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Production of a Monoclonal Antibody to Human α-Fetoprotein and Development of Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assays for Human α-Fetoprotein

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Abstract: This study was attempted to generate a monoclonal antibody against human α-fetoprotein (AFP) and to produce an immunoassay, recognizing AFP in plasma and amniotic fluid. AFP was purified from human amniotic fluid and used to immunize mice. Spleens were taken from the mice and the cells were fused with mouse myeloma cells (Sp2/O-Ag-14) for the production of monoclonal antibodies by employing the hybridoma technology. As a result, a hybridoma cell line producing anti-AFP monoclonal antibody was cloned out and designated as MabF22. From isotyping analysis, it was found that monoclonal antibody MabF22 was IgG type with IgG1 heavy chain and κ light chain. The binding specificity of MabF22 was analyzed by immunoblotting as well as by ELISA. MabF22 was highly specific, reacting with only AFP-containing samples. The binding affinity was determined by ELISA (free-capture mode) and Scatchard analysis. As a result, the value of Kd was 0.8×10^{-10} M. The validity of the MabF22 for AFP assay was examined by two kinds of ELISAs, i.e., non-competitive and competitive ELISA. Both assays revealed that MabF22 reacted well with AFP in sample in a concentration-dependent manner. Standard curve and antibody titration curve were obtained by using purified AFP and MabF22. These results indicate that the monoclonal antibody produced in this study would be useful not only for research purposes but also for further development of immuno-diagnostic kit for the measurement of AFP concentration.

Key Words: Monoclonal, Antibody, ELISA

INTRODUCTION

α-Fetopeotein (AFP) is a well-known embryonal serum protein produced by fetal liver cells, yolk sac cells and some fetal gastrointestinal cells[8]. The clinical significance of human AFP as a marker in the diagnosis and monitoring of certain types of cancer as well as screening for neural tube defects has been extensively reviewed[2,4,11,19,20,23]. AFP has been one of the first tumor-associated antigens proven useful in the diagnosis and evaluation of therapy of hepatocellular carcinoma (HCC) and testicular germ cell tumors. Although it is clinically significant to develop an assay system for the AFP measurement, its development has not been domestically achieved.

Researches on monoclonal antibodies with

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unique specificity for individual binding sites on antigens have been suggested to improve the sensitivity and specificity of AFP determination[1,3,13]. The first FDA-approved monoclonal AFP reagent involving a two-site immunoassay was evaluated by Chan *et al.* in patients with HCC, other malignant tumors, and various non-malignant conditions[3]. As demonstrated with polyclonal antibody-based AFP assay, most HCC patients had increased AFP values, and in most other diseases concentrations were lower, but there was much overlap among the disease groups[15,17,18,21].

Recently, we have produced polyclonal anti-AFP antibodies and an enzyme-linked immunosorbent assay for the measurement of AFP in the previous study[26]. In this work, we report the production of a monoclonal antibody to human AFP and development of an immunoassay, recognizing AFP in plasma and amniotic fluid.

MATERIALS AND METHODS

Preparation of AFP

The dialyzed amniotic fluid with pre-elution buffer, 0.01 M phosphate buffer (pH 6.8) was affinity-chromatographed on anti-AFP antibody-coupled Sepharose 4B. Binding molecules were eluted with elution buffer, 0.1 M glycine buffer (pH 2.5). Peaked fractions of eluates were dialyzed, lyophilized, and loaded onto 10% SDS-polyacrylamide gel for preparative electrophoresis and the contaminant from AFP preparation was removed by electroelution. Purified AFP was dialyzed against 0.9% NaCl and 0.01% EDTA solution (pH 7.4). The purity was confirmed by 10% SDS-polyacrylamide gel electrophoresis (PAGE).

Production of monoclonal antibody to AFP

Female Balb/C mice were immunized initially by intraperitoneal injections with 20 μg of AFP emulsified with an equal volume of Freund's complete adjuvant plus dead *Salmonella* organism. The mice were boosted by intraperitoneal injection of the same amount of antigen emulsified in Freund's incomplete adjuvant after 14 days. Therefore, the mice were boosted at 1-week interval with AFP in phosphate-buffered saline (PBS) until test sera taken from the mice showed good titers against AFP in ELISA. Mice were then given a final intravenous injection of AFP 3 days prior to the fusion.

