in a waterbath (usually 39°C for 60 min). The intact cells and debris were spun down and 30 μl of the supernatant were transferred to the wells of flat-bottomed microtiter dishes (Greiner, no. 655101) all containing 220 μl of water. The plates were shaken and the A_{405} (I) was determined in an automated ELISA-reader (Titertek 310 C). The percentage of lysis (Y) was calculated by:

$$Y = \frac{\mathrm{I} - \mathrm{II}}{\mathrm{III} - \mathrm{IV}} \times 100\%,$$

wherein II refers to the A_{405} of a similarly prepared supernatant of heat-inactivated (56°C, 30 min) serum (control for hemoglobin content) and III and IV to water-lysed (100% lysis) and buffer controls (0% lysis), respectively. To determine the amount of serum corresponding to 50% hemolysis (1 AP50 unit) (Takada et al., 1978) Y-values were transformed according to Van Krogh (Mayer, 1961). The ACP activity of the mouse serum was expressed in AP50 units per ml. All experiments were performed in duplicate and repeated at least twice.

Alternative pathway activators

In 1 experiment mouse ACP levels were also determined in the presence of 1 mg of zymosan A from *Saccharomyces cerevisiae* or 1 U of cobra venom factor (CoF) per test well. The doses used were based on previous dose-response curves. Both zymosan and the starting material for the preparation of CoF (*Naja naja* venom) were obtained from Sigma (St. Louis, MO, U.S.A.). To purify CoF from the crude venom the procedure of Ballow and Cochrane (1969) was followed.

Absorption of mouse serum

To investigate whether natural antibodies to RaE or zymosan could play a role in the hemolytic activities of mouse serum as determined in the absence and presence of zymosan, respectively, mouse serum was submitted to absorption. For this purpose the serum was mixed and put at 4°C with either 25% (v/v) of 3 times washed, packed RaE or 1% (w/v) of sonicated and subsequently washed zymosan for 20 min. After this procedure serum was liberated from cells by centrifugation and micropore (0.45 μm) filtration.

Statistics

Results of similar experiments were arithmetically averaged and the standard of the mean (SEM) was determined. For significancy analysis Student's *t*-test was used. The coefficient of correlation was calculated by the method of least squares.

Results

Influence of erythrocyte variation

The possibility to use other target cells than RaE in the assay of mouse ACP activity was investigated. It appeared that of the untreated cells tested only horse

TABLE I

ALTERNATIVE COMPLEMENT PATHWAY ACTIVITY IN MOUSE [a] SERUM AS DETERMINED WITH DIFFERENT ERYTHROCYTES

Normal erythrocytes	ACP activity (AP50 U/ml)	Neuraminidase-treated erythrocytes	ACP activity (AP50 U/ml)	Control-treated erythrocytes	ACP activity (AP50 U/ml)
Cat	< 10 [b]	[c]			
Chicken	< 10				
Cow	< 10				
Dog	< 10				
Goat	< 10				
Guinea pig	< 10				
Horse	11	Horse	< 10	Horse	11
Human	< 10				
Mouse [a]	< 10	Mouse	< 10	Mouse	< 10
Pig	< 10				
Rabbit	150 ± 15 [d]	Rabbit	152 ± 7 [e]	Rabbit	148 ± 15 [e]
Rat	< 10				
Sheep	< 10	Sheep	52	Sheep	< 10

[a] F1 (BALB/c × Swiss inbred) mice were used.
[b] The detection limit of the assay is 10 AP50 units per ml.
[c] Open places indicate that the erythrocytes were not tested.
[d] Mean ± SEM (n = 10).
[e] n = 3. The difference between neuraminidase- and control-treated RaE was not significant ($P > 0.5$).

