Conjugation with PVP

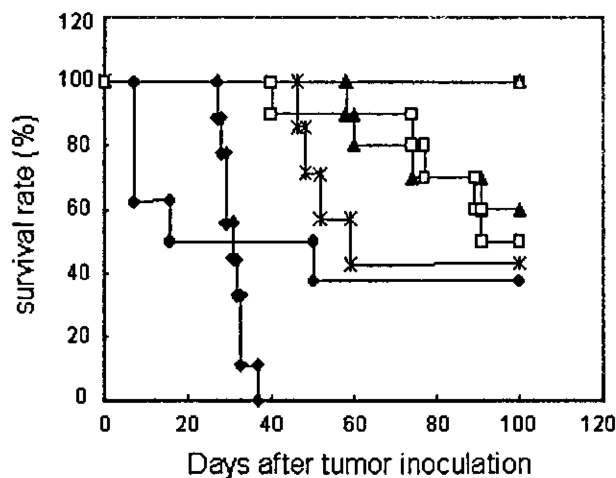
a) PVP- TNF-alpha

To design conjugated proteins with greater efficacy and safety, i.e. to selectively enhance desirable therapeutic activities of bioactive proteins without increasing their side effects, it is important to closely regulate their *in vivo* behavioral characteristics, taking into account their mechanism of action. Thus, the development of polymeric modifiers with useful DDS (Drug Delivery System)-functions, which can regulate their *in vivo* behavioral characteristics such as targeting capability and controlled release, is required. Therefore, we examined other candidates as polymeric modifiers for introduction of these new functions. As mentioned above, polyvinyl pyrrolidone (PVP), to which useful functional groups can be introduced by radical co-polymerization, was found to be retained in blood better than PEG.. Furthermore, PVP is also highly biocompatible, and is widely used as a plasma expander. With this in mind and to assess the usefulness of PVP as a polymeric modifier for protein conjugation, we evaluated the therapeutic potency of PVP-TNF-α compared with that of PEG conjugate (PEG-TNF-α) [21, 22]. The terminal carboxyl-bearing PVP was prepared by radical polymerization of VP. The number-average molecular weight (Mn) of the synthesized PVP was controlled by varying the amount of transfer agent added to the reaction. Previously, we showed that PEG- TNF-alpha and PEG-IL-6, conjugated with PEG (5,000 Mn), possesed marked antitumor activity or thrombopoietic activity, respectively. These conjugates were revealed to have higher therapeutic effects *in vivo* than those prepared with PEG (2,000 Mn) or PEG (12,000 Mn). Therefore, in this study, 6,000 Mn PVP purified by gel filtration was used as a polymeric modifier for the first choice.

Natural human TNF-alpha was conjugated with activated PVP (Mn; 6,000, Mw/Mn =1.14) via amide bonds between lysine amino groups of TNF-alpha and N-hydroxyscuccinimide groups of PVP at the end of the main chain. The resulting PVP- TNF-alpha was purified from native TNF-alpha and separated into five fractions of various molecular sizes by GF-HPLC (protein standard). The activities of PVP- TNF-alphas decreased with increasing of molecular weight and degree of PVP-modification (PVP-attachment to TNF-alpha). This result was also observed when TNF-alpha was conjugated with PEG, and this profile of changes in the bioactivity of PVP-TNF-alphas was similar to that observed with modification of TNF-alpha with PEG (Mn; 5,000, Mw/Mn =1.32).

The anti-tumor effects of PVP-TNF-alphas on S-180 solid tumors were compared with those of native TNF-alpha and MPEG-TNF-alpha by single i.v. injection. The antitumor effects were evaluated by a score of hemorrhagic necrosis 24 hr after sample administration. The antitumor effect of PVP-TNF-alpha fraction 3 (Mn; 101,000), in which 40% of the total lysine amino groups of TNF-alpha were coupled with PVP, was markedly higher than that of native TNF-alpha at a dose of 10,000 JRU/mouse and induced complete regression in two of seven mice(data not shown).

Figure 5. Antitumor effect of PVP-TNF-α by scheduled administration on survival days after Meth-A tumor inoculation. Meth-A-bearing BALB/c mice were given samples on days 7, 10, 14 and 17 after tumor inoculation. Complete regression was defined when tumor was not regrown for >100 days. Statistical significance compared with saline control: *$P < 0.01$.



