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Albumin–IgG complexes in human serum and plasma that inhibit blood platelet adhesion

(artificial surface/protein–protein complex)

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ABSTRACT **Macromolecular albumin–IgG complexes have been isolated from human serum and characterized immunologically. These complexes are shown to be present in plasma also and appear to be normal constituents of blood. The complexes can be demonstrated in normal serum and plasma by immunoelectrophoresis and rocket immunoelectrophoresis, as well as by use of a two-step radioimmunologic binding test. The albumin–IgG complexes inhibit adhesion of human platelets to glass and account for 12–16% of the total protein in serum.**

Platelets, by virtue of their seemingly innate adhesiveness to many nonbiologic materials used in the fabrication of cardiovascular or other blood-contacting prosthetic or diagnostic devices, appear to play an important role in blood–material interactions (1, 2). Adhesion and aggregation of platelets account for a major part of the thrombocytopenia found with the use of extracorporeal or implanted devices that contact blood. Embolization of thrombus fragments or platelet aggregates is a recognized hazard inherent in the use of such devices (2). Increasing clinical application in recent years of prosthetic devices in the cardiovascular and certain other organ systems (3) has induced a number of investigators to develop new artificial surfaces that have enhanced blood compatibility. These clinical developments have also led workers to search for either naturally occurring or synthetic agents that might reduce the adhesion of platelets to artificial surfaces. No single artificial surface yet available has proved to be highly blood compatible, and the ideal antiadhesive agent has not been reported.

One of the major problems in studies of blood–material interactions has been the direct quantitation of platelet adhesion to artificial surfaces *in vitro* or *in vivo*. Such quantitative techniques are needed to evaluate meaningfully the compatibility of test surfaces with blood. Most methods used in the past were either qualitative or measured both adhesion and aggregation (3). The recent availability of more specific methods (4–6) has permitted identification of agents that inhibit platelet adhesion to artificial surfaces. Certain prostaglandins (6), β-lipoproteins, and a number of other plasma proteins (7, 8) have been found to inhibit platelet adhesion selectively.

In earlier studies, we reported the presence of platelet adhesion inhibitory activities in human serum and plasma (8, 9). Several of these plasma components were purified and characterized, while others were not (8).

We report here the isolation, identification, and immunologic characterization of naturally occurring complexes of albumin with IgG from human serum and plasma that inhibit platelet adhesion to glass. The albumin–IgG complexes were first isolated from serum. Next, they were shown to be present in untreated plasma and serum. Finally, a general technique for radioimmunologic detection of these and possibly other protein–protein complexes in plasma and serum was developed.

MATERIALS AND METHODS

Collection and Processing of Blood and Blood Components. Blood from normal healthy donors of either sex, 20–40 years old, who denied receiving medication of any type for at least 1 week preceding venepuncture was drawn by the two-syringe technique into plastic syringes containing 0.108 M sodium citrate (one part anticoagulant to eight parts blood). Platelet-rich plasma and platelet-poor plasma were prepared as described (8, 9).

Platelets were isolated from platelet-rich plasma by gel filtration on Sepharose 2B (10). The separated platelets were suspended in test protein solution in 0.01 M phosphate buffer, pH 7.0/0.133 M NaCl (P_i/NaCl) at a final concentration of 2000 cells per μl for conduct of the test for platelet adhesion. The entire procedure was performed at 23°C.

Serum was obtained from human blood drawn without anticoagulant and allowed to clot at 37°C for 2 hr. Serum was removed and centrifuged at 3000 × *g* for 30 min, incubated at 37°C for 16 hr, and then passed through a Millipore filter (pore size, 0.8 μm). Incubated filtered sterile serum for the isolation of platelet adhesion inhibitory activity and the platelet adhesion test was either used immediately or stored frozen at −70°C.

Determination of Platelet Adhesion. Platelet adhesion to glass was quantitated by an *in vitro* centrifugation method described previously (5).