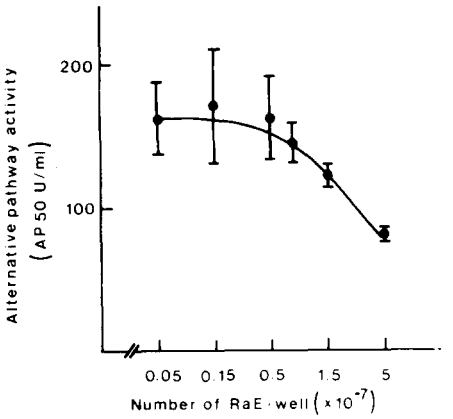

Fig. 1. The effect of varying the number of target cells on F1 (BALB/c × Swiss inbred) mouse serum ACP activity. Vertical bars indicate the SEM. Only the value for 5×10^7 was significantly different from the other values ($P < 0.05$ to < 0.001).

and rabbit erythrocytes were significantly sensitive to lysis by mouse serum via the ACP (Table I). The ACP activity measured with horse erythrocytes, however, was only 7.3% of that determined with RaE. Treatment of horse, mouse, rabbit and sheep erythrocytes with neuraminidase had a differential effect on the sensitivity of the cells to lysis mediated by the mouse ACP. Horse erythrocytes became less sensitive, mouse erythrocytes remained resistant, RaE became slightly more sensitive, whereas sheep erythrocytes were converted from non-activating to activating cells by neuraminidase treatment (Table I). Since RaE were the most sensitive of all erythrocytes tested and neuraminidase did not enhance their sensitivity significantly, only normal RaE were used as target cells in further experiments.

Decreasing and increasing the RaE concentration resulted in a rise and fall of apparent ACP activity, respectively (Fig. 1). Enhanced sensitivity of the assay at lower erythrocyte concentrations, however, showed a concomitant greater inaccuracy. The original number of 7.5×10^6 cells per well seemed to be rather optimal and was consequently also used in further experiments.

Effect of incubation temperature

The influence of the incubation temperature on mouse ACP activity was studied in the range of 31–41°C. A peak was found at 39°C while at both sides of the peak values decreased rather sharply (Fig. 2). Further incubations were performed at 39°C.

Influence of ionic strength and magnesium concentration

The effects of varying the ionic strength and the magnesium concentration on mouse ACP levels were investigated. As shown in Fig. 3 the optimal ionic strength and magnesium concentration were about 200 and 2.5 mM, respectively.

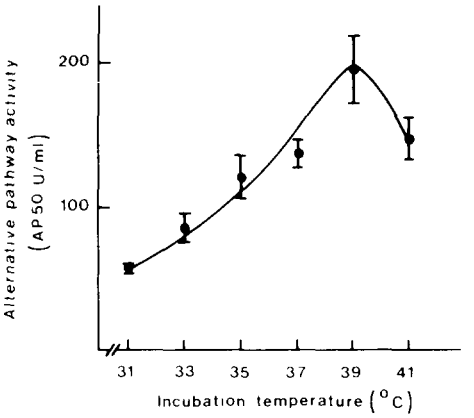

Fig. 2. Influence of the incubation temperature on F1 (BALB/c×Swiss inbred) mouse serum ACP activity. Vertical bars indicate the SEM. The value of 39°C is significantly different from that of 37°C and 41°C (P both < 0.05).

Kinetics of hemolysis in the absence and presence of additional ACP activators

The influence of the incubation time on the outcome of mouse ACP titrations was investigated. As shown in Fig. 4 titers increased with the time till a plateau was reached at 4 h. Since incubation times of 4 h were very impractical, other means were sought to accelerate the hemolytic process. It had been observed earlier that addition of particulate ACP activators such as *Listeria monocytogenes* and inulin might enhance the hemolytic activity of mouse serum via the ACP (Van Kessel et al., 1981). Therefore, the influence of these activators and of zymosan on the kinetics of lysis of RaE by mouse serum via the ACP was investigated. *Listeria monocytogenes* and inulin had a moderate stimulatory effect in the mouse ACP assay (data not

