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Selection of human antibody fragments on the basis of stabilization of the variable domain in the presence of target antigens☆

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Abstract

Here we report a novel method for selecting human antibody fragments from nonimmunized variable domain libraries. The antibody fragments are selected on the basis of stabilization of the variable domain fragment (F_v) in the presence of target antigens (''open sandwich selection''). One variable domain is displayed on phages and another is prepared as soluble molecules. These two reagents are mixed with the biotinylated target molecule and ternary complexes are captured by using streptavidin-conjugated magnet beads. After extensive washing, enriched clones are eluted by using target antigen. Some of the clones selected after 3 rounds are prepared as soluble domains, which then undergo another selection process. We obtained several human antibody fragments specific for human soluble erythropoietin receptor by using this method. Our method minimizes several of the disadvantages associated with human antibody selection through a phage-display system, such as construction of a large-scale library, deletion of genes during selection, and nonspecific binding.

Keywords: Phage display; Human antibody; F_v; In vitro selection; Erythropoietin receptor

A major goal of protein engineering is the design of proteins with novel and/or desired functions, e.g., specific recognition of target molecules. Antibody can be regarded as a protein engineering system that has been perfected by nature for the generation of a virtually unlimited repertoire of complementary molecular surfaces [1–3].

Phage-display systems have been used widely as tools for the selection of peptides and proteins specific for target molecules [4–7]. In vitro selection of antibody fragments (i.e., F_{ab} and single-chain antibody [scF_v]) by using a phage-display system has been reported by several groups. These systems, however, have several disadvantages. (1) *Construction of library.* A library with 10^9 identical clones should be constructed for use in a phage-display system. Technical difficulties regarding efficient transformation of *Escherichia coli* limit the scale of the library. (2) *Low display efficiency for proteins*. Phage-mid display and reducing the size of the molecule displayed on the phages would overcome this problem. (3) *Removal of clones that bind nonspecifically*. (4) *Deletion of genes during selection*. The underlying reason is not yet clear, but the deletion of genes encoding antibody fragments often occurs during panning

☆ *Abbreviations:* F_v, antibody variable domain fragment; V_H, variable region of heavy chain; V_L, variable region of light chain; F_v, single-chain F_v fragment; F_{ab}, antigen-binding fragment; GuHCl, guanidinium hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; β-ME, β-mercaptoethanol; k_{on}, association rate constant; k_{off}, dissociation rate constant; K_d, dissociation constant; CDR, complementarity-determining region; CMD, carboxymethyl dextran; PBS, phosphate-buffered saline; OS, open sandwich; EPO, erythropoietin; s-hEPO-R, soluble human EPO receptor; IMAC, immobilized metal affinity chromatography.

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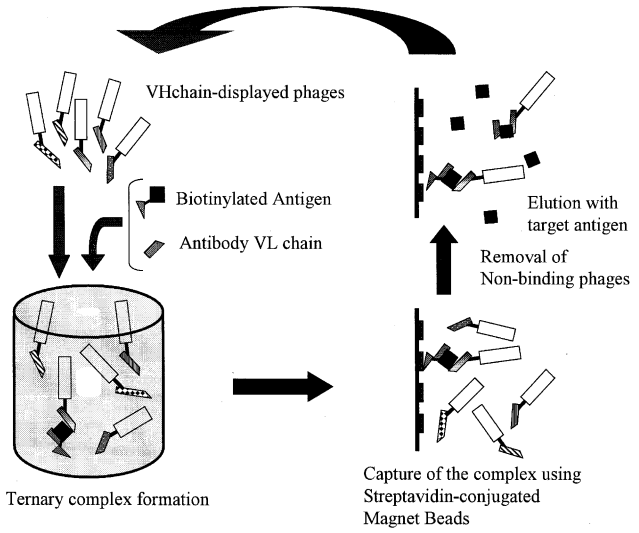

Fig. 1. Schematic representation of "open sandwich selection" (OS selection). Procedures are described in detail in Materials and methods.

procedures. Several improvements have been attempted for overcoming these disadvantages [8–13].

