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Selective removal of human serum amyloid P component from rat blood by use of an immunoaffinity membrane in an extracorporeal circulation system

Tomoko Adachi[a,*], Makio Mogi[b], Minoru Harada[b], Kohichi Kojima[a]

[a]*Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257, Japan*
[b]*Department of Oral Biochemistry, Matsumoto Dental College, Shiojiri, Nagano 399-07, Japan*

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Abstract

We examined the suitability of an immunoaffinity membrane (IAM) bearing specific antibody as a ligand for removing human serum amyloid P component (hSAP) from blood passed through a simple extracorporeal circulation system established in rats. The specific antibody was the most effective of the various ligands tested for removing hSAP from human blood. To determine the value of the hSAP in human or rat plasma, we also developed a simple ELISA. In the rat extracorporeal circulation system, the hSAP level in the inlet blood to the IAM module decreased to 49% of the initial concentration within 60 min. In contrast, the hSAP remained at the initial concentration throughout the study in the module without the IAM. The use of this extracorporeal circulation system in this case allows preclinical evaluation of the ex vivo removal of a human plasma component in an animal model. Biocompatibility of the IAM was also examined. No change in blood cell counts or activation of the coagulation system occurred after contact with the IAM. Non-specific adsorption was not observed, since there was no statistically significant difference in IgG, complement C3, or albumin level between the pre- and post-treatment with this module. The immunological effects of the IAM were also examined using this system. Four weeks after the termination of the extracorporeal circulation, the rats examined showed no detectable antibody titer to the ligand.

Keywords: Amyloid P

1. Introduction

It has been reported that human serum amyloid P component (hSAP) is deposited along with amyloid fibrils in all types of amyloidosis, as well as with cerebral β-protein amyloid associated with Alzheimer's disease [1–3]. Furthermore, hSAP is also a precursor of the amyloid P found in association with amyloid deposits that are the occasional consequence of inflammatory diseases [4]. Although hSAP is not required for amyloid fibrillogenesis in vitro, it may protect the fibrils from degradation in vivo [5]. Human SAP is a normal constituent of plasma and is synthesized by the liver and circulates in the blood at a concentration of 10–40 μg/ml. No deficiency disease of hSAP has been reported in humans. Though the precise mechanisms of amyloid fibril formation, deposition and persistence are not known, it is reasonable that the specific removal of hSAP would result in a decreased risk of amyloid deposi-

*Corresponding author.

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tion. Human SAP is known to bind to a variety of ligands in a calcium-dependent manner [6]. Some of the major ligands and binding sites are 4,6-*o*-(1-carboxyethylidene)-β-D-galactopyranoside, heparin sulfate and DNA but little information is available concerning their use for the effective removal of hSAP from plasma. Therefore, these ligands are not practical for medical application.

Currently, affinity chromatography using an immobilized antibody is widely used and this method has been developed to remove antibodies [7,8] and antigens [9] for medical application. We also previously reported that β_2-microglobulin (β_2-MG) in the plasma of patients on long-term hemodialysis could be removed by high-performance immunoaffinity chromatography [10,11]. Furthermore, specific antibody immobilized on an immunoaffinity membrane (IAM) was highly effective for removal of rat immunoglobulin E (IgE) passed through an extracorporeal circulation system that did not require an efficient plasma separator [12].

When blood makes contact with the surface of a material, several host protection systems are triggered to maintain the host's homeostasis. These systems include the activation of both the coagulation and immune systems [13–15]. Furthermore, immunoadsorption methods also have problems such as leaking of the ligand and immune responses against the ligand. Although rabbits and dogs have been used for models of extracorporeal circulation [16,17], their immunological responses were generally not examined.

In this study, we investigated the removal of injected human protein from the blood of rats having an extracorporeal circulation system. This system involved specific polyclonal antibody (rabbit) for hSAP immobilized on a membrane as the ligand and it was shown to be more effective for removing hSAP from human blood than any of the other ligands tested. The IAM was highly effective for removal of exogenous hSAP in this rat model of an extracorporeal circulation system. Biocompatibility of the specific antibody-bearing IAM was also examined in terms of non-specific binding of other plasma components, blood cell counts, activation of the coagulation system, and humoral response to the specific antibody bound to the IAM.

