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Analysis of the Specific Association of the Eighth and Ninth Components of Human Complement: Identification of a Direct Role for the α Subunit of C8†

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Received January 17, 1985

ABSTRACT: The basis for the physical association between C8 and C9 in solution was examined by isolating the noncovalently associated α–γ and β subunits of C8 and determining their respective affinities for C9. Results indicate that only α–γ associates with C9 and this association, though reversible, is complete at near equimolar ratios of each component. Further experiments using purified α or γ revealed that only α was capable of forming a stable complex with C9. Although the strength of this interaction was dependent on salt concentration, association was observed in buffer of physiological ionic strength and in human serum. These results establish that the domain on C8 responsible for interaction with C9 is located entirely within α. In related experiments, addition of β to preformed dimers of either (α–γ + C9) or (α + C9) resulted in complete association of this subunit. These particular results indicate that there are two physically distinct sites on α that separately mediate association of α with β and with C9. Furthermore, occupation of one site does not impair interaction at the other.

Complement-mediated lysis of cell membranes occurs as a result of specific interaction between C5b, C6, C7, C8, and C9 (Bhakdi & Tranum-Jensen, 1983; Podack & Tschopp, 1984). Assembly of the cytolytic complex on target membranes is initiated by formation of C5b and proceeds in the sequential manner

$$\text{C5b} \xrightarrow{\text{C6}} \text{C5b-6} \xrightarrow{\text{C7}} \text{C5b-7} \xrightarrow{\text{C8}} \text{C5b-8} \xrightarrow{\text{C9}} \text{C5b-9}$$

The high-affinity membrane binding site that mediates association of the nascent complex with cell membranes develops with formation of C5b-7. Subsequently, C8 and several C9 molecules bind to yield C5b-9, the macromolecular complex that produces membrane lysis.

Human C8 consists of three nonidentical subunits: α (M_r 64 000), β (M_r 64 000), and γ (M_r 22 000) (Kolb & Müller-Eberhard, 1976; Steckel et al., 1980). These subunits occur as a disulfide-linked α–γ dimer that is noncovalently associated with β. We previously established that β alone mediates the binding of C8 to C5b-7, as evidenced by the ability of purified β to associate with this complex (Monahan & Sodetz, 1981). Importantly, the resulting C5b-7β complex does not support subsequent incorporation of C9, thus emphasizing that α–γ and β are required for C9 binding (Brickner & Sodetz, 1984). We also established that γ has no direct role in C8 function by demonstrating that C8′, a derivative composed of only α and β, is functionally similar to normal C8 (Brickner & Sodetz, 1984). This derivative is able to interact with C5b-7 to form C5b-8′, a complex that can bind C9 and cause cell lysis. These observations indicate α and β but not γ are essential for incorporation of C9 into C5b-9.

Although C8 is required for C9 binding, there is no conclusive evidence that these proteins physically interact during C5b-9 formation or within the fully assembled cytolytic complex. Indeed, cross-linking studies of intact C5b-9 suggest that few, if any, of the multiple C9 molecules are in close physical proximity to C8 (Monahan et al., 1983). This observation is consistent with two possible explanations for the known requirement for C8. One is that C8 participates only indirectly by inducing a conformational change within the complex or membrane, leading to formation of a C9 binding site or sites that are distant from C8. Another possible explanation is that C8 interacts directly but transiently with C9, facilitating its polymerization and insertion into the membrane.

Current evidence favors at least some direct interaction between C8 and C9. The ability of these proteins to specifically associate in solution has been demonstrated and cited as indirect evidence that interaction occurs during C5b-9 assembly (Kolb et al., 1973; Podack et al., 1982). If so, the inability of β and the requirement for α to mediate incorporation of C9 into the nascent complex suggest that the site of interaction with C9 either is located on α or is shared between α and β. In this study, we investigated these possibilities and

† This research was supported by NIH Grant AI-16856 and by Established Investigator Award 82-121 to J.M.S. from the American Heart Association.
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0006-2960/85/0424-4598$01.50/0

extended previous observations on the association of C8 and C9 to establish whether this interaction is mediated by a specific subunit of C8. In particular, we examined whether the known requirement for α in C9 binding could be explained by a direct interaction between these two components. Further, we discuss the implications of our findings in relation to possible mechanisms of C5b-9 assembly.