Isolation of the Platelet Adhesion Inhibitory Activity. Human serum was lyophilized and then extracted five times with twice the original volume using chloroform/methanol, 2:1 (vol/vol). The isolation method then used consisted of (*i*) salting out at 50% saturation with neutral ammonium sulfate, (*ii*) chromatography of the resultant globulin fraction on Affi-Gel Blue (Bio-Rad), and (*iii*) sequential chromatography of the globulin fraction from Affi-Gel Blue chromatography on Sephadex G-100 and Sepharose 6B. Platelet adhesion inhibitor (PAI) eluted in the void volume of the Sephadex G-100 and Sepharose 6B columns. All columns were equilibrated and eluted with P_i/NaCl at 4–6°C. PAI so purified will be referred to as the albumin–IgG complex or PAI, while PAI in serum or plasma will be termed PAI activity.

Production of Antibodies Against PAI. Antibodies against PAI were raised in New Zealand White rabbits. A PAI preparation (100 μg) was adsorbed on alumina prepared by titration

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Abbreviations: PAI, platelet adhesion inhibitor or albumin–IgG complex; P_i/NaCl, phosphate-buffered saline (0.01 M phosphate, pH 7.0/0.133 M NaCl.

* Deceased, Oct. 20, 1981.

of 10 ml of 10% alum [$AlK(SO_4)_2 \cdot 12\ H_2O$] with 0.25 M NaOH. PAI was added to the alum solution before titration. After 10 min of stirring at 23°C, the PAI–alumina precipitate was separated by centrifugation at 500 × *g* for 10 min and suspended in water to give 10 mg (dry weight) of the precipitate per ml. The PAI–alumina suspension was injected subcutaneously into animals at four sites in the forelimbs and hindlimbs. Injection of antigen was repeated every 15 days for 2 months. In another set of experiments, PAI (100 μg) was injected subcutaneously in the skin of the backs of rabbits at multiple sites after prior emulsification with Freund's complete adjuvant. Injections were repeated weekly for 8 weeks.

All animals were bled from the ear vein. Blood was allowed to clot, and sera were stored at 4°C after addition of sodium azide to a final concentration of 0.01%.

Purification of Monospecific Antibodies. IgG fractions of rabbit anti-human albumin (monospecific by immunodiffusion and immunoelectrophoresis, 5.5 mg of antibody protein per ml) were obtained from Cappel Laboratories (Cochranville, PA) and then further purified by affinity chromatography on Sepharose 4B coupled by means of CNBr with purified albumin according to the manufacturer's instruction (Pharmacia). These preparations will be referred to as monospecific antialbumin.

Goat antiserum against human IgG (γ-chain specific) (Antibodies, Inc.) was fractionated by chromatography on DEAE-Affi-Gel Blue to obtain the IgG fraction. The IgG fractions of γ-chain-specific anti-IgG were then purified by adsorption with (*i*) glutaraldehyde-polymerized human albumin and (*ii*) glutaraldehyde-polymerized rabbit anti-human albumin (IgG fraction). The purified anti-IgG thus obtained was monospecific as determined by immunodiffusion and immunoelectrophoresis tests and will be referred to as monospecific anti-IgG.

Radioimmunologic Technique. PAI, fresh and untreated serum, and plasma were treated with excess monospecific anti-IgG for 16 hr at 4–6°C. The precipitates were centrifuged at 7000 × *g* for 30 min at 4°C and washed three times with 1 ml of P_i/NaCl/0.1% gelatin. The washed precipitates were suspended in 200 μl of P_i/NaCl/gelatin by brief sonication (Ultrasonic Cleaner B52, Smith Kline, New York) and then incubated with 50 μl of ^{125}I-labeled antialbumin [^{125}I-labeled antialbumin (specific activity, 1300 cpm/ng; 8 mg/ml)/unlabeled antialbumin (1.6 mg/ml), 1:20] in P_i/NaCl/gelatin for 16 hr at 4°C. The precipitates were separated by centrifugation at 7000 × *g* for 30 min and washed three times with 1 ml of P_i/NaCl/gelatin/0.2% Tween 20. The radioactivity in each tube was measured in a Beckman Gamma-8000 counter. The counting efficiency for ^{125}I was ≈83%.

Other Procedures. Protein estimation (11), immunoelectrophoresis in Immuno-AgaroSlides (Millipore Biomedica), rocket immunoelectrophoresis (12), polymerization of proteins with glutaraldehyde (13), and iodination of proteins with Na^{125}I using chloramine-T (14) were performed by standard procedures.