Most F_v fragments of immunoglobulins readily dissociate under physiologic conditions but are usually stable in the presence of their antigens. On the basis of this mechanism, we have proposed the "open sandwich" (OS) method for determining antigen concentration [14]. The sensitivity of the method is relatively high and the background is dramatically lower than that with the standard method. Therefore, the OS method can also be applied to the selection of novel antibody fragments (Fig. 1). We recently reported enhancement of the affinity of an anti-hen lysozyme antibody (HyHEL-10) toward a mutated antigen (other lysozymes). This enhancement was accomplished through saturation mutagenesis of 4 residues in the complementarity-determining region 2 of the heavy chain (CDR-H2), followed by selection for affinity to Turkey lysozyme [15]. By using OS selection, we obtained several clones having converted specificity. Therefore, this selection method would be applicable to obtaining a novel antibody fragment from a nonimmunized (i.e., naive) human antibody library, which may widen the range of in vitro selection of human antibody fragments.

Here we describe the selection of an antibody fragment from a nonimmunized library by using the OS selection method. We used human soluble erythropoietin receptor (s-hEPO-R) [16–18] as a model self-antigen. On the basis of results obtained, we discuss the advantages of the OS selection method.

Materials and methods

Materials. The construction of a human antibody library of the variable regions of the heavy and light chains (V_H and V_L) has been described elsewhere [19,20]. Hen-egg-white lysozyme was supplied by Seikagaku-kogyo (Tokyo, Japan). s-hEPO-R was prepared as described elsewhere (Tsumoto, manuscript in preparation). The synthesis of oligodeoxyribonucleotides for polymerase chain reactions was consigned to Nippon Gene Research Laboratories (Sendai, Japan). All enzymes for genetic engineering were obtained from TaKaRa (Kyoto, Japan), Toyobo (Osaka, Japan), Roche (Mannheim, Germany), and New England Biolabs (Beverly, MA).

Preparation of phage and selection of clones from phage antibody libraries. Phage was recovered by precipitation with polyethylene glycol (PEG) [14,21] and resuspended in 500-μl phosphate-buffered saline (PBS) per 20 ml culture. Streptavidin magnet beads (Promega, Madison, WI) were used to select clones from the phage antibody libraries. In the first step in the procedure, 100 μl V_H-displaying phage library (phage-V_H) was mixed with soluble biotinylated target antigens and a purified human V_L fragment at room temperature for 1 h. Next, 50 μl streptavidin-conjugated paramagnetic beads (Promega) were added and rapidly mixed. After an incubation period of 10 min, a magnet (Promega) was used to collect the beads and attached phage. The beads were washed stringently 5 times with 1 ml PBS containing 0.1% Tween 20 and 5 more times with 1 ml PBS. The bound phages were first eluted with 500 μl PBS containing 50 μg target antigen, followed by a second elution with 500 μl of 100 mM glycine (pH 2.0) containing 200 mM NaCl. The eluates were rapidly neutralized with 100 mM Tris–HCl (pH 8.5) containing 500 mM NaCl. Each elute was reinfected into early log-phase cultures of *E. coli* JM109. After 1 h, 1000 μl of each culture was plated and incubated at 37 °C overnight. Clones on the plates were transferred into LB medium containing 100 mg/L ampicillin, infected with M13KO7 helper phage, and incubated overnight at 37 °C. The phage was prepared by centrifugation and PEG precipitation and was subjected to the next round of selection. After 3 rounds of selection, the clones on the plates were cultured and V_H-displaying phages selected were prepared according to the method described previously [14].