Experimental Procedures

Purification of Proteins. Human C8 was purified from Cohn fraction III, which was kindly provided by Cutter Laboratories, Berkeley, CA (Steckel et al., 1980). Isolation of C9 from plasma was accomplished by using a modification of an earlier procedure (Biesecker & Müller-Eberhard, 1980). The noncovalently associated α–γ and β subunits of C8 were separated by gel filtration in high ionic strength buffer as recently reported (Rao & Sodetz, 1985). Molar concentrations of each component were determined from published molecular weights and previously determined $\epsilon_{280}^{1\%}$ values (Monahan et al., 1983). Radioiodinations were performed with iodogen and typically yielded specific radioactivities of (1–8) × 10^7 cpm/nmol (Fraker & Speck, 1980). Separation of ^{125}I-α from ^{125}I-γ was accomplished by limited reduction and modification of ^{125}I-(α–γ) and was followed by ion-exchange chromatography as described in the following paper (Brickner & Sodetz, 1985). The specific radioactivities of purified ^{125}I-α and ^{125}I-γ were estimated from that of the original ^{125}I-(α–γ) on the assumption that the distribution of radiolabel was 4:1 (Brickner & Sodetz, 1984).

Determination of Protein Association. Physical evidence for the specific association of proteins was obtained by ultracentrifugation in 5–10%, 5–20%, or 5–30% (w/w) linear sucrose density gradients. Except where indicated, gradient solutions were prepared in 5 mM imidazole/16.25 mM NaCl/0.15 mM $CaCl_2$/0.5 mM $MgCl_2$/1 mg/mL bovine serum albumin, pH 7.4. In a typical experiment, 1–2 μg of radiolabeled ligand and the desired molar excess of unlabeled protein were mixed and then adjusted with buffer to a final volume of 500 μL. The mixtures were incubated for 10 min at 25 °C. After the samples were adjusted to 2% sucrose, 200 μL of each was applied to 4.0-mL gradients that had been preequilibrated at 4 °C. Centrifugation was performed in a Sorvall TV-865 vertical rotor at 202000*g* for 2 h at 4 °C. Gradients were fractionated from the top, and recovery of total radioactivity after fractionation was consistently >90%.

Ionic strength studies were carried out in the above buffer after adjusting the NaCl concentration as follows: 33.6 (μ = 0.05), 83.6 (μ = 0.10), and 133.6 mM (μ = 0.15). Serum studies were performed with human serum depleted of both C8 and C9 by sequential passage of normal serum through affinity resins containing purified C8 or C9 antibodies (Monahan & Sodetz, 1980; Steckel et al., 1983). Serum experiments at low ionic strength were performed in (C8 + C9)-depleted serum that had been dialyzed against the above buffer (μ = 0.033).

Results

Association of C8 and C9. The criterion used in this study to assess association of C8 or its subunits with C9 was detection of stable complexes following ultracentrifugation on linear sucrose density gradients. Experiments demonstrating the specific association of purified C8 and C9 are shown in Figure 1. Mixtures of C8 and C9 consistently sedimented further than the individual proteins, indicating the formation of a stable complex. Identical results were achieved regardless of which component carried the radiolabel, suggesting the observed interaction is not a function of the iodination procedure. This result confirms previous studies in which association of C8 and C9 was reported. Further, it establishes the use of isotopically labeled components and density gradient centrifugation as valid means for studying this association.

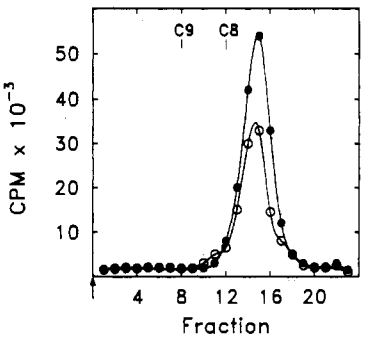

FIGURE 1: Sucrose density gradient analysis of the association of C8 and C9. Mixtures of ^{125}I-C8 and C9 (●) or C8 and ^{125}I-C9 (○) were analyzed on linear 5–30% gradients prepared as described in the text. The sedimentation positions of C8 and C9 markers are shown on the inset. The arrow in this and subsequent figures indicates the top of the gradient.

