Sickle Erythrocytes Adhere to Polymorphonuclear Neutrophils and Activate the Neutrophil Respiratory Burst

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The vasoocclusive process in patients with sickle cell disease (SCD) is complex and involves interactions among sickle erythrocytes (SS-RBC), vascular endothelium, and plasma and cellular components. The role of neutrophils (PMN) in vasoocclusion has not been examined. Patients with SCD appear to have chronically activated PMN. Because the first step in PMN activation is particle recognition, we explored whether normal PMN recognize SS-RBC and whether this recognition results in PMN activation. When SS-RBC were allowed to settle over PMN monolayers, significantly more SS-RBC adhered to the PMN than did normal erythrocytes (AA-RBC; P < .001). Preincubation of erythrocytes with autologous plasma significantly increased the adherence of SS-RBC to PMN but had no effect on AA-RBC (P < .001). When adherence of density fractionated SS-RBC was performed, dense SS-RBC showed greater adherence to the PMN monolayers than did light SS-RBC (P < .001). To determine mechanisms of this adhesion, IgG and Arg-Gly-Asp-Ser (RGDS) receptor sites on PMN were saturated. IgG inhibited adherence of dense SS-RBC, whereas RGDS inhibited adherence in both fractions, although to a greater extent in the light fraction. We measured SS-RBC activation of PMN by incubating SS-RBC with 2',7'-Dichloro-fluorescin Diacetate (DCF)-labeled PMN. Incubation of PMN with SS-RBC resulted in a significant increase in fluorescence compared to AA-RBC. We show here that PMN recognize SS-RBC through multiple mechanisms and that this recognition results in activation of PMN. These findings contribute to the understanding of vasoocclusive crisis in patients with SCD and may have therapeutic implications. © 1996 by The American Society of Hematology.
these rigid and adherent PMN may play a role in the initiation and propagation of VOC by slowing blood flow through the microvasculature, delaying SS-RBC transit time, and increasing the likelihood of Hb S polymerization within the microvasculature. Furthermore, contact between the PMN and the SS-RBC in the microvasculature could lead to activation of the PMN promoting PMN-mediated vascular endothelial damage. We explore here the possibility that normal PMN recognize SS-RBC and that this recognition results in activation of the PMN. We show that SS-RBC specifically bind to normal PMN and that this adherence is IgG- and Arg-Gly-Asp-Ser (RGDS)-mediated. We further show that incubation of SS-RBC with normal PMN activates the PMN respiratory burst.

MATERIALS AND METHODS

Reagents buffers. Phosphate-buffered saline (PBS; 124 mM NaCl, 16 mM Na2HPO4, 5 mM KCl, and 5 mM glucose) and Kreb’s phosphate-buffered saline (KRPG; 120 mM NaCl, 5 mM KCl, 16 mM Na2HPO4, 1 mM CaCl2, 1 mM MgSO4, with 5 mM glucose) were used as the standard buffers. All solutions were made with pyrogen-free H2O (Abbott Laboratories, North Chicago, IL). All standard chemicals were purchased from Sigma Chemical Co (St Louis, MO). RBC were stored at an hematocrit (HCT) of 80% in acid citrate dextrose (ACD; 132 g trisodium citrate [dihydrate], 0.48 g citric acid [monohydrate], and 1.47 g dextrose in 100 mL H2O). The Ficol-Hypaque gradient was prepared by dissolving 36 g of Ficol Type 400 (Sigma) in 454.7 mL (total volume) H2O and 118.3 mL Hypaque 50% (Winthrop Pharmaceuticals, New York, NY). Solutions were stored sterilely at 4°C until used. Dextran (US Chemical Corp, Cleveland, OH; molecular weight, 100,000 to 200,000) was dissolved in PBS and stored for up to 3 weeks at 4°C. RGDS and Gly-Arg-Glu-Ser-Pro (GRGESP) were purchased from Peninsula Laboratories, Inc (Belmont, CA).

Isolation of neutrophils. The PMN were harvested under endotoxin-free conditions from heparinized (Squibb-Marsam Inc, Cherry Hill, NJ; 1000 USP units/mL, 10 U/mL blood) human whole blood obtained from healthy volunteers. The PMN were isolated by dextran sedimentation and Ficol/Hypaque gradient centrifugation according to standard procedures. Excess RBC were lysed with 0.83% ammonium chloride and then washed three times with normal saline. All blood was obtained from healthy volunteers according to the Institutional Review Board guidelines.

