Temperature Dependence of Plasmin-Induced Activation or Inhibition of Human Platelets

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It is known that at 37°C plasmin may have two opposite effects on platelets: at high concentrations (>1.5 caseinolytic units [CU]/mL), plasmin activates platelets; at lower concentrations (0.1 to 1.0 CU/mL) it inhibits platelet activation induced by thrombin, collagen, or calcium ionophore A23187. In this study, we report that when lowering the incubation temperature to 22°C, plasmin at low concentrations (0.1 to 0.5 CU/mL) fully activated platelets. When platelets were treated with 0.2 CU/mL of plasmin, lowering the incubation temperature from 37°C to 22°C resulted in an increase in the expression of fibrinogen receptors, in platelet release and aggregation. Thromboxane A2 was not generated by plasmin treatment at either temperature. Ultrastructural studies showed that platelets responded to low-dose plasmin at 37°C by forming pseudopods, centralizing granules without fibrinogen release, whereas at 22°C the same dose of plasmin caused platelet degranulation with the appearance of α-granule fibrinogen within the lumen of the surface connected canalicular system. In addition, at 22°C plasmin at doses insufficient to induce platelet aggregation potentiated platelet response to thrombin. Thus, we suggest that plasmin may initiate both activating and inhibitory processes within platelets and that the change of temperature could influence this balance. These results may be of clinical relevance, because the fibrinolytic system was found activated during cardiopulmonary bypass in which the temperature of patient’s blood circulation was reduced. This temperature-dependent behavior is also an interesting model for a further study on platelet response to serine proteinases.

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Materials

Human fibrinogen (grade L) and purified plasmin (15 CU per milligram of protein, 1.27 CU/mL equal to 1 μmol/L)

MATERIALS AND METHODS

Materials

Human fibrinogen (grade L) and purified plasmin (15 CU per milligram of protein, 1.27 CU/mL equal to 1 μmol/L)
were purchased from Kabi Laboratory (Stockholm, Sweden). The protein concentration in these preparations was measured using a protein assay kit (Sigma, St Louis, MO) with the Lowry procedure. Bovine thrombin (70 U/mg) was purchased from F. Hoffmann-La Roche Co Ltd (Basel, Switzerland). Aprotinin was from Bayer Parma (Paris, France). Biotin-N-hydroxysuccinimide ester was purchased from Calbiochem (San Diego, CA) and Streptavidin-phycocerythrin (PE) (1:5 diluted) was added to the mixture for another 15 minutes of incubation. The samples were then diluted with Tyrode buffer and fixed with an equal volume of phosphate-buffered saline (PBS) containing 10 mmol/L EDTA and 0.2% glutaraldehyde. Without further washing, the samples were directly analyzed in a Becton Dickinson (Sunnyvale, CA) FACStar flow cytosorter with a matched computer. The platelets were distinguished from debris or “machine noise” and from red blood cells and white blood cells by setting the appropriate forward scatter and side scatter window with a side scatter threshold. PE fluorescence was detected with a 585/42 filter and fluorescence data were obtained with gain setting in the logarithmic mode. Activated platelets appeared in the same forward/side scatter window as nonactivated platelets. The microscopic examination of strongly fluorescent platelets sorted in the machine showed that more than 97% of activated platelets remained in individually separate forms. The expression of antigen on the platelet surface was represented as a mean channel number (MC) of platelet fluorescent intensity after the calculation with the computer program. The data were then calculated using the following formula for plotting:

\[
\text{Specific Fluorescence} \% = \frac{\text{MC of Tested Sample} - \text{MC of Negative Control}}{\text{MC of Positive Control} - \text{MC of Negative Control}} \times 100
\]