2. Experimental

2.1. Materials

Cellulose membrane for size exclusion, pore size 0.8 μm was obtained from Sartorius (Göttingen, Germany). Anti-hSAP IgG (polyclonal, rabbit) was purchased from Dakopatts (Glostrup, Denmark). Horseradish peroxidase and hSAP were purchased from Calbiochem-Novabiochem. (La Jolla, CA, USA). DNA cellulose, heparin agarose, rabbit IgG agarose, rat albumin, and poly(ethylene glycol) 8000 were purchased from Sigma (St. Louis, MO, USA). Sepharose CL-6B and protein A Sepharose were obtained from Pharmacia (Uppsala, Sweden). Rabbit IgG was purified by protein A Sepharose chromatography. Anti-rat IgG, anti-rat complement C3, rat complement C3, anti-rat albumin and peroxidase-conjugated anti-rat IgG were purchased from Organon Teknica (Durham, NC, USA). Rat IgG was from Zymed (South San Francisco, CA, USA). All other chemicals used were analytical grade. Female Sprague–Dawley (SD) rats (12–20 weeks of age, 250–300 g) were used for preparation of the extracorporeal circulation system.

2.2. Sandwich ELISA for hSAP

The preparation of immunoreagents and the assay system were essentially similar to those described previously [12]. The ELISA was conducted with immunoplates (Nunc, Roskilde, Denmark) that had been coated with rabbit IgG anti-hSAP (100 μl per well). Standard solution, rat plasma or human serum was diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (50 μl) and peroxidase-conjugated anti-hSAP IgG (50 μl) was then added. After incubation for 30 min at 37°C, each well was washed five times with PBS-Tween 20 (0.05%). A 100-μl volume of the substrate (0.083 m*M* 3,3′,5,5′-tetramethylbenzidine and 0.01% of H_2O_2 in citrate buffer) was then added. After a 15-min incubation, the reaction was stopped with 1 *M* phosphoric acid and the absorbance was read at 450 nm with an automated microplate reader (Model EL 312e, Bio-Tek, Winooski, VT, USA).

2.3. In vitro test for removal of hSAP from human blood

Preparation of the IAM was carried out as described previously [12]. In short, the cellulose membrane was immersed in a solution of 3% succinic anhydride in pyridine and shaken at room temperature for 24 h. The carboxylated membrane was then reacted with tri-*n*-butylamine and isobutyl chlorocarbonate in dehydrated dioxane for 30 min at 15°C. After having been washed twice with dioxane, the membrane was placed in an aqueous solution of the IgG (anti-hSAP IgG) at pH 9.0 and left there for 3 h at 4°C. Estimation of the amount of anti-hSAP IgG coupled to the membrane was made by the *o*-phthalaldehyde method [18]. The IAM was hydrolyzed in 6 *M* HCl at 110°C for 21 h and the amino acids liberated were reacted with *o*-phthalaldehyde. The fluorescence intensity of the reaction mixture was measured with a spectrofluorophotometer (RF-5000, Shimadzu, Kyoto, Japan). Purified rabbit IgG was used as a standard for protein assay.

The binding rate was measured for the various ligands and gel (IAM, DNA cellulose, heparin agarose, rabbit IgG agarose and Sepharose CL-6B gel). Briefly, 2 ml of heparinized human blood was mixed with 0.066 mg of various ligands coupled to their substratum or with the 0.2 ml of Sepharose CL-6B gel alone. The mixtures were incubated at 37°C for 15, 30, 45, 60 and 90 min and the plasma concentration of SAP was then measured by the ELISA.

2.4. Extracorporeal circulation system

An extracorporeal circulation system was set up as described previously [12]. Each animal received 300 μg of hSAP by intravenous injection 1 h before the system was started since the basal level of hSAP in human serum is 10–40 μg/ml. Blood was pumped at 1.5 ml/min through the empty module, blank membrane module or module containing IAM-immobilized anti-hSAP antibody. The affinity-membrane module was custom-built and consisted of twelve membranes (total surface area 316.8 cm^2) having a total volume of around 3.2 ml.