Preparation of PMN monolayers. Isolated PMN were resuspended to a concentration of 3 x 10⁶/mL in KRPG. To establish PMN monolayers, 200 μL of the PMN suspension was added to each chamber of 8-chamber Permanox culture slides (Nunc Inc, Naperville, IL). The slides were placed on a rotating platform (R4140; Baxter, Mcpharage Park, IL) at 60 RPM and incubated at 37°C in 5% CO2 for 30 minutes. Nonadherent PMN were removed by washing chambers once with 37°C PBS. For experiments involving the blockade of putative PMN-RBC adhesions sites, saturating concentrations of purified human IgG (1 mg/mL IgG; Cappel, Durham, NC), RGDS peptide (500 μg/mL), or controls of human serum albumin (HSA; 1 mg/mL), or control peptide GRGESP (500 μg/mL) were added to the PMN monolayers at 15 minutes of incubation. At 15 minutes, there was near complete formation of the PMN monolayer. The addition of RGDS or IgG at this time had no effect on the overall adhesion of the PMN to the plastic surface. Monolayers had greater than 90% confluence before fixation when examined using phase microscopy.

RBC preparation. Heparinized whole blood was obtained from volunteer donors with sickle cell anemia during steady state and from normal controls and kept on ice until used (within 2 hours of phlebotomy). Whole blood was centrifuged at 2,000g for 5 minutes within 2 hours of phlebotomy. Plasma was stored at -70°C and RBC were washed three times with normal saline. The buffy coat was discarded after each wash. RBC were stored at 4°C in ACD until used in the RBC adherence assays. The RBC were used within 72 hours of collection.

RBC density gradient. Dense and light RBC were separated across a Nycodenz density gradient. Stock Nycodenz gradient solution was prepared by diluting 55.2 g of Nycodenz (Nycomed AS, Oslo, Norway) into 200 mL of 6 mMOL/L PBS with a pH of 7.5. Density solutions were made according to company instructions to final density of 1.106 g/mL and 1.146 g/mL by diluting stock solution with isotonic PBS. The density of Nycodenz solutions was checked using an OCR-D oscillating rheo and density meter (Paar, Graz, Austria). One milliliter of washed RBC (80% HCT) was layered over stacked density gradients (1.5 mL 1.105 g/mL over 1.5 mL 1.145 g/mL Nycodenz) and centrifuged for 20 minutes at 1,000g with slow acceleration and no brake. Clearly defined RBC layers were harvested and washed twice in isotonic PBS. After washing, the RBC were resuspended to a 2% HCT in autologous plasma, ABO-matched heterologous plasma, or PBS with 5% HSA (Armour, Kan-kakee, IL) and incubated for 15 minutes in a 37°C shaking water bath before proceeding to the RBC adherence assay. Control normal erythrocytes (AA-RBC) were subjected to the same gradient centrifugation and handling as SS-RBC.

Adherence of RBC to PMN monolayers. After excess PMN were washed from the PMN monolayers, 150-µL aliquots of suspended RBC were plated over the monolayers and the slides were incubated for an additional 30 minutes at 37°C. The chambers were gently washed four times with warm PBS before fixing the cells for 15 minutes in 4% paraformaldehyde. After washing with normal saline, the slides were stained for hemoglobin using a modification of Raib’s method (sequential staining with 1% benzidine in methanol and peroxide/ EtOH). This process stains hemoglobin brown and leaves PMN unstained.

Quantitation of RBC adherence. The mean number of adherent RBC for 20 fields of each chamber were counted under 40X direct light microscopy. Only RBC that were in contact with PMN were counted.