**Binding of PAC-1 and AC1.2 to platelets and differential releases of platelet granules.** The release of the contents of α-granules, dense granules, and lysosomes and the expression of both fibrinogen receptors and PADGEM on the platelet surface were simultaneously measured as a function of the plasmogen concentration used to activate platelets. One part of ACD anticoagulated PRP was preincubated with \(^{14}\)C-serotonin at 0.6 µmol/L (0.37 kBq/mL PRP) for 30 minutes at room temperature. Then platelets were isolated and washed as mentioned above and resuspended in Tyrode buffer at a concentration of 1.6 \(\times 10^9\)/mL. Three hundred microliters of platelets was incubated for 5 minutes (except for time-dependent experiments) with an equal volume of Tyrode buffer or plasmogen solution at various concentrations. Then plasmogen activity was inhibited by aprotinin at a final concentration of 50 µmol/L. Ten microliters of each sample was added to both PAC-1 and AC1.2 binding assay tubes as described above. EDTA (10 mmol/L) was added to the residual samples and these samples were immediately centrifuged at 4,000g for 3 minutes for the quantifications in the supernatants of β-TG by enzyme-linked immunosorbent assay (Diagnostica Stago, Asnières, France) and N-β-acetyl glucosaminidase (NAG) activity.\(^{24}\) The amount of released β-TG and NAG was expressed as a percentage of the total content in the original platelet suspension lysed with 0.1% Triton X-100 (Touzart et Malignon, Vitry/Seine, France). After the treatment of \(^{14}\)C-serotonin preloaded platelets, 1% formaldehyde was added to stop the release and the platelets were centrifuged. The \(^{14}\)C-serotonin in the supernatants was counted and expressed as a percentage of the total content in the original platelet suspension.

**Measurement of thromboxane B\(_2\).** Washed platelets were exposed to plasmogen at different temperatures for the indicated times. In some experiments, thrombin was added to plasmogen-treated platelets followed by an incubation for another 3 minutes. After an agitation for 3 minutes, the reaction was stopped by the addition of EDTA at 10 mmol/L (final concentration) followed by an immedi-
ate centrifugation at 4°C. Then thromboxane B₂ was measured in the supernatant with duplicate determinations as described.²⁵

Electron microscopy. After the exposure of washed platelets to 0.2 CU/mL plasmin (or buffer for control) at 37°C or at 22°C for 5 minutes, platelets were first fixed by 1% glutaraldehyde in Tyrode buffer, washed three times with PBS, and then alternatively post fixed with 1% osmium tetroxide, stained with uranyl acetate, and embedded in Epon (Ladd Research Industry, Burlington) for standard electron microscopy or embedded in glycol methacrylate for immunoelectron microscopy. Immunostaining for fibrinogen was performed on thin sections as described previously.²⁶ These sections were observed under a Phillips 301 electron microscope.

RESULTS

Influence of temperature on plasmin-induced platelet aggregation. At 37°C plasmin at concentrations higher than 1 CU/mL induced only weak aggregation, whereas at 22°C plasmin at lower concentrations (0.5 to 1 CU/mL) induced an immediate and strong platelet aggregation. Moreover, when preincubating platelets for 5 minutes at 22°C with plasmin before stirring in the aggregometer cuvette, the concentration of plasmin that was needed to induce platelet aggregation was reduced even further. When different concentrations of plasmin were used to activate platelets for 5 minutes at 37°C or at 22°C two dose-dependent aggregation curves were obtained (Fig 1). When platelets were treated with a fixed concentration of plasmin, the occurrence and the intensity of aggregation was temperature dependent (Fig 2). The results in Fig 2 were not modified by the addition of aprotinin and fibrinogen (0.4 mg/mL) at the end of the incubation period, indicating that the absence of aggregation at 37°C was not due to the degradation of platelet fibrinogen by plasmin.

![Fig 1. Plasmin-induced platelet aggregation at 37°C and 22°C.](image1)

Plasmin concentration (CU/ml), log scale

![Fig 2. Effect of temperature on plasmin-induced platelet aggregation](image2)

Table 1. Plasmin-Induced Release of Serotonin and N-P-Acetyl Glucosaminidase From Washed Platelets at Different Temperatures

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>37°C</th>
<th>32°C</th>
<th>28°C</th>
<th>22°C</th>
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<tbody>
<tr>
<td>¹⁴C-serotonin (%)</td>
<td>1.6 ± 1.3</td>
<td>1.9 ± 1.4</td>
<td>7.7 ± 2.3*</td>
<td>9.9 ± 6.0*</td>
</tr>
<tr>
<td>NAG (%)</td>
<td>0.4 ± 0.3</td>
<td>0.2 ± 0.7</td>
<td>1.8 ± 0.6*</td>
<td>5.9 ± 0.77</td>
</tr>
</tbody>
</table>