Materials. Human serum albumin (100% immunologic purity) used in affinity chromatography to purify antialbumin antibodies and in other experiments was from Behring (Somerville, NJ) and will be referred to simply as albumin or as free or noncomplexed albumin. Crystallized human serum albumin used in platelet adhesion tests was obtained from Sigma. Purified human IgG was from Miles and will be referred to simply as IgG or as free or noncomplexed IgG. Both crystallized albumin and purified IgG produced three bands in gradient (2.5–27%) polyacrylamide gel electrophoresis but only one precipitin arc when tested by immunoelectrophoresis with either respective specific antisera or antisera against whole human serum. CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). Antisera against whole human serum raised in rabbits and monospecific antisera against the Fc-fragment of human IgG (2.1 mg of antibody per ml) and human complement-reactive protein raised in goats were from Miles. Specific antisera against other human serum proteins raised in rabbits were obtained from Behring. Agarose (electroendosmosis, relative mobility = 0.13 ± 0.02) was from Bio-Rad.

RESULTS AND DISCUSSION

PAI, first detected in human serum and plasma because of its inhibition of platelet adhesion to glass, was isolated from delipidated human serum as described in *Materials and Methods*. A significant loss of PAI activity occurred at the Affi-Gel Blue chromatography step. PAI activity equal to that in the globulin fraction from Affi-Gel Blue was also present in the fraction retained by the gel. Because Affi-Gel Blue binds albumin and PAI is a complex of albumin and IgG, it may be that the larger portion of PAI activity is lost in the protein fraction that binds to the gel. The protein fraction bound to Affi-Gel Blue is complex, and attempts to purify PAI from this fraction were not successful. Only ≈10 mg of the PAI preparation was recovered from 100 ml (7 g of protein) of fresh normal human serum by the method described.

The PAI preparation migrates as a single band in the α_1–α_2-region in microzone electrophoresis. Its elution from Sepharose 6B in the void volume of the column indicates that PAI is of large molecular size. PAI forms a long single precipitation arc in immunoelectrophoresis with rabbit antisera against either PAI (Fig. 1 *A* and *F*) or human serum (Fig. 1*E*). The arc lay between those produced by human IgG and albumin. PAI also cross-reacted with both monospecific antisera against human albumin and IgG (γ-chain-specific and Fc-fragment specific). The PAI preparation did not react with whole rabbit antisera against

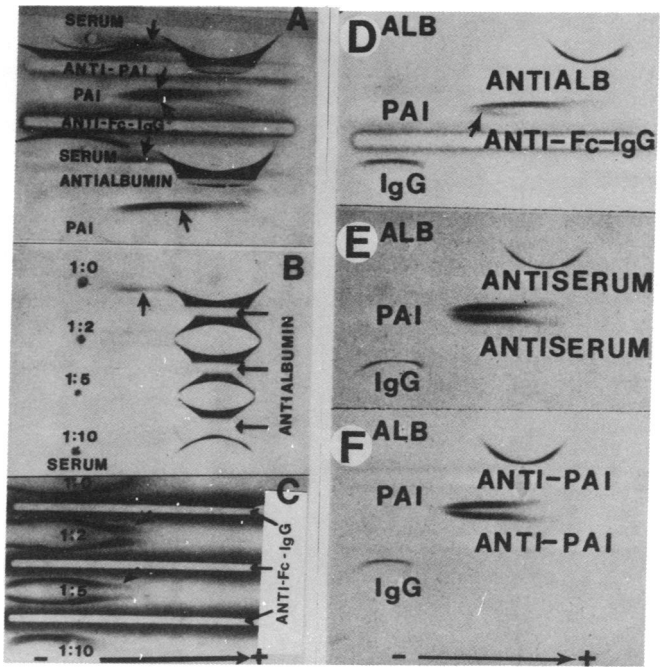

FIG. 1. Immunoelectrophoresis of a PAI preparation, human albumin, fresh and untreated human serum, and human IgG. Each preparation was tested with rabbit anti-human whole serum (antiserum), the IgG fraction of monospecific antihuman albumin (ALB), anti-PAI, and anti-human IgG (Fc-fragment specific). Electrophoresis was conducted on Immuno-AgaroSlides at 100 V for 35 min. Wells contained 1 μl of PAI (3 mg/ml), albumin (2 mg/ml), and IgG (10 mg/ml). (*B* and *C*) Serum (2 μl) was diluted 1:0, 1:2, 1:5, and 1:10. Troughs contained 50 μl of various antisera.

human IgA, IgM, β-lipoprotein, α_1-lipoprotein, α_2-lipoprotein, transferrin, antithrombin III, fibrinogen, complement components C1q and C1s or with whole goat anti-human complement-reactive protein antisera. These observations suggest the presence of both albumin and IgG in the PAI preparation, apparently in the form of a complex.

