Studies of the Nature of the Binding by Albumin of Platelet-activating Factor Released from Cells*

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This work confirms that human umbilical vein endothelial cells activated by A23187 produce platelet-activating factor (PAF) (22.4 ± 9.9 ng/10⁶ cells/h; mean ± S.E.). A proportion of the PAF produced (56%) was released by cells into the medium. The PAF released, however, was not detected without prior organic extraction, and the method of organic extraction was critical for detection. Extraction with 80% ethanol was not successful, but a modified methanol/chloroform extraction method was. These observations may explain some of the conflicting reports in the literature on release of PAF by activated endothelial cells. The requirements for organic extraction may reflect the nature of cell-released PAF's binding by albumin; it was observed that PAF added to identical media could be detected in a bioassay without the requirement for extraction. Such PAF was also readily degraded by PAF-acetylhydrolase added to media, while PAF released from cells was resistant to such degradation, suggesting that it was released in a “protected” configuration. Stimulation of cells was performed in media with albumin as the only extracellular macromolecule. Limited proteolytic digestion of the albumin with trypsin and pepsin showed that PAF released by cells was located exclusively between amino acids 240 and 386 (domain II), while no synthetic PAF added to media was located on this region. These results are identical to those described for the release of PAF by the early embryo. Albumin exposed to embryos had a higher thiol concentration (0.77 ± 0.04 μmol of thiol/μmol of albumin; mean ± S.E.) than control media to which an equivalent amount of synthetic PAF was added (0.59 ± 0.02 μmol of thiol/μmol of albumin) (measured with Ellman’s reagent). Furthermore, albumin from conditioned media was more susceptible to reduction by 10 mM dithiothreitol than control albumin, as assessed by its mobility on PAGE. The protected configuration of released PAF was caused by cell-dependent conformational changes to albumin involving cysteine-cysteine disulfide bonds. Partial reduction with dithiothreitol of albumin exposed to cells resulted in released PAF being able to be detected directly in a bioassay without the requirement for prior organic extraction.

Platelet-activating factor (PAF1; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a biologically active ether phospholipid (1, 2). It has wide tissue distribution and may function in normal physiological processes such as inflammation, neural activity, and reproduction. It may also have a role as a mediator in pathological states such as asthma, ischemia, gastric ulceration, hypertension, atherosclerosis, and shock.

Many studies show that endothelial cells do not produce PAF under basal conditions in vitro, but synthesis can be stimulated by a variety of agonists (see Ref. 3 for review). PAF synthesis occurred in endothelial cells from diverse vascular beds, including aorta, umbilical vein, and pulmonary artery (4). Several reports (5–11) have shown that virtually all PAF synthesized by stimulated endothelial cells remained associated with the cells. By contrast, a number of reports (12–19) show that as much as 25% was released.

A common feature of reports that have failed to detect PAF release from endothelial cells (5–11) is that they have used methods other than a modified chloroform-methanol extraction method (20) for extracting phospholipids prior to assay. A recent study (21) showed that this method of extracting media was required to detect PAF released from preimplantation embryos, due apparently to PAF’s exclusive binding to domain II of albumin. Binding of PAF at that site on albumin resulted in PAF being protected from the actions of PAF-acetylhydrolase (PAF:AH) and also resulted in PAF being undetectable in bioassays without prior extraction. It was suggested that binding of PAF to domain II of albumin resulted in it being presented in a protected form (21). PAF added to medium also bound to albumin but was not found on domain II and was not in a protected form. We were interested to determine whether binding of PAF to albumin in this protected configuration occurs for PAF released by cells other than embryos and also to investigate the nature of the interaction which results in the protected configuration.

In this study we show that activation of endothelial cells results in the production of PAF and its release into medium. It is bound to albumin in a protected form, being exclusively located on domain II of albumin. It is also shown that binding of embryo-derived and endothelial cell-derived PAF to albumin was associated with conformational changes to albumin which involved modification of the cysteine-cysteine disulfide bonds, while the conformation conferred by disulfide bonds was necessary for PAF to remain in its “protected” configuration.