Blood samples were subsequently obtained from the inlet as a subterminal blood at 0.5, 1.0, 1.5 and 2.0 h after the start of the extracorporeal circulation. The hSAP and other plasma component contents were then determined for each sample.

After 2 h of extracorporeal circulation, the module was removed (i.e., from a carotid artery and contralateral jugular vein). The vasa were ligated and the skin sutured and then blood was collected once a week and measured by ELISA for the anti-rabbit IgG titer of the plasma.

2.5. Biocompatibility of IAM

The biocompatibility of the module was examined as follows: (1) possible changes in blood cell counts and activation of the coagulation system were tested in vitro, (2) non-specific adsorption was evaluated by measurement of other plasma component levels during the circulation as a short-term effect and (3) the immunological effect of the IAM was evaluated in terms of the titer of the rat antibody to the ligand (i.e., rabbit IgG) as a long-term effect.

In vitro tests were carried out with human blood. EDTA or sodium citrate was used as an anticoagulant for the measurement of blood cell counts or coagulation system, respectively. The IAM (26.4 cm^2) was incubated with human blood at 37°C for 0.5, 1.0, 1.5 and 2.0 h and the numbers of white blood cells, red blood cells and platelets were counted with an electronic counter (Model S-plus IV, Coulter Electronics, FL, USA). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured with an automatic coagulometer (CA-3000, Toa Medical Electronics, Kobe, Japan).

For evaluation of the short-term effect, rat plasma components IgG, complement C3 and albumin were measured by a turbidimetric assay on a centrifugal analyzer (Cobas Fara, Roche Analytical Instruments, Nutley, NJ, USA). Rat plasma was diluted, 10-fold for complement C3, 50-fold for IgG and 100-fold for albumin, with PBS containing 4% poly(ethylene glycol) 8000 as diluent. Antibodies against the three proteins were diluted 10-fold. Each component was determined by mixing diluted plasma or standard solution with the respective antibody. The change in turbidity was measured at 290 nm. Rat IgG and

albumin were measured with commercially purified protein used as a standard whereas complement C3 was expressed as a percentage of its level in control serum.

For the long-term evaluation, that is, to check a possible immune response against the IAM, rat IgG specific for rabbit IgG (rabbit IgG was ligand for IAM) was quantified by ELISA using purified rabbit IgG immobilized on immunoplates (Nunc). Prior to each subsequent step, the plate was washed five times with PBS–Tween 20. 100 μl of the rat plasma, diluted with the PBS-Tween 20 at 5–200-fold dilutions was added to each well and allowed to interact for 1 h at room temperature. After a wash step, peroxidase-conjugated anti-rat IgG (100 μl) was added and incubation conducted for 30 min at room temperature. The reaction of peroxidase was similar to that for the sandwich ELISA for hSAP described previously. As positive control plasma we used plasma from rats that had been intravenously and/or subcutaneously injected with rabbit IgG.

3. Results

3.1. Evaluation of ELISA for hSAP

The standard curve prepared from the data obtained by the ELISA with the anti-hSAP antibody demonstrated a sigmoidal relationship between the hSAP protein concentration and the absorbance (Fig. 1). The working range of the assay was between 1 and 100 ng/ml for hSAP and the detection limit was 0.5 ng/ml for hSAP. When pooled human plasma was assayed for immunoreactive hSAP protein by the present ELISA, logic transformation of the displacement data generated a line with a slope that was not significantly different from the slope of the line generated by authentic hSAP, indicating the presence of immunoreactive hSAP protein in the plasma samples (Fig. 1). Therefore, this assay can be employed to determine the concentration of hSAP in human plasma. This ELISA was not affected by rat plasma.