Measurement of PMN oxidative burst. Isolated PMN at 2.5 x 10⁶ cells/mL were loaded with 10−4 mol/L 2',7'-Dichloro-fluorescin Diacetate (DCF; Eastman Kodak, Rochester, NY) and then washed with PBS and resuspended in KRPG to 1 x 10⁶ cells/mL. SS-RBC or AA-RBC incubated in autologous plasma were added to 1 mL of PMN in a ratio of SS-RBC to PMN of 1:2. Cells were gently pelleted then resuspended after 2 minutes before incubating suspension at 37°C in 5% CO2 for 30 minutes. The mean fluorescence of the DCF was measured in an SLM 8000C spectrophotofluorometer (SLM Instruments, Urbana, IL) at an excitation wavelength of 485 nm and an emission wavelength of 510 nm. Phorbol myristate acetate (PMA; Sigma; 10−9 mol/L), a potent stimulus of the PMN respiratory burst, was added to an aliquot of DCF-loaded PMN as a positive control. DCF-loaded PMN incubated without RBC with and without PMA were used as unstimulated controls. PMA was added 5 minutes before measuring fluorescence.

Statistical methods. The graphics are representative of at least three separate similar experiments. Error bars are the standard error of the mean (SEM) calculated from duplicate or triplicate measurements. Differences in sample means were tests using an analysis of variance.

RESULTS

Adherence of SS-RBC to PMN monolayers. The initial step in the recognition of SS-RBC by PMN is attachment.
Fig 1. SS-RBC adhere to PMN monolayers. SS-RBC and AA-RBC were incubated in autologous plasma (■) or 5% HSA (□) and then plated over the PMN monolayers. SS-RBC in autologous plasma were more adherent than SS-RBC in 5% HSA (P < .001; N = 2). AA-RBC in either autologous plasma or 5% HSA had equivalent adherence (P = .2) and were significantly less adherent than SS-RBC under either condition (P < .001; N = 2).

To determine whether SS-RBC specifically attach to PMN, we measured the number of SS-RBC that adhered to PMN monolayers. SS-RBC incubated in PBS containing 5% HSA adhered to the PMN monolayer significantly more than similarly treated AA-RBC (Fig 1, P < .001). Because sickle plasma enhances the attachment of SS-RBC to endothelium, we next examined whether sickle plasma would enhance the attachment of SS-RBC to the PMN monolayers. In the presence of autologous plasma, twice as many SS-RBC attached to the PMN monolayers compared with those incubated in 5% HSA (P < .001). Furthermore, autologous plasma did not significantly increase the adherence of AA-RBC to PMN (Fig 1). This finding suggests that SS-RBC are intrinsically more adherent to PMN than are AA-RBC and, furthermore, that plasma from patients with SCD contains factors that promote adhesion of the SS-RBC to PMN.

To determine whether this effect of sickle plasma is specific to SS-RBC or if sickle plasma also promotes the adherence of AA-RBC to PMN monolayers, AA-RBC were incubated in the presence of ABO-matched sickle plasma. This treatment had no effect on the adherence of AA-RBC to the PMN. Similarly, normal plasma did not increase the adherence of SS-RBC above that of SS-RBC incubated in 5% HSA (Fig 2), indicating that the plasma-dependent increase in adherence is specific for SS-RBC and sickle plasma.

After repeated cycles of sickling, a dense population of SS-RBC emerges. These dense SS-RBC are felt to play a role in the propagation of vasoocclusion. Because dense and light SS-RBC display different adhesive properties to vascular endothelium, we compared the adherence of light and dense fractions of SS-RBC to PMN. Dense SS-RBC incubated in 5% HSA were significantly more adherent to the PMN monolayers than were light SS-RBC (P < .001). The increased adherence of the dense SS-RBC fraction was more apparent after incubating the erythrocytes in autologous plasma. Dense SS-RBC incubated in autologous plasma were more than twice as adherent to PMN monolayers as light SS-RBC (Fig 3, P < .001). Thus, although SS-RBC are intrinsically more adherent to PMN monolayers than AA-RBC, autologous plasma significantly increases this adherence to PMN. This effect is specific for SS-RBC and, although seen in both the dense and light fractions, is greater in the dense fraction.

IgG-mediated attachment of SS-RBC to PMN monolayers. Dense SS-RBC are coated with IgG that is directed against specific surface proteins. Because neutrophils have distinct surface receptors that recognize the Fe portion of IgG, we sought to establish if the adherence of SS-RBC to PMN monolayers is mediated by IgG. Figure 4 shows a partial inhibition of SS-RBC attachment to the PMN monolayers.