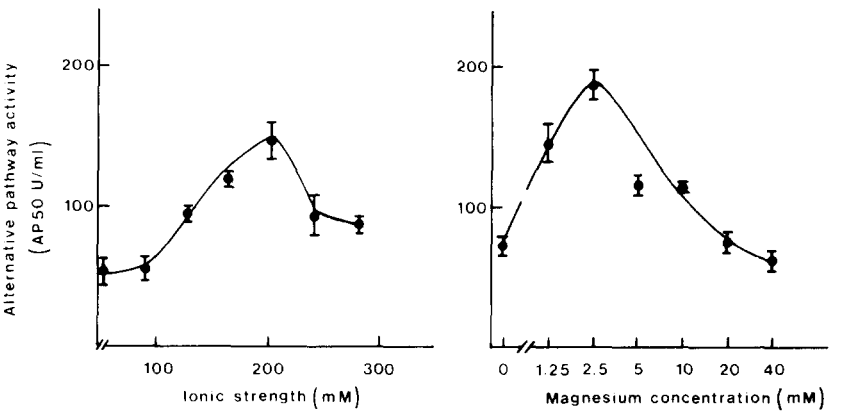

Fig. 3. Influence of varying the (calculated) ionic strength and magnesium concentration on F1 (BALB/c×Swiss inbred) mouse serum ACP activity. Vertical bars indicate the SEM. The values for 205 and 2.5 mM respectively were significantly different from the other values ($P < 0.05$ to < 0.001).

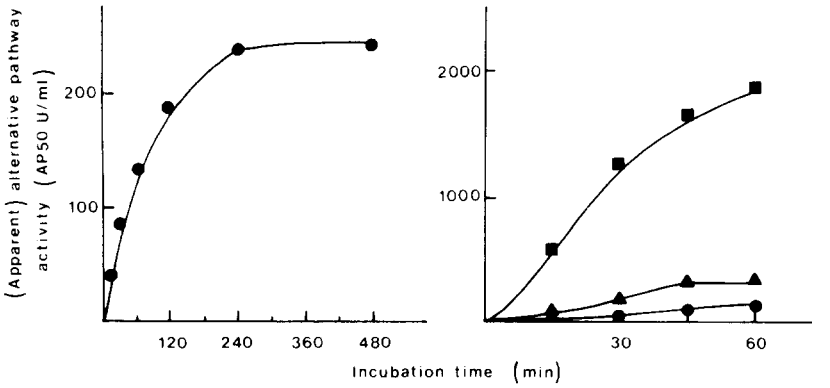

Fig. 4. Effects of the incubation time and additional C activators on F1 (BALB/c × Swiss inbred) mouse serum ACP levels. Symbols refer to the normal ACP assay (●) and to assays potentiated by 1 mg of zymosan (▲) and 1 U of CoF (■) per test well. The mean values of 3 independent experiments were represented. The relative SEMs of the potentiated assays were similar to that of the normal ACP assay (8%).

shown). At an optimal dose of 1 mg per test well, however, zymosan did not only enhance but also accelerate lysis of RaE via the mouse ACP (Fig. 4). In this case a plateau was reached at 45 min of incubation. The maximal titer reached in the presence of zymosan was about 25% higher than the end-point titer obtained with RaE only.

Since the phenomenon of zymosan-induced increase and acceleration of hemolytic activity was very likely to be due to bystander lysis of the erythrocytes, another inducer of this phenomenon, CoF, was also tested for enhancing and accelerating activity. As also shown in Fig. 4, CoF caused a 13-fold increase in hemolytic activity, but no measurable acceleration (not shown).

TABLE II

EFFECT OF ABSORPTION OF MOUSE [a] SERUM ON ACP-MEDIATED HEMOLYTIC ACTIVITIES

Mouse serum	Apparent ACP activity (AP50 U/ml)	
	Testing particles	
	RaE only	RaE + zymosan
Untreated	157 ± 12 [b]	298 ± 15 [c]
RaE-absorbed	127 ± 10	324 ± 14 [c]
Zymosan-absorbed	80 ± 17	179 ± 38

[a] F1 (BALB/c × Swiss inbred) mice were used as serum donors.
[b] Mean ± SEM (n = 4).
[c] The increase after RaE-absorption is not significant ($P > 0.05$).
All other data are significantly different ($P < 0.05$).