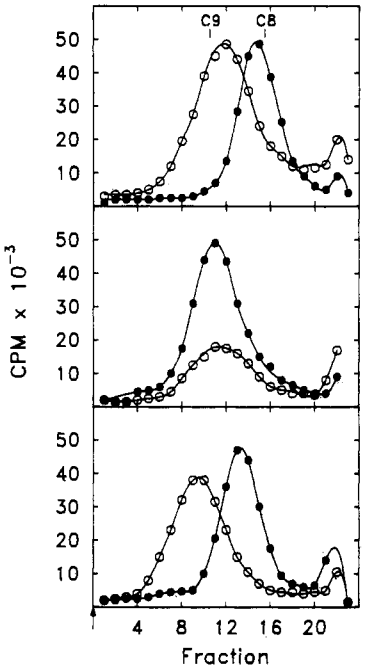

FIGURE 2: Analysis of the association of α–γ and β with C9. Samples of α–γ or β were incubated with C9 as indicated in the text and analyzed on 5–10% sucrose density gradients. The sedimentation positions of C8 and C9 markers are shown on the inset. (Top) Sedimentation profiles of ^{125}I-(α–γ) (○) and a mixture consisting of ^{125}I-(α–γ) and a 1.5-fold molar excess of unlabeled C9 (●). (Middle) Sedimentation profiles of ^{125}I-β (○) and a mixture consisting of ^{125}I-β and a 1.5-fold molar excess of unlabeled C9 (●). (Bottom) Sedimentation profiles of ^{125}I-C9 and a 1.5-fold molar excess of unlabeled α–γ (●) or β (○).

Association of C8 Subunits and C9. To further probe this interaction, the noncovalently associated α–γ and β subunits of C8 were purified, radiolabeled, and individually tested for their ability to associate with C9. Results from these experiments are shown in Figure 2. The top panel shows the sedimentation profile obtained for a mixture containing ^{125}I-(α–γ) and an excess of C9. A high molecular weight complex is observed that sediments at a position approximating that of a C8 marker (M_r 151 000). This is consistent with formation of a dimer that appears to be quite stable since it forms at nearly equimolar concentrations of each component

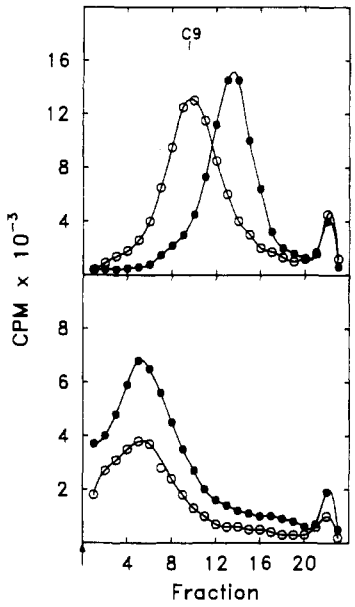

FIGURE 3: Analysis of the association of α and γ with C9. Purified ^{125}I-α or ^{125}I-γ was incubated with a 3-fold molar excess of unlabeled C9 and analyzed on 5–10% sucrose density gradients. The position of a C9 marker is shown on the inset. (Top) Sedimentation profiles of ^{125}I-α (○) and a mixture of ^{125}I-α and C9 (●). (Bottom) Sedimentation profiles of ^{125}I-γ (○) and a mixture of ^{125}I-γ and C9 (●).

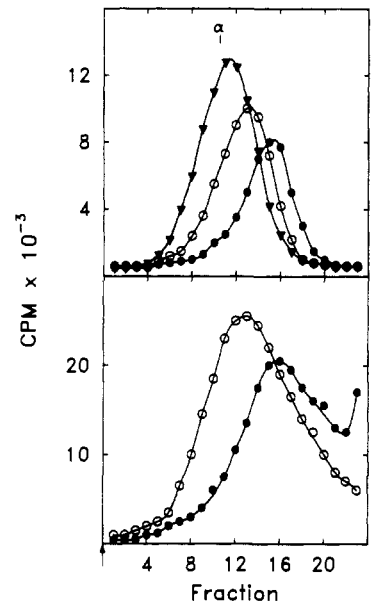

FIGURE 4: Effect of ionic strength on the association of α and C9. (Top) Sedimentation profiles are shown for a mixture of ^{125}I-α and a 3-fold molar excess of C9 in buffers with the following ionic strengths: μ = 0.05 (●), 0.10 (○), or 0.15 (▼). The inset shows the sedimentation position of an α marker, which remained unchanged at all ionic strengths tested. (Bottom) Samples of ^{125}I-α and a 3-fold molar excess of C9 were added to (C8 + C9)-depleted serum of μ = 0.033 (●) or 0.15 (○) and analyzed as above.