Inter- and intra-assay variations for the ELISA were evaluated for samples that contained 16–53 μg/ml ($n=8$) of hSAP. Intra-assay variation based on the within-day C.V. ranged from 2.99% to 6.97%

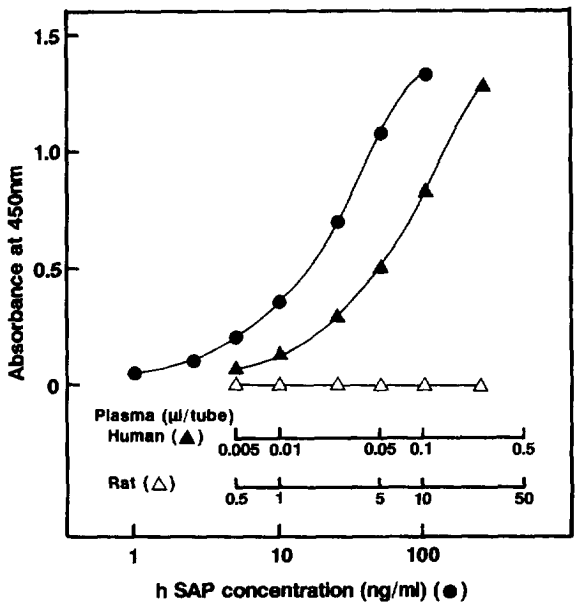

Fig. 1. Standard curves of hSAP generated by our sandwich ELISA. Authentic hSAP (●) was incubated in triplicate and indicated volumes of human serum (▲) or rat serum (△) were incubated in duplicate.

($n=8$). Inter-assay variation derived from the between-day C.V. ranged from 2.48% to 9.97% ($n=5$). Furthermore, the recovery of hSAP added to rat plasma was 92–114%.

3.2. Evaluation of IAM used with model samples

The density of IgG coupled to the membrane was 11.1±3.6 mg/cm^2. The membrane trapped the hSAP efficiently and the content was 1.45±0.15 μg hSAP/cm^2 when we employed a model sample of 80 μg of purified hSAP dissolved in 2 ml of PBS. Based on our calculations, the binding capacity of the support was 131 μg hSAP/mg immobilized IgG. The time course of trapping of hSAP by the IAM indicated that 90% of the initial concentration was complexed within 15 min (data not shown).

The strength of hSAP binding to the IAM was assessed with 0.1 *M* glycine-HCl buffer (pH 2.5) used as a regenerating solvent. Because of the tight binding of hSAP to the antibodies, all hSAP (99.9% of the initial concentration) remained on the IAM. Therefore, reusage of the IAM was difficult in practice. By immediate equilibration with PBS after

preparation of the IAM, the membrane could be stored at 4°C in PBS containing 0.01% NaN_3 and no loss of hSAP binding capacity of the IAM was observed over a period of six months as assessed by the same type of in vitro experiment. The specificity of the antibody coupled to the IAM was ascertained as follows: when human plasma was subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with the anti-hSAP used for the IAM, a single band (at 23 kDa) appeared and its mobility was identical to that of authentic hSAP (data not shown).

3.3. Evaluation of binding test using human serum

The time-course of the trapping of hSAP by the IAM was examined and compared with that of trapping by the various ligands and Sepharose CL-6B gel (Fig. 2). No trapping of hSAP in human blood by rabbit IgG agarose or Sepharose CL-6B gel was observed. However, DNA cellulose and heparin agarose trapped 50% of the hSAP. In contrast, anti-hSAP rabbit IgG as a ligand on the IAM (26.4 cm^2 surface area) trapped 95% of the initial concentration within 90 min.

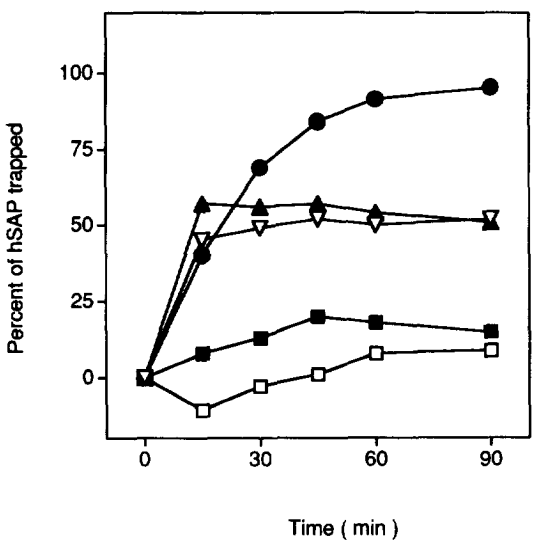

Fig. 2. Time course of adsorption of hSAP by the IAM or various other ligands. Human blood (2 ml) containing 15.8 μg/ml hSAP was applied to various ligands of equal weight: the IAM (●), DNA cellulose (▲), heparin agarose (▽), rabbit IgG agarose (□) and Sepharose CL-6B gel (■).