Characterization of the phage-V_H fragments selected. Dideoxy reactions for DNA sequencing were performed using an auto-read sequencing kit (Amersham Bioscience, Tokyo) according to the manufacturer's recommendations. The analysis of DNA sequences was performed by using the ALF Express auto-read sequencer (Amersham). Open-sandwich ELISA was performed according to the method described by Ueda et al. [14]. We added 100 μl of 10-μg/ml human V_L fragments in PBS to each well of the microtiter plates and incubated them for 1 h at room temperature. After removing the solution, 200 μl SuperBlock (Pierce, Rockford, IL) was added to each well and the plates were incubated for 1 h at room temperature. After the buffer was discarded, 100 μl V_H-phage, which had been mixed with target antigens and prediluted with 1 volume binding buffer 30 min before, was added to each well, and the plates were incubated for 1 h at room temperature. The wells were washed twice with PBST, 100 μl horseradish-peroxidase (HRP)-conjugated anti-M13 (Pharmacia; diluted 1:5000 in PBS) was added to each well, and the plates were incubated for 1 h at room temperature. After washing the plates 3 more times with PBST, we added 200 μl of 50 mM sodium succinate buffer containing both 10 mg/ml 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Wako Fine Chemicals, Osaka, Japan) and 0.01% H_2O_2 to each well and incubated the plates for 1 h at room temperature. Absorbance at a wavelength of 415 nm was measured with a microplate reader (model 55, Bio-Rad); the absorbance at 630 nm was used as the control.

Expression and purification of the selected F_v fragments. The expression vectors for V_H and V_L (designated as pUT-VH, and pUT-VL, respectively) were constructed as described previously. In brief, *E. coli* BL21(DE3) cells [22] transformed with pUT-VH or pUT-VL were cultured in shaking flasks at 28 °C in LB medium containing 100 μg/ml ampicillin to an optical density of 0.8 at 600 nm and induced by addition of 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After induction, the temperature was raised to 37 °C and the cultures were grown for another 12–16 h. Cells were harvested and resuspended in 20 ml of 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. The suspension was

sonicated for 15 min at 150–170 W and centrifuged for 20 min at 9000 rpm. The pellet obtained was dissolved in 10 ml of 20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, and 5 mM imidazole containing 6 M guanidinium hydrochloride (GuHCl) and applied to His-Bind Resin (Novagen, Madison, WI) preequilibrated in the same buffer as that used for pellet solubilization. The $(His)_6$-tagged variable-region fragments were eluted with the same buffer containing 60 mM imidazole. The molecular weights of the purified V_H and V_L fragments were confirmed through SDS–PAGE in the buffer system described by Laemmli [23].

Refolding and purification of the F_v fragments selected. Purified variable-region fragments in denaturing conditions (i.e., 6 M GuHCl) were diluted to a concentration of about 7.5 μM and reduced by addition of β-mercaptoethanol (β-ME). In the case of F_v fragments whose domains were expressed separately, V_H and V_L were mixed stoichiometrically at the same concentration. Procedures for the refolding of insoluble antibody fragments described by Tsumoto et al. [24] were followed. After 2–5 h, the concentration of GuHCl in the dialysis buffer was decreased gradually (3, 2, 1, 0.5, and 0 M), and 75 μM of an oxidizing reagent (glutathione, oxidized form; Sigma, St. Louis, MO) and 0.4 M L-Arg were added during the final dialysis stage. The supernatants containing refolded proteins were concentrated to approximately 10 μM by using a Centriplus YM-10 ultrafiltration membrane, followed by a Centricon YM-10 membrane (Millipore, Bedford, MA). The F_v fragment obtained was purified on a Superdex 75 pg ($16 \times 400\ mm^2$) column equilibrated with 50 mM Tris–HCl (pH 7.5) containing 200 mM NaCl. Finally, the F_v fragment was dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.2) containing 200 mM NaCl.

Determination of binding affinities of variable-region fragments for target antigen. Kinetic and equilibrium constants for the interactions between target antigens and the variable-region fragments selected were measured with an IAsys Auto+ optical biosensor (Affinity Sensors, Cambridge, UK). The s-hEPO-R was immobilized on the surface of a carboxymethyl dextran (CMD) cuvette according to manufacturer's recommendations. Solutions of variable-region fragments at various concentrations in PBS were added to the target-antigen-immobilized cuvette and the interactions were monitored instantaneously with IAsys 3.0 software (Affinity Sensors). The resulting association curves were processed as described previously [25]. In brief, the association rate constant (k_{on}) was obtained by plotting the slope, k_s, of the dR/dt vs R plot for all concentrations of the selected F_v against the F_v concentration. The slope of this new plot is the k_{on}, and the intercept, in theory, is the dissociation rate constant (k_{off}).