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1 The abbreviations used are: PAF, platelet-activating factor; PAF:AH, PAF-acetylhydrolase; PAGE, polyacrylamide gel electrophoresis; HUVEC, human umbilical vein endothelial cells; BSA, bovine serum albumin; ECCM, endothelial cell-conditioned medium; ECM, embryo-conditioned medium; HTFM, human tubal fluid medium; RIA, radioimmunoassay; ANOVA, analysis of variance.

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EXPERIMENTAL PROCEDURES—Human Umbilical Vein Endothelial Cells—Human umbilical vein endothelial cells (HUVEC) were collected (Maternity Unit, Royal North Shore Hospital of Sydney, NSW, Australia) as described (22). Blood was flushed from the vein, and cells were removed by 0.05% (w/v) collagenase (ICN Biomedicals, Costa Mesa, CA) in Hanks’ balanced salt solution, pH 7.2 (calcium/magnesium-free, Sigma). The cells were washed by centrifugation and resuspended in HEPES growth medium containing medium 199, ICN Biomedicals) supplemented with 20% (v/v) fetal calf serum (ICN Biomedicals), 50 μg of heparin/ml (Sigma), and 100 μg of endothelial cell growth supplement/ml (from bovine neural tissue; Sigma). They were plated onto 75-cm² flasks (Corning) coated with gelatin (from porcine skin; Sigma) and re-fed every second day until confluent. Following 4–6 passages, cells were transferred to 35-mm Petri dishes (Lux; Nunc, Naperville, IL) coated with gelatin at a seeding density of 3 × 10⁵ cells/1.5 ml. At confluence, dishes were washed three times with medium 199 + 3 mg of bovine serum albumin/ml (BSA (crystalized, Pentex); Miles, Kankakee, IL) (basal medium). The endothelial cell-conditioned medium (ECCM) was collected, and the cells (washed free of incubation medium) were resuspended in 1 ml of fresh basal medium. ECCM and cells were stored at −20°C.

Marine Embryo Culture—Two-cell mouse embryos were collected from superovulated Swiss outbred albino mice (Department of Veterinary Physiology, University of Sydney, NSW, Australia) as described (21). Thirty-two-cell embryos were cultured for 24 h at 37°C in 5% CO₂ in air. These embryos were cultured in HEPES culture medium (HTFM; Ref. 22) containing 3 mg BSA/ml. Embryos were removed after 24 h, and the embryo-conditioned medium (ECM) was stored at −20°C.

PAF Extraction and Assay—PAF in media or cell samples was extracted by a modified version (20, 24) of the Bligh-Dyer organic extraction method (25), followed by partial purification by TLC (24). Cells were sonicated for 1 s prior to extraction. Quantitative measurement of PAF was performed by either a platelet aggregation bioassay (25) or a radioimmunoassay (RIA) (26).

Preparation of ECM and ECCM Pools—Individual ECM and ECCM media samples were assayed. Where PAF was detected, samples were pooled to give large volumes of identical media with which to carry out characterization studies. Controls for ECM were prepared in the same media used for the stimulation of endothelial cells containing 2.5 μM A23187, or an equivalent volume of vehicle in basal medium (0.25 μl of Me₂SO/ml of basal medium). The endothelial cell-conditioned medium (ECCM) was collected, and the cells (washed free of incubation medium) were resuspended in 1 ml of fresh basal medium. ECCM and cells were stored at −20°C.

Binding of PAF to Albumin—The PAF receptor antagonist, WEB 2170 (6-(2-chloro-phenyl)-1-ethyl-8-(4-morpholinylcarbonyl)-4H,7H-cyclopenta[4,5]-thieno[3,2-b]pyridine-1,2-2H)-dithionitrobenzoic acid to 1 ml of ECM or control medium, containing 3 mg BSA/ml. Embryos were removed after 24 h, and the embryo-conditioned medium (ECM) was stored at −20°C.

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Binding of PAF to Albumin

RESULTS

PAF Production and Release by Endothelial Cells—Stimulation of endothelial cells with 2.5 μM A23187 induced all cultures to produce PAF (n = 18). Total PAF production was 22.4 ± 9.9 ng of PAF/10^6 cells/h (mean ± S.E.), of which 56% was released into the medium. The amount of PAF released into the media by 1 × 10^5 cells was 12.7 ± 1.4 ng (mean ± S.E.). PAF could not be detected in media in which cells were treated with basal media alone or basal media with vehicle (Me2SO).