and remains intact during sedimentation. Additionally, no further increase in sedimentation was observed when the molar ratio of C9 was adjusted to 1:10 (data not shown). These data indicate that no further association between components occurs once an equimolar complex is formed. Results from corresponding experiments using ^{125}I-β and C9 are shown in the middle panel of Figure 2. No complex is detected at a ratio of 1:1.5, nor was any observed at 1:10 (data not shown). These results indicate that specific association of C8 and C9 in solution is mediated exclusively by α–γ.

To further confirm this, an excess of unlabeled α–γ or β was added to buffer containing ^{125}I-C9 before analysis on sucrose density gradients. Results in the bottom panel of Figure 2 show that when a 1.5-fold molar excess of α–γ is added, there is a significant increase in sedimentation of ^{125}I-C9 relative to the control. Conversely, when β was tested at the same molar ratio, no difference in sedimentation was observed. Even at a molar ratio of 10:1, β caused no shift in the ^{125}I-C9 profile (data not shown). This demonstrates clearly that association of C8 and C9 is attributable to the specific affinity of C9 for α–γ and is not an artifact of radiolabeling.

Association of α or γ with C9. We next attempted to establish if the domain on C8 responsible for interaction with C9 could be localized in either the α or γ chain of the disulfide-linked α–γ dimer. To do this, radiolabeled α and γ were purified after limited reduction and modification of interchain disulfides in ^{125}I-(α–γ). Purity was assessed by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels under nonreducing conditions. Although complete separation of ^{125}I-α from ^{125}I-γ was achieved, a trace amount of unmodified ^{125}I-(α–γ) was observed in the ^{125}I-α preparation. However, this contaminant represented <2% of the total recovered radioactivity in the ^{125}I-α pool and therefore was not a significant factor in the gradient analyses.

Radiolabeled α and γ were tested individually for their ability to associate with C9, and the results from these experiments are shown in Figure 3. The top panel contrasts the sedimentation profile of a mixture of ^{125}I-α and excess C9 with that of ^{125}I-α alone. The affinity between α and C9 at a molar ratio of 1:3 is evident from the appearance of a stable complex sedimenting at a position corresponding to a dimer of the two components. Association is essentially complete under these conditions since no further increase in sedimentation was observed when C9 was increased to a molar ratio of 1:10 (data not shown). Results from parallel experiments with a mixture of ^{125}I-γ and C9 are shown in the bottom panel of Figure 3. At a molar ratio of 1:3, sedimentation of the mixture is identical with that of ^{125}I-γ alone, indicating that under these conditions γ and C9 do not associate. Importantly, identical results are observed when the molar ratio of C9 is increased to 1:10 (data not shown). On the basis of these experiments, we conclude that γ has no significant affinity for C9 and the functional domain of C8 that specifically associates with C9 is contained entirely within α.

Effect of Ionic Strength on Association of α and C9. Experiments described above were all performed in buffer of relatively low ionic strength (μ = 0.033). To determine whether comparable results are obtainable under more physiological conditions, the association of α and C9 was examined at higher ionic strengths. Specifically, mixtures of ^{125}I-α and a 3-fold molar excess of unlabeled C9 were prepared in buffers of μ = 0.05–0.15 and analyzed on sucrose density gradients. Results from this experiment are shown in the top panel of Figure 4. The sedimentation profiles indicate a shift in equilibrium toward free α with increasing ionic strength. Although this indicates a gradual weakening of the interaction, the slightly lower sedimentation position of ^{125}I-α in the presence of C9 at μ = 0.15 is reproducible and indicative of at least some association. This demonstrates that dimer formation can still occur at physiological ionic strength. The bottom panel of Figure 4 shows that similar results are obtained when α and C9 interaction is examined in (C8 + C9)-depleted serum.