	Single i.v. injection dose (JRU/mouse)	Survival time (Days)	Complete regression
◆ Saline	0	31 ±1.0	0/9
● Native TNF-α	10,000	>48 ±15.9	3/8
■ MPEG-TNF-α	200	>100 ±0*	10/10
*	100	>72 ±10.0*	3/7
△ MPVP-TNFαFr.3	200	>100 ±0*	10/10
▲	100	>88 ±5.5*	6/10
□	50	>87 ±6.1*	5/10

To investigate the usefulness of PVP as a polymeric modifier and PVP-TNF-alpha fraction 3 as a systemic antitumor agent, we compared the antitumor potency of PVP-TNF-alpha fraction 3 to those of native TNF-alpha and MPEG-TNF-alpha with scheduled i.v. injections on Meth-A solid tumors. As shown in Figure 5, PVP-TNF-alpha fraction 3 and MPEG-TNF-alpha at a dose of 200 JRU showed the maximal antitumor effects without any toxic side effects (such as sudden death and others) and had antitumor effects superior to that of native TNF-alpha at a dose of 10,000 JRU. On the other hand, only 50 JRU of PVP-TNF-alpha fraction 3 was needed to exhibit a marked antitumor potency, and tumor growth was completely inhibited for the observation period, as in 10,000 JRU native TNF-alpha and 100 JRU MPEG-TNF-alpha. These results indicated that PVP-TNF-alpha fraction 3 was

approximately 200- and 2-fold more potent an antitumor agent than native TNF-alpha and MPEG-TNF-alpha, respectively. The plasma half-life of PVP-TNF-alpha fraction 3 (360 min) was about 80- and 3-fold longer than those of native TNF-alpha (4.6 min) and MPEG-TNF-alpha (122 min), respectively. As described above, anti-tumor effects of TNF-alpha are due not only to direct cytotoxicity against tumor cells, but also to specific injury of the tumor vascular and effective activation of anti-tumor immune cells. TNF-alpha selectively enhances the vascular permeability of tumor vessels. The enhancement of TNF-alpha half-time may lead to a decrease in its distribution to the liver and spleen, which are the major sites of side effects, and would selectively increase its anti-tumor effects. Therefore, we are confident that the increased anti-tumor potency of PVP-TNF-alpha Fr.3 relative to native TNF-alpha and MPEG- TNF-alpha may be attributed to increased half-life. These results suggest that PVP is a useful polymeric modifier for conjugation of TNF-alpha to increase its antitumor potency, and multifunctionally conjugated TNF-alpha may be a potenciated antitumor agent for clinical use.

b) PVP-IL-6

Next, we applied PVP for polymer conjugated-IL-6 and assesed its usefulness as a novel protein modifier [41]. PVP-IL-6 was synthesized and separated into three fractions with different molecular size to assess the appropriate degree of modification for the highest *in vivo* thrombopoietic potency. To investigate the effects of PVP-modification on therapeutic activity (thrombopoietic activity) of IL-6 *in vivo*, we administered PVP-IL-6s of various molecular sizes subcutaneously to mice every 2 days for 8 days. Administration of native IL-6 at 50ug/day caused a 30% increase in platelet number with respect to the most potent enhancer of thrombopoieses among the three fractions of PVP-IL-6s examined. No severe side effects such as body weight loss were observed in PVP-IL-6-treated mice. All mice treated with native IL-6 had acute phase reactions and increase in plasma IgG1 level. This result suggested that PVP is efficient polymer for many cytokines.