In another sample, medium conditioned by 5 × 10^5 endothelial cells released 3.0 ± 0.2 ng of PAF (mean ± S.E.). However, direct assay of the medium (addition to bioassay without prior organic extraction) failed to detect any PAF present. By contrast, synthetic-PAF added to such media was readily detected without prior extraction. Samples of the same medium were also extracted with 80% ethanol. The ethanol was removed under nitrogen and lipids resuspended with HTFM with 3 mg of BSA/ml. PAF was not detected following this extraction procedure, yet it was readily recovered and detected following Bligh-Dyer extraction.

Pharmacological Characterization of PAF Released from Endothelial Cells—WEB 2170 (100 ng/ml) completely inhibited platelet aggregation induced by 3 ng of synthetic PAF/ml (Fig. 1, inset). This concentration of WEB 2170 also significantly (p < 0.001, t test) inhibited platelet aggregation induced by endothelial cell-derived PAF, following its extraction and partial purification by TLC. The antagonism of endothelial cell-derived PAF was equivalent to that of the same concentration of synthetic PAF (Fig. 1).

Susceptibility of Endothelial Cell-derived PAF to PAF-AH—Synthetic PAF or ECCM were exposed to 10% (v/v) untreated or acid-treated serum for 24 h (Fig. 2). PAF was still present in ECCM after a 24-h exposure to serum containing PAF-AH, and the PAF concentration was not significantly different from that found after exposure to acid-treated serum (in which PAF-AH was deactivated). By comparison, synthetic PAF in similar media exposed to serum was degraded after 24 h, but synthetic PAF exposed to acid-treated serum was unaffected.

Limited Proteolysis of BSA with Pepsin and Trypsin—ECCM or control medium was subjected to limited proteolysis with pepsin or trypsin in a similar manner to that previously described (21). The digestion products are referred to as P-44 (pepsin digestion, 44.4 kDa) and T-23 (trypsin, 36.9 kDa). Amino-terminal sequencing (21) showed that P-44 corresponded to amino acids 1–386 of albumin and T-23 to amino acids 240–583. There was a stoichiometric recovery of endothelial cell-derived PAF on both P-44 and T-23 after digestion (Fig. 3), suggesting that it was bound to a site that was common to both albumin fragments: amino acids 240–386, which correspond to domain II of albumin (29–31). By contrast, PAF added to control media was not found at this location. This suggests that PAF added to solution does not readily bind to that site on albumin accessed by cell-released PAF.

Susceptibility of Albumin to Reduction by Dithiothreitol—The results of the foregoing experiments are identical to those found for the association of embryo-derived PAF with albumin (21). We were interested to learn whether the binding of cell-released PAF to albumin in this apparently protected configuration involved conformational changes to albumin. The conformation of albumin is largely governed by 17 disulfide bonds in its structure (36). The status of these bonds were examined by observing the susceptibility of albumin to reduction with dithiothreitol. The reduction of disulfide bonds causes an increase in the hydrodynamic volume of the protein in SDS. The consequent reduced electrophoretic mobility (giving the appearance of increased molecular weight) is an indirect measure of the susceptibility to reduction.

Using this strategy, ECM and control media (with synthetic PAF added) were incubated with 10 μl dithiothreitol at 25 °C for increasing time. Albumin exposed to embryos showed a faster increase in apparent molecular weight than did control media (Fig. 4). After reduction with dithiothreitol for 20 min, the electrophoretic mobility of BSA that had not been exposed to embryos had not changed (68 kDa), while the apparent molecular mass of BSA in ECM had increased to 76 kDa. This difference between BSA in embryo-conditioned and control medium may suggest that exposure of albumin to embryos caused it to be more susceptible to reduction with dithiothreitol than was BSA not exposed to embryos.

Determination of the Thiol Concentration in Albumin—To assess whether this apparently greater susceptibility was due to a change in the thiol status of albumin, the thiol concentra-
Binding of PAF to Albumin

FIG. 3. The recovery of endothelial cell-derived PAF (in comparison to synthetic PAF recovery) on sham-digested BSA (■), peptic fragment P-44 (residues 1-386, □), or trypic fragment T-23 (residues 240–583, △) after correction for protein recovery. PAF was extracted and measured by bioassay after fragments were separated by preparative native PAGE. Histograms represent mean ± S.E. of 3 replicates for P-44 and representative data for T-23.