If, indeed, albumin and IgG are present in the form of macromolecular complexes in PAI preparations and these complexes are present in serum and plasma and have not been formed as a result of the various isolation procedures used, then it should be possible to demonstrate their presence in untreated serum and plasma. To show this, a PAI preparation, untreated fresh serum, and plasma were first allowed to crossreact with excess monospecific anti-IgG to precipitate PAI–anti-IgG. This precipitate was washed three times with P_i/NaCl/gelatin to remove noncomplexed albumin and possibly other contaminating proteins. The washed PAI–anti-IgG precipitate was then allowed to crossreact with ^{125}I-labeled antialbumin. The ^{125}I-labeled antialbumin–PAI–anti-IgG formed was separated by centrifugation and washed three times with P_i/NaCl/gelatin/0.2% Tween 20. The results indicate that the PAI preparation binds first to anti-IgG and then to ^{125}I-labeled antialbumin to form precipitates (Fig. 2). The binding of the washed PAI–anti-IgG complexes to ^{125}I-labeled antialbumin was linear up to 20 μg of PAI. Both untreated serum and plasma showed the presence of components similar to those in the PAI preparation when examined by this radioimmunologic test. In five normal human sera studied so far, the PAI complexes ranged from 9.5 to 12 mg per ml, which account for 12–16% of the total protein in serum. The plateaus obtained in tests with serum and plasma are not identical. This difference may be due to interference with binding by citrate or other components present in plasma.

Data presented in Table 1 show that free albumin, free IgG, or a mixture of free albumin and IgG in experiments similar to those described above did not show significant binding with ^{125}I-labeled antialbumin. When identical amounts of PAI were added to free albumin, free IgG, or the mixture, binding of ^{125}I-labeled antialbumin occurred, and the binding was of the same order in each case. The recovery of PAI was ≥93% in each instance, assuming binding in the absence of albumin or IgG to be 100%.

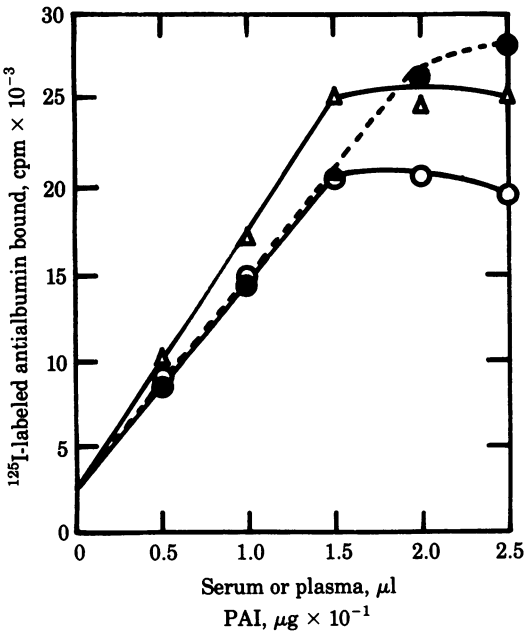

FIG. 2. Immunoreactivity of a PAI preparation (albumin–IgG complexes) (●), fresh and untreated human serum (△), and plasma (○) treated first with monospecific anti-IgG and then with ^{125}I-labeled antialbumin.