The fusion was carried out according to the method of Galfre *et al.* using $5x10^7$ myeloma cells (Sp2/0-Ag-14) and $1\sim2x10^8$ spleen cells[7]. After fusion, cells were resuspended in RPMI 1640 (Sigma) containing 10% fetal bovine serum and final concentrations of 10 mM glucose, 20 mM sodium bicarbonate, 20 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and $5x10^{-5}$ M 2-mercaptoethanol in addition, and distributed into flat-bottom 96-well culture plates (100 μl per cells/well) over a mouse macrophage feeder layer ($3x10^5$ cells/well). The cultures were maintained in a humid incubator containing 5% CO_2. On the following day, 100 μl of a selective medium (HAT; $1x10^{-4}$ M hypoxanthine, $4x10^{-7}$ M aminopterin and $3x10^{-5}$ M thymidine) of double strength was added to each well. Hybrid clones began to appear macroscopically $7\sim15$ days after the fusion, supernatants were screened for the antibodies to AFP by a non-competitive ELISA method (see below), and the positive clones were finally obtained by serial limiting dilutions.

Isotyping analysis

The immunoglobulin class and subclass of each monoclonal antibody were assessed by antigen-independent ELISA using a commercialized kit (Pierce #37501). For antigen-independent analysis, goat anti-mouse Ig (G+A+M) was coated to microtiter plate at 4℃ overnight and culture supernatant of monoclonal antibody was added to the wells and incubated at 37℃ for 1 hr. ELISA was done using one of rabbit antisera raised against isotypes of mouse im-

munoglobulins at 37℃ for 1 hr. Horseradish peroxidase (HRP)-conjugated, goat anti-rabbit IgG (H+L) was added to the wells and the plate was incubated 37℃ for 1 hr. The absorbances at 450 nm were determined by ELISA reader (BIO-RAD, USA).

Immunoblotting

Immunoblotting was performed according to the method of Towbin *et al.*[24]. Protein samples from SDS-PAGE were transferred to nitrocellulose membrane at 40V-constant condition for 6 hrs. After transfer, membrane was soaked in a blocking buffer (10 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, 1% bovine serum albumin, pH 7.5) for 1 hr at room temperature and washed 3 times with a washing buffer (10 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Next, the membrane was incubated for 3 hrs at room temperature in the washing buffer containing culture supernatant of monoclonal antibody (500-fold dilution). After washing once, the membrane was incubated in the washing buffer containing goat anti-mouse IgG coupled with HRP for 1 hr at room temperature and then washed again. Next, a freshly prepared solution of peroxidase substrates, 4-chloro-1-naphthol and H_2O_2, was added for the visualization of positive bands.

Affinity measurement using ELISA (Free-capture mode)

The antigen at various concentrations was first incubated in solution with the antibody at constant concentration until equilibrium was reached. The concentration of free antibody was then determined by an indirect ELISA. The antigen at various concentrations ($4x10^{-10}$ M to $2x10^{-7}$ M) was mixed with a constant amount of antibody ($3x10^{-10}$ M), in 0.1 M potassium phosphate, 2 mM EDTA, pH 7.8, supplemented with 1% BSA. After overnight incubation at 4℃, 150 μl of each mixture was transferred into the wells of a microtiter plate previously coated with AFP (150 μl per well, at 1 μg/ml in 50 mM sodium carbonate, pH 9.6 overnight at 4℃) and incubated overnight at 4℃, in which free antibody is captured by binding to antigen on the well. After washing with PBS supplemented with 0.5% Tween 20, the bound immunoglobulins were detected by adding rabbit Ig with specificity against mouse IgG coupled with HRP and measuring the HRP activity retained in each well. Dissociation constant was calculated by the modified Scatchard equation.