FIG. 4. Comparison of the apparent molecular mass of BSA under reducing conditions for BSA in ECM and that which not been exposed to embryos but contained synthetic PAF. Medium was incubated with dithiothreitol (10 mM) for increasing time and the reaction stopped with iodoacetamide (0.1 M). The graph shows the molecular mass (kDa) of albumin in synthetic PAF medium (■) and in ECM (□) over time. Data shown are representative results. Electrophoresis was on 20% SDS-PAGE.

FIG. 5. The thiol concentration (micromolar) in BSA in control medium supplemented with PAF (■) or ECM (□), measured over a range of protein concentrations, using the 5,5’-dithionitrobenzoic acid spectrophotometric assay. Results are mean ± S.E. of 3 replicates. Assay validation was performed by incubating increasing concentrations of cysteine with 5,5’-dithionitrobenzoic acid for 20 min at room temperature (n = 3) (inset). The correlation between calculated thiol concentration (y axis) and cysteine concentration (x axis) was y = 0.996x + 0.048, r = 1.000.

treated embryo- or endothelial cell-derived media, but was detected in preparations following modified Bligh-Dyer extraction. PAF activity was detected in media treated with dithiothreitol for 30 min (being approximately 70–80% of that detected following organic extraction). Following treatment with dithiothreitol for 60 min, the amount of PAF detected was less than that observed at 30 min, while no PAF was detected after 90 min of treatment. Synthetic PAF activity added to control medium (which had been treated with 10 mM dithiothreitol for 0 or 30 min before assay) was unaffected (results not shown), indicating that dithiothreitol had no effect on the assay or on PAF bioactivity. The results suggest that reduction of disulfide bonds in albumin in cell-conditioned medium altered the association of PAF and albumin in such a way that PAF became detectable in the bioassay.

The loss of bioactivity after longer periods of exposure to dithiothreitol (60 and 90 min) may have been caused by the reduction of albumin occurring to a degree that it was no longer a suitable carrier for PAF. A consequence of this may be that PAF was lost from solution onto the hydrophobic surfaces of the incubation vessels. This possibility was tested by the use of a strategy designed to allow for the convenient recovery of any PAF that was lost from albumin following its reduction with dithiothreitol. Dithiothreitol was added to ECCM in the presence of a slurry of Amberlite resin XAD-2 chromography beads. The beads were to provide a high surface area hydrophobic binding site for PAF released from albumin. PAF was recovered by washing the beads with organic solvents and PAF was measured by RIA. In the absence of dithiothreitol, no endothelial cell-derived PAF was recovered from the XAD-2 beads, suggesting that under such conditions albumin had a higher affinity for PAF. After reduction with dithiothreitol for 60 min, approximately 60% of the estimated PAF present in ECCM was recovered from XAD-2 beads (Table I).

**DISCUSSION**

The results confirm that human umbilical vein endothelial cells activated by A23187 produce PAF. While a significant proportion of the PAF remained associated with the cells, much of it was released into medium. This PAF was not bioactive in...
media in a platelet aggregation assay, nor was it recovered following ethanolic extraction. This behavior of endothelial cell-derived PAF contrasts with that of synthetic PAF added to media under the same conditions.

Synthetic PAF was readily detected by platelet aggregation without the requirement for extraction, and was also readily extracted by ethanol (80%). We have shown previously (21) that this behavior of synthetic PAF is independent of the method of its preparation. Cell-released PAF was also apparently different from PAF retained by cells, which can be readily extracted by 80% ethanol treatment (11). The reason why the modified Bligh-Dyer method was able to extract PAF from ECCM but 80% ethanol could not is not clear. The relative volume of methanol used was much greater, being 1:19 (media:methanol) compared with only 1:4 (media:ethanol). We have found that the very slow dropwise addition of media to the larger volume of methanol followed by incubation at room temperature is required for successful extraction. The subsequent secondary extraction of methanol with chloroform may also be important in the successful recovery of PAF. Systematic study of the variables is needed to define the minimum requirements for successful extraction of PAF from media released from cells.