Interaction of β with (α–γ + C9) or with (α + C9). We next examined whether distinct functional domains on α mediate its interaction with C9 and β by examining the ac-

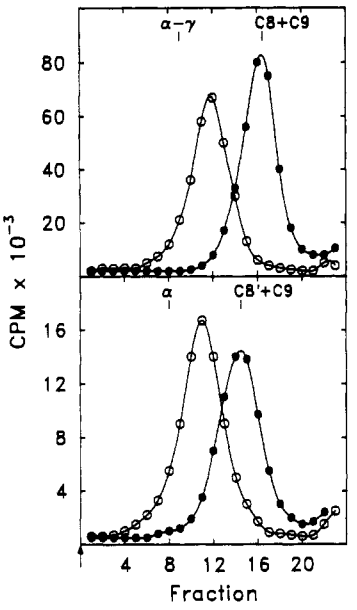

FIGURE 5: Association of β with preformed (α–γ + C9) or (α + C9) complexes. (Top) A sample of ^{125}I-(α–γ) was mixed with a 1.5-fold molar excess of C9, and a portion was taken for analysis on 5–20% sucrose density gradients (○). A parallel sample of this mixture was further incubated with a 1.5-fold molar excess of β for 10 min at 25 °C and analyzed on the same gradient system (●). The inset indicates the sedimentation positions of an α–γ marker and a standard composed of C8 + C9. (Bottom) A sample of ^{125}I-α was mixed with a 3-fold molar excess of C9, and a portion was taken for analysis on sucrose density gradients (○). A parallel sample of this mixture was further incubated with a 3-fold molar excess of β for 10 min at 25 °C before analysis on the same gradient system (●). The inset indicates the sedimentation positions of an α marker and a standard composed of C8′ + C9.

cessibility of the β binding site when α is associated with C9. To do this, ^{125}I-(α–γ) or ^{125}I-α was mixed with an excess of C9 under conditions that yield complete dimer formation. These samples were then incubated with an excess of β and analyzed on sucrose density gradients. The results of these experiments are shown in Figure 5. Association of either ^{125}I-(α–γ) (top panel) or ^{125}I-α (bottom panel) with C9 is indicated by the shift in radiolabel relative to α–γ or α markers. Subsequent addition of excess β results in a further increase in sedimentation for both ^{125}I-(α–γ + C9) and ^{125}I-(α + C9), indicating that, within these complexes, the site of β interaction remains accessible. Importantly, the final sedimentation positions correspond exactly to standards of (C8 + C9) and (C8′ + C9), thereby confirming association of a stoichiometric amount of β with each preformed complex.

Discussion

Results in this study indicate that association of C8 and C9 in solution is mediated strictly through α, as evidenced by the ability of the isolated subunit to form a stable dimer with C9. This interaction is specific since association is observed at dilute concentrations and in the presence of excess serum albumin. Additional experiments not reported indicate that association is independent of concentration since similar results were observed when the concentration of each component was increased 4–40-fold while keeping the molar ratio constant. Significantly, this range encompasses the concentrations of C8 and C9 normally found in serum. Results also showed that although the stability of the dimer is dependent on ionic strength, some association does occur in buffer of physiological ionic strength and in human serum. Further, the association of α and C9 did not affect the known ability of α to recombine with β, indicating that two mutually exclusive sites on α are responsible for these interactions.

This report extends earlier observations on the association of C8 and C9 in solution by establishing that the site of interaction on C8 is localized in a single subunit. Moreover, the fact that α retains this ability after purification indicates that a stable, well-defined structural domain is likely involved. Importantly, the affinity of C9 for α provides a physical basis for the known requirement for α in C9 binding. This accounts for the earlier observation that β alone on C5b-7 cannot mediate C9 incorporation into this complex. It is also consistent with the observation that γ is not required for C9 binding to the nascent complex. Instead, direct interaction of α and C9 appears to be an essential step in the assembly of C5b-9.