3.4. Extracorporeal circulation system

Aliquots of the arterial blood at the inlet of the IAM as subterminal blood were taken for the determination of the hSAP concentration. Three experiments were performed and similar results were obtained in each case. As shown in Fig. 3, the plasma level of hSAP in the blood that passed through the system equipped with an empty module or blank membrane (without immobilized anti-hSAP IgG) did not change for up to 2 h. In contrast, when the IAM was used in the system, the hSAP blood level showed a sharp decrease. The subterminal blood hSAP level decreased to 49% of the initial concentration within 60 min and reached 24% of it within 2 h of the start of circulation. The immunoadsorption of hSAP did not saturate the capacity of the immunoadsorbent during the experiment. The flow-rate did not affect the trapping rate for hSAP over the range of 0.5–1.5 ml/min.

3.5. Biocompatibility of IAM

Possible changes in blood cell counts and activation of the coagulation system effected by the IAM were examined in vitro by incubation of human

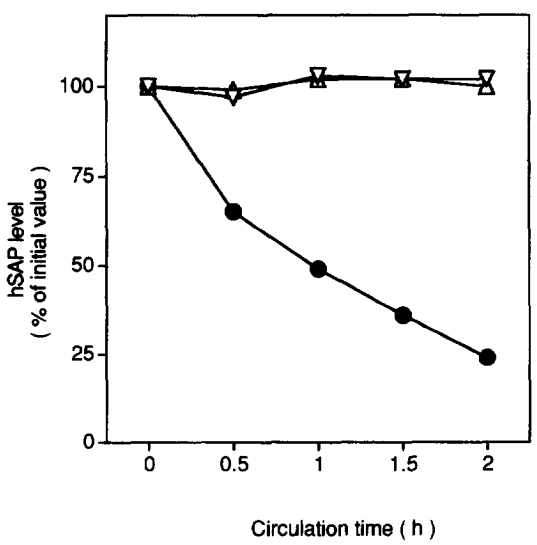

Fig. 3. Blood levels of hSAP during extracorporeal circulation through IAM, blank membrane or empty module. Blood hSAP level is given as a percentage of the initial concentration: IAM (●), blank membrane (△), empty module (▽).

Table 1
Hematological and coagulation changes after incubation of IAM with human blood

	Percentage of control values			
	0.5 h[a]	1 h	1.5 h	2 h
White blood count	102	98	96	100
Red blood count	98	97	96	97
Platelet count	94	97	97	95
PT	100	100	98	106
APTT	102	103	104	110

[a] Incubation time.

blood with the IAM. There was no significant difference in the number of white blood cells, red blood cells, or platelets and no effect on PT or APTT was observed (Table 1).

The precision and analytical range of the assays used for the rat plasma components assessed for non-specific binding to the IAM are shown in Table 2. Evaluation of possible changes in other plasma components aside from hSAP, i.e., a comparison with the initial profile, is essential for a validation of the present system. First of all, there was no significant change in IgG, complement C3 or albumin level in the plasma of blood that had circulated through the blank membrane or the IAM (Table 3 and Table 4,

Table 2
Analytical range and precision of turbidimetric assays used for the three plasma components evaluated

Item	Analytical range	Precision (C.V., %)	
		Within-run	Between-run
IgG	25–300 μg/ml	3.40–7.20	2.19–4.36
C3	5–40% of control serum	0.14–0.99	2.10–4.50
Albumin	50–500 μg/ml	0.11–0.34	1.55–3.21

Table 3
Evaluation of non-specific adsorption to blank membrane at various times of circulation

	Percentage of initial concentration			
	0.5 h[a]	1 h	1.5 h	2 h
IgG	101	102	95	96
Complement C3	94	95	90	89
Albumin	96	100	87	101

[a] Circulation time.