TABLE III

COMPARISON OF THE CoF-POTENTIATED WITH THE ORIGINAL ACP ASSAY

Serum donors	ACP activity (AP50 U/ml)	
	Original	CoF-potentiated
BALB/c	124 [a]	1500
C3H/HeJ	118	1381
C3HeB/FeJ	98	1368
DBA/2 [b]	< 10	< 10
F1 (BALB/c × Swiss inbred)	175	1769
Swiss inbred	176	1465
		Coeff. of corr.: 0.918–0.929 [c]

[a] Mean of 2 entirely independent observations in duplicate.
[b] C5-deficient.
[c] Dependent on the values for DBA/2 serum.
The correlation between the 2 assays is significant ($P < 0.01$).

Role of antibodies in the ACP-mediated lysis of RaE in the absence and presence of zymosan

The role of natural antibodies to RaE and zymosan in the lysis of RaE by mouse serum via the ACP was studied by testing the hemolytic activities of untreated and absorbed mouse serum in the normal and zymosan-potentiated ACP assay. In both tests an incubation time of 60 min was used. As shown in Table II absorption with RaE decreased the ACP-mediated hemolytic activity in the normal ACP assay and caused a non-significant, but reproducible stimulation of hemolytic activity in the assay with zymosan. Prior absorption with zymosan caused proportional decreases in the hemolytic activities in both assays.

Comparison of the normal and CoF-potentiated mouse serum ACP assay

Sera of 6 genetically different groups of mice were tested in the normal and CoF-stimulated ACP assay using an incubation time of 60 min. A significant, positive correlation between the results in both assays was observed (Table III).

Discussion

The optimal reaction conditions for hemolytic assay of mouse ACP activity were investigated using pooled serum of male F1 (BALB/c × Swiss inbred) mice as C source. Of heterologous erythrocytes of 12 different species only RaE appeared to be useful as target cells. The sensitivity of only RaE confirms earlier studies wherein 3 types of erythrocytes, namely those of mice, rabbits and sheep were compared (Van Dijk et al., 1980a). Sheep erythrocytes became sensitive on neuraminidase treatment which is in line with results of Fearon (1978) and colleagues (Kazatchkine et al., 1979) regarding the human ACP. The decrease in sensitivity to ACP-mediated lysis

of horse erythrocytes after incubation with neuraminidase may be explained by sialic acid-specific natural antibodies present in mouse serum. We could not confirm the findings of Joiner et al. (1979), who described an 8–10-fold increase in sensitivity of RaE to lysis by the mouse ACP upon pretreatment with neuraminidase. These authors, however, used a chromium-release test, which possibly could explain the discrepancy. Our results are concordant with a low substitution of untreated RaE with sialic acid (Aminoff et al., 1976).

The independence of the response on the target cell concentration, as far as low numbers of RaE were concerned (Fig. 1), is in contrast to the increasing sensitivity of the assays for classical pathway (Berden et al., 1978a) and total C (Van Dijk et al., 1980b) activity in mouse serum. It is in line with the idea that, in case of the ACP, rather spontaneous than cell-induced activation is involved. The number of 7.5×10^6 RaE per well, corresponding to 1.5×10^7 per test tube in the semi-microassay (Van Dijk et al., 1980a), appeared to be optimal when both response and accuracy were concerned.

The optimal incubation temperature of 39°C for mouse ACP activity determination is high when compared to the 37°C used by others for the human ACP (Takada et al., 1978; Joiner et al., 1983); 37°C appeared also in our microsystem the optimal temperature for human serum. Furthermore, it is high in relation to the normal body temperature of mice which is 37°C (Bernstein, 1966). This may suggest that the ACP functions more efficiently at elevated temperatures and could, perhaps, account for the enhanced resistance of mice to bacterial and viral infections at higher temperatures (reviewed by Ashman and Müllbacher, 1984).