Fig 2. Normal plasma does not affect adherence of SS-RBC. SS-RBC were incubated in autologous plasma (■), ABO-compatible normal plasma (□), or 5% HSA (△) and then plated over PMN monolayers. SS-RBC in normal plasma or 5% HSA had equivalent adherence and were less adherent than SS-RBC in autologous plasma (P < .001; N = 2). AA-RBC had minimal adherence under all conditions.

Fig 3. Dense SS-RBC adhere more than light SS-RBC. Dense and light SS-RBC were incubated in autologous plasma (■) or 5% HSA (△) and were plated over PMN monolayers. Dense SS-RBC in autologous plasma were significantly more adherent to the PMN monolayers than were light SS-RBC (P < .001; N = 2) or dense SS-RBC in 5% HSA. Light SS-RBC in autologous plasma adhered more than in 5% HSA (P < .001; N = 2).
Fig 4. IgG partially blocks adherence of SS-RBC to PMN. SS-RBC and AA-RBC were incubated in autologous plasma and then plated over PMN monolayers treated with HSA (■) or saturating doses of IgG (■). Adherence of SS-RBC to the IgG-treated PMN was significantly less than that of control PMN (P < .001; N = 2). Treatment of the PMN monolayers with IgG had no effect on the adherence of AA-RBC.

Fig 5. IgG HSA its predominant affect on dense SS-RBC. Density fractionated SS-RBC incubated in autologous plasma were plated over IgG treated PMN monolayers. Dense SS-RBC had a significant decrease in adherence to IgG-treated PMN (■) compared with control PMN (■; P < .001; N = 2), whereas IgG treatment had no significant effect on the adherence of light SS-RBC.

Fig 6. RGDS peptide partially blocks adherence of SS-RBC to PMN monolayers and is additive to inhibition by RGDS. SS-RBC incubated in autologous plasma were plated over PMN monolayers treated with IgG, RGDS, control peptide (GRGESP), or IgG with each peptide. Adherence of SS-RBC was significantly reduced in IgG- and RGDS-treated wells (P < .001; N = 2). The combination of RGDS and IgG treatment of PMN monolayers had an additive effect, yielding a significant reduction in adherence of SS-RBC compared with either IgG-treated or RGDS-treated monolayers (P < .001; N = 2).

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after incubating the PMN with 1 mg/mL of nonspecific IgG. Doubling the concentration of IgG showed no further inhibition of SS-RBC adherence, suggesting that the PMN IgG binding sites had become saturated. Experiments using purified Fc fragments yielded similar results. Dense SS-RBC incubated in autologous plasma showed a greater than 50% decrease in their attachment to PMN monolayers treated with IgG (P < .001). Light SS-RBC incubated in autologous plasma showed no significant change in the extent of their adhesion to the IgG-treated PMN monolayers (Fig 5). Conversely, adhesion of AA-RBC to the PMN monolayers was unaffected by pretreating the PMN monolayers with IgG (data not shown). We conclude that the specific enhancement of SS-RBC adherence to PMN is mediated in part by IgG bound to the SS-RBC surface. However, because this adherence is only partially blocked by saturating amounts of IgG, other adhesion mechanisms must also be involved.

RGDS-mediated attachment of SS-RBC to PMN. Integrins are involved in many adhesion processes and proteins containing the RGDS motif are involved in the attachment of SS-RBC to endothelium.34,35 We explored the possible role of RGDS on the adherence of SS-RBC by saturating potential RGDS binding sites on the PMN monolayers. For nondensity separated SS-RBC, pretreatment of the PMN monolayers with saturating concentrations of RGDS peptide resulted in at least a 50% reduction in the attachment of SS-RBC to the monolayers (P < .001; Fig 6). Adherence studies were repeated with density fractionated SS-RBC. Blockade of RGDS sites on the PMN reduced the adherence of the light fraction of SS-RBC to the PMN monolayer. Although attachment of SS-RBC from the dense fraction also was inhibited by RGDS preincubation of the PMN monolayer, the effect was less pronounced (Fig 7). For both fractions, the inhibition of attachment to the PMN monolayer was incomplete.