Table 4
Evaluation of non-specific adsorption to IAM at various times of circulation

	Percentage of initial concentration			
	0.5 h[a]	1 h	1.5 h	2 h
IgG	98	95	98	122
Complement C3	96	92	88	88
Abumin	97	93	97	90

[a] Circulation time.

respectively). These proteins remained constant at more than 80% of their initial concentration throughout the entire circulation period. Secondly, neither Western blotting nor dot blotting detected (detection limit was 0.1 ng/dot) any loss of rabbit IgG from the membrane (data not shown). Moreover, neither clotting nor hemolysis was observed under our experimental conditions.

The titer of rat IgG toward rabbit IgG (rabbit IgG anti-hSAP was immobilized on the IAM) was negligible for four weeks after termination of the extracorporeal circulation (Fig. 4) but rats injected with rabbit IgG intravenously and/or subcutaneously as a positive control showed an immunological response (antibody production) after two injections.

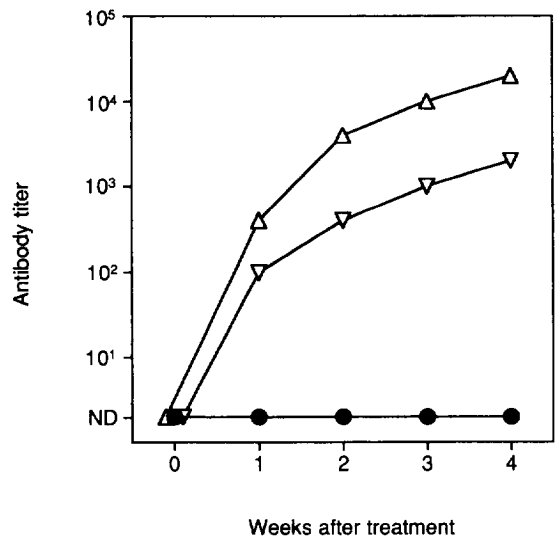

Fig. 4. Time-course of immune response to rabbit IgG. Three rats per group were used to assess the immunogenicity of the IAM (●), of intravenously injected rabbit IgG (▽) and of subcutaneously injected rabbit IgG (△).

4. Discussion

The use of the IAM technique is simple, economical and easy compared with high-performance immunoaffinity chromatography and this method is a practical procedure for medical application. The current study is in line with a previous report showing that IAM (immobilized rabbit IgG specific for rat immunoglobulin E [IgE]) was effective for removal of IgE from plasma of rats injected with recombinant IgE [12]. If the goal is to apply IAM-immobilized specific antibody to human proteins or components for clinical use, evaluation of the efficacy of this approach for removing human proteins or components from the circulation is necessary. The result obtained with the IAM here indicates that this method is excellent for removing hSAP directly from the blood of rats intravenously injected with hSAP. Also, the specific antibody-immobilized IAM was more effective than other ligands or gel in removing hSAP directly from human blood in the presence of heparin.

We chose the rat as a model animal for assessment of the IAM in an extracorporeal circulation system because it is easily used in small-scale experiments, its immunological profile has been well characterized and inbred strains are easily available. In this study, when 300 μg of hSAP was injected, the plasma level of hSAP in the rat was equivalent to the normal human plasma level and remained constant at >97% in the rat. The use of this extracorporeal circulation system in this case allows preclinical evaluation of the ex vivo removal of a human plasma component in an animal model. While a variety of different disease models [19] or transgenic models using rats have been established and characterized [20], other types of experimental models like the present one can also hopefully be of use for eventual clinical application.

Immunoadsorption methods may be problematic in terms of antibody responses to the ligand. However, the IAM did not elicit anti-rabbit IgG (immobilized IAM) rat IgG during the 4-week observation period after termination of the extracorporeal circulation. Although many animal models of extracorporeal circulation have been established using rabbits or dogs [16,17], immunological responses were not tested. Furthermore, larger animals represent expensive running costs in one-time experiments and the evaluation of immunological responses would require an even larger number of such animals. Thus, the rat circulation model is advantageous for removal of human protein by the immunoadsorption method and provides an easy means to examine immunological responses.

In conclusion, the IAM using specific antibody was highly effective for removal of hSAP passed through an extracorporeal circulation system of the rat. Further study of this system may lead to eventual clinical application. We are currently using this system to evaluate various kinds of immobilized antibodies and immune responses to it.

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