Results and discussion

Selection of clones with high affinity for s-hEPO-R

Human antibody V_H libraries were subjected to selection on the basis of F_v fragment stabilization under coexistent biotinylated antigens (i.e., OS selection) by using streptavidin-conjugated magnetic beads (Fig. 1). We mixed phage-V_H with soluble target antigens and purified human V_L fragments. The resulting ternary complex was captured by using streptavidin-conjugated paramagnetic beads. We then washed the beads meticulously and recovered the adsorbed phage particles by competitive elution, followed by elution at low pH. Then, the eluted phages were reinfected into early log-phase *E. coli* cultures. The prepared phage was subjected to the next selection stage. ELISA analysis [14] using selected phages and soluble V_L fragment clearly indicated that clones with increased affinity for the target antigen were enriched and the number of clones increased during selection (Table 1). After 3 rounds of panning, we selected random clones from the culture plates and phages displayed V_H have been prepared. OS-ELISA analyses using phage-V_H indicated that some of the clones selected showed a marked signal for the target antigen (data not shown). We determined the DNA sequences of the clones enriched (Table 2). Interestingly, the deduced amino acid sequence of clone 3B1 is most homologous to an autoantibody specific for myosin [26].

Bacterial expression, purification, and refolding of selected V_H

Because most of the clones selected cannot be obtained in large quantities by using a secretory expression system, we adapted a cytoplasmic expression system [24], which enabled us to obtain the gene products as inclusion bodies. V_H and V_L, whose expressions were controlled by an inducible T7 promoter and terminator, were fused with a $(His)_6$ tag at their carboxyl termini to facilitate purification. *E. coli* BL21(DE3) cells transformed with pUT-VH were propagated to early log-phase at 28 °C and induced by the addition of IPTG. Insoluble materials including the recombinant proteins were obtained from the sonicated cell mixture as pellets, which were solubilized in a denaturation buffer containing 6 M GuHCl.

To purify the proteins, supernatants containing solubilized variable domain fragments were subjected to immobilized metal chelate affinity chromatography

Table 1
Binding of phage-V_H fragments during OS selection[a]

Rounds of panning	Input number (CFU)[b]	Number of clones recovered	
		Competitive elution[c]	From beads[d]
1	6.0×10^7	Not done	1.1×10^3
2	7.8×10^6	1.1×10^4	3.0×10^4
3	Not determined	5.1×10^4	3.0×10^4

[a] Experimental procedures are described in Materials and methods.
[b] The procedure for estimating these values is that of [24].
[c] The number of CFUs after elution with 100 μg/ml antigen.
[d] The number of CFUs remaining on the beads.

Table 2
Kinetic parameters of the interactions between V_H fragments selected and s-hEPO-R[a]

Clone	CDR-H1[b]	CDR-H2[b]	CDR-H3[b]	k_{ass} ($\times 10^4\,M^{-1}\,s^{-1}$)	k_{diss} ($\times 10^{-3}\,s^{-1}$)	K_d[c] ($\times 10^{-7}$ M)
3B1	NYPIS	GIIPVLGIQNDAQKFQD	GSGGDTGYF------DL	1.83	3.90	2.13
3C2	GYEMN	YISSSGSTIFYADSVKG	DHMTTVTPF------DS	0.46	1.70	3.69
3G5	DYYRT	NIYN-SGINKYNPSLES	WGPGYFNGGSCYSF-DH	1.24	7.60	6.12

[a] See Materials and methods for details of experimental procedures. Data are mean values from triplicate determinations.
[b] Deduced CDR sequences of each clone are shown.
[c] The dissociation constants (K_d) were calculated by using the equation $K_d = k_{diss}/k_{ass}$.

(IMAC). From the SDS–PAGE analysis, all variable region fragments were eluted in a very pure state by using 200 mM imidazole. The methods used for refolding the variable region fragments [24] were based on gradually decreasing the concentration of GuHCl from 6 to 0 M. We began the refolding reaction by using a 1:1 stoichiometric mixture of the V_H selected to the V_L mixture under reducing conditions. Little aggregation was found in either refolding solution, indicating that the antibody fragments undergoing refolding primarily were present in the supernatant. We obtained more than 5 mg F_v from 1 L culture medium.