Washed platelets were preincubated at the indicated temperatures for 5 minutes. Then they were incubated with 0.2 CU/mL of plasmin for another 5 minutes at the indicated temperatures. After a centrifugation, released ¹⁴C-serotonin and NAG in the supernatants were measured. The data were calculated as a percentage of the total amount released from triton-lysed original platelets. Means ± SD for four different donors. The significance of the difference between means was calculated by two-way analysis of variance. *P < .05 to values obtained at 37°C. **P < .01 to values obtained at 37°C.
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Fig. 3. Enhancement of plasmin-induced expression of PADGEM antigen on platelet surface by lowering the temperatures. Washed platelets were preincubated at the indicated temperatures for 10 minutes. Then platelets were incubated with plasmin (0.2 CU/mL) or Tyrode buffer for 5 minutes. Control nonactivated platelets (A), platelets treated with plasmin at 37°C (B), at 32°C (C), or at 22°C (D). Ten microliters of each sample was used for AC1.2 antibody-binding assay. Plasmin activity was neutralized by addition of aprotinin (final concentration, 50 μmol/L) in AC1.2 binding assay tubes. Ten thousand cells were analyzed in the flow cytometer.

Effect of plasmin on the binding of PAC-1 and AC1.2 to platelets and on the degranulation of platelets at 22°C. As plasmin degrades fibrinogen, the fibrinogen receptors induced by plasmin on platelets were studied with MoAb PAC-1, which recognizes specifically the fibrinogen receptors on GP IIb/IIIa. The amount of PAC-1 bound to the platelets treated with plasmin at 22°C was compared to the platelets activated with thrombin and ADP. The three agonists were added to the platelet suspension at relatively high concentrations to get maximal expression of fibrinogen receptors. Hirudin and aprotinin were added to neutralize thrombin and plasmin activities, respectively. As shown in Fig 4, the amount of PAC-1 bound to platelets activated by plasmin was similar to that induced by thrombin and much higher than that induced by ADP. This indicates that plasmin and thrombin are capable of inducing more fibrinogen-binding sites on platelets than ADP.

Figure 5 showed that platelets released their contents from α-granules (PADGEM expression), dense granules (serotonin), and lysosomes (NAG), and expressed fibrinogen-binding sites (PAC-1–binding sites) in response to plasmin at 22°C dose dependently. Expression of PADGEM correlated well to the curve of β-TG release (not shown). The binding of PAC-1 and AC1.2 MoAbs to platelets was found to be parallel during activation induced by plasmin at 22°C (Fig 6), suggesting that during platelet activation by plasmin at 22°C there was a large exposure of fibrinogen receptors on GP IIb/IIIa initially located in α-granules, as already described for thrombin-treated platelets.

Thromboxane B2 synthesis by plasmin-treated platelets. It has been reported previously that plasmin does not induce thromboxane synthesis. Schafer and Adelman14 found that the arachidonic acid metabolism of platelets was impaired by plasmin treatment, and they suggested that this impair-

Fig. 4. Comparison of PAC-1 binding on the platelets activated by ADP, thrombin, and plasmin. Washed platelets (10⁶/ml) were incubated with Tyrode buffer (A), 40 μmol/L ADP (B), 2 U/ml thrombin (C), or 2 CU/mL plasmin (D) for 5 minutes at 22°C, then 10 μL of each sample was transferred to PAC-1 binding assay tubes, and 10,000 cells were analyzed in the flow cytometer. The indicated concentrations are the final concentrations.