Table 1. Comparison of binding of anti-IgG followed by that of ^{125}I-labeled antialbumin in tests with human albumin, IgG, an albumin–IgG mixture, and a PAI preparation

Protein used, μg			Radioactivity bound, cpm	% recovery of PAI
PAI	Albumin	IgG		
0	0	0	2,634	—
15	0	0	18,815	100
0	20	0	2,567	—
15	20	0	18,021	93.0
0	0	7.5	2,760	—
15	0	7.5	18,353	96.2
0	20	7.5	2,492	—
15	20	7.5	18,921	101.4

To each of the various proteins or mixtures of proteins in P_i/NaCl/gelatin (total vol, 200 μl), 50 μl of monospecific anti-IgG (γ-chain specific; 2.1 mg of antibody protein per ml) were added. The mixtures were incubated at 4°C for 16 hr, and then the tubes were centrifuged at 7000 × *g* for 1 hr at 4°C. The supernatants were decanted as completely as possible. The precipitates were washed three times with 900 μl of P_i/NaCl/gelatin by suspending them by brief sonication and then centrifuging at 7000 × *g* for 30 min. Finally, the precipitates were suspended in 200 μl of P_i/NaCl/gelatin by brief sonication. To the precipitates was added 50 μl of monospecific ^{125}I-labeled antialbumin [^{125}I-labeled antialbumin (specific activity, 1300 cpm/ng; 8 mg/ml)/unlabeled antialbumin (1.6 mg/ml), 1:20]. The mixtures were incubated at 4°C for 16 hr. The resultant precipitates were separated and washed three times in P_i/NaCl/gelatin/0.2% Tween 20, and the radioactivity was measured. Values are averages of triplicate samples.

These observations indicate that appreciable amounts of albumin and IgG are present as macromolecular complexes in both serum and plasma. The findings further show that these albumin–IgG complexes have not been formed during the procedures used to isolate the PAI. It is curious that separate precipitin arcs corresponding to the complex have not been described previously in immunoelectrophoresis of serum when this test is conducted with either antialbumin or anti-IgG antisera. Immunoelectrophoresis of human serum in 1% agarose gels normally produces only one precipitin arc with antialbumin or with anti-IgG. Consequently, electrophoresis was performed using different amounts of serum. Immunoelectrophoretic patterns for four different strengths of normal untreated fresh human serum in Immuno-AgaroSlides with monospecific antialbumin and with monospecific anti-Fc-IgG are shown in Fig. 1 *B* and *C*, respectively.

Immunoelectrophoresis of undiluted human serum (2 μl) produced two arcs with antialbumin, one of them characteristic of albumin; these two arcs were partially identical. Similar studies with anti-IgG produced only one arc (Fig. 1*C*). Failure to see a precipitation arc in routine immunoelectrophoresis of serum versus anti–IgG may be due to the following reasons: (*i*) the arc remains fused with the IgG–anti-IgG arc, (*ii*) the amount of IgG in the PAI complex is small and does not form a separate visible arc with anti-IgG, or (*iii*) both of these. To verify these possibilities, electrophoresis of serum was conducted using larger volumes of serum than are normally loaded. The results (Fig. 1*C*) did not clearly demonstrate the presence of another arc corresponding to PAI. However, the arcs produced with anti-IgG were extended when 2 μl of undiluted serum or serum diluted 1:2 were used. The extended portions of the arcs occupied the region in which arcs corresponding to PAI appeared. Therefore, the extended portions of the arcs may be due to PAI. The second possibility, that the amount of IgG in the PAI complex is less than that of albumin seems plausible,

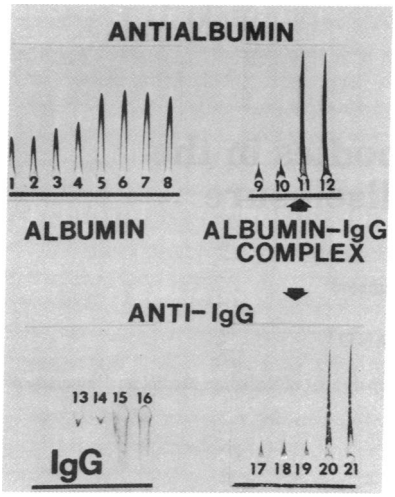

FIG. 3. Rocket immunoelectrophoresis (12) in 1% agarose gels of a human albumin–IgG complex preparation (PAI), human albumin, and human IgG against separate monospecific antisera to human albumin and to IgG. Five-microliter samples of antigens at various concentrations were subjected to electrophoresis for 16 hr at 100 V per plate (10 × 20 cm). Wells: 1 and 2, 3 and 4, and 5–8, an immunologically pure human albumin preparation (4 g/100 ml) diluted 1:300, 1:200, and 1:100, respectively; 13 and 14, purified human IgG (1 g/100 ml) diluted 1:100; 15 and 16, purified human IgG (1 g/100 ml) diluted 1:10; 9, 10, and 17–19, PAI preparation (2.5 mg/ml) diluted 1:10; 11, 12, 20, and 21, PAI preparation (2.5 mg/ml) diluted 1:0. Each plate contained 1 ml of either monospecific antialbumin or monospecific anti-IgG. Results are representative examples of five experiments.