The failure of previous reports to detect the release of PAF from endothelial cells may have been due to the measurement of PAF without extraction, or following simple ethanolic extraction procedures, or using various other mixtures of chloroform and methanol (5–12). It can be concluded that extraction of cell-conditioned media with the methods used in this report are required before release of PAF by cells can be excluded. It also shows that recovery of synthetic PAF added to media does not act as a suitable positive control for the recovery of cell-released PAF.

One study (37) showed that stimulated endothelial cells produce both PAF and its sn-1-acyl analogue (acyl-PAF), with acyl-PAF being the predominant product. That study measured the concentration of PAF and acyl-PAF remaining associated with the cells but did not determine whether either was released into medium. It was suggested that acyl-PAF can mimic the biological actions of PAF, but was substantially less potent. Thus measurement of the bioactive material released by endothelial cells does not exclude the possibility that it was induced by acyl-PAF. The antibody used in the RIA in the current study is more selective for PAF (38) than that used in earlier studies (39). The good agreement on the amount of PAF released by cells detected by the bioassay and the RIA is unlikely if the bioactivity was due to acyl-PAF. The amount of released PAF detected by the RIA was similar after treatment of extracted material with phospholipase A₁ (results not shown). Since acyl-PAF, but not PAF, is sensitive to digestion with phospholipase A₁ (37), we conclude that most of the bioactivity detected from endothelial cells was PAF. This does not exclude the possibility that acyl-PAF may also be released and bind in a similar fashion to PAF. This question could be addressed by mass spectrometric analysis of the released phospholipids.

It has previously been demonstrated that extracellular albumin is an acceptor for PAF released by cells (40), presumably by removing PAF from the lipophilic environment of the membrane. We observed (21) that the release of PAF by preimplantation embryos was exclusively at domain II of albumin, and the current study shows that this is also the binding site for PAF released by endothelial cells.

BSA (583 amino acids) has 80% sequence homology with human serum albumin (41), and most structural studies have been performed on human serum albumin. It is a “heart-shaped” globular molecule, similar to an equilateral triangle. It is proposed that there are three cylindrical peptide segments (domains I, II, and III) connected by solvent exposed α-helical chains, which are common sites of proteolytic cleavage. Domain II contains a hydrophobic core, which can provide a binding region for hydrophobic molecules (42, 43). Clay et al. (44) provided kinetic evidence that albumin possessed four binding sites for PAF which had an average dissociation constant of 0.1 μM. The rabbit PAF receptor has an estimated kₐ for PAF of −0.5 nM (45). Synthetic PAF added to control culture medium caused platelet aggregation in whole blood down to a concentration of 0.5 ng/ml (−0.9 nM). However, at a concentration of 3.0 ng/ml (−5.6 nM), endothelial cell-derived PAF in untreated culture medium did not cause platelet activation. This concentration was well below the stated kₐ for PAF binding by albumin, yet above the kₐ of the platelet receptor. It might therefore...
be expected that the kinetics would favor PAF transfer from albumin to the PAF-receptor, as was the case for synthetic PAF. The absence of this suggests that PAF released by cells either binds to sites on albumin that have a much higher affinity than those described by Clay et al. (44), or that the conformation of albumin causes PAF bound at domain II to be solvent- or sterically protected in a way that does not occur for PAF binding to albumin in the absence of cells.

The secondary and tertiary structure of albumin is highly dependent on the presence of 17 interchain disulfide bonds, which link 34 (of the available 35) cysteine residues. The free cysteine residue is generally at amino acid 34 in domain I of albumin. This should be detected as 1 μmol of thiol/μmol of BSA, yet approximately 0.5 μmol of thiol/μmol of albumin is normally detected (32, 46). This may be the result of dimerization of albumin (47) in solution or be due to the reactive Cys-34 being “solvent-protected” by the helices in some albumin molecules (48). BSA exposed to embryos expressed more reactive thiol residues than untreated medium with PAF added, showing that binding of PAF per se did not induce this increase in thiol concentration. The increase in thiol concentration was therefore apparently due to a cell-dependent process. The observation that albumin was more readily reduced by dithiothreitol after incubation with embryos suggests that the cell-dependent process involved conformational changes to albumin involving cysteine-cysteine disulfide bonds. It has been shown (49) that interaction of albumin with cells or surfaces caused a conformational change resulting in a mixed population of albumin molecules. While the mechanisms of the change are not well understood, it appears to cause a flattening of the molecule giving it a greater surface area when bound to cells and a higher binding affinity. It has been proposed (49) that this conformational change promotes dissociation of passenger fatty acids, facilitating transfer from albumin to the cell. It will be of interest to determine whether a similar mechanism operates in reverse for the removal of PAF from cells.