Fig. 5. Effect of plasmin at 22°C on the expression of fibrinogen receptors and PADGEM on platelet surface, and on the differential release of granules from platelets: dose-response curves. Three hundred microliters of washed platelet suspension (1.6 x 10⁹/mL) was incubated at 22°C for 5 minutes with 0.3 mL plasmin (final concentrations indicated). The platelets were tested for PAC-1 and AC1.2 binding by flow cytometric analysis as described in the Materials and Methods section. The amount of bound antibodies was presented as a percentage of the highest value (mean channel number) of fluorescent intensity obtained in platelets stimulated by 1.0 U/ml thrombin. After centrifugation, the supernatants were analyzed for ³⁵S-serotonin released from dense granules and for N-acetyl β-glucosaminidase activity released from lysosomes. The data for serotonin and NAG were obtained from triplicate determination and were expressed as a percentage of the total amount released from a 0.1% Triton X-100-lysed sample of washed platelets. Similar results were obtained from two other repeated experiments.
different temperatures. The results (Table 2) indicate that plasmin did not reduce the thromboxane synthesis in plasmin-treated platelets at 37°C nor did it at 22°C. To further test the activity of the arachidonic acid metabolism in plasmin-treated platelets under our experimental conditions, thrombin was added to plasmin-treated platelets to induce thromboxane synthesis. The results in Table 3 show that plasmin did not reduce the thromboxane formation after thrombin stimulation.

Ultrastructural observation of platelets treated by plasmin at different temperatures. Electron microscopy observation of control platelets at 22°C and at 37°C showed no sign of dense bodies. At 22°C pretreatment of platelets with plasmin at concentrations lower than that inducing platelet aggregation (0.01 CU/mL) potentiated platelet aggregation by thrombin (Fig 7A and B). At 37°C all the platelets had a discoid shape, no pseudopods, the marginal band of microtubules was located in the periphery, and granules were scattered in the cytoplasm. At 22°C some pseudopods were observed. After being exposed to 0.2 CU/mL plasmin at 37°C, activation was evidenced by the loss of discoid form, pseudopod formation, and centralization of the granules surrounded by differently oriented microtubules (Fig 7C). When platelets were exposed to the same concentration of plasmin at 22°C, the morphology of platelets was different because apart from the emission of pseudopods, a process of degranulation was clearly shown: the channels of the surface connected canalicular system (SCCS) were filled with dense material, and a large number of platelets were almost empty (Fig 7D). Fibrinogen was restricted in α-granules (Fig 8A) and was absent in SCCS after plasmin treatment at 37°C. When platelets were treated with plasmin at 22°C, few fibrinogen-containing granules remained in the cytoplasm and a large amount of fibrinogen was present in the lumen of the SCCS (Fig 8B). However, dense bodies were often observed (Fig 8C).

Plasmin-induced platelet aggregation has been studied in vitro at different temperatures because platelets may be exposed to plasmin in a clinical condition, like open heart surgery using cardiopulmonary bypass.27 and in thrombolytic therapy.7 However, published results of in vitro studies indicate the inconsistent platelet responses to different concentrations of plasmin. Some investigators reported12-14 that plasmin induced platelet aggregation and degranulation. Others15-16 showed that thrombolytic agents

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Thromboxane B₂ (ng/10⁶ platelets)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal Control</td>
</tr>
<tr>
<td>Thrombin 0.06</td>
<td>36.4 ± 35.5</td>
</tr>
<tr>
<td>Thrombin 0.1</td>
<td>219.8 ± 49.2</td>
</tr>
<tr>
<td>Thrombin 0.4</td>
<td>1,688.0 ± 424.6</td>
</tr>
</tbody>
</table>

Washed platelets preincubated with Tyrode buffer in 37°C were incubated with either 0.3 CU/mL plasmin or Tyrode buffer at 37°C for 15 minutes, then aprotinin (final concentration 10 μmol/L) and thrombin were added. After agitation for 3 minutes in the aggregometer, the reaction was stopped by addition of EDTA at 10 mmol/L (final concentration), and the samples were centrifuged immediately. Thromboxane B₂ was measured in the supernatants. Means ± SD for three different donors.