PVP-derivates

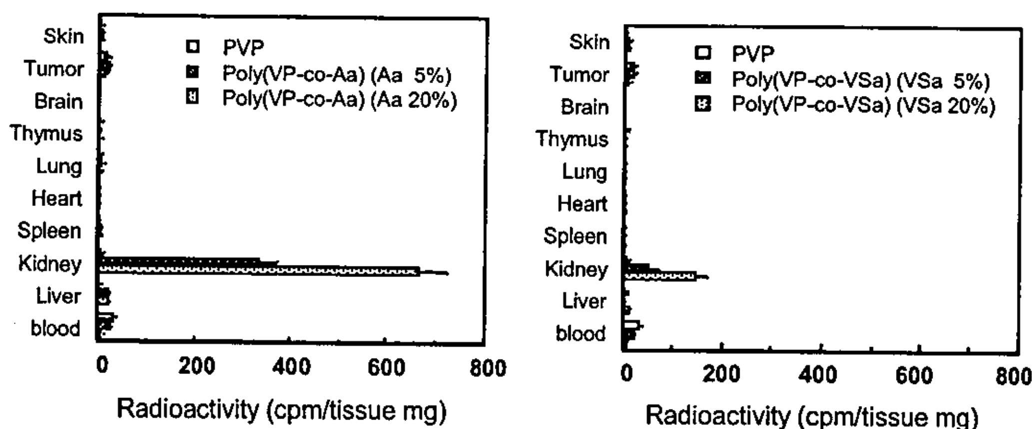
In the DDS area, development of targeting polymers for specific tissues is very important. In order to deliver a conjugated drug to a targeted tissue, the conjugate must display desirable pharmacokinetic characteristics such as plasma clearance and tissue distribution. Therefore, to control the *in vivo* behavior of conjugated drugs with polymeric modifiers, the pharmacokinetic characteristics of the polymeric modifiers themselves must be assessed. Based on our studies mentioned above, selection of the optimal polymeric modifiers for manifestation of specific characteristics of drugs and for the purpose of drug therapeutic regimens will be obtained.

Polyvinyl pyrrolidone (PVP) can be easily mixed with various co-monomers by radical polymerization, in order to control its physicochemical properties and to add functions such as targeting or sustained release. Additionally PVP is the most suitable polymeric modifier for prolonging the circulation lifetime of a drug and localizing the conjugated drug in blood, as described above. Previously, we found that nonionic polymers did not interact with endothelial cells, but the increase of interaction between polymers and endothelial cells is parallel to the amount of charge or hydrophobic groups. To evaluate the relationship between pharmacokinetics and their anionic functional groups, copolymers of carboxylated PVP [poly(vinylpyrrolidone-co-acrylic acid)] and sulfonated PVP [poly(vinylpyrrolidone-co-vinylsulfonic acid)] were synthesized by radical polymerization [23]. The plasma clearance of PVP and anionized PVPs were compared in mice bearing S-180 solid tumors. The anionized PVP derivatives in the circulation decreased as the number of anionic groups in the PVP increased. Anionized PVPs were cleared more quickly from the blood than PVP, and the clearance of carboxylated PVP from blood was similar to that of sulfonated PVP with the same content of anionic groups. Endothelial cells and the glomerular capillary wall of the kidney are known to be coated with highly polyanionic sialoprotein [42]. Therefore, anionic charged polymers, such as anionized dextran, are generally considered to be cleared more slowly from the circulation than nonionic and cationic polymers despite having the same molecular weight [43]. The reason for this discrepancy is not clear, but it may be partially due to the differences in the structures of anionized polymers. Polysaccharides, such as dextran, have often been used to characterize the pharmacokinetics of polymeric modifiers with various characteristics [44]. Nonionic and cationic polysaccharides are rapidly captured by the cells of the reticuloendothelial system (RES) mainly in the liver after their i.v. injection, but anionized polysaccharides are barely taken up by the RES due to the electrostatic repulsion between the negative charge of the polymer and the vascular wall [13]. Consequently, the plasma half-lives of anionized polysaccharides are longer than those of nonionic and cationic polysaccharides, even if anionized polysaccharides are distributed in the kidney. By contrast, PVP did not show any specific tissue distribution and mainly remained in blood. Thus, the anionized PVPs are suitable for assessing the effects of anionic groups on pharmacokinetic characteristics.

The tissue distribution of PVP and anionized PVPs of the same molecular size were measured 3 h after i.v. injection (Figure 6). Anionized PVPs accumulated more in the kidney than in other tissues, although PVP showed little tissue-specific localization. In particular, carboxylated PVPs showed higher renal accumulation than sulfonated PVPs, and about 30% of the administered dose of 20%-carboxylated PVP was observed in the kidney. In contrast, PAA (100%-carboxylated) and PVP (0%-carboxylated) showed little accumulation in the kidney. These results suggest that carboxylated PVP that contains the optimal number of carboxyl groups might exhibit the highest accumulation in the kidney. The blood levels of all polymers decreased rapidly 24 h after i.v. injection. PVP did not accumulate in the kidneys, while anionized PVPs accumulated in the kidneys over the period studied.