Non-competitive ELISA

96-well microplates were coated with 100 μl of sodium carbonate buffer (pH 9.6) containing protein samples overnight at 4℃. The wells were washed 3 times with 200 μl of a washing buffer (PBS containing 0.05% BSA and 0.05% Tween 20). A blocking buffer (1% BSA in PBS) was added, the plates were incubated for 1 hr at room temperature with gentle agitation, and then the wells were washed 3 times with the washing buffer. 100 μl of culture supernatant of monoclonal antibody was added to each well and the plates were incubated for 1 hr at 37℃. After washing, 100 μl of HRP-conjugated goat anti-mouse IgG was pipetted into the wells, the plates were incubated for 1 hr at 37℃ and then washed again. 100 μl of freshly prepared solution of *o*-phenylenediamine and H_2O_2 was added to all the wells, and the absorbances at 450 nm were determined by ELISA reader.

Competitive ELISA

96-well microplates were coated with 0.1 ml of PBS containing 5 μg/ml of AFP overnight at 4℃. The wells were washed 3 times with 0.2 ml of a washing buffer (PBS containing 0.5 mg/ml of BSA and 0.05% Tween 20). A blocking buffer (1% BSA in PBS) was added, the plates were incubated for 1 hr at room temperature with gentle agitation, and then the wells were washed 3 times with the washing

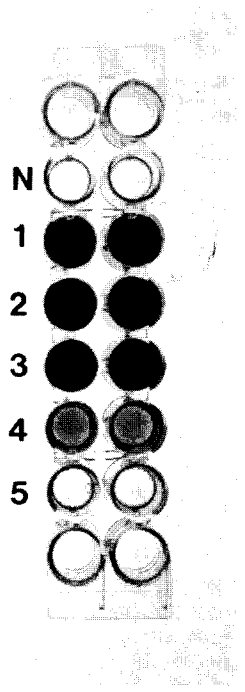

Fig. 1. Examination of antibody production from test blood before sacrificing the mouse for fusion of its spleen cells with the myeloma cells. Blood was taken by eye-bleeding and the anti-AFP antibody production was examined by ELISA on AFP-coated wells. Antiserum was used after serial 10-fold dilutions, i.e., 1, 10^{1} dilution. N is the negative control where mouse serum taken before the immunization was used for the ELISA.

buffer. 50 μl of standard or samples were added, followed by immediate addition of 50 μl of diluted anti-AFP antiserum. The plates were incubated overnight at 4℃. The following steps were performed as described in the non-competitive ELISA procedure above.

RESULTS

Production of monoclonal antibody

The fusion was done by using the Sp2/0-Ag-14 mouse myeloma cell line and the splenocytes of a Balb/c mouse immunized with human AFP. Prior to the removal of spleen from the mouse, the antibody production was confirmed (Fig. 1). After fusion, cell growth was observed in all the 270 wells, unfused cells

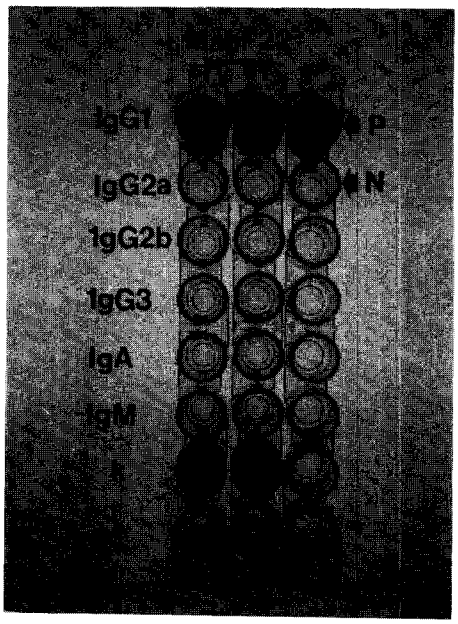

Fig. 2. Picture of a microtiter plate showing the result of isotyping analysis of MabF22 by ELISA. The ELISA was done by antigen-independent style, as described in the legend of table 1. P, postitve control; N, negative control.

were growth-retarded by addition of HAT selection medium, and the culture supernatants were tested for anti-AFP antibody production by ELISA. The cells in the positive wells were transferred to new plates and cloned after serial limiting dilutions. Among the 12 stable hybridomas isolated, one clone that secreted IgG-type antibody specific for AFP was finally selected and designated as MabF22.