**DISCUSSION**

The plasmin-induced platelet activation has been studied in vitro at different temperatures because platelets may be exposed to plasmin in a clinical condition, like open heart surgery using cardiopulmonary bypass, and in thrombolytic therapy.7 However, published results of in vitro studies indicate the inconsistent platelet responses to different concentrations of plasmin. Some investigators reported12-14 that plasmin induced platelet aggregation and degranulation. Others15-16 showed that thrombolytic agents...
Fig 7. Ultrastructure of plasmin-treated platelets at different temperatures. Washed platelets were preincubated at the indicated temperatures for 5 minutes. Then platelets were incubated with plasmin (0.2 CU/mL) or buffer at the different temperatures for 5 minutes. (A) Control platelets at 37°C: they exhibit an even and regular elliptic shape and display numerous α-granules and scant SCCS (arrows); (B) control platelets at 22°C: they show uneven shape with few pseudopods (arrowheads); (C) platelets treated with plasmin at 37°C: signs of platelet activation, e.g., numerous pseudopods (arrowheads) and granule centralization. The SCCS is empty (arrows); (D) platelets treated with plasmin at 22°C: platelet activation is intense with long and numerous pseudopods (arrowheads), less granules in the cytoplasm, and large SCCS cisternae filled with dense material (arrows). (Original magnification × 20,000.)
led to an inhibited platelet aggregation and degranulation in response to thrombin, ADP, collagen, and A23187.

Our experiments performed at 37°C confirm previous observations: platelet aggregation did not occur when incubating washed platelets with plasmin at low concentrations (<1.0 CU/mL) for 5 minutes, but did occur at plasmin concentrations higher than 1.0 CU/mL. However, we found that the change in the incubation temperature could modify the platelet response to plasmin stimulation as shown in Figs 1 and 2 with a temperature-dependent platelet aggregation, and in Table 1 showing a temperature-dependent release reaction. At 22°C with incubation, plasmin induced strong aggregation even at a low concentration (<0.1 CU/mL). We also found that at 22°C plasmin potentiated platelet response to thrombin. The decrease in platelet response to plasmin at 37°C is not related to the digestion of platelet fibrinogen or of fibrinogen receptors by plasmin. These can be excluded for two reasons: Firstly, the addition of fibrinogen plus aprotinin in aggregation tests failed to induce aggregate formation of platelets pretreated with plasmin at 37°C. Secondly, the addition of fibrinogen into platelets that were treated with a high concentration of plasmin for at least 2 hours triggered perfect aggregation, indicating that fibrinogen receptors resisted plasmin digestion. Thus, these findings demonstrate for the first time that plasmin-induced platelet activation is temperature dependent.

Our experiments performed at 22°C showed a much stronger positive response of washed platelets to plasmin than those reported in the literature in which the studies were conducted at 37°C. At a concentration of plasmin below 1 CU/mL, we showed a dose-dependent platelet aggregation accompanied by an expression of fibrinogen receptors (PAC-1 binding) and by a release of α-granules with PADGEM expression, dense granules, and lysosomes. Furthermore, at 22°C the amount of fibrinogen receptors on platelets activated by plasmin was similar to that on platelets activated by thrombin and was much higher than that on platelets induced by ADP. The fibrinogen receptors on platelet-treated platelets could be detected at plasmin concentrations below 0.05 CU/mL. Thus, plasmin can probably induce the conformational change of GP IIb/IIIa when release reaction has not yet occurred. Further exposure of PAC-1 binding sites on platelet-treated platelets was noted at the higher concentrations of plasmin and accompanied by expression of PADGEM antigen on plate-
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Fig 9. Induction of platelet aggregation by a combination of plasmin with thrombin. Washed platelets (4 × 10⁶/mL) were incubated with plasmin for 5 minutes at 22°C. Aggregation was triggered by the addition of thrombin and stirring. (A) Platelets were incubated with plasmin at a fixed final concentration (0.01 CU/mL) or buffer and platelet aggregation was triggered by various concentrations of thrombin (at indicated final concentrations). (B) Platelets were incubated with various concentrations of plasmin (at indicated final concentrations) and platelet aggregation was triggered by stirring in the absence or presence of a fixed concentration of thrombin (0.01 CU/mL). Aggregation rate was calculated with the velocity of each curve and expressed as a percentage of the velocity obtained from thrombin (0.5 U/mL) stimulated platelet aggregation. Means ± SD for six donors in each panel.