Kinetics of the interactions between s-hEPO-R and the variable-region fragments selected

The binding affinities of F_v fragments selected toward s-hEPO-R were estimated by using an optical biosensor. Bacterially expressed and refolded s-hEPO-R was immobilized onto the sensor surface. The observed response (R) of the immobilized protein was 500 arc second, equivalent to a concentration of approximately 0.50 μM within the CMD matrix. We subjected the sensor surface to various concentrations of the F_v fragments selected and calculated the kinetic constants from the binding profiles as described in Materials and methods. Fig. 2A shows a typical response curve for the binding of the antibody fragment (100–500 nM for clone 3B1) to the immobilized s-hEPO-R. Kinetic parameters obtained from the binding curves are shown in Table 2. The values of the affinity constants for clones 3B1, 3C2, and 3G5 as estimated by using k_{diss}/k_{ass} were 213, 369, and 612 nM, respectively.

Enhancement of affinity for s-hEPO-R by selection of V_L fragment by using selected V_H

As shown in the previous section, the selected V_H fragment showed moderate affinity for the target antigen. To enhance the affinity of the selected V_H fragment for the target, we performed selection for V_L chains by using one of the soluble V_H fragments (3B1) (Table 3). After the third selection, phages were recovered from the magnetic beads by competitive elution under several antigen concentrations (1, 10, and 100 μg/ml) as well as

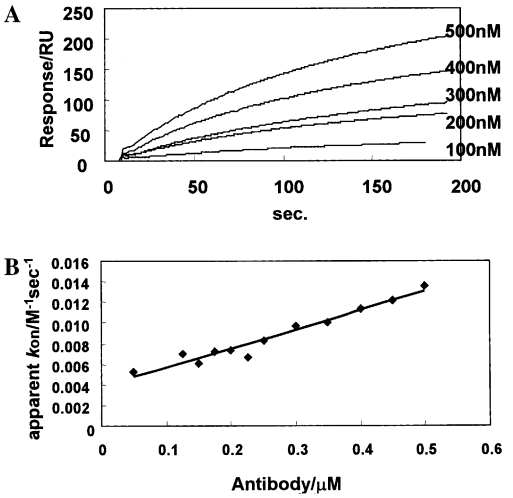

Fig. 2. Surface plasmon resonance analyses of the interactions between 3B1 F_v and s-hEPO-R. The range of concentrations of 3B1 F_v used was 100–500 nM. (A) Concentration-dependence of the response. (B) k_{on}-Concentration plot based on the dR/dt plot.

low pH. We randomly selected and DNA-sequenced several of the clones that had been enriched after 3 rounds of selection (Table 4). Results of ELISA analyses indicated that the clones selected yielded a marked signal to each antigen (data not shown). Some of the clones were prepared as soluble molecules, as in the case of selection of V_H.

We estimated the binding affinities of several F_v fragments selected toward s-hEPO-R and the calculated kinetic constants of the clones selected are shown in Table 4. The results clearly indicate that "fine-tuning" of the V_L chains by the second round selection led to 5–10-fold increases in affinity for the target antigen. We also estimated the affinity of these clones for soluble mouse EPO-R (Fig. 3, Table 4). Despite the high (~80%) sequence homology between mouse EPO-R and s-hEPO-R, clones, 3a1 and 3d4, showed 10–15-fold decreased affinity for mouse EPO-R compared with that for s-hEPO-R, suggesting specific recognition by the clones selected for s-hEPO-R. The decreased affinity of the clones for mouse EPO-R seems to originate from the dissociation rate constant. These clones did not show specific binding curves for several proteins (e.g., BSA, hen lysozyme), suggesting the specificity of selected clones for the target protein.