Results from analysis of the ionic strength dependency of this interaction indicate it is reversible and can occur under physiological conditions. Although the equilibrium is shifted toward free α and C9 at $\mu = 0.15$, the ability to detect even a small amount of association at this ionic strength is significant. One reason is that only interactions of relatively high affinity can be detected by the method of density gradient centrifugation. Therefore, the intrinsic affinity of α for C9 must be substantial since at least some population of dimer is observed on gradients. Secondly, one must consider that when cell lysis occurs under physiological conditions, incorporation of C9 into C5b-9 is a multistep process with the first step involving recognition of C9 by C5b-8. Results here clearly indicate that specific recognition occurs through the α subunit of C8 while earlier results argue against involvement of C5, C6, and C7 (Kolb et al., 1973). The observation that the affinity of C9 for α is lower at physiological ionic strength does not diminish the significance of this interaction but must be interpreted in relation to the C5b-9 assembly process as a whole. Following recognition of C9 by C5b-8, subsequent steps lead to the irreversible insertion and/or polymerization of C9 in the membrane. If these latter steps drive the overall process toward C9 incorporation, then the affinity for α need only be sufficient to direct C9 onto C5b-8.

When considering the detailed mechanism by which C8 mediates C9 binding, there are several possibilities that can be proposed. Two of these specifically exclude direct interaction between C8 and C9. The first proposes that C8 binding induces conformational changes within the complex that expose sites of interaction for C9 that are distant from C8. The second suggests that α, which is known to interact directly with the target membrane bilayer (Steckel et al., 1983), exposes lipid sites within the membrane that facilitate C9 incorporation. The latter mechanism would presumably involve only C9–lipid interactions. Our results argue against both mechanisms since neither allows for direct association of α with C9.

Two other mechanisms that do involve at least limited interaction between C8 and C9 can be proposed. One is that every C9 within C5b-9 is associated in a stable complex with α. Although consistent with the affinity of C9 for α, this mechanism cannot account for the incorporation of multiple C9 molecules relative to C8 since each α contains only one C9 binding site. Earlier results from cross-linking studies also argue against this proposal, since they indicate that few, if any, of the multiple C9 molecules bound within C5b-9 are in close physical proximity to C8 (Monahan et al., 1983).

An alternative mechanism suggests that C9 incorporation into the nascent complex involves association of only one C9 with α. This interaction can then produce further C9 incorporation either by serving as a site for C9 self-polymerization,

as has been previously suggested (Podack et al., 1982), or through a transient interaction in which individual C9 molecules are successively shunted from α into the membrane. Such an alternative mechanism is most consistent with our results because it requires only a single C9 binding site on α.

Although our results indicate direct interaction of C8 with C9 during C5b-9 formation, they do not allow us to conclude whether these proteins remain associated within the fully assembled complex. Regarding this, two possibilities must be considered. One is that C9 can dissociate from C8 once formation of C5b-9 is complete. This is supported by the previous observation that membranolytic oligomers of C9 can exist independently of C5b-8 within synthetic membrane bilayers (Tschopp & Podack, 1981). It is also consistent with our inability to detect cross-linking between C8 and C9 within C5b-9. However, inherent limitations of the latter study render it questionable whether association between C8 and only one of the multiple C9 molecules in the complex could be detected. The second possibility is that, once formed, oligomeric C9 remains physically associated with C5b-8. There is direct evidence that this can occur, although the site of interaction on C5b-8 has not been established (Tschopp et al., 1982). Our results suggest that such an interaction would likely involve stable association of α with one of the multiple C9 molecules.

This study also extends our knowledge of functional domains within C8. We previously identified two distinct domains on α that separately mediate its insertion into the membrane bilayer and its specific interaction with β in C8 (Monahan & Sodetz, 1981; Steckel et al., 1983; Brickner & Sodetz, 1984). This study has identified a previously unknown third domain on α that mediates its stable association with C9. This site is physically distinct from that which mediates β interaction since its occupation by C9 does not affect the ability of α and β to recombine. The C9 binding site on α is also distinct from the site of membrane interaction, since α is inserted in the membrane in C5b-8 and remains inserted in C5b-9. More recently, a fourth distinct functional domain on α has been postulated. Details regarding this site, which is involved in interactions with γ, are presented in the following paper (Brickner & Sodetz, 1985).

Registry No. Complement C8, 80295-58-5; complement C9, 80295-59-6.

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