Platelet membrane, and the binding of these two MoAbs (AC1.2 and PAC-1) was parallel in a time-dependent manner. Associated with the fact that expression of PAC-1 was much lower on ADP-stimulated platelets, this result suggests that the large expression of fibrinogen-binding sites on plasmin-stimulated platelets is the result of an externalization of GP IIb/IIIa from α-granules. In addition, maximal expression of PADGEM antigen on plasmin-activated platelets was noticed when dense-granule release had hardly begun. This is similar to the result obtained in thrombin-induced platelet activation.

The results of the biologic assays were confirmed by ultrastructural observation with electron microscopy. The difference between platelets treated with the same low dose of plasmin at 37°C and 22°C was evident: at 22°C, platelet organelles were fused with the channels of the SCCS and a large amount of granules was extruded, as shown by the presence of fibrinogen in the lumen of the SCCS; at 37°C, only centralization of granules and few pseudopods were observed, but extrusion was rarely noticed. This observation suggests that the absence of platelet aggregation and the attenuated degranulation in response to low-dose plasmin at 37°C is related to an inhibited extrusion after centralization of granules.

Platelet activation is a complex process. The initiation of thromboxane synthesis in platelets is a common feedback pathway that provokes full activation. The results showed that the treatment of platelets by plasmin either at 37°C or at 22°C failed to induce the thromboxane generation, whereas a normal amount of thromboxane B₂ was obtained with plasmin-treated or nontreated platelets after thrombin stimulation. Thus, strong platelet activation induced by plasmin at 22°C was not mediated by the thromboxane B₂ pathway.

Taking our results and other published results together, we suggest that plasmin may initiate concomitantly both activating and inhibitory processes that are influenced by temperature. Adnot et al. found that plasmin may induce an elevation of the local cAMP concentration, indicating the possibility that cAMP is implicated in this inhibitory process.

Although full understanding of the biologic basis of plasmin-induced platelet activation and inhibition deserves further investigation, these results might help in understanding platelet activation during open-heart surgery using cardiopulmonary bypass (CPB). The hemostatic defect leading to heavy blood loss and prolonged postoperative bleeding in this type of surgery is a well-known clinical observation. A concomitant increase in fibrinolytic activity and platelet activation during CPB has been described. Activated platelets were detected using flow cytometric analysis during CPB. Our results lead us to suggest that platelet activation during CPB is related to the influence of temperature on platelet activation in response to generated plasmin, because the temperature of patient's blood circulation using extracorporeal circulation (ECC) is maintained around 28°C to 32°C. Tanaka et al. related the decrease in platelet count to the activation of fibrinolysis and coagulation during CPB despite the use of heparin. Our results provide further evidence that platelets are more easily activated in the presence of generated plasmin plus thrombin at the temperature maintained during CPB. As a consequence, by exposure of the receptors for fibrinogen (or) and PADGEM antigen, which mediate adhesion of activated platelets to neutrophils and monocytes, these activated platelets may be quickly eliminated from the blood circulation.

In this context, the temperature-dependent influence of
plasmin on platelet function may be an additional explanation for the mechanism, by which aprotinin, a serine proteinase inhibitor with strong plasmin-inhibiting capacity, effectively reduces blood loss in CPB.  

Recently, attention has also been drawn to the role of plasmin in rethrombosis after thrombolytic therapy. Theoretically, one can suggest that platelet activation induced by a high local concentration of plasmin (generated by thrombolytic agents) may reduce the rate of thrombolysis. To minimize the rate of reocclusion, the administration of antiplatelet agents, including aspirin and prostaglandin E1, has been advocated together with plasminogen activators. Antiplatelet GP IIb/IIIa antibody was also tested in animals. The data provided by these investigations showed that the use of platelet inhibitors both enhanced the rate of thrombolysis and reduced the occurrence of reocclusion.

In brief, our studies show, for the first time, the importance of temperature in determining the occurrence of platelet activation induced by plasmin. This phenomenon may be related to the alteration by temperature of plasmin triggered platelet activating and inhibitory processes. This finding is relevant to the clinical practice such as CPB, blood platelet preservation in the cold and thrombolytic therapy. The interaction of the fibrinolytic system and platelets may be an important mechanism regulating the balance between hemostasis and thrombolysis. There is no doubt that further detailed investigations of this mechanism will yield substantial benefit for medical practice.

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