The heavy chain and light chain isotypes of monoclonal antibody, MabF22, was analyzed by employing the antigen-independent ELISA. ELISA showed that MabF22 possesses IgG1 isotype of heavy chain and κ light chain (Fig. 2 and Table 1).

Characteristics of monoclonal antibody MabF22

The specificity of MabF22 to human AFP was analyzed by immunoblotting and ELISA. For immunoblotting, samples were subjected to 10% SDS-PAGE and transferred to nitrocellu-

Table 1. Result of isotyping analysis of anti-AFP monoclonal antibody by an antigen-independent ELISA

Class/Subclass	A_{450}	
	MabF22	P or N
IgG1	1.372	1.226 (P)
IgG2a	0.219	0.213 (N)
IgG2b	0.238	
IgG3	0.241	
IgA	0.242	
IgM	0.220	
κ	1.261	
λ	0.196	

Goat anti-mouse Ig (G+M+A) was coated to microtiter plate and culture supernatant containing MabF22 was added to the wells. Subclass-specific rabbit anti-mouse immunoglobulins were separately added to each wells and HRP-conjugated, goat anti-rabbit IgG (H+L) was added in sequence, subsequently. The absorbance readings at 450 nm are shown

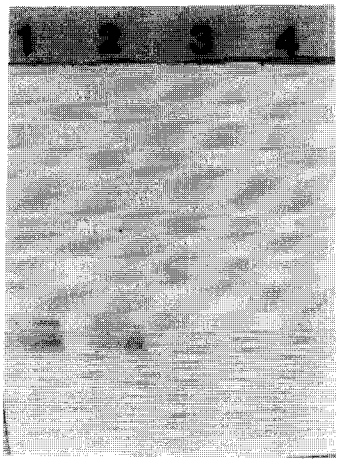

Fig. 3. Analysis of the specificity of monoclonal antibody, MabF22 by immunoblotting. Proteins were separated electrophoretically in 10% gel for the subsequent immunoblotting analysis. 1, AFP; 2, amniotic fluid; 3, normal human serum; 4, human serum albumin.

lose membrane for reaction with the monoclonal antibody. As a result, MabF22 was found out to react specifically with isolated AFP as well as with amniotic fluid, and no-detectable reaction was seen with normal human serum

Table 2. Result of the specificity of anti-AFP monoclonal antibody by ELISA

Antigens	A_{450}
AFP	2.635
Amniotic fluid	1.997
Serum from HCC patients	1.874
Normal human serum	0.231
Human serum albumin	0.080
Blank	0.057

Microtiter plate wells were coated with each antigen and then culture supernatant containing MabF22 and HRP-conjugated, anti-mouse IgG were added in sequence, subsequently

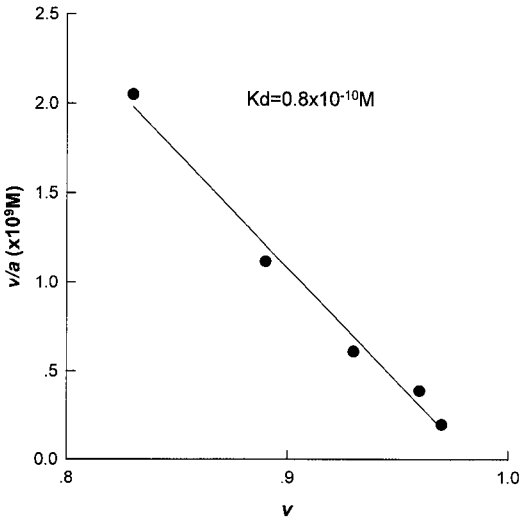

Fig. 4. Result of the affinity constant of MabF22 by ELISA and Scatchard analysis. υ is the fraction of the bound antibody and α the concentration of free antigen at equilibrium. υ corresponds to $(A_o\text{-}A)/(A_o)$. A_o is the absorbance measured in the absence of AFP.

or human serum albumin (Fig. 3). For ELISA, MabF22 showed specific bindings with AFP, amniotic fluid and serum from HCC patients but no signal was obtained when normal human serum or human serum albumin was used for the analysis (Table 2).