The maximal renal levels occurred 3h after treatment and slowly declined thereafter. In particular, the level of renal 20%-carboxylated PVP was thirty-two times higher than that of PVP 24 h after i.v. injection. These results suggest that carboxylated PVP was rapidly distributed to the kidney and was gradually excreted into the urine, whereas sulfonated PVPs were quickly excreted in urine. In contrast, PVP was effectively retained in the blood and gradually excreted into the urine without concentrating in the kidneys.

Figure 6. Tissue distribution of PVP and anionized PVP derivatives at 3h after i.v. injection in mice. Mice were intravenously injected with 125I-labeled polymers. After i.v. injection, mice were killed and the organs were collected. The radioactivity was measured by γ-counter. Mice were used in groups of five. Each value is the mean ±S.D.



Few studies have examined the effects of carboxyl and sulfonic groups attached to a polymer on the pharmacokinetic characteristics of drug conjugates. We assessed the accumulation sites of anionized PVPs in the kidney by tissue-section analysis, and found that carboxylated PVPs were effectively localized and retained in the renal proximal tubular epithelium cells (data not shown). The mechanism of uptake of carboxylated PVPs in the proximal tubular epithelium cells in vivo is currently under investigation.

Our approach for designing polymeric modifiers involved only a few steps. In the present study, the effects of anionic charge and other physicochemical properties of the polymeric modifier were investigated. The relationship between several biological factors and physicochemical disposition is necessary to determine the biopharmaceutical characteristics of polymeric modifiers. This approach may facilitate the optimum molecular design of polymeric modifiers for drug delivery systems.

Conclusions

One of the most efficient ways of improving therapeutic potency of proteins has been to modify them with polymeric modifiers, as typified by PEG PEGylated IFN-alpha, that has clinically been shown to have marked antiviral activity against hepatitis C. Until recently, we have also shown the usefulness of conjugation. However, clinical application of most PEGylated proteins has not yet been successful. In most cases, PEGylation occurs randomly at multiple lysine residues in the proteins, some of which may be located in or near the protein active site. The resultant PEGylated proteins are therefore heterogeneous and composed of various positional isomers with distinct specific activities. Additionally, their bioactivity is markedly lower than of the unmodified proteins. To overcome these drawbacks, we attempted to develop a strategy for site-specific PEGylation [45]. The PEGylation system in which the N terminus of the protein is specifically PEGylated after creation of lysine-deficient mutants with full bioactivity through the use of phage libraries have been successfully established. This N-terminal mono-PEGylated mutant TNF-alpha had comparable bioactivity to unmodified TNF-alpha *in vitro*, and other properties including plasma half-life, antitumor activity, and toxicity were greatly improved. This technology will open a new window in the area of polymer-conjugation and increase the possibility for clinical application of conjugated proteins, by adopting polymeric modifiers with useful DDS-functions as described in this review.

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Fusogenic Liposome can be Used as an Effective Vaccine Carrier for Peptide Vaccination to Induce Cytotoxic T Lymphocyte (CTL) Response

Toshiki SUGITA,[a,e] Tomoaki YOSHIKAWA,[a,e] Jian-Qing GAO,[a] Mariko SHIMOKAWA,[e] Atushi ODA,[a,e] Takako NIWA,[a,e] Mitsuru AKASHI,[b,e] Yasuo TSUTSUMI,[c,e] Tadanori MAYUMI,[d] and Shinsaku NAKAGAWA*,[a,e]

[a] *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan:* [b] *Department of Molecular Chemistry, Graduate School of Engineering, Osaka University; 2–1 Yamadaoka, Suita, Osaka 565–0871, Japan:* [c] *National Institute of Health Science, Osaka Branch Fundamental Research Laboratories for Development of Medicine; 1–1–43 Hoenzaka, Chuo-ku, Osaka 540–0006, Japan:* [d] *Department of Cell Therapeutics, Graduate School of Pharmaceutical Sciences, Kobe-Gakuin University; 518 Arise, Igawadani, Nishi-ku, Kobe 651–2180, Japan: and* [e] *CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation; Tokyo, 102–8666, Japan.*