Pretreatment of the PMN monolayer with saturating concentrations of both IgG and RGDS resulted in a significantly greater reduction in adherence of the SS-RBC to the PMN monolayers than either IgG or RGDS alone (Fig 6). Despite this significant decrease in the attachment of the SS-RBC to the PMN monolayers, the adherence of SS-RBC to PMN remained significantly greater than the adherence of AA-RBC. Thus, although PMN recognize SS-RBC by both IgG- and integrin-mediated mechanisms, these mechanisms do not entirely account for the increased adherence of SS-RBC to PMN.
Activation of PMN by SS-RBC. Phagocyte-mediated recognition of particles is a potent activating signal for PMN. Activation of PMN produces free oxygen radicals that further upregulate adhesion molecules on the vascular endothelium and cause vascular injury. Because this process may play a role in the vascular damage of SCD, we sought to determine whether PMN recognition of SS-RBC resulted in activation of the PMN. PMN were loaded with 10 μmol/L DCF, a sensitive probe that increases in fluorescence in response to the production of free radicals and permits measurement of the respiratory burst with relatively little interference from hemoglobin. These DCF-loaded PMN were then incubated with SS-RBC or AA-RBC in the presence of autologous plasma. SS-RBC induced a significant increase in fluorescence compared with AA-RBC (Fig 8, P < .001), indicating that SS-RBC but not AA-RBC activate the respiratory burst in PMN. Measurement of DCF fluorescence change stimulated by PMA in the presence or absence of SS-RBC and AA-RBC indicated that scavenging of oxidant species by RBC was not sufficient to explain differences in DCF fluorescence between SS-RBC and AA-RBC.

DISCUSSION

The mechanisms leading to VOC in patients with SCD are complex and involve interactions among SS-RBC, vascular endothelium, platelets, hemostatic plasma factors, and, quite likely, PMN. The possibility that PMN participate in the VOC process has not previously been explored. Specifically, the mechanism by which PMN become activated in patients with SCD and how these activated PMN may participate in VOC has not been studied. We have hypothesized that PMN become activated in SCD, in part, through specific interactions with SS-RBC and, as a result, participate in the events instigating or promoting VOC.

Because the initial step in PMN activation is recognition and because SS-RBC adhere to monocytes and vascular endothelium, it seemed plausible that direct attachment of SS-RBC could activate neutrophils in SCD. Neutrophils bind weakly to the microvasculature via the adhesion molecule P-selectin even when quiescent. Under such conditions of low shear at the microvascular wall, PMN come in direct contact with erythrocytes. In this study, we show that sickle erythrocytes incubated in the absence of plasma adhere to PMN, whereas AA-RBC show no significant attachment. Furthermore, when density fractionated SS-RBC were allowed to settle over the PMN monolayers, the dense fraction was more adherent than the light fraction. The mechanism of non–plasma-mediated SS-RBC attachment to endothelium has been related to cell shape and surface charge characteristics. Although we did not specifically study these mechanisms, surface charge is a well-known mediator of nonspecific attachment to PMN.

Sickle plasma increases the adhesion of SS-RBC to vascular endothelium. When we examined adherence of SS-RBC to PMN monolayers in the presence of autologous plasma, there was a dramatic increase in attachment that was not shown with AA-RBC. Because dense SS-RBC are opsonized with IgG directed against specific surface antigens on the RBC surface, these SS-RBC could attach to the PMN Fc receptor. Our data show that blocking the Fc receptors on the PMN monolayers results in a significant reduction in the number of attached SS-RBC after incubation in autologous plasma. Furthermore, when density fractionated SS-RBC were studied, the inhibition of SS-RBC attachment by IgG was limited to the dense fraction, consistent with the known distribution of IgG on SS-RBC fractions. We also show that this IgG-mediated process is specific for SS-RBC and sickle plasma. When AA-RBC were incubated with ABO-matched sickle plasma, there was no increase in the adherence of the AA-RBC. Similarly, SS-RBC incubated in normal plasma showed adherence equivalent to that of SS-RBC in buffer.

Although saturating PMN Fc receptors decreased SS-RBC
adherence, IgG did not completely inhibit attachment, indicating that other mechanisms are involved. Sickle plasma as well as specific soluble plasma factors, such as von Willebrand factor, fibrinogen, and thrombospondin, which all contain the RGDS motif, increase the adherence of SS-RBC to vascular endothelium. Blocking the endothelial adhesion receptors significantly reduces SS-RBC adherence to vascular endothelium. We found that incubation of PMN monolayers with saturating concentrations of RGDS peptide resulted in decreased adherence of both dense and light fractions of SS-RBC, although the effect was more prominent in the light fraction. This is consistent with previous findings that integrin-mediated adherence to vascular endothelium is more pronounced for sickle reticulocytes. Thus, integrin-mediated adherence appears to have a role in SS-RBC attachment to PMN.