In contrast to the results of Joiner et al. (1983) with respect to the human ACP we found an optimal ionic strength of about 200 mM. The optimal ionic strength for the human ACP was also in our hands much lower (Joiner et al., 1983; unpublished). The optimal magnesium concentration, however, is in the same order as for the human ACP (Joiner et al., 1983). The concentration of 2.5 mM magnesium is half the concentration published before (Klerx et al., 1983) which was optimalized in the semi-microassay. The difference between the 2 optima may be due to surface effects.

Also with respect to the optimal incubation time the human and mouse ACP behave different. The human ACP-mediated lysis of RaE reaches a plateau at 60 min (Joiner et al., 1983). In mouse serum it lasted 4 h before a constant level was achieved. A similar observation was done by Joiner et al. (1979). We found, however, that the incubation time could be shortened to 45 min when 1 mg of zymosan per test well was added. This accelerating effect of zymosan must be ascribed to the rapid formation of a zymosan-C5-convertase which, indirectly, gives rise to bystander lysis of the target cells. A similar phenomenon was described by us for the ACP activators *Listeria monocytogenes* and inulin (Van Kessel et al., 1981). Prior absorption of mouse serum with RaE or zymosan on ACP titrations in the absence and presence of zymosan gave information on the possible role of natural antibodies in mouse ACP activity. The suppressive effect of RaE absorption on normal mouse ACP activity may point to the presence and importance of natural antibodies to RaE. This was noticed before (Van Dijk et al., 1980a; Miletic et al., 1983). The slightly stimulatory effect of RaE absorption on mouse ACP activity determined in

the presence of zymosan must be explained by the inhibitory activity of RaE-specific antibodies on the bystander hemolysis (Van Kessel et al., 1981). The proportional depleting effect of zymosan absorption on mouse ACP levels determined in the presence and absence of zymosan suggests that natural antibodies to zymosan do not play a predominant role in the mouse ACP titration in the presence of zymosan. This would discern mouse serum from human serum (Schenkein and Ruddy, 1981). Antibody independence is also an advantage of the zymosan-potentiated over the normal mouse ACP assay.

CoF did not accelerate the lysis of RaE via the mouse ACP, as did zymosan, but it enhanced it to a higher degree. The mechanism by which this is thought to occur is the formation of a fluid phase CoF-C5-convertase, which gives, again, indirectly rise to the phenomenon of bystander hemolysis (Pickering et al., 1969; Vogel and Müller-Eberhard, 1984; Klerx et al., 1985). Obviously, the generation of the CoF-C5-convertase proceeded more slowly than the zymosan equivalent. Moreover, the mechanism by which CoF activates the C-cascade is different from that by RaE and zymosan. For the mouse system it was shown that the formation of the CoF-C5-convertase is rather independent on C3 and goes on in the presence of EDTA (Goldman et al., 1979). Despite the different reaction mechanisms, however, ACP levels in 6 mouse inbred strains determined in the presence and absence of CoF showed a significant correlation. On the other hand, in our system EDTA reduced the lytic activity of mouse serum towards RaE in the presence of CoF for more than 90% (data not shown) pointing to, at least, an enhancing role of magnesium in the CoF-potentiated ACP assay. With respect to the magnesium concentration it must be stressed that 2.5 mM is not necessarily optimal for the CoF-induced enhanced hemolytic activity of mouse serum. Whatever the optimal magnesium concentration and other reaction conditions, however, the enhancing activity of CoF in the mouse ACP assay can be very welcome in determining C levels at immunologically relevant, C-poor body sites, such as the peritoneal cavity. It is clear that, at least for the mouse ACP assay, CoF does not need to be covalently coupled to the target cells as described for guinea pig and rabbit C (Okada and Tanaka, 1981).

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