Exposure of embryo-conditioned media and endothelial cell-conditioned media to dithiothreitol, under conditions expected to cause reduction of albumin, resulted in a large proportion of the expected PAF activity present to be detected in a direct assay of platelet aggregation (without prior organic extraction). Control experiments confirmed that the aggregation was not caused by the dithiothreitol itself, nor did dithiothreitol reduce the sensitivity of the assay to PAF. This result suggests that binding of PAF to domain II of albumin, which results in its protected configuration, involves protein conformation that is dependent upon the disulfide bonds that can be reduced by dithiothreitol.

The observation that PAF activity was lost with prolonged exposure to dithiothreitol may have several possible explanations. One is that as albumin was reduced (and thus changed conformation) it lost its affinity for PAF, resulting in PAF being adsorbed by the surfaces of the holding tube, as is known to occur in protein-free media (50). Using different types of vessels may reduce this loss of activity. Confirmation that the cause of the time-dependent loss of activity was its loss to hydrophobic surfaces, was the ability to recover PAF from XAD-2 chromatography beads after incubation with dithiothreitol treated ECCM. The results indicate that as the conformation of albumin is altered with reduction, PAF becomes “exposed” and hence available to bind to the platelet receptor, causing platelet aggregation in the bioassay. As reduction proceeds, PAF seems to be readily lost from albumin and bound by other hydrophobic surfaces, such as the test tube or XAD-2 beads.

These experiments provide indirect evidence that the protected nature of PAF's binding to albumin involves disulfide bonds between cysteine molecules. The observation that breaking these disulfide bonds is necessary for making PAF accessible in vitro infers that some form of disulfide isomerization or reduction may be required for PAF's “release” from the cell and binding on domain II of albumin. The changed thiol status of albumin following exposure to cells implicates cellular enzymes in this process.

Studies with embryo-conditioned medium show that, while embryo-derived PAF in untreated media is inactive in vitro (21), upon injection into animals it can induce thrombocytopenia (51). Such studies show that cellular-dependent factors must be involved in the creation and processing of cell-released PAF in its protected form and its exposure in vivo to allow it to be bioactive. Further experiments are required to determine the nature of this mechanism. One possibility may be the presence of cell surface protein disulfide isomerases (52, 53), which might cause reduction of some disulfides resulting in solvent exposure of domain II, facilitating the loading of PAF at this site. It might be speculated that a similar process, in reverse, may be required to allow PAF to be made available at target cells.

While there is some controversy regarding the ability of some cell types to release PAF, for some other cell types such as activated basophils, there is general agreement that PAF is released upon cellular activation. This might suggest that PAF released from some cell types, or under some conditions, is not in the protected configuration described in this report. Systematic investigations are required to assess this possibility.

This study used defined culture media with albumin as the only extracellular macromolecule. The next important question to investigate will be to determine whether PAF binds to albumin in this protected form when a complex protein source such as serum is present. Several studies have detected PAF in blood (54, 55). In view of the high PAF:AH levels present in blood, this is a surprising result, inferring that this PAF may be protected from PAF-AH.

In conclusion, PAF released from embryos and endothelial cells binds to albumin at domain II (amino acids 240–386), protecting it from the hydrolytic effects of PAF:AH in vitro. PAF added to solution does not bind to this site on albumin in vitro. This binding makes extracting and measuring cell-released PAF difficult, but may also act to increase the half-life of PAF released from these cell types. Should such cryptic binding also occur in vivo, it may well influence PAF's half-life and hence its potential to act as a circulating mediator. The impact of such binding by albumin on the kinetics of its recognition by the PAF-receptor in vivo requires investigation.

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