as the arc produced by PAI and anti-IgG is weaker and shorter than that produced by PAI and antialbumin, anti-whole serum, or anti-PAI. The latter possibility has not been proven at this time, but these observations suggest that PAI contains less IgG than albumin.

Further immunoelectrophoretic and immunoadsorption studies of PAI preparations were conducted to rule out the possibility that albumin and IgG were present in noncomplexed form. In immunoelectrophoretic studies in 1% agarose, the PAI preparation produced a single long precipitin arc having a mobility between that of albumin and IgG when tested with either anti-PAI, monospecific antialbumin, or monospecific anti-IgG (Fig. 1 *D* and *F*); similar results were obtained in tests with antiserum against whole human serum (Fig. 1*E*). In rocket immunoelectrophoretic studies of PAI preparations (Fig. 3), cigar-shaped rockets characteristic of free IgG with cathodal migration were not observed in the anti-IgG region of the gel. The preparation did, however, react with anti-IgG and produced rockets that migrated toward the anode. These observations suggest that IgG is not present in free form in PAI preparations.

Additional studies of PAI preparations were carried out with insoluble immunoadsorbents prepared from either monospecific antialbumin or monospecific anti-IgG crosslinked by glutaraldehyde. An aliquot of a PAI preparation (1 mg of protein per ml) was incubated with each immunoadsorbents separately. Both the polymerized antialbumin and the polymerized anti-IgG removed immunologically detectable PAI completely.

PAI preparations were observed to inhibit platelet adhesion to glass (Table 2), whereas albumin alone had slight inhibitory effect and IgG alone or in a mixture with albumin was not inhibitory. In addition, the platelet adhesion inhibitory activity of the PAI preparation was abolished completely by treatment with either antialbumin or anti-IgG immunoadsorbents. Platelet adhesion values fixed at 50% by adjusting the concentration of the PAI preparation to 1 mg/ml increased to 98.8% and 98.6%, respectively, after treatment of the preparation with

Table 2. Inhibition of platelet adhesion to glass by PAI

Test material	Protein, mg/ml	% platelet adhesion Mean ± SD	*n*
PAI	3	15.9 ± 2.3	6
Serum*	69–78	17.3 ± 4.1	65
Albumin	50	78.3 ± 3.4	5
IgG	10	95.2 ± 4.6	5
Albumin/IgG	50:10	98.6 ± 3.8	5
None	0	95.4 ± 2.3	73

Test materials were in P_i/NaCl.
* Preincubated at 37°C for 16 hr and then filtered.

either of the immunoadsorbents, indicating virtually complete loss of PAI. Quantitative removal of both albumin and IgG antigens and simultaneous loss of platelet adhesion inhibitory activity after separate treatment of PAI preparations with polymerized antialbumin or polymerized anti-IgG would occur only if albumin and IgG were present in the form of macromolecular albumin–IgG complexes.

These studies should prove helpful in developing assays for screening the sera of patients for abnormal levels of albumin–IgG complexes (PAI). Deficiency of these complexes in a patient's plasma might lead to enhanced platelet adhesion to artificial surfaces, a condition encountered in extracorporeal circulation and use of vascular prosthetic or other devices that contact blood. Such a situation could result in thromboembolic complications. By coating artificial surfaces with a PAI preparation or by adding concentrated solutions of the preparation to blood that subsequently will contact an artificial surface, it may be possible to reduce platelet adhesion and thereby improve the compatibility of such surfaces with blood.

The general principles of the radioimmunologic procedure used to detect albumin–IgG complexes (PAI) may have application to the study of other protein–protein complexes in blood or other body fluids.

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