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We reported previously that fusogenic liposome (FL) introduced antigen protein encapsulated in the liposome directly into the cytoplasm of the antigen presenting cells, and that it induced immune responses. In the present study, we encapsulated TAX38-46, an HTLV-I derived protein and an antigen peptide model, into FL. The ability to induce effective cytotoxic T lymphocytes (CTL) responses in immunized mice was evaluated. Results showed FL could induce CTL response effectively and suggested that FL is a potential peptide vaccine carrier.

Key words fusogenic liposome; cytotoxic T lymphocyte (CTL); peptide vaccine

Induction of cytotoxic T lymphocytes (CTL) that kill tumor cells is a critical role of immunotherapeutic agents for cancer. Most cancer vaccine strategies have focused on induction of CTL and various approaches, including DNA, virus vector or peptide vaccine, have been tested.[1] In general, the advantages of a peptide vaccine are the induction of CTL by the epitope and the safety, stability and simplicity of peptide production. However, peptides by themselves are rather weak immunogens. Peptides usually require the addition of an adjuvant for inducing immunogenicity, and recently, incomplete Freund's adjuvant (IFA) has been widely used as a vaccine adjuvant in clinical research.[2] However, IFA is only useful for inducing humoral immunity and thus it does not induce effective cell-mediated immune responses. In contrast, complete Freund's adjuvant (CFA) is used for CTL induction, although it cannot be applied clinically due to serious side effects, such as inflammation.[3]

To induce cell-mediated immune responses, a peptide must be delivered through the cytoplasm to the MHC class I processing pathway. However, peptides are unable to pass through the cytoplasm alone.[4] Therefore, we hypothesized that a vaccine carrier is required, which can deliver antigens into the cytoplasm and which exhibits adjuvant activity. We reported previously that fusogenic liposome (FL) introduces antigen protein encapsulated in the liposome directly into the cytoplasm, and that it can induce effective immune responses.[5—7] FL is a fusion liposome in which proliferating ability is inactivated, but which retains its cell membrane fusing ability. Therefore, FL can deliver encapsulated molecules into cells. Furthermore, FL possesses immune stimulating activity.[5] In this context, we considered FL as an ideal peptide vaccine carrier.

In the present study, we chose the TAX protein epitope (TAX 38-46; H-2D^{k-} restricted epitope in TAX, amino acid residues 38-46, sequence ARLHRHALL.[10]), which is an immunodominant target antigen derived from the human T-cell leukemia virus type 1 (HTLV-I), as a model peptide. HTLV-I, a retro virus, is known to cause Adult T-cell leukemia (ATL).[8] ATL is characterized by poor prognosis after chemotherapy and no effective therapy exists. Immunotherapy, which can induce strong anti HTLV-I CTL, has been proposed as an optimal approach to ATL treatment.[9] However, an effective immunotherapeutic approach has not been developed to date.

MATERIALS AND METHODS

Cells and Animals L929 cells were cultured with RPMI-1640 containing with 10% fetal calf serum (FCS). Female C3H mice were purchased from Nippon SLC (Kyoto, Japan) and used at 6 weeks-old stage.

Fusogenic Liposome Encapsulated TAX 38-46 TAX 38-46 and FITC conjugated TAX 38-46 were purchased from SIGMA (Japan). FL was prepared as described previously.[5—7] Briefly, lipid mixture (L-α-dimyristoyl phosphatidic acid/phosphatidylcholine/cholesterol in molar ratio of 1:4:5) was hydrated with phosphate buffered saline (PBS) or PBS containing TAX 38-46 or FITC conjugated TAX 38-46. Peptides containing liposome was prepared from these hydrated mixture by using a hand-held extruder with two layers of cellulose acetate membranes (pore size, 800 nm in diameter) (ADVANTEC, Osaka, Japan), and washed with PBS by centrifugation (20000 rpm, 40 min, 4 °C) in order to remove free peptides. These liposomes were mixed with UV-inactivated Sendai virus and incubated at 37 °C for 2 h with shaking. FL was purified by sucrose gradient centrifugation (24000 rpm, 2 h, 4 °C). The diameter of FL was detected by using ZETASIZER 3000HS (Malvern, U.K.). The concentration of peptide in FL was determined by measuring the fluorescence intensity of FITC.