The ELISA was also applied to the determination of the affinity constant in solution. The result obtained from Scatchard plot is shown in Figure 4. The Kd value deduced from the slope calculated from linear regression is 0.8x

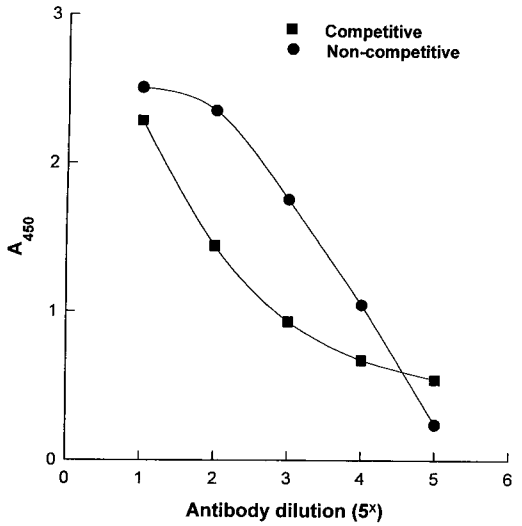

Fig. 5. Titration curve of anti-AFP, monoclonal antibody. The culture supernatant was diluted as indicated on the abscissa and used in the ELISAs for human AFP. The arrows indicate dilution point where 50% maximum binding was obtained.

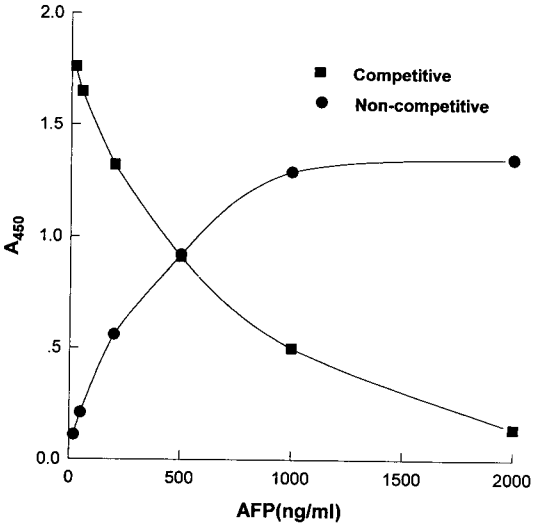

Fig. 6. Standard curve of the monoclonal antibody-based, non-competitive and competitive ELISAs for human AFP. For detailed description of the assay conditions, refer to MATERIALS AND METHODS.

10^{-10} M. The only requirement is that the total antigen concentration should be sufficiently higher than the antibody concentration.

Application of MabF22 in ELISAs

The usefulness of the monoclonal antibody in AFP assay was assessed by two kinds of ELISAs, i.e., non-competitive and competitive methods as described in MATERIALS AND METHODS.

In non-competitive assay where samples coated directly on microtiter plates were used for AFP analysis, the optimal dilution of monoclonal antibody from culture supernatant of the hybridoma cells was determined to be about 500-fold (Fig. 5). The working range calibrated from standard curves was 20~1,000 ng/ml of AFP concentrations (Fig. 6). The minimum detectable concentration of AFP in this assay was 20 ng/ml.

In competitive assay where a fixed amount of AFP is precoated onto the microtiter plates and used with samples to compete for the binding with antibody, the optimal dilution of MabF22 was determined to be about 100-fold (Fig. 5). Standard curve was drawn from ELISA results obtained by using different concentrations of AFP prepared by a serial dilution of standard AFP whose concentration was previously determined (Fig. 6). The working range calibrated from standard curve was 20~2,000 ng/ml of AFP concentrations. The minimum detectable concentration of AFP in the assay was 20 ng/ml and this competitive assay showed much broader working range of AFP concentrations in samples to analyze compared with non-competitive assay.

DISCUSSION

Monoclonal antibodies against AFP have been very useful in studies of its structure, properties and function. Anti-AFP monoclonal antibodies have also been used in biochemical analysis for AFP in plasma and amniotic fluid in addition to histological, ultrastructural and immunological studies.