When the PMN monolayers were incubated with both IgG and RGDS peptide, the inhibition of SS-RBC adherence was additive but incomplete, suggesting that additional mechanisms are involved. Repeated sickling of SS-RBC results in significant alterations in membrane properties. Phosphatidylserine (PS) is exposed from the inner lipid layer to the outer membrane. Exposed PS has been shown to cause adherence of SS-RBC to monocytes. Although not addressed here, PS may play a role in the recognition of SS-RBC by PMN and could be another mechanism of SS-RBC-PMN interaction. Charge distribution and shape change of SS-RBC also have been suggested as mechanisms of adhesion to vascular endothelium and could represent another mechanism for attachment of SS-RBC to the PMN monolayers. Whereas the attachment of RBC was measured using PMN that had spread on a surface and therefore were likely activated, the fact that the respiratory burst was triggered in suspension with SS-RBC suggests that spreading of PMN on a surface is not required for recognition of SS-RBC by PMN.

The ability of PMN to recognize SS-RBC may have clinical significance. Attachment of SS-RBC or PMN to microvascular endothelium decreases flow rate and promotes polymerization of SS Hb within the capillary. These flow conditions permit erythrocytes to have direct contact with neutrophils, which could allow attachment of SS-RBC to PMN. This recognition of SS-RBC could result in activation of the PMN and enhancement of the vasoocclusive process. Activation of PMN results in upregulation of CD11b/CD18 on the PMN surface. This complex mediates PMN adherence to the vascular endothelium via the adhesion molecule ELAM-1. Adhesion of activated PMN can directly damage vascular endothelium through the production and release of superoxide radicals. These toxic oxygen radicals from activated PMN also stimulate upregulation of VCAM-1 on the vascular endothelium, a known attachment site for sickle reticulocytes. Consistent with this model, we were able to show that PMN recognition of SS-RBC resulted in significant activation of the PMN oxidative burst and production of toxic oxygen radicals. Although these measurements were not made in a flow chamber, the strength of attachment that triggered the respiratory burst was at least strong enough to withstand washing and stirring during the assay procedure. Furthermore, in conditions of complete microvasculature occlusion, PMN could be in contact with SS-RBC for prolonged periods of time. Thus, activation of PMN may play a role in the pathophysiology of VOC through direct damage to the vascular endothelium as well as upregulating the vascular adhesion molecule VCAM-1 on the surface of the postcapillary venule.

Direct interaction of SS-RBC with PMN represents only one mechanism of PMN activation in SCD. Tissue injury and ischemia result in local production of inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1, that are potent mediators of PMN activation. These inflammatory cytokines, which are elevated in the plasma of a subpopulation of patients with SCD, also stimulate upregulation of ELAM-1 and ICAM-1 on the vascular endothelium, promoting adherence of PMN and sickle reticulocytes to the microvasculature. Thus, inflammatory cytokines, produced as a result of local tissue injury in SCD, could establish the conditions for further vasoocclusion. Infection, which is clinically associated with VOC, produces these inflammatory cytokines as well as other PMN activators, such as C5a, leukotriene B4, and bacteria-derived peptides. Therefore, chronic inflammation and infection are other mechanisms of neutrophil activation and also may set up conditions in the microvasculature that favor vasoocclusion.

We have presented a theoretical framework for the participation of neutrophils in the initiation and propagation of vasoocclusive crisis and have shown that PMN can become activated through direct interaction of sickle erythrocytes with PMN. Although the interaction of SS-RBC with neutrophils leading to PMN activation does not fully explain the complex pathophysiology of VOC, PMN may have a significant part in the cascade of events leading to crisis. Therapeutic interventions aimed at reducing SS-RBC-PMN adhesion and inhibiting PMN activation may prove significant in ameliorating the sequelae of both acute crisis and chronic vasoocclusion in patients with SCD.


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