^{51}Cr Release Assay For the CTL assay, TAX38-46 (50 μg), empty FL, TAX38-46 emulsified with CFA (contains 50 μg

* To whom correspondence should be addressed. e-mail: nakagawa@phs.osaka-u.ac.jp © 2005 Pharmaceutical Society of Japan

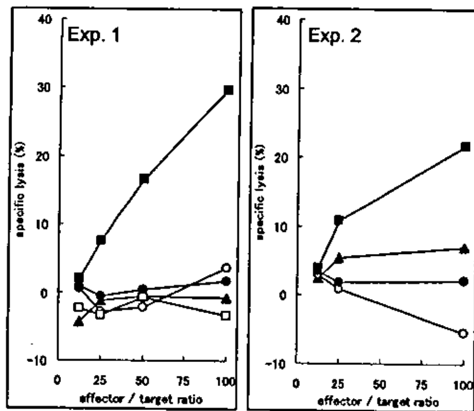

Fig. 1. TAX Peptide Encapsulated in FL Induced TAX Specific CTL

Nine days after the final immunization, mononuclear cells from the spleen of mice immunized with PBS (closed circle), TAX 38-46 alone (open circle), empty FL (open square), TAX 38-46 emulsified with CFA (closed triangle), or TAX 38-46 encapsulated in FL (closed square) were isolated and restimulated with MMC-treated TAX38-46 pulsed L929 for 5 d to enhance the frequency of antigen specific CTLs. CTL activity against TAX38-46 pulsed L929 (positive targets) or L929 (negative targets) was measured by ^{51}Cr release assay. Results were expressed as a percentage of specific lysis. Percentage of specific lysis=(percentage of positive target lysis)−(percentage of negative target lysis).

TAX38-46), or TAX38-46 encapsulated in FL (contains 30 μg TAX38-46) diluted to in total were injected into back of C3H mice (i.d.), respectively. Mice were immunized once a week for three weeks, and the spleen was harvested 9 d after the last immunization. Splenocytes were mixed with mitomycin C (MMC) treated TAX38-46 pulsed L929 for 5 d, and CTL assay was determined as follows. L929 cells (5×10^6) were pulsed with TAX 38-46 (positive targets) for 1 h at 37 °C or not (negative targets), and labeled with 200 μCi of ^{51}Cr for 2 h at 37 °C. Splenocytes were incubated with target cells (positive or negative targets) for 2 h at 37 °C. CTL activity was determined by measuring ^{51}Cr levels in the supernatants using a gamma counter. The specific lysis was determined as follows: percentage of specific lysis= (percentage of positive target lysis)−(percentage of negative target lysis).

RESULTS AND DISCUSSION

We prepared the FL encapusulating TAX 38-46. The diameter of FL was 880.1±9.5 nm. We calculated the concentrarion of peptide in FL using FITC conjugated TAX38-46. One milliliter of FL suspension at OD_{540} of 1.0 contained 29.8 μg of TAX 38-46. FL encapsulating TAX 38-46 was immunized with 100 μl at OD_{540} of 10.0.

Figure 1 demonstrates that the induction of TAX-specific CTL occurred only in response to TAX38-46-FL. CTL induction could not be detected in the TAX38-46-CFA administered group, in the TAX38-46 group, or in the empty FL group. Likewise, there was no CTL response in the group immunized with the mixture of TAX 38-46 and empty FL (data not shown). Previous reports have demonstrated that FL can deliver peptide directly into cytoplasm and that it possesses immune stimulating ability.

In a future study, we will investigate the control of peptide distribution in the cytoplasm and attempt to induce a stronger CTL response. Peptides that target the endoplasmic reticulum (ER) are able to induce stronger CTL responses because MHC class I molecules are expressed on the ER membrane.[11—13] However, because FL cannot control peptide distribution in the cytoplasm, we were unable to target the ER specifically. Therefore, the use of both FL and ER targeting sequences will more effectively induce CTL.

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