Table 3
Binding of phage-V_L fragments during OS selection[a]

Rounds of panning	Input number (CFU)[b]	Number of clones recovered	
		Competitive elution[c]	Remaining on beads[d]
1	9.0×10^7	Not done	1.9×10^3
2	1.1×10^8	1.1×10^5 (100)	3.0×10^5
3	4.8×10^8	5.1×10^4 (1)	1.1×10^6
		2.0×10^5 (10)	
		2.5×10^5 (100)	

[a] Experimental procedures are described in Materials and methods.
[b] The procedure for estimating values is followed by Tsumoto et al. [24].
[c] The number of CFUs by elution with antigen is shown. In parentheses are the concentrations of the antigen (μg/ml) used for elution.
[d] The number of CFUs remaining on the beads is shown.

Table 4
Kinetic parameters of the interactions between 3B1-V_L fragments selected and EPO-R[a]

Clone	EPO-R	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_d[b] (M)
3B1–3a1[c]	Human	3.64×10^4	1.60×10^{-3}	4.39×10^{-8}
3B1–3d4[d]	Human	2.75×10^4	0.40×10^{-3}	1.45×10^{-8}
3B1–3a1[c]	Mouse	1.70×10^4	1.40×10^{-2}	8.23×10^{-7}
3B1–3d4[d]	Mouse	1.25×10^4	1.30×10^{-3}	1.04×10^{-7}

[a] See details for experimental procedures. Data are mean values from triplicate determinations.
[b] The dissociation constants (K_d) were calculated by using the equation $K_d = k_{off}/k_{on}$.
[c] Competitively eluted by using 1 μg/ml s-hEPO-R. Deduced CDR sequences are RASQGISNYLA, GASTLQI, and QQLT for L1, L2, and L3, respectively.
[d] A clone left on the beads. Deduced CDR sequences are KSSQSVLYSFNNKNKLA, WASTRES, and QQFYSHPLT for L1, L2, and L3, respectively.

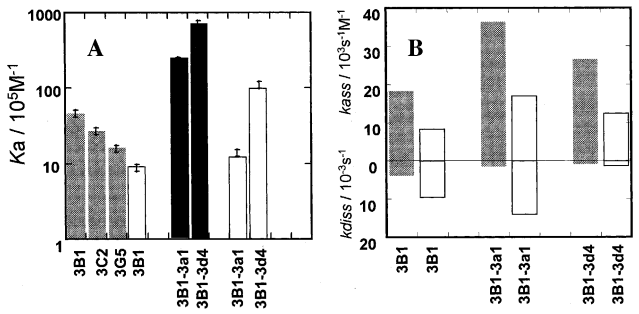

Fig. 3. Kinetic parameters of the interactions between F_v fragments selected and EPO-R. Values for each bar are from Tables 3 and 4. Clones 3B1, 3C2, and 3G5 are the V_H fragment-containing clones obtained after the first selection and clones 3B1–3a1 and 3B1–3d4 are F_v fragments obtained after the 2-step selection process. Solid bar, h-sEPO-R; open bar, mouse EPO-R.

Applicability of OS selection to the preparation of human antibody fragments

Although a phage-display system is useful for in vitro selection of antibody fragments, the method is associated with several drawbacks as described above, limiting the applicability of this system. Using an F_v fragment would overcome the problems associated with phage-display systems. Only 1 chain (V_H or V_L) is selected and therefore a smaller-scale library can be constructed. Upon comparison with display of scF_v or F_{ab} on phages, our F_v system requires the display of only 1 domain on phages thereby reducing the effects of display efficiency on the selection and deletion of genes during selection. In addition, the improved procedure reported here is based on the mechanism of F_v stabilization in the presence of antigens—a scenario that might remove nonspecific clones. These improvements make stable and efficient selection possible. We have used this method successfully to select several human antibody fragments specific for various antigens (KT, HW, and IK, manuscript in preparation).

Antibody molecules from an unimmunized library have an affinity of approximately 10^6 for target antigens (reviewed in [3]) and this affinity can be enhanced further through affinity maturation in vivo. Recent technical progress in methods for random mutation by using error-prone PCR [27] and cassette mutagenesis would make it feasible to mimic hypermutation in vivo and OS selection can be applied in these situations to enhance the affinity of selected clones. Further, OS selection would be applicable to various artificially constructed antibody libraries, such as a focus library in which the some of the residues in CDRs are randomly mutated [28], semi-synthetic antibody libraries [29], and CDR-shuffling antibody libraries [30].

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