Many research groups abroad have generated and characterized anti-human AFP monoclonal

antibodies[16,22,25]. In the present report, I have described the production and application of monoclonal antibody against AFP, which is designated as MabF22.

From the fusion for monoclonal antibody production, we initially obtained more hybridoma clones producing anti-AFP monoclonal antibody. But from isotyping analysis, only the one clone was found to produce IgG-type antibody and others produced IgM-type antibodies. IgM antibodies are kind of immature antibodies formed in the early stage of the antibody response and need to have pentameric structure of Y for the antigen-binding active form[9], which is disadvantageous for large-scale production of single effective molecules. Therefore, this IgG-type of monoclonal antibody was focused on for the subsequent studies. The reason why IgM-type of antibody survived to be produced through repeated booster injections in the immunization protocol is not quite clear but the interval periods (10 days each) between the booster injections may not have been long enough to induce full secondary response of IgG production.

For the monoclonal antibody to be used in immunological studies of human AFP, the immunochemical specificity of monoclonal antibody must be firmly established and the antibody must be capable of binding all the AFP-containing samples. On this regard, I confirmed the binding of MabF22 to epitopes that was expressed on native AFP particles by immunoblotting and by ELISA on microtiter plates where human amniotic fluid and isolated AFP fractions were precoated. MabF22 was very specific to human AFP and didn't show any detectable cross-reaction with human serum albumin nor with normal human serum. Monoclonal antibody prepared in this experiment can react with AFP from plasma as well as from amniotic fluid since there is no immunological distinction between AFP derived from HCC and that of any other origin[5].

The dissociation constant for monoclonal AFP antibody was determined using the free-capture mode[27]. This method is a particularly simple way to estimate Kd. A Scatchard plot of this result provides values of 0.8×10^{-10} M. The value by this method is very similar to the dissociation constant obtained by other researchers[10,12,14].

Monoclonal antibody showing the unique intramolecular specificity is homogeneous in quality and advantageous for application such as immunoassay for quantification of serum AFP or amniotic AFP levels, compared with polyclonal antibodies being heterogeneous and of limited production[1,3,13]. Here, we developed two kinds of monoclonal antibody-based ELISAs for AFP, i.e., competitive and non-competitive assays. The use of monoclonal antibody permitted a wider working range of AFP concentrations and less dilution of samples, compared with the case of using polyclonal antibodies[26]. Monoclonal antibody-based ELISAs, especially the competitive one, are thus believed to decrease the analytical error caused by sample dilution.

In conclusion, monoclonal antibody against human AFP has been very useful in studies of AFP structure and function and been particularly true in the cases of epitope characterization and immunoassay development. Therefore, it is expected that MabF22 of this study is very useful not only for research purposes but also for reliable diagnosis of several cancer diseases.

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=국문초록=

인간 α-fetoprotein에 대한 모노클로날 항체의 제조 및 모노클로날 항체를 이용한 효소면역분석법의 개발

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본 연구에서는 혈장이나 양수에 있는 α-fetoprotein (AFP)을 인식할 수 있는 모노클로날 항체를 제조하고, 모노클로날 항체를 이용한 효소면역분석법을 개발하고자 하였다. 양수로부터 얻은 AFP를 쥐에 주사한 후 비장을 분리하여 종양세포 (Sp2/O-Ag-14)와 융합하였고, 하이브리도마 기술을 이용하여 모노클로날 항체를 제조하였다. 모노클로날 항체를 클로닝하였으며, 생성된 항체를 MabF22로 명명하였고, IgG1 중사슬과 κ 경사슬의 isotype을 나타냈다. 또한 immunoblotting 방법과 ELISA로 특이도를 조사한 결과 모노클로날 항체는 AFP와만 반응하였고, 결합 친화상수는 $0.8x10^{-10}$ M이었다. 두 종류의 효소면역분석법 - 경쟁적 또는 비경쟁적 분석 - 을 이용하여 항체의 효용성을 조사하였으며, 두 방법 모두 AFP와 농도에 비례하여 반응하였다. 따라서 본 연구에서 생산된 모노클로날 항체는 연구목적으로 뿐만 아니라 AFP 농도를 측정하기 위한 면역진단시약의 개발에도 유용할 것으로 생각된다.

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